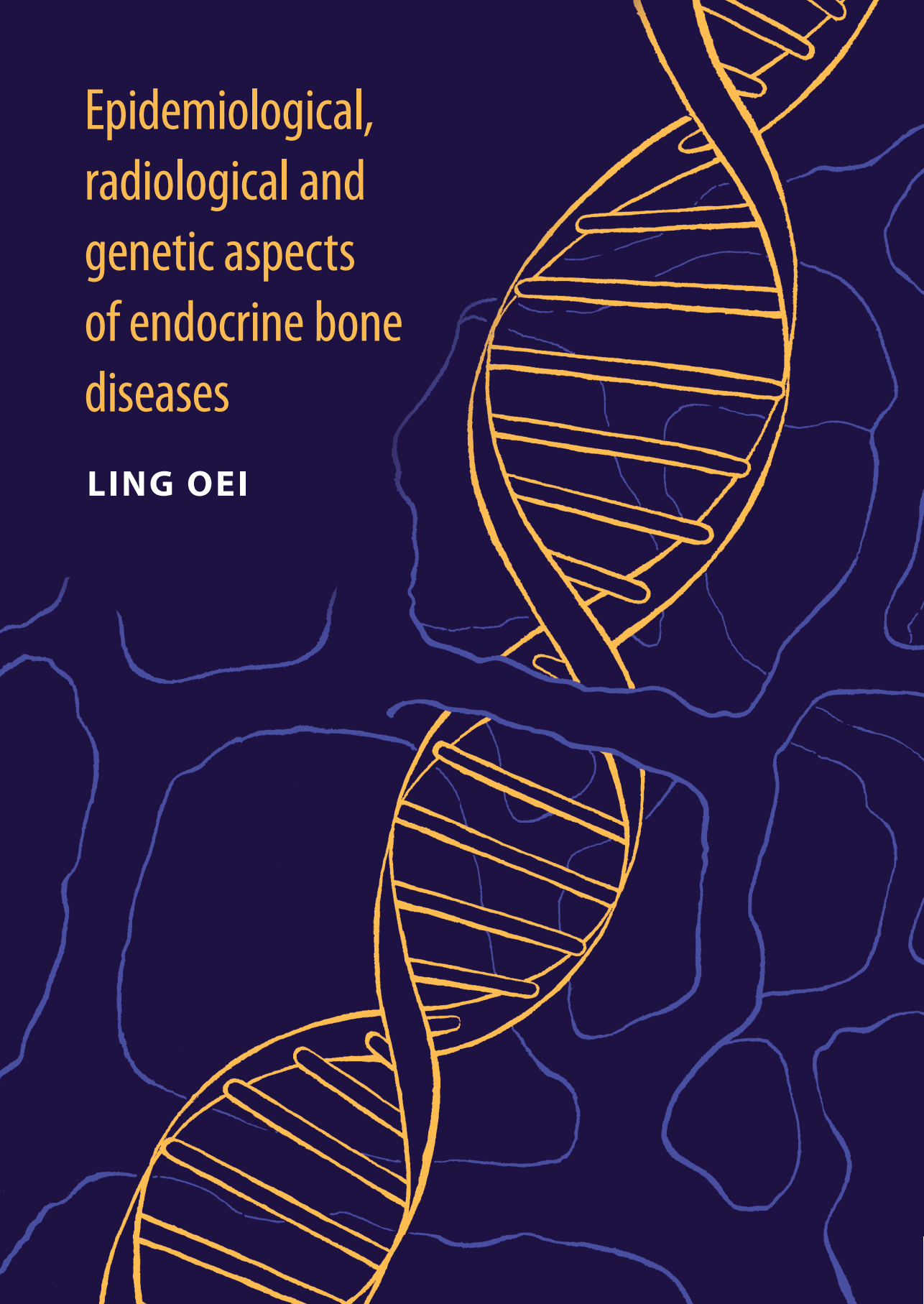
Epidemiological, radiological and genetic aspects of endocrine bone diseases

LING OEI



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Epidemiological, Radiological and Genetic Aspects of Endocrine Bone Diseases

Epidemiologische, radiologische en genetische aspecten van endocriene botziekten

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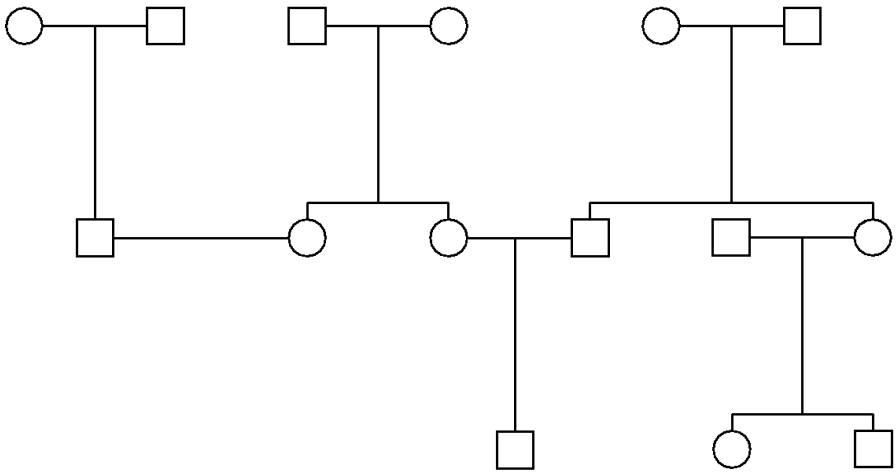

Voor hen die in mij geloven
For those who believe in me

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Chapter 2.2

Castaño-Betancourt MC, **Oei L**, Rivadeneira F, de Schepper EIT, Hofman A, Bierma-Zeinstra S, Pols HAP, Uitterlinden AG, van Meurs JBJ. "Association of lumbar disc degeneration with osteoporotic fractures; the Rotterdam Study and meta-analysis from systematic review." Bone. 2013;57(1):284-9. PMID: 23958823

Chapter 2.3

Oei L, Campos-Obando N, Dehghan A, Oei EHG, Stolk L, van Meurs JBJ, Hofman A, Pols HAP, Uitterlinden AG, Franco OH, Zillikens MC, Rivadeneira F. "Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study." Osteoporosis International. 2014;25(4):1247-54. PMID: 24337661

Chapter 2.4

Ma L*, **Oei L***, Jiang L, Estrada K, Chen H, Wang Z, Yu Q, Zillikens MC, Gao X, Rivadeneira F. "Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies." European Journal of Epidemiology. 2012;27(5):319-32. PMID: 22451239

Chapter 2.5

Oei L, Zillikens MC, Dehghan A, Buitendijk GHS, Castaño-Betancourt MC, Estrada K, Stolk L, Oei EHG, van Meurs JBJ, Janssen JAMJL, Hofman A, van Leeuwen JPTM, Witteman JC, Pols HAP, Uitterlinden AG, Klaver CCW, Franco OH, Rivadeneira F. "High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study." Diabetes Care. 2013;36(6):1619-28. PMID: 23315602

Chapter 2.6

Oei L, Rivadeneira F, Zillikens MC, Oei EHG. "Diabetes, diabetic complications, and fracture risk." Current Osteoporosis Reports. 2015;13(2):106-15. PMID: 25648962

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Oei L, Zillikens MC, Rivadeneira F, Oei EHG. "Osteoporotic vertebral fractures as part of systemic disease." Journal of Clinical Densitometry. 2016;19(1):70-80. PMID: 26376171.

Chapter 3.1

Oei L, Ly F, Breda SJ, Zillikens MC, Uitterlinden AG, Krestin GP, Rivadeneira F, Oei EHG. "Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings." European Radiology. 2013;23(2):476-86. PMID: 22892721

Chapter 3.2

Oei L*, Breda SJ*, Koromani F, Schousboe JT, Clark EM, Ly F, van Meel ER, Dogterom EJ, de Kok LGC, van Meurs JBJ, Hofman A, Pols HAP, Waarsing E, Kiel DP, Bouxsein ML, Black DM, van Rooij FJA, Hunink MG, Uitterlinden AG, Zillikens MC, Krestin GP, Oei EHG**, Rivadeneira F**. "Osteoporotic vertebral fracture prevalences vary widely between radiological assessment methods: the Rotterdam Study." (In preparation)

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Chapter 3.4

Oei L, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJA, Uitterlinden AG, Zillikens MC, Rivadeneira F, Oei EHG. "Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses." Quantitative Imaging in Medicine and Surgery. 2013;3(5):249-255. PMID: 24273742

Chapter 3.5

Makurthou AA, **Oei L**, El Saddy S, Breda SJ, Castaño-Betancourt MC, Hofman A, van Meurs JBJ, Uitterlinden AG, Rivadeneira F, Oei EHG. "Scheuermann's disease: evaluation of radiological criteria and population prevalence." Spine. 2013;38(19):1690-4. PMID: 23759818

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Breda SJ, **Oei L**, Oei EHG, Zillikens MC. "Clinical lesson. Osteoporotic vertebral fractures or Scheuermann's disease." Dutch Journal of Medicine. 2013;157(45):A6479. PMID: 24191924

Chapter 4.1

Estrada K*, Styrkarsdottir U*, Evangelou E*, Hsu YH*, Duncan EL*, Ntzani EE*, **Oei L***, Albagha OM, Amin N, Kemp JP, Koller DL, Li G, Liu CT, Minster RL, Moayyeri A, Vandenput L, Willner D, Xiao SM, Yerges-Armstrong LM, Zheng HF, Alonso N, Eriksson J, Kammerer CM, Kaptoge SK, Leo PJ, Thorleifsson G, Wilson SG, Wilson JF, Aalto V, Alen M, Aragaki AK, Aspelund T, Center JR, Dailiana Z, Duggan DJ, Garcia M, Garcia-Giralt N, Giroux S, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Khusainova R, Kim GS, Kooperberg C, Koromila T, Kruk M, Laaksonen M, Lacroix AZ, Lee SH, Leung PC, Lewis JR, Masi L, Mencej-Bedrac S, Nguyen TV, Nogues X, Patel MS, Prezelj J, Rose LM, Scollen S, Siggeirsdottir K, Smith AV, Svensson O, Trompet S, Trummer O, van Schoor NM, Woo J, Zhu K, Balcells S, Brandi ML, Buckley BM, Cheng S, Christiansen C, Cooper C, Dedoussis G, Ford I, Frost M, Goltzman D, González-Macías J, Kähönen M, Karlsson M, Khusnutdinova E, Koh JM, Kollia P, Langdahl BL, Leslie WD, Lips P, Ljunggren Ö, Lorenc RS, Marc J, Mellström D, Obermayer-Pietsch B, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Ridker PM, Rousseau F,

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Chapter 4.2

Oei L*, Zheng HF*, Ntzani EE*, Trajanoska K*, Morris J*, Nielson CM*, Estrada K*, Styrkarsdottir U*, Ridker PM*, Leong A, Ackert-Bicknell CL, van de Peppel J, Medina-Gómez C, Hsu YH, Duncan EL, Yang J, Esko T, Atanasovska B, Kaptoge S, Pettersson-Kymmer U, Nordström P, Garcia M, Aragaki AK, Enneman AW, Lehtimäki T, Trompet S, Eriksson J, Amin N, Kung AW, Tsilidis K, Thorleifsson G, Rose LM, Zmuda J, Liu CT, Smith AV, Srikanth P, Wilson SG, Clark G, Viikari J, Mihailov E, Moayyeri A, Li G, Kammerer CM, Lorentzon M, Rivera N, Xiao S, Pers T, Tranah GJ, Evans DS, Siggeirsdottir K, Oei EHG, Stefansson K, Leo PJ, Aalto V, Willner D,Wareham N, Minster RL, Bis J, Lyytikäinen LP, Brandi ML, Heckbert S, Cheung CL, Eriksson N, Cheng S, Sitlani C, Cooper C, Khusainova R, van Hul W, Dedousi G, McGuigan FE, Hocking LJ, Koh JM, Kollia P, van Schoor NM, Khusnutdinova E, Lips P, Langdahl BL, Grigouriou E, van Duijn CM, Boyle A, Snyder MP, Herrera L, Nogues X, Koller DL, Vandenput L, Cupples LA, Panagoula K, Zoe D, Aspelund T, Riancho JA, Mellström D, Obermayer-Pietsch B, Olmos JM, Reid DM, Raitakari O, Grinberg D, LaCroix AZ, Åkesson K, Khaw KT, Christiansen C, Formosa MM, Xuereb-Anastasi A, Jones G, Dailiana Z, Giroux S, Lacroix AZ, Frost M, Lorentzon M, McCloskey E, Robbins J, Liu Y, Breda SJ, Tang NL, Szulc P, Husted LB, Prince RL, Lewis JR, Nethander M, Rousseau F, Luben R, Cauley JA, Arnold A, Reppe S, Hibbs MA, Stolk L, Pasco J, Grundberg E, Gautvik KM, Yadav V, Choi K, van Leeuwen JPTM, Pols HAP, Hofman A, Lewis JR, Masi L, Shen J, van Meurs JBJ, Lee SH, Sham PC, Psaty BM, Harris TB, Reeve J, Jukema JW, Metspalu A, Kahonen M, van der Velde N, Brown MA, Ralston SH, Gudnason V, Ioannidis JP, Uitterlinden AG, Cummings SR, Spector TD, Karasik D, Zillikens MC, Visscher P, Michaëlsson K, Jackson RD**, Thorsteinsdottir U**, Chasman D**, Orwoll E**, Forgetta V**, Kiel DP**, Ohlsson C**, Richards JB**, Rivadeneira F**, for the GEFOS consortium. "Large-scale genome wide meta-analysis identifies genetic determinants for fracture risk." (In preparation)

Chapter 4.3

Oei L, Hsu YH, Styrkarsdottir U, Nandakumar K, Eussen BH, de Klein A, Peters MJ, Halldorsson B, Liu CT, Alonso N, Kaptoge SK, Thorleifsson G, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Kruk M, Lewis JR, Patel MS, Scollen S, Svensson O, Trompet S, van Schoor NM, Zhu K, Buckley BM, Cooper C, Ford I, Goltzman I, González-Macías J, Langdahl BL, Leslie WD, Lips P, Lorenc RS, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Slagboom PE, Garcia-Ibarbia C, Ingvarsson T, Johannsdottir H, Luben R, Medina-Gómez MC, Arp P, Nandakumar K, Palsson STh, Sigurdsson G, van Meurs JBJ, Zhou Y, Hofman A, Jukema JW, Pols HAP, Prince RL, Cupples LA, Marshall CR, Pinto D, Sato D, Scherer SW, Reeve J, Thorsteinsdottir U, Karasik D, Richards JB, Stefansson K, Uitterlinden AG, Ralston SH, Ioannidis JPA, Kiel DP, Rivadeneira

F, Estrada K. "A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus." Journal of Medical Genetics. 2013; 51(2):122-31. PMID: 24343915

Chapter 4.4

Oei L, Estrada K, Duncan EL, Christiansen C, Liu CT, Langdahl BL, Obermayer-Pietsch B, Riancho JA, Prince RL, van Schoor NM, McCloskey EV, Hsu YH, Evangelou E, Ntzani EE, Evans DM, Alonso N, Husted LB, Valero C, Hernandez JL, Lewis JR, Kaptoge SK, Zhu K, Cupples LA, Medina-Gómez MC, Vandenput L, Kim GS, Lee SH, Castaño-Betancourt MC, Oei EHG, Martinez J, Anna Daroszewska A, van der Klift M, Mellström D, Herrera L, Karlsson MK, Hofman A, Ljunggren O, Pols HAP, Stolk L, van Meurs JBJ, Ioannidis JPA, Zillikens MC, Lips P, Karasik D, Uitterlinden AG, Styrkarsdottir U, Brown MA, Koh J, Richards JB, Reeve J, Ohlsson C, Ralston SR, Kiel DP, Rivadeneira F. "Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus." Bone. 2014;59:20-7. PMID: 24516880

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van der Eerden BCJ, **Oei L**, Hoenderop JGJ, van Schoor NM, Pettersson U, Koedam M, Uitterlinden AG, Hofman A, Suzuki M, Ohlsson C, Rivadeneira F, Bindels RJM, van Leeuwen JPTM. "TRPV4 deficiency causes sexual dimorphism in bone metabolism and osteoporotic fracture risk." Bone. 2013;57(2):443-54. PMID: 24084385

Chapter 4.6

Campos-Obando N, **Oei L**, Simon MEH, Zillikens MC. "Osteoporotic vertebral fractures during pregnancy: be aware of a potential underlying genetic cause." Journal of Clinical Endocrinology and Metabolism. 2014;99(4):1107-11. PMID: 24423337

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Oei L, Zillikens MC, Rivadeneira F, Oei EHG. "Genetics of osteoporotic vertebral fractures." Journal of Clinical Densitometry. 2016;19(1):23-8. PMID: 26376172

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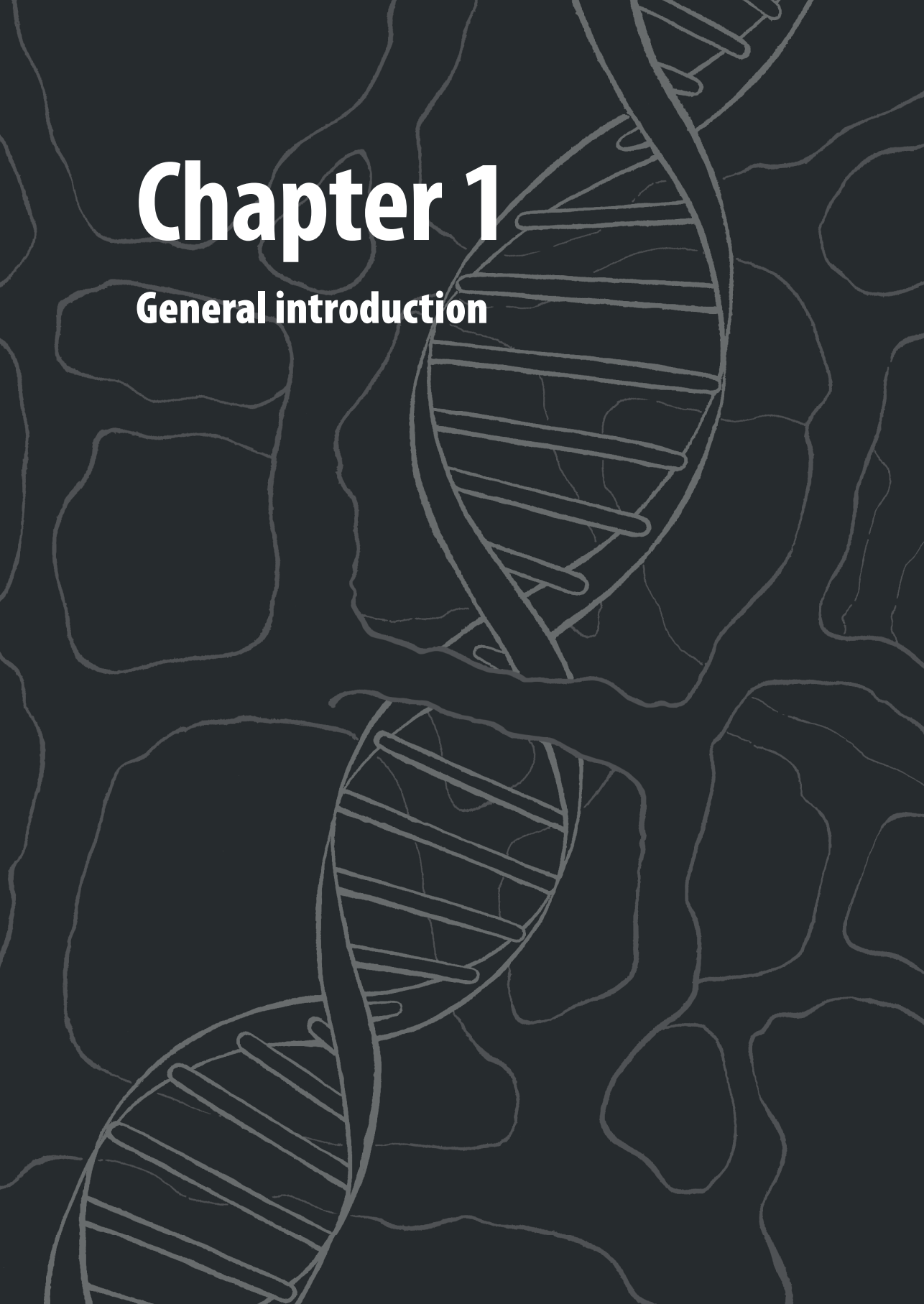
*These authors contributed equally

Chapter 1

General introduction



THE SKELETON AND BONE TISSUE

A typical adult human skeleton consists of 206 bones of varying shapes and sizes to match their functions. Why do we have bones? The skeleton provides a frame to support the body and protects our inner organs. Without our bony structures, we would be a jelly-fish-like mass unable to sit or stand upright. Sites where bones connect to each other are called joints and muscles are attached to the bones by tendons. Altogether, this musculoskeletal system enables movement. Further, bone acts as a reservoir for minerals, such as calcium and phosphorus, salts, and growth factors including insulin-like growth factor-I (IGF-I).[1, 2] Additionally, the marrow located in the interior of certain bones produces red and white blood cells. Finally, in recent years it has become evident that we should regard bone tissue as a metabolically active endocrine organ, which produces hormones such as fibroblast growth factor – 23 (FGF-23)[3] and osteocalcin[4].

There are two types of bone tissue in the human skeleton, namely cortical and trabecular (Figure 1). Most human bones consist of an outer shell of cortical bone that surrounds the inner trabecular elements. Cortical bone tends to be harder, stiffer and heavier than trabecular bone. Nevertheless, load sharing between cortical and trabecular bone compartments varies between skeletal sites; for example cortical bone contributes relatively more to bone strength in the femoral necks than it does in the vertebral bodies.[5, 6] Bone is a dynamic organ, constantly undergoing remodeling, which involves a coupled process of resorption by bone-destroying cells, i.e., osteoclasts and bone formation by bone-building cells, i.e., osteoblasts. When osteoblasts become embedded in the matrix that they secrete, they become osteocytes, which compose the far majority of all cells in adult bone (Figure 2).[3] The bone remodeling balance is under control of various stimulatory and inhibitory effects at the same time. Mechanical loading through increased Wnt/beta-catenin signaling is a very important stimulatory factor; for instance, astronauts lose an average of more than 1% of their bone mass per month when weightless in space. Also, hormonal effects have a very prominent stimulatory role on bone, including sex steroids, vitamin D, parathyroid hormone, calcitonin, the aforementioned IGF-I and insulin.[7, 8] Inhibitory factors are, for example, exposure to toxic substances, glucocorticoids and inflammation.[9-12] Furthermore, different factors may influence the efficiency of aforementioned regulatory processes. For instance, high glucose and advanced glycation endproducts (AGEs) may blunt the stimulatory actions of IGF-I on osteoblasts[13-15] and, additionally, they can directly compromise bone material properties.[16]

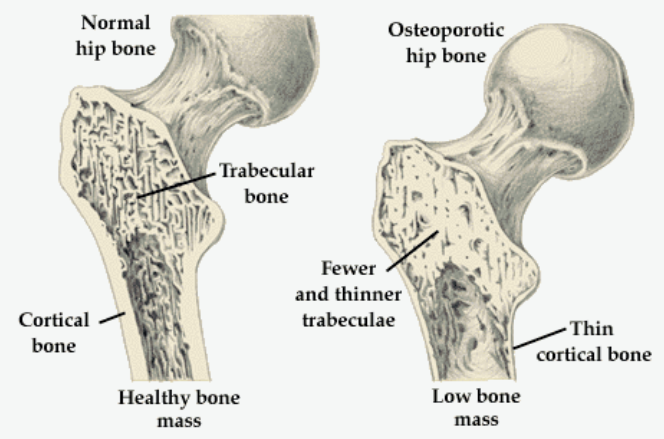


Figure 1. Cross-sections of a normal (left) and an osteoporotic (right) hip bone showing cortical and trabecular components. Adapted from http://www.hughston.com/hha/a.osteo.htm

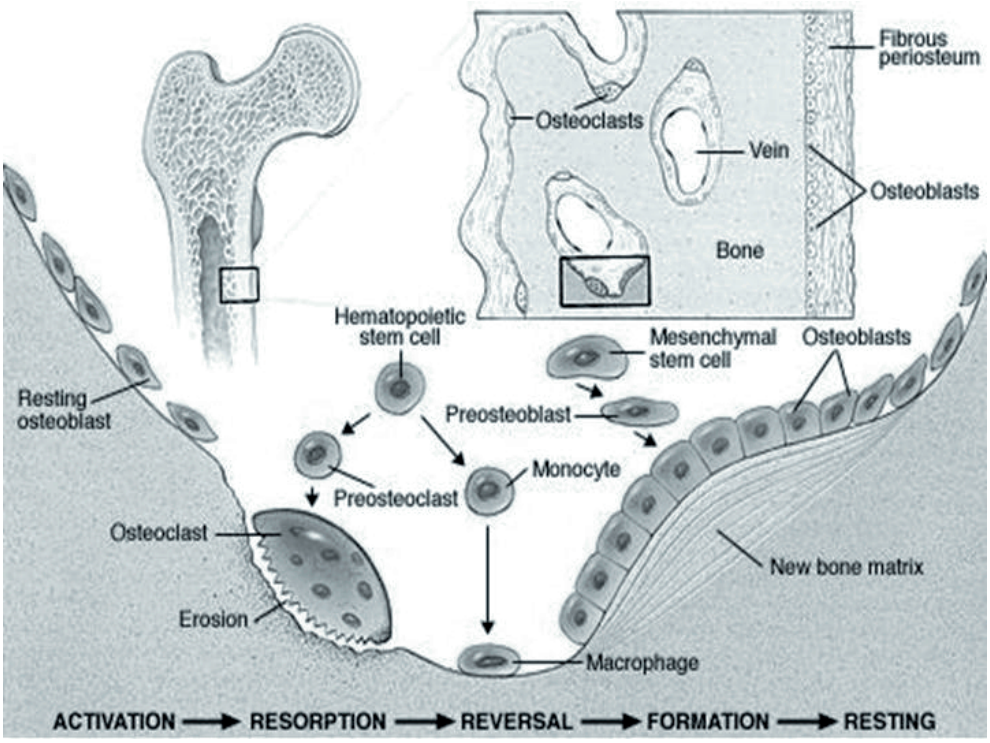


Figure 2. Stages of the bone remodeling process depicting bone-destroying cells, i.e., osteoclasts, bone-building cells, i.e., osteoblasts, and osteocytes. Source http:\\www.medscape.com

OSTEOPOROSIS AND FRACTURES

Normally, bone resorption and bone formation are well-balanced through regulation by hormones, growth factors and cytokines.[17] Resorption is necessary for bone repair, as illustrated by the condition pycnodysostosis, a form of osteopetrosis due to defective osteoclasts, where patients have an increased fracture risk despite a high bone mass.[18] However, more commonly, when bone resorption is higher than bone formation, this leads to bone loss. Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk.[19] It is the most common metabolic bone disease, with the most common skeletal sites of fracture being the spine, hip and wrist (Figure 3).[20] Of the population aged 50–84 years, approximately 50% are classified as having osteopenia and 20% as having osteoporosis and it has been estimated that after the age of 50 years up to 1 in 2 women and 1 in 4 men will suffer a major osteoporotic fracture in their remaining lifetime.[21, 22] These fractures are associated with a high morbidity and mortality.[23] Worldwide, osteoporosis causes more than 9 million fractures annually, which is equivalent to an osteoporotic fracture every 3 seconds.[24] The greatest number of osteoporotic fractures occur in Europe, where osteoporotic fractures account for more disability-adjusted life years (DALYs, a measure for disease burden) lost than common cancers with the exception of lung cancer.[24] Moreover, the death rate for all low-trauma fractures in older men and women is increased immediately post-fracture with a quarter of hip fracture patients dying within 6 months due to complications, but mortality remains

elevated also for many years thereafter.[25, 26] Given the "greying" (i.e., ageing) of populations, osteoporotic fractures are likely to become an even increasingly important health issue. The annual cost of treating all types of fracture has been projected at $17 billion in the United States and is predicted to have increased 50% by 2025.[27]

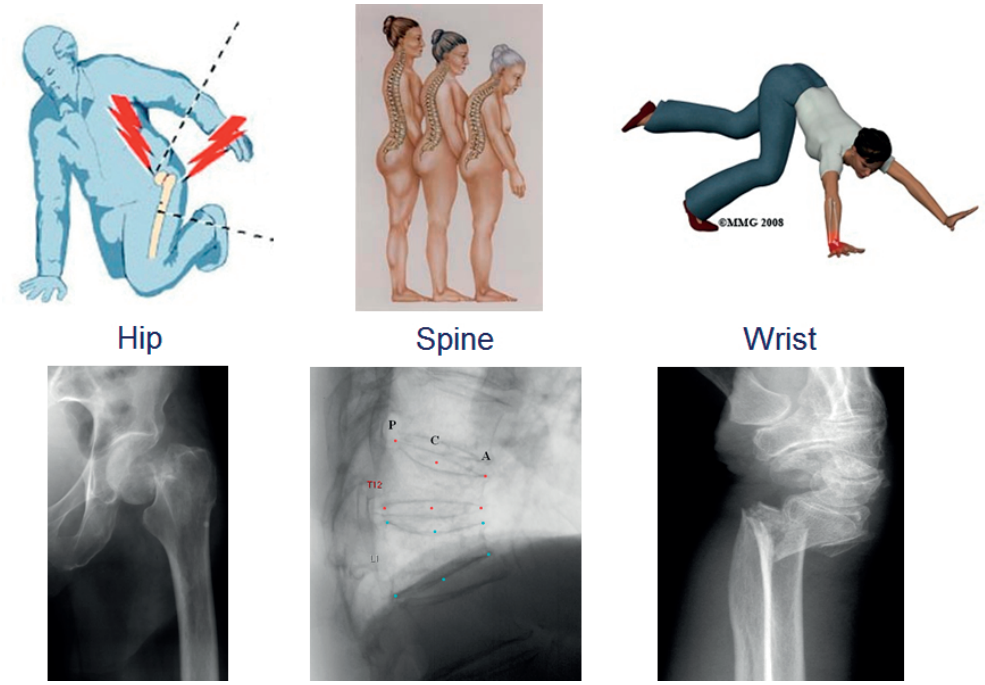


Figure 3. Image representations at the top and radiographs at the bottom for the most common skeletal sites of osteoporotic fractures, i.e. the spine, hip and wrist. Parts obtained and adapted from http://www.orthopaedicsurgeon.com.sg/what-you-need-to-know-about-osteoporosis/ and http://www.medicalmultimediagroup.com/

SKELETAL DIAGNOSTICS

The most well-studied clinical risk factors for osteoporosis and fractures include age, lower body mass index,[28] smoking,[29] alcohol consumption[30] and glucocorticoid use[31]. Additionally, a positive family history confers an increased risk of fracture.[32] The term secondary osteoporosis refers to disorders that are strongly associated with osteoporosis;[33] these include rheumatoid arthritis, diabetes, hypogonadism (including premature menopause), malnutrition and malabsorption. Furthermore, prior fracture[34-36] and bone mineral density[37] are particularly strong predictors of future osteoporotic fractures. Several clinical risk score calculators are available which enable physicians to calculate the future risk of osteoporotic fractures in patients, such as the World Health Organization's (WHO) fracture risk assessment tool (FRAX).[38] Although these algorithms represent major advances in clinical practice, clinicians should be aware that the calculations do not accommodate all known risk factors and there are more fracture determinants remaining to be discovered.[39]

Dual-energy X-ray absorptiometry (DXA) at the lumbar spine and hip (Figure 4) to measure BMD is a routine investigation in osteoporosis, because, as stated before, BMD constitutes one of the strongest predictors of future fracture with each standard deviation (SD) decrease of BMD being associated with

a two-fold increase in fracture risk.[21, 38] The BMD measured is most commonly expressed as the T score, the number of standard deviations above or below the mean for a healthy 30 year old adult of the same sex and ethnicity as the patient. Subsequently, osteoporosis is defined as a T score≤-2.5 and osteopenia as a T score≤-1.0 at any skeletal site. No upper reference value has been proposed, as the adverse health effects of having an increased BMD have been poorly studied. Intriguingly, the far majority of fractures occur in individuals without an abnormal clinically assessed bone mineral density.[21] In addition, several diseases are paradoxically known to be associated with a higher fracture risk despite a higher bone mineral density, such as diabetes-related bone disease and degenerative disease. There is increasing evidence supporting an association between type 2 diabetes and increased fracture risk, even though individuals with type 2 diabetes have on average a high BMD.[40-43] Also, the increased BMD in subjects with lumbar disc degeneration (LDD) would theoretically protect against fractures, however, a few studies which examined the relationship between LDD and fractures found conflicting results.[44-47] Another valuable evaluation in osteoporosis is vertebral fracture assessment on lateral DXA or radiography (Figure 5). However, there is currently no gold standard for osteoporotic vertebral fracture diagnosis[48] and several radiological scoring methods exist, each using different criteria for diagnosing and grading fractures. Such grading definitions are currently under debate. In addition, there are a number of differential diagnoses that have to be considered in individuals with vertebral deformities,[49] such as Scheuermann's disease and degenerative changes.[50] Scheuermann's disease is a form of osteochondrosis of the spine of unknown etiology characterized by increased posterior rounding of the thoracic spine in association with structural deformity of the vertebral elements.[51, 52] Experimental assessments for osteoporosis are being developed in the research setting by either post-processing of radiographic or DXA data or involving more advanced imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI) to appraise bone microarchitecture or bone geometry (i.e., biomechanical characteristics of a bone's size and shape).[53-59] Finally, an arsenal of biochemical markers derived from serum, urine, DNA or bone biopsy sampling have found their way into clinical practice as they are helpful for the initial clinical assessment and for the monitoring of treatment.[60] Ideally, we would like to further improve and

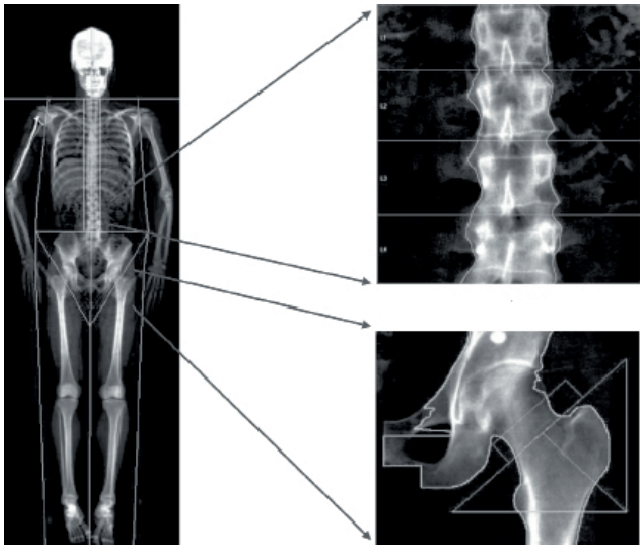


Figure 4. Dual-energy X-ray absorptiometry (DXA) of the total body (left panel), at the lumbar spine (upper right panel) and femoral neck (lower right panel) to measure bone mineral density.

expand our current diagnostic panels to facilitate precision medicine, the customization of healthcare tailored to the individual patient.

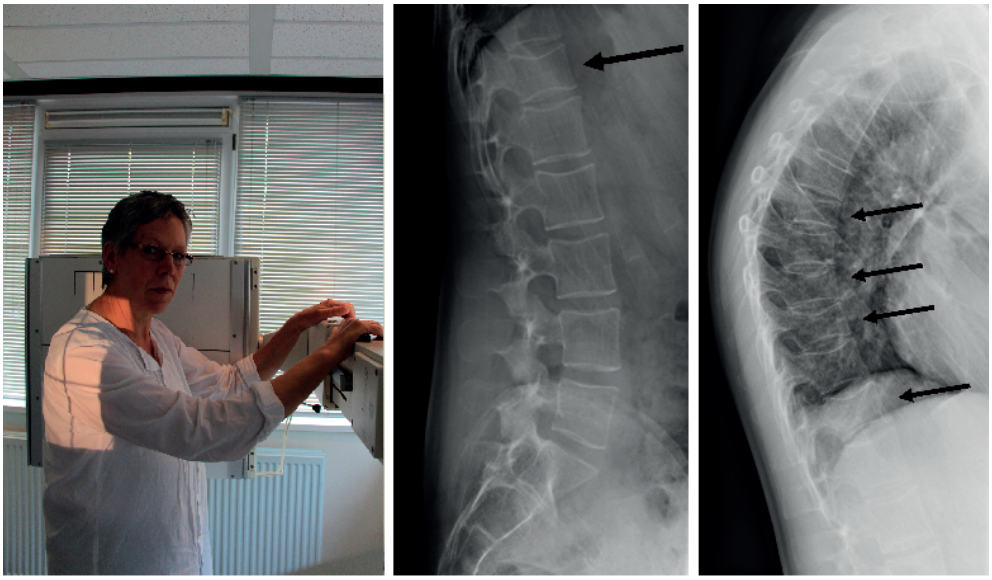


Figure 5. Radiological imaging by means of lateral radiographs (X-rays) of the thoracolumbar spine to assess the presence of osteoporotic vertebral fractures. Arrows indicate osteoporotic vertebral fractures of vertebral levels T7, T9, T10 and T12. The simulated patient photo (left panel) is a courtesy of Hannie van den Boogert.

EPIDEMIOLOGICAL STUDIES

Epidemiology is the science of health and disease in populations; epidemiological research investigates frequencies of phenomena of health, the occurrence of illness and related states and events.[61] To discover the causes of diseases in the elderly we should study risk factors of those diseases.[62] Most association studies presented in this thesis are based on the Rotterdam Study (Figure 6), a prospective population-based cohort study of determinants of diseases in elderly men and women in Rotterdam city's district of Ommoord.[63] Participant enrolment started in 1990 by inviting all inhabitants aged 55 years and over to take part in the study, of which 7,983 subjects (response rate 78%) entered the study (RS-I). In 2000, 3,011 participants (response rate 67%) who had become 55 years of age or moved into the study district since the start of the study were added to the cohort (RS-II). In 2006, a further extension of the cohort (RS-III) was initiated in which 3,932 subjects (response rate 65%) aged 45 years and over were included. The participants were interviewed at home (2 hours) and had an extensive set of examinations (a total of 5 hours) in a specially built research facility in the center of the Ommoord district. These clinically state-of-the-art examinations were repeated every 3–4 years. Radiological imaging and collection of bodily fluids for molecular and genetic analyses were performed. Additionally, very comprehensive patient health information was obtained from pharmacy medication histories, general practitioners' records and hospital registries. The research projects within the Rotterdam Study endeavor to bring light to the prevalences, incidences, risk factors, biomarkers, correlates and consequences of various diseases including musculoskeletal and endocrine diseases such as osteoporosis and diabetes.

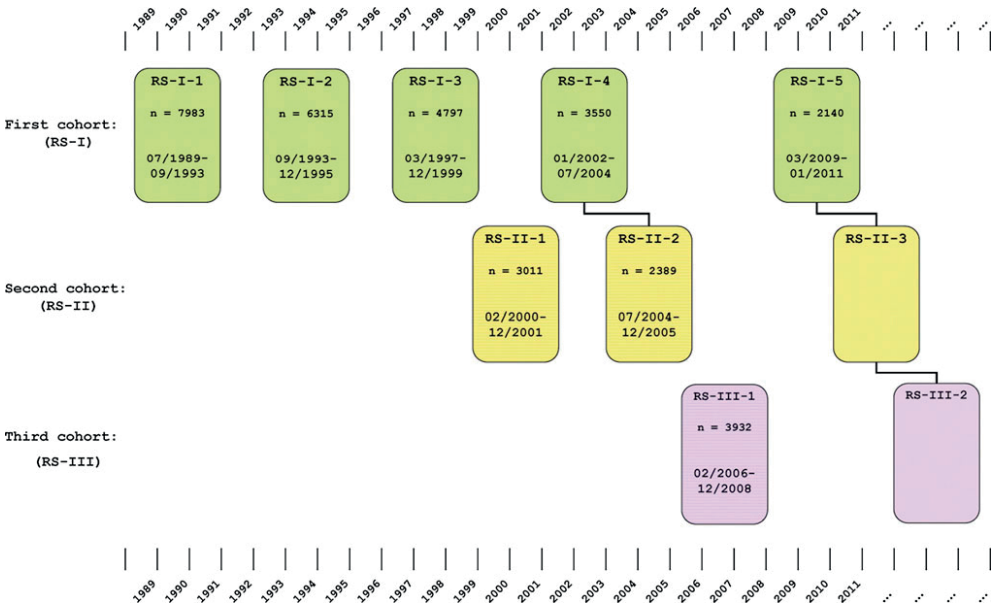


Figure 6. Diagram of examination cycles of the Rotterdam Study (RS); five visit cycles have been conducted until now and are denoted as ERGO-1 up to ERGO-5. RS-I-1 to RS-I-5 refer to the examinations of the original cohort. RS-II-1 and RS-II-2 relate to the extension of the cohort with persons in the study district that became 55 years since the start of the study or those of 55 years or over that migrated into the study district. RS-III-1 denotes the baseline examination of all persons aged 45 years and over living in the study district that had not been examined before in a second extension cohort. Examinations RS-I-4 and RS-II-2 feature an identical research program (ERGO-4), and so will examinations RS-I-5, RS-II-3, and RSIII-2 share the same program items (ERGO-5).

GENETIC EPIDEMIOLOGY

As mentioned before, one of the most important risk factors for osteoporosis and fractures is a positive family history, and reinforces that genetics is at the basis of liability to osteoporosis and fractures.[64] Heritability studies have reported estimates of BMD and fractures of up to 66% and 46%, respectively.[65, 66] Nucleotides are the building blocks of the genome. One human genome counts roughly 3 billion (3,000,000,000) nucleotides. The genetic alphabet is made up of the four nucleotide bases letters A, T, G and C. When a single letter (base) in the sequence is swapped for another letter, this is called a single nucleotide polymorphism (SNP). Only ~1% (~3,000,000) seems to be variable per genome when comparing different people.[67-70] Because of this limited variability, sets of SNPs tend to co-occur; these are called haplotype blocks. Technologies for SNP genotyping include enzyme-based methods (e.g., polymerase chain reaction [PCR]-based), hybridization-based methods (e.g., microarrays) and next-generation sequencing. It has been shown that SNPs underlie differences between people, including the variability in disease risks, and recent genome-wide association studies (GWAS) have vastly expanded our knowledge in this area.[71] For instance, previous GWAS have identified 24 loci that influence BMD variation explaining ~3% of trait variance[72-78] of which several variants have also been nominally associated with fracture risk.[79, 80]

In a typical GWAS a large number of SNPs (thousands to millions) are genotyped by microarrays and analyses are run to see if particular areas in the genome (i.e., loci) relate to certain phenotypes in

hundreds or thousands of people.[81] In a standard case-control disease GWAS, SNP allele frequencies are compared between disease cases and healthy control subjects to see if certain SNPs are more prevalent in persons with the disease of interest (Figure 7).[82] As so many tests are performed in a GWAS, by chance, false-positives may arise. Therefore, a Bonferroni correction assuming one million independent tests, representing the estimated number of haplotype blocks, is applied to the P value threshold for declaring statistical significance: $\alpha = 5x10^{-8}$ (i.e., P=0.05/1,000,000).[83] Good practice is to follow-up the initial set of promising susceptibility loci identified by discovery GWAS to a meta-analytical replication stage, in which the SNPs identified are examined in additional independent studies.[84] Apart from developing our understanding of disease etiology, expectations are that these genetic markers will be useful in disease diagnostics and prediction, form potential drug targets and potentially modulate treatment response.[85]

AIMS AND OUTLINE OF THIS THESIS

This thesis covers studies that investigate the epidemiology of hormonal and skeletal diseases. We have focused on osteoporosis, diabetes-related bone disease and Scheuermann's disease. The objectives were to discover novel risk factors for these conditions, deepen our understanding of known determinants, expand our knowledge about the prevalences of the diseases in the general population, refine our tools for clinical assessments including diagnostics such as radiological imaging and make the first

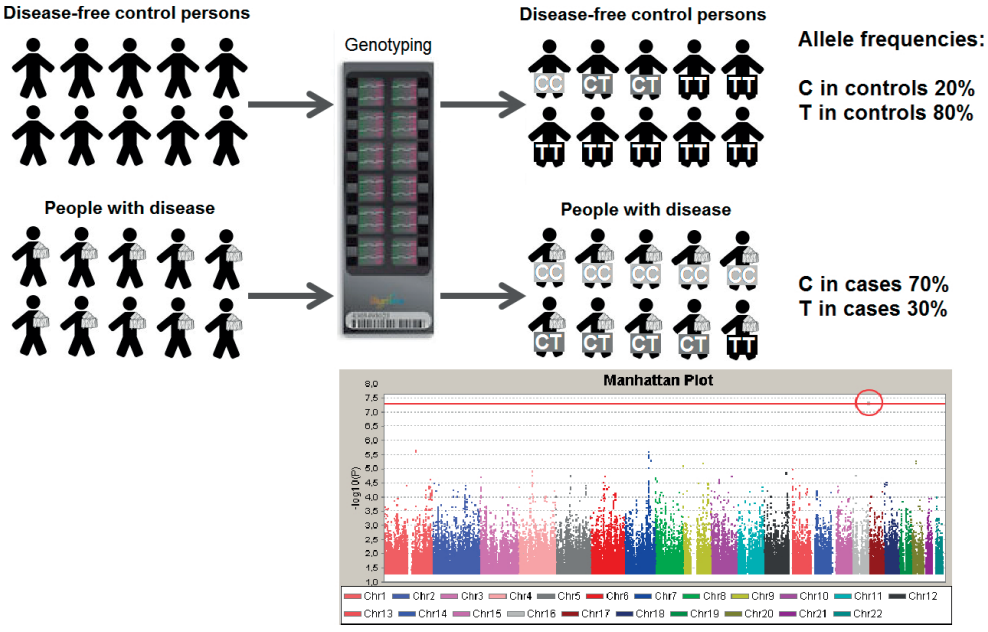


Figure 7. A schematic representation of a disease case-control genome-wide association study (GWAS). All study participants are genotyped to investigate if certain genetic variants are associated with the disease of interest. Then, single nucleotide polymorphism (SNP) allele frequencies are compared between people with the disease (cases) and healthy control subjects free of the disease being studied. In this example the C allele of a SNP mapping to chromosome 16 is associated with the disease, i.e., osteoporotic fractures, as it appears to be more frequent in the group with the disease than in the disease-free control group: 70% versus 20%. At the bottom right the results are displayed in a "Manhattan plot" where the statistical significance P values for association are mapped on the Y-axis by chromosomal position on the X-axis. Each color denotes a specific chromosome and each dot represents a single SNP.

steps from bench-to-bedside and back. Chapter 2 encompasses epidemiological studies of fractures and bone mineral density performed in the Rotterdam Study. Chapter 3 brings a comparative appraisal of radiological scoring methods for osteoporotic vertebral fractures. Chapter 4 reports various genetic epidemiological studies for osteoporotic fracture risk, with the majority being large-scale projects executed within the framework of the genetic factors for osteoporosis (GEFOS) and genetic markers for osteoporosis (GENOMOS) consortia. Chapter 5 presents a treatise on the future role of personalized sequencing in medicine in general.

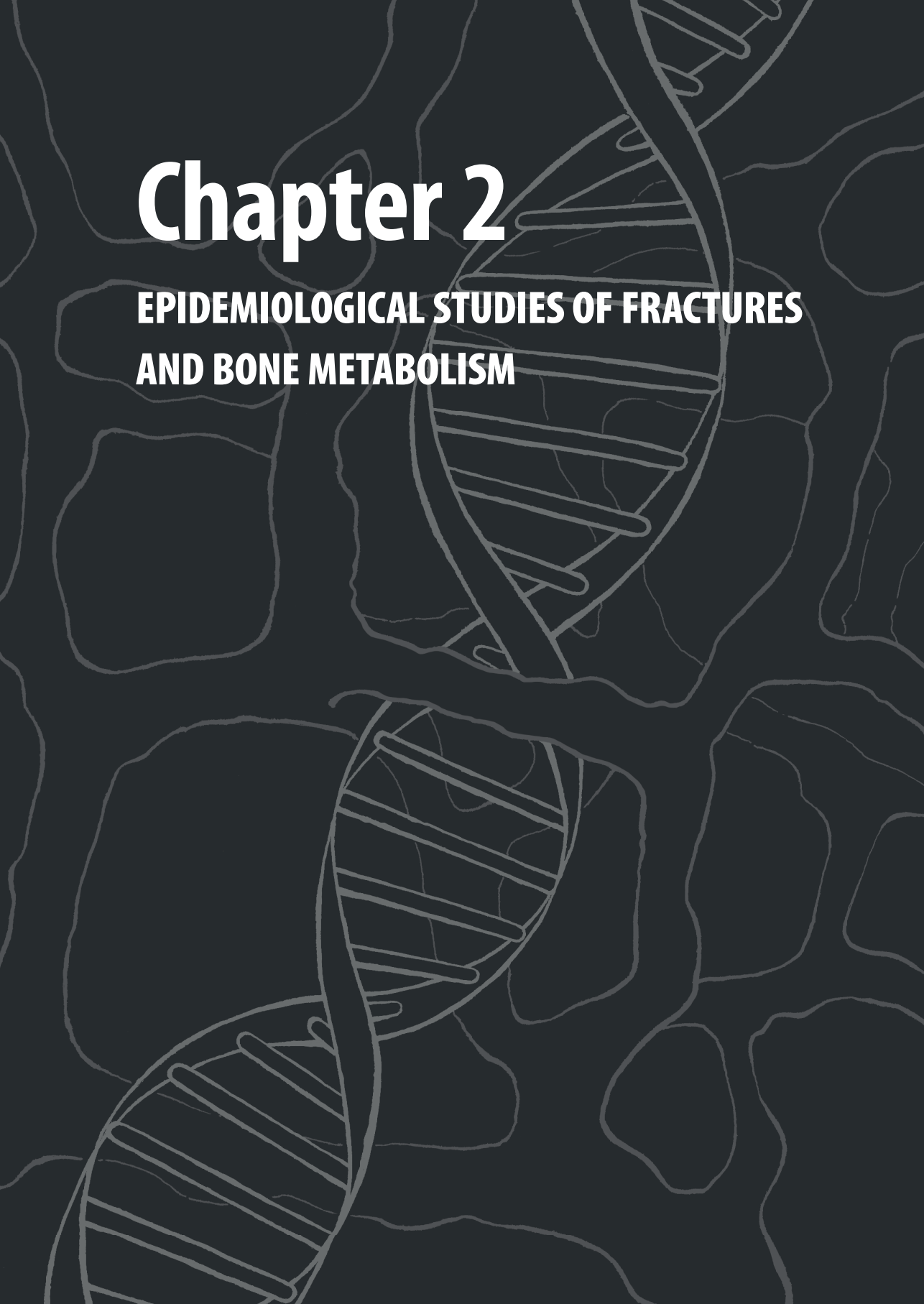
REFERENCES

1. Rosen CJ. Insulin-like growth factor I and bone mineral density: experience from animal models and human observational studies. Best Pract Res Clin Endocrinol Metab. 2004 Sep;18(3):423-35.
2. Rosen CJ, Donahue LR. Insulin-like growth factors and bone: the osteoporosis connection revisited. Proc Soc Exp Biol Med. 1998 Oct;219(1):1-7.
3. Dallas SL, Prideaux M, Bonewald LF. The Osteocyte: An Endocrine Cell and More. Endocr Rev. 2013 Apr 26.
4. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, et al. Endocrine regulation of energy metabolism by the skeleton. Cell. 2007 Aug 10;130(3):456-69.
5. Holzer G, von Skrbensky G, Holzer LA, Pichl W. Hip fractures and the contribution of cortical versus trabecular bone to femoral neck strength. J Bone Miner Res. 2009 Mar;24(3):468-74.
6. Eswaran SK, Gupta A, Adams MF, Keaveny TM. Cortical and trabecular load sharing in the human vertebral body. J Bone Miner Res. 2006 Feb;21(2):307-14.
7. Rodan GA. Introduction to bone biology. Bone. 1992;13 Suppl 1:S3-6.
8. Canalis E. Insulin-like growth factors and osteoporosis. Bone. 1997 Sep;21(3):215-6.
9. Malluche HH. Aluminium and bone disease in chronic renal failure. Nephrol Dial Transplant. 2002;17 Suppl 2:21-4.
10. Potula V, Kaye W. Report from the CDC. Is lead exposure a risk factor for bone loss? J Womens Health (Larchmt). 2005 Jul-Aug;14(6):461-4.
11. Chakkalakal DA. Alcohol-induced bone loss and deficient bone repair. Alcohol Clin Exp Res. 2005 Dec;29(12):2077-90.
12. Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol. 2009 Jun;201(3):309-20.
13. Terada M, Inaba M, Yano Y, Hasuma T, Nishizawa Y, Morii H, et al. Growth-inhibitory effect of a high glucose concentration on osteoblast-like cells. Bone. 1998 Jan;22(1):17-23.
14. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation. 2006 Aug 8;114(6):597-605.
15. Hernandez CJ, Tang SY, Baumbach BM, Hwu PB, Sakkee AN, van der Ham F, et al. Trabecular microfracture and the influence of pyridinium and non-enzymatic glycation-mediated collagen cross-links. Bone. 2005 Dec;37(6):825-32.
16. Yamaguchi T, Sugimoto T. Bone metabolism and fracture risk in type 2 diabetes mellitus [Review]. Endocr J. 2011;58(8):613-24.
17. Pacifici R. Cytokines, estrogen, and postmenopausal osteoporosis--the second decade. Endocrinology. 1998 Jun;139(6):2659-61.
18. Edelson JG, Obad S, Geiger R, On A, Artul HJ. Pycnodysostosis. Orthopedic aspects with a description of 14 new cases. Clin Orthop Relat Res. 1992 Jul(280):263-76.
19. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. World Health Organ Tech Rep Ser. 1994;843:1-129.
20. Cooper C, Melton LJ, 3rd. Epidemiology of osteoporosis. Trends Endocrinol Metab. 1992 Aug;3(6):224-9.
21. Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone. 2004 Jan;34(1):195-202.

22. Kanis JA, Johnell O, Oden A, Sembo I, Redlund-Johnell I, Dawson A, et al. Long-term risk of osteoporotic fracture in Malmo. Osteoporos Int. 2000;11(8):669-74.
23. Sambrook P, Cooper C. Osteoporosis. Lancet. 2006 Jun 17;367(9527):2010-8.
24. Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int. 2006 Dec;17(12):1726-33.
25. Cooper C, Atkinson EJ, Jacobsen SJ, O'Fallon WM, Melton LJ, 3rd. Population-based study of survival after osteoporotic fractures. Am J Epidemiol. 1993 May 1;137(9):1001-5.
26. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
27. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. 2007 Mar;22(3):465-75.
28. De Laet C, Kanis JA, Oden A, Johanson H, Johnell O, Delmas P, et al. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporos Int. 2005 Nov;16(11):1330-8.
29. Kanis JA, Johnell O, Oden A, Johansson H, De Laet C, Eisman JA, et al. Smoking and fracture risk: a meta-analysis. Osteoporos Int. 2005 Feb;16(2):155-62.
30. Kanis JA, Johansson H, Johnell O, Oden A, De Laet C, Eisman JA, et al. Alcohol intake as a risk factor for fracture. Osteoporos Int. 2005 Jul;16(7):737-42.
31. Kanis JA, Johansson H, Oden A, Johnell O, de Laet C, Melton IL, et al. A meta-analysis of prior corticosteroid use and fracture risk. J Bone Miner Res. 2004 Jun;19(6):893-9.
32. Kanis JA, Johansson H, Oden A, Johnell O, De Laet C, Eisman JA, et al. A family history of fracture and fracture risk: a meta-analysis. Bone. 2004 Nov;35(5):1029-37.
33. Miazgowski T, Kleerekoper M, Felsenberg D, Stepan JJ, Szulc P. Secondary osteoporosis: endocrine and metabolic causes of bone mass deterioration. J Osteoporos. 2012;2012:907214.
34. van der Klift M, de Laet CE, McCloskey EV, Johnell O, Kanis JA, Hofman A, et al. Risk factors for incident vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2004 Jul;19(7):1172-80.
35. Kanis JA, Johnell O, De Laet C, Johansson H, Oden A, Delmas P, et al. A meta-analysis of previous fracture and subsequent fracture risk. Bone. 2004 Aug;35(2):375-82.
36. Black DM, Arden NK, Palermo L, Pearson J, Cummings SR. Prevalent vertebral deformities predict hip fractures and new vertebral deformities but not wrist fractures. Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1999 May;14(5):821-8.
37. Johnell O, Kanis JA, Oden A, Johansson H, De Laet C, Delmas P, et al. Predictive value of BMD for hip and other fractures. J Bone Miner Res. 2005 Jul;20(7):1185-94.
38. Dawson-Hughes B, Tosteson AN, Melton LJ, 3rd, Baim S, Favus MJ, Khosla S, et al. Implications of absolute fracture risk assessment for osteoporosis practice guidelines in the USA. Osteoporos Int. 2008 Apr;19(4):449-58.
39. Silverman SL, Calderon AD. The utility and limitations of FRAX: A US perspective. Curr Osteoporos Rep. 2010 Dec;8(4):192-7.
40. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007 Sep 1;166(5):495-505.
41. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.
42. de Liefde II, van der Klift M, de Laet CE, van Daele PL, Hofman A, Pols HA. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporos Int. 2005 Dec;16(12):1713-20.
43. van Daele PL, Stolk RP, Burger H, Algra D, Grobbee DE, Hofman A, et al. Bone density in non-insulin-dependent diabetes mellitus. The Rotterdam Study. Ann Intern Med. 1995 Mar 15;122(6):409-14.
44. Schneider DL, Bettencourt R, Barrett-Connor E. Clinical utility of spine bone density in elderly women. J Clin Densitom. 2006 Jul-Sep;9(3):255-60.
45. Roux C, Fechtenbaum J, Briot K, Cropet C, Liu-Leage S, Marcelli C. Inverse relationship between vertebral fractures and spine osteoarthritis in postmenopausal women with osteoporosis. Ann Rheum Dis. 2008 Feb;67(2):224-8.

46. Sornay-Rendu E, Allard C, Munoz F, Duboeuf F, Delmas PD. Disc space narrowing as a new risk factor for vertebral fracture: the OFELY study. Arthritis Rheum. 2006 Apr;54(4):1262-9.
47. Arden NK, Griffiths GO, Hart DJ, Doyle DV, Spector TD. The association between osteoarthritis and osteoporotic fracture: the Chingford Study. Br J Rheumatol. 1996 Dec;35(12):1299-304.
48. Guglielmi G, Diacinti D, van Kuijk C, Aparisi F, Krestan C, Adams JE, et al. Vertebral morphometry: current methods and recent advances. Eur Radiol. 2008 Jul;18(7):1484-96.
49. Link TM, Guglielmi G, van Kuijk C, Adams JE. Radiologic assessment of osteoporotic vertebral fractures: diagnostic and prognostic implications. Eur Radiol. 2005 Aug;15(8):1521-32.
50. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
51. Hart ES, Merlin G, Harisiades J, Grottkau BE. Scheuermann's thoracic kyphosis in the adolescent patient. Orthop Nurs. 2010 Nov-Dec;29(6):365-71; quiz 72-3.
52. Ali RM, Green DW, Patel TC. Scheuermann's kyphosis. Curr Opin Pediatr. 1999 Feb;11(1):70-5.
53. Burghardt AJ, Link TM, Majumdar S. High-resolution computed tomography for clinical imaging of bone microarchitecture. Clin Orthop Relat Res. 2011 Aug;469(8):2179-93.
54. Krug R, Burghardt AJ, Majumdar S, Link TM. High-resolution imaging techniques for the assessment of osteoporosis. Radiol Clin North Am. 2010 May;48(3):601-21.
55. Link TM. Osteoporosis imaging: state of the art and advanced imaging. Radiology. 2012 Apr;263(1):3-17.
56. Van Goethem JW, Maes M, Ozsarlak O, van den Hauwe L, Parizel PM. Imaging in spinal trauma. Eur Radiol. 2005 Mar;15(3):582-90.
57. Wilmink JT. MR imaging of the spine: trauma and degenerative disease. Eur Radiol. 1999;9(7):1259-66.
58. Bouxsein ML, Karasik D. Bone geometry and skeletal fragility. Curr Osteoporos Rep. 2006 Jun;4(2):49-56.
59. Beck TJ, Ruff CB, Warden KE, Scott WW, Jr., Rao GU. Predicting femoral neck strength from bone mineral data. A structural approach. Invest Radiol. 1990 Jan;25(1):6-18.
60. Naylor K, Eastell R. Bone turnover markers: use in osteoporosis. Nat Rev Rheumatol. 2012 Jul;8(7):379-89.
61. Miettinen O. A conversation with Olli Miettinen. Interview by James A. Hanley. Epidemiology. 2012 May;23(3):495-9.
62. Peto R, Doll R. There is no such thing as aging. BMJ. 1997 Oct 25;315(7115):1030-2.
63. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol. 2015 Aug;30(8):661-708.
64. Kanis JA. Diagnosis of osteoporosis and assessment of fracture risk. Lancet. 2002 Jun 1;359(9321):1929-36.
65. Sigurdsson G, Halldorsson BV, Styrkarsdottir U, Kristjansson K, Stefansson K. Impact of genetics on low bone mass in adults. J Bone Miner Res. 2008 Oct;23(10):1584-90.
66. Michaelsson K, Melhus H, Ferm H, Ahlbom A, Pedersen NL. Genetic liability to fractures in the elderly. Arch Intern Med. 2005 Sep 12;165(16):1825-30.
67. Finishing the euchromatic sequence of the human genome. Nature. 2004 Oct 21;431(7011):931-45.
68. Chen R, Mias GI, Li-Pook-Than J, Jiang L, Lam HY, Miriami E, et al. Personal omics profiling reveals dynamic molecular and medical phenotypes. Cell. 2012 Mar 16;148(6):1293-307.
69. Lam HY, Clark MJ, Chen R, Natsoulis G, O'Huallachain M, Dewey FE, et al. Performance comparison of whole-genome sequencing platforms. Nat Biotechnol. 2012 Jan;30(1):78-82.
70. Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, et al. A map of human genome variation from population-scale sequencing. Nature. 2010 Oct 28;467(7319):1061-73.
71. Hindorff LA, Junkins HA, Hall PN, Mehta JP, Manolio TA. A catalog of published genome-wide association studies. 2010; Available from: http://www.genome.gov/gwastudies.
72. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008 May 3;371(9623):1505-12.
73. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008 May 29;358(22):2355-65.

74. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
75. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. New sequence variants associated with bone mineral density. Nat Genet. 2009 Jan;41(1):15-7.
76. Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, et al. An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet. 2010 Jun;6(6):e1000977.
77. Kung AW, Xiao SM, Cherny S, Li GH, Gao Y, Tso G, et al. Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies. Am J Hum Genet. 2010 Feb 12;86(2):229-39.
78. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
79. Richards JB, Kavvoura FK, Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, et al. Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture. Ann Intern Med. 2009 Oct 20;151(8):528-37.
80. van Meurs JB, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, et al. Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. JAMA. 2008 Mar 19;299(11):1277-90.
81. Wu MC, Kraft P, Epstein MP, Taylor DM, Chanock SJ, Hunter DJ, et al. Powerful SNP-set analysis for case-control genome-wide association studies. Am J Hum Genet. 2010 Jun 11;86(6):929-42.
82. Stranger BE, Stahl EA, Raj T. Progress and promise of genome-wide association studies for human complex trait genetics. Genetics. 2011 Feb;187(2):367-83.
83. Panagiotou OA, Ioannidis JP. What should the genome-wide significance threshold be? Empirical replication of borderline genetic associations. Int J Epidemiol. 2012 Feb;41(1):273-86.
84. Kraft P, Zeggini E, Ioannidis JP. Replication in genome-wide association studies. Stat Sci. 2009 Nov 1;24(4):561-73.
85. Manolio TA. Genomewide association studies and assessment of the risk of disease. N Engl J Med. 2010 Jul 8;363(2):166-76.



Chapter 2

EPIDEMIOLOGICAL STUDIES OF FRACTURES AND BONE METABOLISM

Chapter 2.1

The effect of thiazide and loop diuretics on urinary levels of free deoxypyridinoline: an osteoclastic bone-resorption marker

Ruiter R, Oei L, Visser LE, Peltenburg HG, Hofman A, Zillikens MC, Uitterlinden AG, Rivadeneira F, Stricker BH

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ABSTRACT

What is known and Objective Diuretics can cause changes in calcium levels due to renal effects. Moreover, calcium levels can also vary as a result of changes in intestinal absorption and in the activity of osteoclastic cells. A marker of osteoclastic bone resorption activity is the level of urinary free deoxypyridinoline (FDP). Deoxypyridinoline (DP) acts as a cross-link between adjacent collagen chains to provide structural rigidity. Our aim was to investigate the association between use of thiazides and loop diuretics and urinary levels FDP.

Methods In this follow-up study, data were obtained from the Rotterdam Study, a large population-based prospective cohort study. For a subset of 658 participants, urinary levels of FDP were measured at baseline. Linear regression analysis was performed to assess the association between the use of thiazides and loop diuretics and the urinary levels of FDP.

Results In women, current use of loop diuretics for less than 42 days was associated with an increased level of urinary FDP (+3.43 nmol deoxypyridinoline per mmol urinary creatinine; 95% CI: 1.85–5.02) compared with no use. However, use for a period of more than 42 days was not associated with an increased level of FDP, nor was past use of loop diuretics. For thiazide diuretics, no statistically significant associations were found.

Conclusion In women, short-term use of loop diuretics is associated with an increased level of FDP, reflecting increased bone resorption by osteoclasts. As the difference disappears with longer term use, the clinical significance is unclear and the value of FDP as a biomarker in this setting is not established. The molecular mechanism for the observed differences in bone fracture rates with use of diuretics remains unclear.

INTRODUCTION

Thiazides increase serum calcium levels, and use of diuretics has been associated with a decreased risk of fractures.[1] In 2003, we described that, relative to non-use, current use of thiazides for more than 365 days was statistically significantly associated with a lower risk of hip fracture. This lower risk disappeared after thiazide use was discontinued.[2] In contrast, use of loop diuretics has been associated with decreased bone mineral density (BMD)[3] and with an increased risk of fractures.[4] Although loop diuretics can also be used in the treatment of hypertension, they are more frequently used for the management of patients with heart failure. More recently, it was investigated whether the risk of fractures varied between use of different antihypertensive drugs.[5] Analyses were also performed for use of thiazide and loop diuretics. In users of thiazide diuretics, fracture rates were significantly lower compared with users of loop diuretics.[5]

Thiazide diuretics can cause hypercalcemia, mainly through effects on the distal convoluted tubule inducing enhanced calcium reabsorption.[6] In contrast to thiazides, loop diuretics (e.g., furosemide) cause hypocalcaemia due to decreased reabsorption of calcium in the loop of Henle.[7] Calcium levels can, however, also vary by changes in intestinal absorption and by changes in activity of osteoclastic cells. A marker of osteoclastic bone resorption activity is the level of urinary free deoxypyridinoline (FDP) crosslinks.[8] Deoxypyridinoline is one of the two pyridinium cross-links that provide structural rigidity to type I collagen, which represents 90% of the organic matrix of bone.[9] Deoxypyridinoline is only found in significant quantities in bone,[10] and is not absorbed from the diet[11] and is not metabolized by the liver. During osteoclastic bone resorption, deoxypyridinoline is released into the circulation and is excreted in the urine in free and peptide-bound forms.[12] However, it has been published that measurement of free cross-links only provides similar information to that from the total amounts.[13] Hence, it was shown that measurement of urinary FDP provides a reasonably accurate estimate of bone resorption.[8, 14] In addition, an association between high levels of FDP and an increased risk of fractures, independent of BMD, has been demonstrated.[15-17]

We wished to explore if, in addition to the known renal effects, osteoclastic activity is involved in diuretic-related effects on bone metabolism. Therefore, we aimed to verify the association between use of thiazides and loop diuretics and the urinary levels of FDP. We hypothesized that use of thiazides is associated with decreased levels, whereas the use of loop diuretics might be associated with increased levels of FDP. To this end, we studied the association in a subgroup of subjects from a large prospective cohort study.

METHODS

Setting

Data were obtained from the Rotterdam Study, a large population-based prospective cohort study. The objectives and design were extensively described earlier.[18] In summary, as of 1991, inhabitants of the suburb Ommoord, aged 55 years or older were invited to participate. Of all 10,275 invited subjects, 7,983 entered the study (78%).

Baseline examinations consisted of a home interview and a clinical work up at the research center. During follow-up, additional interviewing, laboratory assessments, clinical examinations and imaging procedures were carried out every 3–4 years. As all pharmacies which serve the Ommoord district are on one computer network, detailed information on drug dispensing was available for all participants as of January 1, 1991. The vital status of the participants was obtained regularly from the municipal population registry. Morbidity and mortality were assessed by information from the general practitioner, or, in case of hospitalization, by discharge reports from the medical specialists. The study was approved by the Medical Ethics Committee of the Erasmus MC and all participants gave written informed consent.

For a subset of 658 participants, the urinary levels of FDP were measured at baseline. This subset was used for a case-control analysis in which participants with a non-vertebral fracture (N=376) were compared with participants without this type of fracture.[19] To ensure a cohort of incident users of diuretics, only participants with a prescription-free period of 3 months in the period before baseline were eligible.

Exposure

Use of diuretics (ATC-code C03C) in the Rotterdam Study was categorized into two groups: use of thiazides and use of loop diuretics.[20] The duration of a prescription was calculated as the total number of units delivered divided by the prescribed daily number of units. Participants could contribute cumulative exposure time to both categories. Cumulative exposure was calculated from start of drug use until the measurement of the urinary FDP level (index date). When participants did not use a diuretic on the index date, but did use a diuretic in the past, the cumulative number of days of use was calculated until the last day of the last prescription; past use since discontinuation was also used as determinant. Participants who did not use a diuretic during the study period were used as reference. To assess the effect of dose in current users, the average number of 'defined daily doses' (DDD) over all previous prescriptions was calculated.[20]

Outcome

Bone resorption was evaluated by the measurement of FDP in the first morning void of an overnight fasting urine. Urine samples were collected at baseline and stored until measurement at -20°C. Urinary FDP was determined by an automated chemiluminescence immunoassay (ACS: 180 DPD, Chiron Diagnostics, Medfield, MA, USA).[21] To correct for dilution, results were normalized against urinary creatinine and expressed as nmol deoxypyridinoline per mmol urinary creatinine (nmol/mmol).[22]

Covariables

The following covariables were assessed as potential confounders and/or effect modifiers: age, sex, body mass index (BMI; kg/m^2), smoking status (no, current or past smoking), diabetes mellitus and presence of an earlier myocardial infarction, stroke or non-vertebral fracture. Of these, height, weight and the presence of a non-vertebral fracture were assessed at baseline at the research center. Other factors were assessed via an interview at baseline. In addition, the effect of use of other drugs known to have an effect on the calcium level on the index date was assessed. Use of the following drugs (ATC-code) was assessed as present use (yes/no) at the index date: corticosteroids for systemic use (H02), thyroid therapy (H03), drugs affecting bone structure and mineralization (M05B), estrogens and/or progestagens (G03C, G03D, G03F), drugs involved in calcium homeostasis (H05), antithrombotic agents (B01AA or B01AB), vitamin A and/or D (A11C), retinoids for systemic treatment (D10BA01), certain antineoplastic and immunomodulating agents (ciclosporin [L04AD01], methotrexate [L01BA01, L04AX03] and ifosfamide [L01AA06]), antiepileptics (N03A), antidepressants (N06A), thiazolidinediones (A10BG), statins (C10), antivirals for treatment of HIV infections (J05AR), nitric oxide (R07AX01) and beta-blocking agents (C07).[20]

Statistical analyses

Linear regression analysis was performed to assess the association between the use of thiazides and loop diuretics and the urinary levels of FDP. Covariables that changed the point estimate by more than 10%, or covariables which were considered to be clinically relevant, were included in the full model.[23] A stepwise model was used to include all other potential relevant confounders.

As levels of FDP differ between men and women, analysis was stratified for sex. Differences between the groups were tested for significance with ANOVA and for categorical variables with a Chisquared test.

The effect of current use or past use of thiazides and loop diuretics was assessed. Current use, determined as cumulative days of use of diuretics at the date of collection of the urine sample, was compared with never use at the date of collection of the urine sample. Current use was categorized in current use for 1–42 days, current use for 43–365 days and current use for more than 365 days. The cut-off point of 42 days was used as in the first 6 weeks of thiazide use, a decrease in circulating volume can occur; after 42 days, the circulating volume in most patients is within normal limits again.[2] Although in our analyses, FDP levels were normalized against urinary creatinine levels, this decrease in circulating volume might potentially influence this ratio. Past use, determined as cumulative days of use of thiazides and loop diuretics assessed at the date of collection of the urine sample, was compared with never use at the date of collection of the urine sample as well. Past use was categorized in past use after discontinuation for more than 120 days before the index date, between 60 and 120 days before the index date and for less than 60 days before the index date. These time frames were chosen because they were used in an earlier study.[2]

The effect of dose was assessed in current users as average cumulative dosage at the date of collection of the urine sample. Analyses were performed using SPSS software (IBM SPSS statistics, version 170; New York, NY, USA); all P values are two sided and were considered significant if $P<0.05$.

RESULTS

For 658 participants in the Rotterdam Study, the urinary levels of FDP were measured at baseline. However, for 120 participants, a prescription-free period of 3 months could not be obtained leaving 538 participants for the analysis. As can be seen from Table 1, the majority of the participants were female; there were, however, no statistically significant deviations between the different drug categories. Age did differ between the different drug users, with users of loop diuretics being older than those using thiazide diuretics or those not using any diuretic.

Table 1. Baseline characteristics for current users of thiazides and loop diuretics compared with those not using diuretics (N,% unless otherwise stated).

		No diuretic (N=461)	Thiazide (N=50)	Loop diuretic (N=26)
Female sex[a]		373 (80.9)	47 (94.0)	20 (76.9)
Age at start (years, SD)[b]		71.8 (9.1)	73.7 (9.0)	81.2 (7.2)
BMI (SD)[c]		26.0 (4.0)	28.2 (3.4)	27.5 (3.8)
Stroke at baseline[a]		18 (3.9)	3 (6.0)	1 (3.8)
MI at baseline[a]		31 (6.7)	5 (10.0)	7 (26.9)
Diabetes at baseline[a]		39 (8.5)	9 (18.0)	4 (15.4)
Smoking[a]	Current	88 (19.1)	10 (20.0)	6 (23.1)
	Former	159 (34.5)	15 (30.0)	11 (42.3)
	Never	209 (45.3)	24 (48.0)	7 (26.9)
	Missing	5 (1.1)	1 (2.0)	2 (7.7)
History of a non-vertebral fracture[a]		220 (47.7)	24 (48.0)	13 (50.0)
Mean average dosage (SD)		NA	0.81 (0.27)	1.33 (1.80)
Mean average duration (SD)		NA	42.7 (27.2)	36.2 (23.9)

[a]P value not significant following Chi-squared test. [b]P value following ANOVA significant.
[c]P value following ANOVA not significant.

In crude analyses (men + women), current use of loop diuretics was associated with an increased level of FDP (+2.27 nmol deoxypyridinoline per mmol urinary creatinine; 95% CI: 1.23–3.32). Use of thiazides was statistically non-significantly associated with a decreased level of FDP (-0.29 nmol deoxypyridinoline per mmol urinary creatinine; 95% CI: -1.08–0.50). In Table 2, the effects of past use and current use of thiazides and loop diuretics on the FDP levels, per mmol urinary creatinine, are shown. Results were adjusted for age and BMI, which were the only two variables that changed the point estimate by more than 10% for thiazide diuretics as well as loop diuretics. Using stepwise modelling, we further included the covariates regarding previous myocardial infarction and use of beta-blocking agents for both diuretics. Adjustment for smoking status, the presence of a previous stroke, the diabetic status of the patient and use of other drugs than beta-blocking agents known to have an effect on the calcium level was not deemed necessary using this approach. As mentioned in the methods section, our intention was to stratify results for men and women; however, as numbers for men were too low, these analyses were not performed. The adjusted results for women, categorized on duration, are shown in Table 2. In women, current use for less than 42 days of loop diuretics was associated with an increased level of FDP (+3.43 nmol deoxypyridinoline per mmol urinary creatinine; 95% CI: 1.85–5.02). However, use for a period of 43–365 days was not associated with an increased level of FDP, nor was past use of loop diuretics. In contrast, the use of thiazides was not associated with a change in FDP levels.

Table 2. The effects of past use and current use of thiazides and loop diuretics on the free deoxypyridinoline levels per mmol urinary creatinine.

Diuretic	Duration of (past) use	Women	
		N	Change in level with 95% CI
Thiazides	No use		Reference
	Current use for < 42 days	23	−0.74 (−1.85; 0.37)
	Current use for 43–365 days	24	0.27 (−0.81; 1.35)
	Current use for > 365 days	0	NA
	Past use since < 60 days	9	0.23 (−1.47; 1.93)
	Past use since 60–120 days	3	+1.59 (−1.31; 4.49)
	Past use since > 120 days	23	+0.01 (−1.09; 1.11)
Loop diuretics	Current use for < 42 days	11	+3.43 (1.85; 5.02)[a]
	Current use for 43–365 days	9	+1.08 (−0.58; 2.75)
	Current use for > 365 days	0	NA
	Past use since < 60 days	9	+1.50 (−0.37; 3.38)
	Past use since 60–120 days	1	+0.36 (−4.51; 5.23)
	Past use since > 120 days	6	+0.57 (−1.64; 2.78)

[a]P value $<2.5\times10^{-5}$. Results are adjusted for age, BMI, previous myocardial infarction and use of beta-blocking agents.

Dosage analyses were performed in current users of diuretics. In women, neither the dosage of thiazides (change in DDD -0.09 nmol deoxypyridinoline per mmol urinary creatinine; 95% CI -0.28–0.16) nor the dosage of loop diuretics (change in DDD +0.30 nmol deoxypyridinoline per mmol urinary creatinine; 95% CI -0.27–1.17) was associated with the levels of FDP.

Post-hoc power analyses were performed to verify whether we had sufficient power for especially the thiazide analyses. Using a P value of 0.05, and given the number of predictors in the model, the observed R2 of the model and the samples sizes in the different models used, the power in women for the thiazide analyses was >0.80 and for the loop diuretics analyses, power was 0.67.

DISCUSSION

Short-term use for less than 42 days of loop diuretics is associated with an increased level of FDP, reflecting increased bone resorption by osteoclasts. Longer term use was not associated with increased levels nor was past use of these drugs associated with a deviation in the urinary levels of FDP. For users of thiazides, no statistically significant associations were found. Moreover, no dose-dependent associations could be described.

When it is hypothesized that the use of drugs has an instant effect on the excretion of FDP, the lack of association with past use of loop diuretics can be explained. However, the absence of a relationship with longer duration of current use cannot readily be explained. The cut-off point for short-term use was 42 days (for thiazide diuretics), a decrease in circulating volume can occur during this period. With longer term use, the circulating volume in most patients returns to normal limits again.[2] This would explain relatively higher urinary levels of FDP during the first 42 days of use of thiazides and might possibly explain why associations could not be observed in our study. However, the fact that in our analyses, FDP levels were normalized against creatinine levels would argue against this hypothesis. Moreover, whether a similar mechanism could explain the lack of association between long-term use of loop diuretics and urinary deoxypyridinoline levels remains questionable as for loop diuretics, this decrease in circulating volume after 42 days has not been described. Population-based cohort studies may be affected by selection bias, information bias and confounding. Selection bias probably did not occur as cases with a non-vertebral fracture were ascertained independent of their diuretic exposure status within a large population-based cohort study. However, with regard to the external validity, the analyses were performed in a subgroup of this cohort and these analyses could only be performed in women. We had too few men, and hence inadequate statistical power, to test the associations in men. However, when pooled analyses were performed, with adjustment for sex, the point estimate for short term use of loop diuretics remained statistically significant (data not shown). Another limitation of our study is the relatively limited number of users of diuretics. Information bias is unlikely as all information was gathered prospectively and without knowledge of the research hypothesis. With regard to confounding, we were able to adjust for several potential confounding factors, which did not change the point estimate. Confounding by indication could also be present. Recently, heart failure has been reported to be associated with an increase in rate of major fractures (independent of traditional risk factors and BMD).[24] After adjustment for use of loop diuretics, this association weakened but remained statistically significant.[24] Therefore, the association described in our study could also be explained by confounding by indication. However, in our opinion, this is less likely as in earlier studies, including a randomized controlled trial, use of loop diuretics was associated with increased bone turnover; as well as with decreased BMD and with increased risk of fractures.[3, 4, 25, 26]

Loop diuretics increase the excretion of calcium.[7] Calcium reabsorption in the thick ascending limb of the loop of Henle results from a positive lumen potential. Loop diuretics block the Na-K-chloride transporter, which decreases the positive lumen potential, and consequently diminishes calcium reabsorption.[7] To compensate, the decrease in serum calcium induces an increase in parathyroid hormone, which subsequently stimulates the intestinal absorption of calcium as well as the absorption from bone through increased activity of osteoclasts.6 Our finding of increased levels of FDP is therefore consistent with the hypothesis that the use of loop diuretics has an impact on the osteoclastic activity.

However, we could not find a statistically significant association between change in urinary levels of FDP and the use of thiazide diuretics. The use of thiazide diuretics reduces urinary calcium excretion and consequently, thiazides have been associated with increased serum calcium levels.[7] Thiazide diuretics in-

crease calcium reabsorption in the distal tubule by increasing the activity of the Na+/Ca^{2+}-ATPase on the basolateral membrane, subsequently lowering the Ca^{2+} concentration so that more Ca^{2+} is reabsorbed from the lumen. This increase arises from an increase in proximal calcium reabsorption in parallel with an increase in reabsorption of sodium and water, hypothesized to be caused by the parathyroid hormone.[7] The increased calcium level may result in decreased intestinal calcium absorption and suppression of the osteoclastic activity.[7] In addition, it has been suggested that thiazides in vitro also directly inhibit osteoclastic activity, and consequently bone resorption.[27] However, others could not confirm this.[28] In our study, the hypothesized lower osteoclastic activity could not be explained by decreased levels of urinary excretion of FDP. This may suggest that other (yet unknown) factors are involved in the association between thiazides, calcium levels and osteoclastic activity. As numbers of subjects in our study were low, this might also be explained by insufficient power.

In a large Cochrane meta-analysis on the relationship between thiazides and risk of hip fracture, marked heterogeneity was found.[29] Part of this variation could be explained by the use of multiple diuretics.[30] We aimed to analyze the association between use of multiple diuretics and the effect on levels of FDP, but as only 10 participants used multiple diuretics, these analyses could not be performed. In addition, the effect of use of diuretics other than loop diuretics and thiazides could not be evaluated due to low numbers. Although our findings seem to be consistent with the hypothesis that the use of loop diuretics has an impact on the osteoclastic activity, we cannot exclude the possibility that other factors may influence the possible association between the use of loop diuretics and the increased risk of fractures.[4]

CONCLUSION

Short-term use of less than 42 days of loop diuretics is associated with an increased level of FDP, reflecting increased bone resorption by osteoclasts. Whether the increased levels of FDP are induced directly by the loop diuretics, indirectly via decreased serum calcium levels, through a compensatory increase in parathyroid hormone, or via other mechanisms, requires further exploration. Also, the impact of comorbidity and other drugs used on the bone needs to be further evaluated. The value of FDP as an additional biomarker for the assessment of bone resorption in diuretic-induced effects on bone metabolism is not yet established.

REFERENCES

1. Hale WE, Stewart RB, Marks RG. Thiazide and fractures of bones. N Engl J Med. 1984 Apr 5;310(14):926-7.
2. Schoofs MW, van der Klift M, Hofman A, de Laet CE, Herings RM, Stijnen T, et al. Thiazide diuretics and the risk for hip fracture. Ann Intern Med. 2003 Sep 16;139(6):476-82.
3. Lim LS, Fink HA, Kuskowski MA, Taylor BC, Schousboe JT, Ensrud KE. Loop diuretic use and increased rates of hip bone loss in older men: the Osteoporotic Fractures in Men Study. Arch Intern Med. 2008 Apr 14;168(7):735-40.
4. Rejnmark L, Vestergaard P, Mosekilde L. Fracture risk in patients treated with loop diuretics. J Intern Med. 2006 Jan;259(1):117-24.
5. Solomon DH, Mogun H, Garneau K, Fischer MA. Risk of fractures in older adults using antihypertensive medications. J Bone Miner Res. 2011 Jul;26(7):1561-7.
6. Ruppe MD. Medications that affect calcium. Endocr Pract. 2011 Mar-Apr;17 Suppl 1:26-30.
7. Grieff M, Bushinsky DA. Diuretics and disorders of calcium homeostasis. Semin Nephrol. 2011 Nov;31(6):535-41.

8. Delmas PD. Biochemical markers of bone turnover. J Bone Miner Res. 1993 Dec;8 Suppl 2:S549-55.
9. Eyre DR, Dickson IR, Van Ness K. Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxypyridinium residues. Biochem J. 1988 Jun 1;252(2):495-500.
10. Eyre DR, Koob TJ, Van Ness KP. Quantitation of hydroxypyridinium crosslinks in collagen by high-performance liquid chromatography. Anal Biochem. 1984 Mar;137(2):380-8.
11. Colwell A, Russell RG, Eastell R. Factors affecting the assay of urinary 3-hydroxy pyridinium crosslinks of collagen as markers of bone resorption. Eur J Clin Invest. 1993 Jun;23(6):341-9.
12. Yu SL, Ho LM, Lim BC, Sim ML. Urinary deoxypyridinoline is a useful biochemical bone marker for the management of postmenopausal osteoporosis. Ann Acad Med Singapore. 1998 Jul;27(4):527-9.
13. Robins SP, Woitge H, Hesley R, Ju J, Seyedin S, Seibel MJ. Direct, enzyme-linked immunoassay for urinary deoxypyridinoline as a specific marker for measuring bone resorption. J Bone Miner Res. 1994 Oct;9(10):1643-9.
14. Eastell R, Colwell A, Hampton L, Reeve J. Biochemical markers of bone resorption compared with estimates of bone resorption from radiotracer kinetic studies in osteoporosis. J Bone Miner Res. 1997 Jan;12(1):59-65.
15. Akesson K, Vergnaud P, Gineyts E, Delmas PD, Obrant KJ. Impairment of bone turnover in elderly women with hip fracture. Calcif Tissue Int. 1993 Sep;53(3):162-9.
16. Garnero P, Hausherr E, Chapuy MC, Marcelli C, Grandjean H, Muller C, et al. Markers of bone resorption predict hip fracture in elderly women: the EPIDOS Prospective Study. J Bone Miner Res. 1996 Oct;11(10):1531-8.
17. van Daele PL, Seibel MJ, Burger H, Hofman A, Grobbee DE, van Leeuwen JP, et al. Case-control analysis of bone resorption markers, disability, and hip fracture risk: the Rotterdam study. BMJ. 1996 Feb 24;312(7029):482-3.
18. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
19. Weel AE. Skeletal and genetic determinants of osteoporosis. Enschede, The Netherlands: Erasmus Medical Center. Print Partners Ipskamp; 2001.
20. WHO. Collaborating Centre for Drug Statistics Methodology. [June 2012]; Available from: http://www.whocc.no/atc_ddd_index/.
21. Seibel MJ, Woitge HW, Farahmand I, Oberwittler H, Ziegler R. Automated and manual assays for urinary crosslinks of collagen: which assay to use? Exp Clin Endocrinol Diabetes. 1998;106(2):143-8.
22. Rosano TG, Peaston RT, Bone HG, Woitge HW, Francis RM, Seibel MJ. Urinary free deoxypyridinoline by chemiluminescence immunoassay: analytical and clinical evaluation. Clin Chem. 1998 Oct;44(10):2126-32.
23. Vandenbroucke JP, von Elm E, Altman DG, Gotzsche PC, Mulrow CD, Pocock SJ, et al. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. Ann Intern Med. 2007 Oct 16;147(8):W163-94.
24. Majumdar SR, Ezekowitz JA, Lix LM, Leslie WD. Heart failure is a clinically and densitometrically independent risk factor for osteoporotic fractures: population-based cohort study of 45,509 subjects. J Clin Endocrinol Metab. 2012 Apr;97(4):1179-86.
25. Mazziotti G, Canalis E, Giustina A. Drug-induced osteoporosis: mechanisms and clinical implications. Am J Med. 2010 Oct;123(10):877-84.
26. Rejnmark L, Vestergaard P, Heickendorff L, Andreasen F, Mosekilde L. Loop diuretics increase bone turnover and decrease BMD in osteopenic postmenopausal women: results from a randomized controlled study with bumetanide. J Bone Miner Res. 2006 Jan;21(1):163-70.
27. Hall TJ, Schaueblin M. Hydrochlorothiazide inhibits osteoclastic bone resorption in vitro. Calcif Tissue Int. 1994 Oct;55(4):266-8.
28. Aubin R, Menard P, Lajeunesse D. Selective effect of thiazides on the human osteoblast-like cell line MG-63. Kidney Int. 1996 Nov;50(5):1476-82.
29. Aung K, Htay T. Thiazide diuretics and the risk of hip fracture. Cochrane Database Syst Rev. 2011(10):CD005185.
30. Isakova T, Anderson CA, Leonard MB, Xie D, Gutierrez OM, Rosen LK, et al. Diuretics, calciuria and secondary hyperparathyroidism in the Chronic Renal Insufficiency Cohort. Nephrol Dial Transplant. 2011 Apr;26(4):1258-65.

Chapter 2.2

Association of lumbar disc degeneration with osteoporotic fractures; the Rotterdam study and meta-analysis from systematic review

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ABSTRACT

Objective To investigate the relation between lumbar disc degeneration (LDD) and all type of osteoporotic (OP) fractures including vertebral.

Methods This study is part of the Rotterdam study, a large prospective population-based cohort study among men and women aged 55 years and over. In 2,819 participants spine radiographs were scored for LDD (osteophytes and disc space narrowing (DSN)) from L1 till S1, using the Lane atlas. Osteoporotic (OP) fracture data were collected and verified by specialists during 12.8 years. We considered two types of vertebral fractures (VFx): Clinical VFx (symptomatic fractures recorded by medical practitioners) and Radiographic VFx (using the McCloskey–Kanis method). Meta-analysis of published studies reporting an association of LDD features and VFx was performed. Differences in bone mineral density (BMD) between participants with and without LDD features were analyzed using ANOVA. Risk of OP-fractures was analyzed using Cox regression.

Results In a total of 2385 participants, during 12.8 years follow-up, 558 suffered an OP-fracture. Subjects with LDD had an increased OP fracture risk compared to subjects without LDD (HR: 1.29, 95% CI: 1.04–1.60). LDD-cases have between 0.3 and 0.72 standard deviations more BMD than non-cases in all analyzed regions including total body BMD and skull BMD (P<0.001). Only males with LDD had increased risk for OP-fractures compared to males without LDD (adjusted-HR: 1.80, 95% CI: 1.20–2.70, P=0.005). The risk was also higher for VFx in males (HR: 1.64, 95% CI: 1.03–2.60, P=0.04). The association LDD–OP-fractures in females was lower and not significant (adjusted-HR: 1.08, 95% CI: 0.82–1.41). Meta-analyses showed that the risk of VFx in subjects with LDD has been studied only in women and there is not enough evidence to confidently analyze the relationship between LDD-features (DSN or/and OPH) and VFx due to low power and heterogeneity in phenotype definition in the collected studies.

Conclusions Male subjects with LDD have a higher osteoporotic fracture risk, in spite of systemically higher BMD.

INTRODUCTION

Lumbar disc degeneration (LDD) and osteoporosis are two age-related skeletal diseases which are very prevalent in elderly and known to be related to pain, increased morbidity and disability in this population.[1, 2] In Europe, the mean prevalence of vertebral fractures (VFx) in women between 60 and 64 years is 17% and this increases up to 35% when they are aged 75 years or more.[1] Both, osteoporotic (OP) fractures and LDD occur also in men however, it has been more studied in women.[3, 4]

The relationship between LDD and bone health is unclear. As it has been previously shown, the presence of LDD is associated with higher spine bone mineral density (BMD).[5-7] In addition, LDD has been found associated with higher BMD of the femoral neck, which suggests a systemic increased BMD in subjects affected by LDD.[5, 6, 8] In this respect LDD behaves very similar to knee or hip osteoarthritis (OA), where also an increased systemic BMD has been found.[9, 10]

In theory, the higher BMD found in subjects with LDD should corresponds to lower fracture risk compared to subjects without LDD. However, the few studies examining the relationship between LDD and vertebral fractures (in women) found conflicting results.[7, 11-14] In part this might be explained by the different radiological definitions used for LDD (based on the presence of osteophytes (OPH) and/or disc space narrowing (DSN)) and vertebral fractures (scored by different methods). Additionally, there are no studies examining the relationship between LDD and all types of OP fractures which would indicate whether the increased BMD found in LDD cases corresponds to a decreased fracture risk.

Therefore, we investigated the relation between LDD and all type of osteoporotic fractures including vertebral, in a large prospective cohort that includes men and women. In addition, we performed a systematic review of previously published studies.

METHODS

The Rotterdam study

This study is part of the Rotterdam study (RS), a large prospective population-based cohort study among men and women 55 years of age and older. The study design and rationale are described elsewhere in detail.[15] The objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly. The baseline measurements were conducted between 1990 and 1993. In total, 7,983 participants were examined. The current study was performed in 2,385 study participants for whom data on incident vertebral fractures, BMD and LDD was available. The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant.

Data collection for potential risk factors

Home interviews on medical history were performed by trained interviewers. Smoking habit was categorized binary as current or former versus never. The lower limb disability index used was composed of the mean score from six different questions regarding activities of daily living, using a modified version from the Stanford Health Assessment Questionnaire.[16]

At baseline measurement, medical information and physical examination including height and weight were obtained. Body mass index (BMI) was calculated by dividing weight by height squared (kg/m^2). Radiographic assessment of LDD Each vertebral level from L1 to S1 was reviewed for the presence and severity of osteophytes (OPH) and vertebral narrowing (disc space narrowing (DSN)), using the Lane atlas.[3, 4] In this atlas the categories are as follows: grade 0=none; grade 1=mild; grade 2=moderate; and

grade 3=severe. DSN was defined as present when there was a grade 1 narrowing at two or more vertebral levels. Because of the small proportion of subjects without osteophytes, we used a higher cut-off value for this feature; OPH was positive when there were osteophytes of at least grade 2 at two or more vertebral levels. When DSN and OPH were both positive and present at 2 or more levels, the participant was assigned as "LDD case". The definition suggested for LDD was previously found as the best related to clinical symptoms including lumbar pain.[17] A severity score for each participant was calculated adding the individual scores of DSN and OPH (1–3) of all intervertebral levels.

BMD measurements

DXA BMD (g/cm^2) of the right proximal femur and lumbar spine was measured at baseline using a Lunar DPX-L densitometer (Lunar Radiation Corp., Madison, WI, USA). Total body scans were performed at the third follow-up visit (mean follow-up 6.5 years) using a ProdigyTM fan-beam densitometer (GE Lunar Corporation Madison, WI) and analyzed with EncoreTM software. The software employs an algorithm that divides body measurements into areas corresponding to total body, head, trunk, arms and legs. Other methodological details have been described previously.[18]

Assessment of osteoporotic fracture

Follow-up started either on January 1, 1991 or at the time of inclusion into the study. For this analysis, follow-up ended either at January 1, 2007 or, when earlier, at the participant's death or loss to follow-up. For ~80% of the study population, medical events were reported through computerized general practitioner diagnosis registers. For the remaining 20%, research physicians collected data from the general practitioners' medical records of the study participants. All collected fractures were verified by reviewing discharge reports and letters from medical specialists. Fracture events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10). Finally, an expert in osteoporosis reviewed all coded events for final classification. Fractures coded as incident vertebral fractures were considered clinical fractures if they were identified on radiographs when subjects with symptoms (principally pain) visited the medical practitioner. All fractures that were considered not osteoporotic (fractures caused by cancer and all hand, foot, skull, and face fractures) were excluded. The period of follow-up was calculated as the time from enrollment in the study to the first fracture, death, or the end of the planned follow-up period, whichever occurred first. The participants were followed for the occurrence of fracture for approximately 12.8 years (± 3.1 SD yr).

Assessment of prevalent and incident radiographic vertebral fracture

Radiographic vertebral fracture: both at baseline, between 1990 and 1993, and at the second follow-up visit, between 1997 and 1999, a trained research technician obtained lateral radiographs of the thoracolumbar spine of subjects who were able to come to the research center. The follow-up radiographs were available for 2819 individuals, who survived an average of 6.3 years after their baseline center visit and who were still able to come to our research center. All follow-up radiographs were evaluated morphometrically in Sheffield by the McCloskey–Kanis method, as described previously.[19] If a vertebral fracture was detected, the baseline radiograph was evaluated as well. If the fracture was already present at baseline, it was considered a prevalent fracture. All vertebral fractures were confirmed by visual interpretation by an expert in the field to rule out artifacts and other etiologies, such as pathological fractures.[20] Participants with missing data on one or more risk factors were excluded (N=434).

Literature study

Relevant articles were identified by a systematic search using the database of PubMed with the words ["spine osteoarthritis" or "spine OA" or "disc degeneration"] and [fracture] as keywords in the title or abstract. The following inclusion criteria applied for this review are: (1) listed in PubMed, (2) publication in the English language, (3) study in humans, (4) the article represents original data, (5) subjects with and without disc degeneration features are compared in the study in relation to vertebral and/or osteoporotic fractures and (6) the full-text article was available. Methodological quality assessment is found in the Supplementary material.

Statistical analysis

We compared the baseline characteristics of the study population and the FN- and LS-BMD between the LDD cases and controls using analysis of variance (ANOVA). Categorical variables were analyzed using chi squared test. Cox's proportional hazards regression was used to assess association between LDD and OP fractures (or only clinical vertebral fractures). The analyses were adjusted by gender (or stratified by gender), age, BMI, lower limb disability and FN-BMD as continuous variables. Departure from additive effect of the risk factors was tested using interaction terms in the model. All these analyses were made using SPSS V. 15.0.Meta-analyzed results and forest plots included in the literature study were obtained using the Comprehensive Meta-analysis Software Version 2, Biostat, Englewood NJ (2005). Power calculations were done with PS version 2.1.31.

RESULTS

Study population

Characteristics of the cohort comprising 2,385 participants with data for the two major outcomes: LDD and vertebral fracture are shown in Table 1. At baseline, 362 participants had LDD (moderate OPH and mild-DSN) in two or more intervertebral levels. Subjects with LDD were older and heavier than controls. Also, LDD subjects had 0.72 and 0.32 SD higher LS- and FN-BMD at baseline compared to controls (Figure 1, P<0.001 for both FN and LS-BMD differences). Additionally, Figure 1 shows that total body-BMD and skull-BMD (measured at a later time point) were also significantly increased in subjects with lumbar disc degeneration. All BMD analyses were adjusted for age, gender, BMI, and lower limb disability. The number of prevalent radiographic vertebral fractures was not different in the LDD group compared to the group without LDD after adjustment for age and gender (Table 1, P=0.83). The mean LDD severity score was higher in males than in females (mean=6.6 (SD=4.3) for males and 5.9 (SD=4.4) for females (P<0.001, adjusted for age and BMI)). However, there was no statistically significant association between LDD-severity score and all type of OP-fractures (P=0.13).

Osteoporotic fracture risk

During 12.8 (SD=3.12) years of follow-up, 558 participants suffered an osteoporotic (OP) fracture. Subjects with LDD had an increased risk of OP fractures compared to subjects without LDD (HR: 1.29, 95% CI:1.04–1.60). The risk slightly decreased after adjustment for age, gender, BMI, lower limb disability and FN-BMD (HR: 1.24 (0.99–1.55)). We found a significant interaction between gender and LDD on fracture risk suggesting differences in OP fracture risk between genders (P for interaction term: 0.03). Therefore, we stratified the analysis according to gender and observed that only males with LDD had an increased

Table 1. Baseline characteristic for subjects according to LDD features in two or more levels.

Variables	Controls* N=2,023 (82)	LDD** N=362 (18)	P value
Female	1161 (57)	207 (57)	0.9
Age (years)	63.7 (5.7)	65.9 (6.2)	<0.001
BMI (kg/m^2)	26.2 (3.7)	26.9 (3.8)	0.04
FN-BMD (g/cm^2)	0.88 (0.13)	0.91 (0.14)	<0.001
Total-BMD (g/cm^2)	1.10 (0.12)	1.12 (0.13)	0.001
Head-BMD (g/cm^2)	1.93 (0.28)	2.01 (0.29)	<0.001
Smoking (current/former)	1367 (68)	233 (64)	0.18
Lower limb disability	0.16 (0.38)	0.21 (0.33)	<0.001
Falling (yes)	241 (12)	52 (14.5)	0.29
Prevalent radiological VFx*	88 (6.6)	22 (7.4)	0.83

Abbreviations: LDD: Lumbar disc degeneration, **defined as mild disc space narrowing (DSN) and moderate/severe osteophytes (OPH) in two vertebral levels. *Controls: Participants with less than 2 levels affected by DSN and OPH per level. Values presented are mean and standard deviations (SD) for each continuous variable and numbers and percentage (%) for categorical variables. BMI (body mass index), smoking, LLD (lower limb disability), falling and prevalent radiographic-vertebral fracture (VFx) comparisons were adjusted for age and gender. All bone mineral density (BMD) analyses were adjusted for age, gender, BMI and LLD.

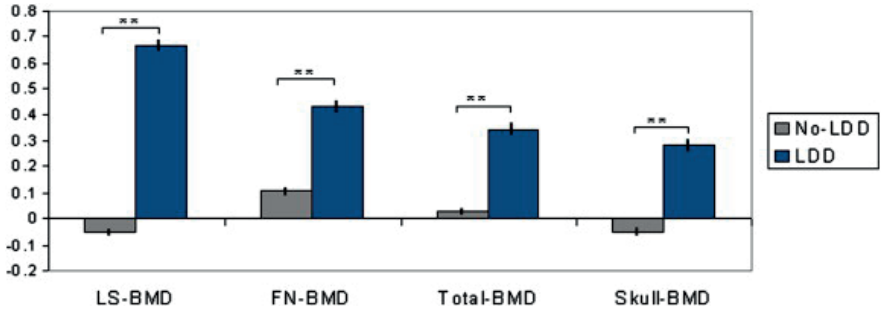

Figure 1. Bone mineral density (BMD; Z-scores) differences between subjects with lumbar disc degeneration (LDD) and without LDD in four different regions: lumbar spine (LS), femoral neck (FN), and total body and skull. Estimates were adjusted by age, gender, height and BMI. **P value ≤0.001. Total body-BMD and skull-BMD were measured in a subset of 1,649 participants at a second follow-up.

OP fracture risk (Table 2, adjusted HR: 1.80 (1.20–2.70), P=0.005 for males and HR: 1.08, 95% CI: 0.82–1.41, P=0.59 for females).

Clinical vertebral fracture

After the follow-up time, 21% of participants having fractures (N=116) had a clinically defined vertebral fracture. Participants with LDD had an increased hazard of having a clinical vertebral fracture during the follow-up (Table 2, adjusted-HR: 1.64, 95% CI: 1.03–2.60, P=0.04). As it was for overall OP-fractures, the hazard for a clinical vertebral fracture was higher only for males with LDD (HR: 2.34, 95% CI: 1.09–5.04 in males and 1.39, 95% CI: 0.78–2.50 for females).

Radiographic vertebral fracture

During 6.3 years of follow-up, 106 participants had an incident radiographic-vertebral fracture. After adjustment for age, gender, BMI, FN-BMD and prevalent radiographic vertebral fracture, subjects with

Table 2. Risk of vertebral and osteoporotic fracture in participant with lumbar disc degeneration (LDD).

N.LDD/N	Clinical vertebral fractures (HR & 95% CI)				Osteoporotic fractures (HR & 95% CI)			
	Unadjusted	**P**	**Adjusted risk**	**P**	**Unadjusted**	**P**	**Adjusted risk**	**P**
All (362/2,385)	1.53 (0.98–2.40)	0.06	1.64 (1.03–2.60)	0.04	1.29 (1.04–1.60)	0.02	1.24 (0.99–1.55)	0.06
Males	1.41 (0.68–2.90)	0.36	2.34 (1.09–5.04)	0.03	1.88 (1.27–2.79)	0.002	1.80 (1.20–2.70)	0.005
Females	1.22 (0.71–2.10)	0.47	1.39 (0.78–2.50)	0.26	1.11 (0.85–1.43)	0.45	1.08 (0.82–1.41)	0.59

Risk for osteoporotic and clinical vertebral fractures in participants with Lumbar disc degeneration (LDD) defined as categorical variable according to the presence of at least mild disc space narrowing (DSN ≥ 1) and moderate/severe osteophytosis (OPH≥2) per intervertebral level in at least two intervertebral levels. Lumbar disc degeneration was evaluated only at lumbar spine (L1–L5). Risk of vertebral and osteoporotic fracture are hazard ratio (HR) and 95% confidence interval (CI) unadjusted or adjusted for baseline characteristics (gender, age, BMI, lower limb disability, femoral neck bone mineral density (FN-BMD)). Number of clinical vertebral fractures=116; number of osteoporotic fractures=558.

LDD had 2.14 increased odds of having a radiographic vertebral fracture. However, this was not statistically significant and the broad confidence interval revealed low power in the analysis (95% CI: 0.82–5.58, P=0.12). Hence, we reviewed the existent literature on the relationship between LDD and (vertebral) fractures.

Results of the literature study: separate LDD features and risk for radiographic vertebral fractures

From 36 studies selected on bases of the search terms in abstract/title, a total of 28 studies were excluded because the abstract clearly showed that the study did not analyze the studied relationship LDD/spine OA-fracture. Based on the criterion 4 studies were excluded: no comparison was made between subjects with and without disc degeneration in relation to vertebral and/or osteoporotic fractures (criteria e). A total of five studies (four from the literature search + current results from our study) analyzing the relation LDD and radiographic vertebral fractures were included in this review (Supplementary Table 1). There were no studies analyzing the relation of LDD with other types of fractures in accordance with the selection criteria previously explained. Further details of the selection procedure of studies can be found in the Supplementary material. From these five selected studies, two were done in the same population therefore, only the most recent "longitudinal prospective" (Sornay-Rendu et al.) was included in the meta-analysis.[12] These studies fulfilled the inclusion criteria requirements and methodological quality assessment, including adjustment for age, gender, BMI and BMD in the analysis. Only one study (Roux et al.) did not perform BMD adjustment in the analysis of vertebral fracture risk.[11]

All selected studies were done in postmenopausal women, one of them in women with osteoporosis.[11] In all studies, radiographic-LDD features were evaluated from the first till fifth lumbar segment (L1–L5). LDD was defined as the presence of osteophytes (OPH), or disc space narrowing (DSN) in at least one intervertebral level. A detailed description of the studies and definition of LDD and vertebral fracture assessment is presented in Supplementary Table 1.

Table 3 shows the results of the studies reviewed. Prevalence of at least minimal/mild osteophytes in the studied populations differs between 56 and 90% (Table 3). In the study of Roux et al. there was also a protective effect of OPH for vertebral fractures, however in that study, the association was not adjusted for BMD; what seems to modify the relationship of osteophytes–vertebral fractures (Table 2). Post-hoc power calculation demonstrated that to have 80% power to detect OR N=1.2 having an incidence of radiographic vertebral fractures of around 5%, a sample size of around 4,200 participants would be

Table 3. Results of the reviewed studies for association of LDD and risk of vertebral fractures.

Reference	Definition of VFx	N. of VFx	OR (confidence interval) unadjusted risk	OR (confidence interval) (BMD adjusted)	P adj.
Arden[13]	Quantitative McCloskey	41	Not shown	LS-OPH: 0.46 (0.21–0.99)	<0.05
				Thoracic-OPH: 3.57 (1.55–8.24)	
Sornay-Rendu[14]	Semi-quantitative Genant	48	Lumbar spine	Lumbar spine	Not shown
			OPH≥1: 0.8 (0.4–1.6)	1.0 (0.5–2.1)	
			DSN≥1: 3.4 (1.5–7.8)	3.5 (1.5–8.3)	
			OA grade 2: 1.6 (0.9–2.3)	1.7 (0.9–3.2)	
Sornay-Rendu[12]	Semi-quantitative Genant	42	LS-DSN: Not shown	LS-DSN: 3.27 (1.19–8.98)	Not shown
			LS and thoracic spine	LS and thoracic spine	
			DSN≥1: 6.88 (1.64–28.9)	DSN≥1: 6.59 (1.36–31.94)	
			OPH≥1: 1.39 (0.18–10.7)	OPH≥1: 0.94 (0.10–8.97)	
			OA grade 2: 1.57 (0.81–3.01)	OA grade 2: 0.92 (0.42–1.99)	
**Roux[11]	Semi-quantitative Genant	215	DSN≥1: 0.83 (0.55–1.26)	DSN≥1: 0.67 (0.43–1.04)	0.071
			OPH≥1: 0.45 (0.21–0.99)	OPH ≥1: 0.38 (0.17–0.86)	0.02
			LS-DSN≥2: 0.86 (0.56–1.33)	LS-DSN≥2: 0.74 (0.47–1.16)	0.191
			DSN≥1: 1.26 (0.72–2.20)	DSN≥1: 1.27 (0.71–2.26)	0.42
This study	Quantitative McClosckey	108	OPH≥1: 1.10 (0.43–2.84)	OPH≥1: 1.27 (0.48–3.34)	0.63
			OA grade 2: 1.00 (0.57–1.74)	OA grade 2: 1.05 (0.59–1.87)	0.86
			LDD: 1.14 (0.61–2.15)	LDD: 1.14 (0.57–2.29)	0.7

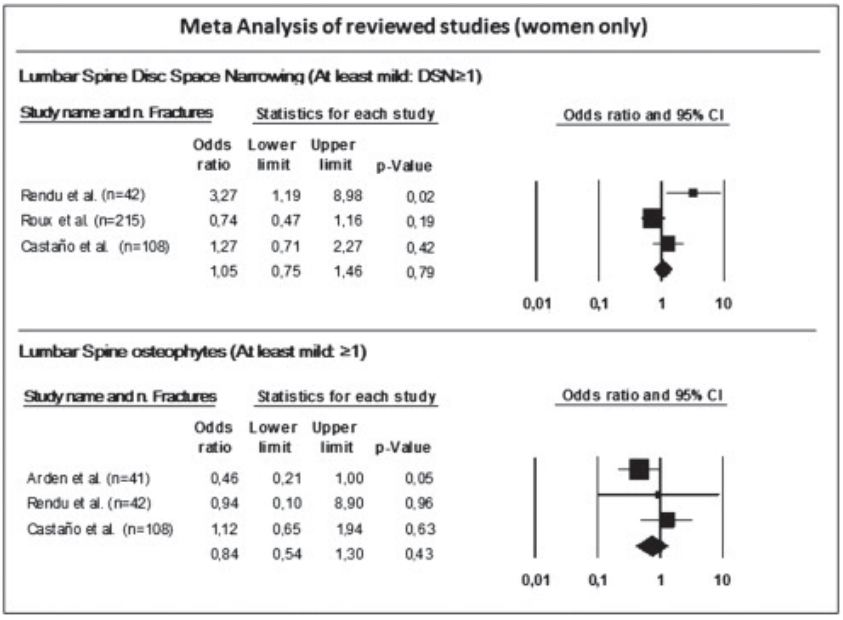

Figure 2. Meta-analysis of lumbar disc degeneration features and their relation with vertebral fractures in women. Values are odds ratios (OR) and 95% confidence intervals. Abbreviations: DSN≥1=mild disc space narrowing, OPH≥1=minimal or mild osteophytes. Studies were included when they had similar adjustments.

needed. Consequently, confidence intervals are wide and associations of separate features of combined LDD definition did not reveal conclusive evidence (Figure 2, OR: 0.70, 95% CI: 0.39–1.25, P: 0.23 and OR: 1.05, 95% CI: 0.75–1.46, P: 0.79 for the presence of at least mild osteophytes and disc space narrowing, respectively).

DISCUSSION

This study shows that individuals affected with lumbar disc degeneration (LDD), in spite of having a systemically higher BMD, are not protected of osteoporotic fractures. Contrarily, male subjects with LDD had an increased risk for osteoporotic fractures, including clinical vertebral fractures. Results of the meta-analysis of the literature showed that LDD has been studied only in women for its association with radiographic vertebral fractures. Additionally, this is the first study analyzing the association of LDD with all type of fractures in males and females. The results of the meta-analysis were not conclusive regarding the relation of separate LDD features (osteophytes and disc space narrowing) and risk of radiographic vertebral fractures in females.

We found a higher systemic BMD in participants with LDD. Participants with LDD had a higher BMD not only in lumbar spine (where BMD measurements are known to be influenced by the presence of osteophytes), but also in femoral neck, total body and skull. This is in line with previous findings of higher BMD in LDD patients not only in lumbar spine but also in other body regions.[6, 7] Measurement of skull-BMD has been shown to be less subjective to change during aging and influence of environmental and mechanical factors (strain and weight bearing).[21] Higher skull-BMD in subjects with LDD suggests that a systemically higher BMD might be present before LDD.

In spite of higher BMD in participants with LDD, we found a higher osteoporotic fracture risk. It is possible that the increased BMD in subjects with LDD is not enough to compensate for other detrimental effects of disc degeneration on trunk stability and flexibility that might result in an increased fracture risk. Loading on the spine is determined by a person's height, weight, muscle forces, and activity, but can also be affected by intervertebral disc degeneration.[22-24] Loss of disc height and its properties produce high tensile strains in the endplate and they have been shown as causal factors for "failure of the vertebra".[25, 26] Additionally, disc degeneration can affect other structures (vertebra itself, muscles and ligaments) producing modification in the distribution of compressive and tensional forces through the column that in normal conditions are evenly distributed. Ligaments of the anterior region have changes as a consequence of LDD, causing its remodeling and thickening.[27, 28] Consequently ligaments lose elasticity and the trunk's flexibility decreases; this becomes evident during aging where the range of spine movement is severely affected. Individuals with LDD have more stiffness in trunk and lower legs that could increase the reaction time during falling and other demanding occupational activities which are major situations where fractures occur in elderly.[29, 30]

We found a higher osteoporotic fracture risk in males. Severity of LDD was higher in males, principally because of higher severity of disc space narrowing. Additionally, there is some evidence for an association between disc space narrowing and lower back pain especially in men proportionally increasing with a higher number of affected intervertebral disc spaces.[17] However, severity itself did not explain the increased OP fracture risk in males. Neither was the risk explained by other factors such as lower limb disability, which was found to be higher in males with LDD and other common risk factors including age and falling risk. In older males (N=65 years), clinical vertebral fractures are caused by no known trauma or by low-energy trauma. It is known that most fractures occur in men with normal BMD and clinical

vertebral fractures are particularly common in the oldest men.[31] Clinical vertebral fractures have been also related to important comorbidities, negative effects on quality of life and increased mortality.[32-34]

The systematic review showed some important aspects. Previous studies analyzing the association between LDD and fractures have been done only in females, the majority had small sample size and they defined LDD using only separate features. There were also important methodological differences between these studies in type (cross-sectional versus longitudinal) and fracture assessment. In spite of trying to meta-analyze the results of studies included in this review, using homogeneous definitions, power was still insufficient to draw significant conclusion on the relation of separate LDD features and radiological vertebral fractures.

The review made evident that there is a need for consensus in the definition of radiological LDD. In our opinion, more stringent radiological definitions are needed; some studies only consider osteophytes to define LDD. Osteophytes are a common feature in older populations and its prevalence depends of how stringent the definition is, reaching 90% for the presence of minimal/mild osteophytes. The presence of disc space narrowing and osteophytes (at least moderate) in the same intervertebral level should be considered when LDD is defined because it has been shown to be more clinically relevant; this combination of radiological definition was found to best correlate with clinical symptoms: lumbar pain and stiffness.[17, 29]

There are strengths and limitations in this study. This study is unique in examining the relation of LDD, osteoporotic and vertebral fractures in a large prospective cohort that includes males and females. In addition, the composed definition of several radiographic features used in this study is an advantage because it is more stringent and clinically relevant. We also examined separate LDD features in order to compare results with earlier published studies. However, we concluded that even after the meta-analysis the number of radiographic vertebral fractures was insufficient to get conclusive evidence in the relation of radiographic vertebral fractures with separate LDD features.

CONCLUSION

To conclude, we consider that subjects with LDD in spite of having higher systemic BMD are at higher risk of osteoporotic fractures, especially males for whom LDD seems more severe. The exact mechanisms to explain this association merits further investigation, considering that both, LDD and clinical vertebral fractures are common, associated with comorbidities and decreasing quality of life.

ACKNOWLEDGMENTS

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REFERENCES

1. Cummings SR, Melton LJ. Epidemiology and outcomes of osteoporotic fractures. Lancet. 2002 May 18;359(9319):1761-7.
2. Engel CC, von Korff M, Katon WJ. Back pain in primary care: predictors of high health-care costs. Pain. 1996 May-Jun;65(2-3):197-204.
3. Lawrence JS. Disc degeneration. Its frequency and relationship to symptoms. Ann Rheum Dis. 1969 Mar;28(2):121-38.
4. Miller JA, Schmatz C, Schultz AB. Lumbar disc degeneration: correlation with age, sex, and spine level in 600 autopsy specimens. Spine (Phila Pa 1976). 1988 Feb;13(2):173-8.
5. Jones G, Nguyen T, Sambrook PN, Kelly PJ, Eisman JA. A longitudinal study of the effect of spinal degenerative disease on bone density in the elderly. J Rheumatol. 1995 May;22(5):932-6.
6. Livshits G, Ermakov S, Popham M, Macgregor AJ, Sambrook PN, Spector TD, et al. Evidence that bone mineral density plays a role in degenerative disc disease: the UK Twin Spine study. Ann Rheum Dis. 2010 Dec;69(12):2102-6.
7. Schneider DL, Bettencourt R, Barrett-Connor E. Clinical utility of spine bone density in elderly women. J Clin Densitom. 2006 Jul-Sep;9(3):255-60.
8. Miyakoshi N, Itoi E, Murai H, Wakabayashi I, Ito H, Minato T. Inverse relation between osteoporosis and spondylosis in postmenopausal women as evaluated by bone mineral density and semiquantitative scoring of spinal degeneration. Spine (Phila Pa 1976). 2003 Mar 1;28(5):492-5.
9. Bergink AP, Uitterlinden AG, Van Leeuwen JP, Hofman A, Verhaar JA, Pols HA. Bone mineral density and vertebral fracture history are associated with incident and progressive radiographic knee osteoarthritis in elderly men and women: the Rotterdam Study. Bone. 2005 Oct;37(4):446-56.
10. Hart DJ, Cronin C, Daniels M, Worthy T, Doyle DV, Spector TD. The relationship of bone density and fracture to incident and progressive radiographic osteoarthritis of the knee: the Chingford Study. Arthritis Rheum. 2002 Jan;46(1):92-9.
11. Roux C, Fechtenbaum J, Briot K, Cropet C, Liu-Leage S, Marcelli C. Inverse relationship between vertebral fractures and spine osteoarthritis in postmenopausal women with osteoporosis. Ann Rheum Dis. 2008 Feb;67(2):224-8.
12. Sornay-Rendu E, Allard C, Munoz F, Duboeuf F, Delmas PD. Disc space narrowing as a new risk factor for vertebral fracture: the OFELY study. Arthritis Rheum. 2006 Apr;54(4):1262-9.
13. Arden NK, Griffiths GO, Hart DJ, Doyle DV, Spector TD. The association between osteoarthritis and osteoporotic fracture: the Chingford Study. Br J Rheumatol. 1996 Dec;35(12):1299-304.
14. Sornay-Rendu E, Munoz F, Duboeuf F, Delmas PD. Disc space narrowing is associated with an increased vertebral fracture risk in postmenopausal women: the OFELY Study. J Bone Miner Res. 2004 Dec;19(12):1994-9.
15. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
16. Lawton MP, Moss M, Fulcomer M, Kleban MH. A research and service oriented multilevel assessment instrument. J Gerontol. 1982 Jan;37(1):91-9.
17. de Schepper EI, Damen J, van Meurs JB, Ginai AZ, Popham M, Hofman A, et al. The association between lumbar disc degeneration and low back pain: the influence of age, gender, and individual radiographic features. Spine (Phila Pa 1976). 2010 Mar 1;35(5):531-6.
18. Burger H, van Daele PL, Algra D, van den Ouweland FA, Grobbee DE, Hofman A, et al. The association between age and bone mineral density in men and women aged 55 years and over: the Rotterdam Study. Bone Miner. 1994 Apr;25(1):1-13.
19. McCloskey EV, Spector TD, Eyres KS, Fern ED, O'Rourke N, Vasikaran S, et al. The assessment of vertebral deformity: a method for use in population studies and clinical trials. Osteoporos Int. 1993 May;3(3):138-47.
20. van der Klift M, de Laet CE, McCloskey EV, Johnell O, Kanis JA, Hofman A, et al. Risk factors for incident vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2004 Jul;19(7):1172-80.
21. Turner AS, Maillet JM, Mallinckrodt C, Cordain L. Bone mineral density of the skull in premenopausal women. Calcif Tissue Int. 1997 Aug;61(2):110-3.

22. Pollintine P, Dolan P, Tobias JH, Adams MA. Intervertebral disc degeneration can lead to "stress-shielding" of the anterior vertebral body: a cause of osteoporotic vertebral fracture? Spine (Phila Pa 1976). 2004 Apr 1;29(7):774-82.
23. Polga DJ, Beaubien BP, Kallemeier PM, Schellhas KP, Lew WD, Buttermann GR, et al. Measurement of in vivo intradiscal pressure in healthy thoracic intervertebral discs. Spine (Phila Pa 1976). 2004 Jun 15;29(12):1320-4.
24. Adams MA, Dolan P. Spine biomechanics. J Biomech. 2005 Oct;38(10):1972-83.
25. Fields AJ, Lee GL, Keaveny TM. Mechanisms of initial endplate failure in the human vertebral body. J Biomech. 2010 Dec 1;43(16):3126-31.
26. Hulme PA, Boyd SK, Ferguson SJ. Regional variation in vertebral bone morphology and its contribution to vertebral fracture strength. Bone. 2007 Dec;41(6):946-57.
27. Iida T, Abumi K, Kotani Y, Kaneda K. Effects of aging and spinal degeneration on mechanical properties of lumbar supraspinous and interspinous ligaments. Spine J. 2002 Mar-Apr;2(2):95-100.
28. Fujiwara A, Tamai K, An HS, Shimizu K, Yoshida H, Saotome K. The interspinous ligament of the lumbar spine. Magnetic resonance images and their clinical significance. Spine (Phila Pa 1976). 2000 Feb 1;25(3):358-63.
29. Scheele J, de Schepper EI, van Meurs JB, Hofman A, Koes BW, Luijsterburg PA, et al. Association between spinal morning stiffness and lumbar disc degeneration: the Rotterdam Study. Osteoarthritis Cartilage. 2012 Sep;20(9):982-7.
30. Grisso JA, Kelsey JL, Strom BL, Chiu GY, Maislin G, O'Brien LA, et al. Risk factors for falls as a cause of hip fracture in women. The Northeast Hip Fracture Study Group. N Engl J Med. 1991 May 9;324(19):1326-31.
31. Freitas SS, Barrett-Connor E, Ensrud KE, Fink HA, Bauer DC, Cawthon PM, et al. Rate and circumstances of clinical vertebral fractures in older men. Osteoporos Int. 2008 May;19(5):615-23.
32. Cauley JA, Thompson DE, Ensrud KC, Scott JC, Black D. Risk of mortality following clinical fractures. Osteoporos Int. 2000;11(7):556-61.
33. Oglesby AK, Minshall ME, Shen W, Xie S, Silverman SL. The impact of incident vertebral and non-vertebral fragility fractures on health-related quality of life in established postmenopausal osteoporosis: results from the teriparatide randomized, placebo-controlled trial in postmenopausal women. J Rheumatol. 2003 Jul;30(7):1579-83.
34. Adachi JD, Ioannidis G, Olszynski WP, Brown JP, Hanley DA, Sebaldt RJ, et al. The impact of incident vertebral and non-vertebral fractures on health related quality of life in postmenopausal women. BMC Musculoskelet Disord. 2002 Apr 22;3:11.

Chapter 2.3

Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study

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ABSTRACT

Summary Serum high-sensitivity C-reactive protein (CRP) is an inflammatory biomarker. We investigated the relationship between CRP and bone health in the Rotterdam Study. Serum high-sensitivity CRP was associated with fracture risk and lower femoral neck bending strength. Mendelian randomization analyses did not yield evidence for this relationship being causal.

Introduction Inflammatory diseases are associated with bone pathology, reflected in a higher fracture risk. Serum high-sensitivity C-reactive protein CRP is an inflammatory biomarker. We investigated the relationship between CRP and bone mineral density (BMD), hip bone geometry and incident fractures in the Rotterdam Study, a prospective population-based cohort.

Methods At baseline, serum high-sensitivity CRP was measured. A weighted genetic risk score was compiled for CRP based on published studies (29 polymorphisms; Illumina HumanHap550 Beadchip genotyping and HapMap imputation). Regression models were reported per standard deviation increase in CRP adjusted for sex, age and BMI. Complete data was available for 6,386 participants, of whom 1,561 persons sustained a fracture (mean follow-up, 11.6 years).

Results CRP was associated with a risk for any-type of fracture (hazard ratio [HR]=1.06; 95% confidence interval [CI]: 1.02–1.11), hip fractures (HR=1.09; 1.02–1.17) and vertebral fractures (OR=1.34; 1.14–1.58). An inverse relationship between CRP levels and section modulus (-0.011 cm^3; -0.020 to -0.003 cm^3) was observed. The combined genetic risk score of CRP SNPs was associated with serum CRP levels ($P=9\times10^{-56}$), but not with fracture risk (HR=1.00; 0.99–1.00; P=0.23).

Conclusions Serum high-sensitivity CRP is associated with fracture risk and lower bending strength. Mendelian randomization analyses did not yield evidence for this relationship being causal. Future studies might reveal what factors truly underlie the relationship between CRP and fracture risk.

INTRODUCTION

Chronic inflammatory diseases are frequently associated with bone loss, usually translated as a higher fracture risk.[1, 2] Independent of the type of inflammatory process the effects arise due to alterations on the bone remodeling cycle, through direct (e.g., pro-inflammatory cytokines like TNF-alpha stimulating osteoclastogenesis through regulation of RANKL expression in osteoblasts or inhibiting bone formation through DKK1 action); or indirect mechanisms (e.g., it is well-established that the remodeling cycle is controlled by mechanical strains and a variety of endocrine and immunological mechanisms.[3]

The most commonly measured inflammatory serum biomarker is C-reactive protein (CRP), a protein found in the blood which levels rise in response to inflammation.[4] To date, several publications have reported on serum CRP levels in relation to BMD,[2, 5-9] non-vertebral fractures,[2, 8] radiographic vertebral fractures and bone microarchitecture at the distal radius.[8] To our knowledge, there have been no studies investigating CRP in relation to hip bone geometry. In summary, some,[5-7, 9] but not all, studies have found serum CRP to be associated with lower BMD; a few studies with fracture data found an increased risk for higher CRP levels,[2, 8] which intriguingly appeared independent of BMD or trabecular microarchitecture.[8] Nonetheless, the causal mechanism is largely unknown.

Observational studies are well-suited to identify risk factors, it is, however, difficult to establish causal relationships, because they are prone to confounding and reverse causation.[10] Analytical techniques are available to assess causality in observational data using genotypes as instrumental variables. If we know of genetic determinants closely linked to a particular disease risk factor, where the genetic determinants do not have direct effects on the disease, it can be reasonably assumed that these genetic determinants fully represent the risk factor and are themselves not affected by confounding factors.[11] This approach, which is based on the fact that genotypes are transmitted randomly from parents to offspring during meiosis (Mendel's second law), is called the Mendelian randomization approach.[12] The heritability of serum CRP levels has been estimated to range between 25% to 40%,[13-15] suggesting that genetic factors are influencing variation in CRP levels. So far, 18 genetic loci have been identified by genome-wide association studies (GWAS) as associated at genome-wide significant level with serum CRP levels and explaining approximately 5% of the trait variance.[16] An example of a well-conducted Mendelian randomization study is the report by Zacho et al. examining the relationship between polymorphisms in the *CRP* gene and ischemic vascular disease.[17]

Our study's aim was to investigate the relationship between serum high-sensitivity CRP levels and bone health using data from the Rotterdam Study, a prospective population-based cohort. First, we performed association analyses between CRP and different bone-related outcomes, such as fracture risk, bone mineral density (BMD) and hip geometry. Secondly, we explored the potential causality of serum CRP levels on the skeletal outcomes using a Mendelian randomization approach by applying regression of CRP-SNP genotypes on the fracture risk outcome.

METHODS

The Rotterdam Study

The Rotterdam Study is a prospective population-based cohort that aims to investigate the determinants of chronic diseases and disability in Dutch men and women. Both the objectives and the study design have been described previously.[18] The study targets investigations on endocrine diseases like osteoporosis amongst others. In short, all inhabitants aged 55 years and over of the Ommoord district in the city of Rotterdam in The Netherlands were invited to participate from January 1990 onwards (re-

sponse rate 78%). Between 1990 and 1993, a baseline home interview on medical history and risk factors for chronic diseases and medication use was taken by trained interviewers. Falling was assessed using structured personal interviews and falling frequency was recorded as never, less than once a month, and more than once a month. Smoking habits were coded as "current", "former", and "never". Subsequently, participants were invited to the research center for clinical examination. During the baseline visit height and weight were measured with indoor clothing and no shoes. Body mass index (BMI) was calculated as weight (in kg)/height (in m^2).

Serum CRP measurement

At the baseline visit, high-sensitivity CRP was measured in non-fasting frozen serum samples of 6,658 study participants using a rate near-infrared particle immunoassay (Immage Immunochemistry System, Beckman Coulter, Fullerton, CA, USA). This system measures concentrations from 0.2 to 1.440 mg/l, with a within-run precision <5.0%, a total precision <7.5%, and a reliability coefficient of 0.995. More details are described elsewhere.[19]

Skeletal assessments

All events, including incident fractures and death were reported by general practitioners (GPs) in the research area (covering 80% of the cohort) by means of a computerized system. All reported events were verified by two trained research physicians, who independently reviewed and coded the information. Subsequently, all coded events were reviewed by a medical expert for final classification. Subjects were followed from their baseline visit until January 1, 2007 or until a first fracture or death occurred. In addition, during the second follow-up visit between 1997 and 1999 all Rotterdam Study participants underwent lateral spine radiographic screening. Spinal radiographs obtained at the follow-up visit 6.3 years after baseline were evaluated morphometrically for the presence of prevalent vertebral fractures in Sheffield, UK, using the McCloskey-Kanis method as described previously.[20, 21] Femoral neck (FN) and lumbar spine (LS) BMD were measured at baseline by dual-energy X-ray absorptiometry (DXA), using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA).[22] Hip structural analysis[23] was used to measure hip bone geometry from the DXA scans of the femur narrow-neck region as described previously.[24] One intensively trained interview assistant carried out a one hour home interview including the Stanford Health Assessment Questionnaire (HAQ) [25] to assess lower limb disability, as described previously.[26] Briefly, it measures the ability or disability and difficulty to perform different categories of daily life activities, such as rising, walking, bending and getting in and out of a car, summarized in an index ranging from 0 (no impairment) to 3 (severe impairment).

Genotyping

The Rotterdam Study participants were genotyped using the Illumina (Hayward, CA, USA) Infinium HumanHap550 Beadchip in the Genetic Laboratory of Erasmus MC Department of Internal Medicine, The Netherlands, following manufacturers' protocols and quality control standards. Further single nucleotide polymorphism (SNP) imputations were performed based on the HapMap CEU reference panel (release 22, build 36).[27, 28] The genotypes of the 29 variants reported to be associated with CRP levels were extracted from the GWAS dataset.[16]

Statistical analysis

All effect estimates are reported per standard deviation of increase in CRP. Additionally, CRP values were log-transformed to account for the non-normal distribution and effect estimates expressed per unit log-transformed CRP (Results presented in Supplementary Materials). Risk of incident any-type of fractures was evaluated for association with CRP levels in Cox proportional hazard regression models. Proportional hazards assumption was tested via Schoenfeld residuals. The association with risk of vertebral fractures was assessed in logistic regression models. The relationships between CRP levels and continuous measures of BMD and hip bone geometry parameters were tested in linear regression models. Models for fracture, BMD and hip geometry were corrected for potential confounders, including: sex, age and BMI; fracture analyses had additional adjustments for myocardial infarction, prevalent type 2 diabetes mellitus, dementia, prevalent cancer, lower limb disability, use of systemic corticosteroids and risk of falling. In addition, the fracture analyses were adjusted for femoral neck and lumbar spine BMD. Change in effect estimates of 10% or more and/or loss of statistical significance after correction was examined. To further study relationships with confounders, analyses were performed stratified across gender and smoking status, and across age and BMI tertiles. Potential interaction of CRP levels with both BMD and BMI was tested by adding an interaction term to the models, to explore if an effect of CRP on fracture risk dependent on BMD levels could be detected. Subsequently, we performed stratified analyses per unadjusted and age-adjusted BMD tertiles.

For the Mendelian Randomization approach a weighted genetic risk score was constructed for each subject using all SNPs reported as associated with serum CRP levels (Supplementary Table 1)[16]. The cumulative effect of the CRP SNPs was tested in relation to CRP levels and risk of fracture by including the allele score on the statistical models. To declare a causal relationship, the following requirement should be met: $\beta_{\text{CRP gene scorefor serum CRP}} \times \beta_{\text{serum CRP for fracture}} \approx \beta_{\text{CRP gene score for fracture}}$. In addition, association statistics were computed for each SNP separately and to account for multiple testing the significance threshold was corrected according to Bonferroni (0.05/29=0.002 with corresponding 99.8% confidence intervals). SPSS statistics software version 20 (IBM, Armonk, NY, USA) and R software version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) were used for the analyses.

Table 1. Population baseline characteristics.

Age (years)	69.1	(8.9)
Height (m)	1.67	(0.09)
Weight (kg)	73.1	(12)
CRP (mg/L)	3.31	(6.69)
Log-transformed CRP	0.61	(1.04)
Femoral neck BMD (g/cm^2)	0.87	(0.14)
Lumbar spine BMD (g/cm^2)	1.09	(0.2)
Use of corticosteroids (%)	1.9	
Current smoking (%)	23.1	

Data presented as mean (SD) or percentages from the total study population (N=6,386) assessed at baseline (%). CRP=C-reactive protein; BMD=bone mineral density.

Table 2. Association statistics between serum high-sensitivity CRP and fracture risk.

	Outcome Any-type of fracture	Total sample size (number of cases)	Hazard ratio per unit increase in log-CRP	95% Confidence interval	P value
Model 1		6,386 (1,561)	1.06	1.02–1.11	0.005
Model 1	Hip fracture	6,386 (390)	1.09	1.02–1.17	0.008
Model 1	Wrist fracture	6,386 (363)	1.06	0.97–1.16	0.21
Model 1	Vertebral fracture	3,246 (359)	1.34[a]	1.14–1.58[a]	0.0004
Model 2	Any-type of fracture	5,399 (1,311)	1.08	1.03–1.13	0.003
Model 3	Any-type of fracture	5,420 (1,324)	1.07	1.02–1.13	0.003
Model 4	Any-type of fracture	6,234 (1,529)	1.06	1.01–1.11	0.018
Model 5	Any-type of fracture	6,304 (1,542)	1.07	1.02–1.12	0.003

Model 1: adjusted for age, sex and BMI; Model 2: adjusted for age, sex, BMI and FN-BMD; Model 3: adjusted for age, sex, BMI and LS-BMD; Model 4: adjusted for age, sex, BMI, myocardial infarction, prevalent type 2 diabetes mellitus, dementia, prevalent cancer and lower limb disability and use of systemic corticosteroids; Model 5: adjusted for age, sex, BMI and risk of falling. FN-BMD=femoral neck bone mineral density; LS-BMD=lumbar spine bone mineral density. [a]Odds ratios for prevalent radiographic vertebral fractures.

RESULTS

Association between serum CRP and fracture risk

Complete data was available for 6,386 participants (59% women), of whom 1,561 persons sustained a fracture during a mean follow-up duration of 11.6 years (SD=4.5 years; incidence=0.024 / person years). Serum CRP levels were significantly associated with increased risk of any-type of fracture (hazard ratio [HR] per unit increase in log-CRP: 1.06; 95% confidence interval [CI]: 1.02-1.11; P=0.005) (Table 2). For the gender-stratified analyses effect estimates did not all reach statistical significance, but were of similar magnitude (male HR=1.11; 95% CI: 1.02-1.22; P=0.02; female HR=1.05; 95% CI: 1.00-1.11; P=0.05). Furthermore, the main analysis was repeated stratifying according to age tertiles, where the effect was found foremost in the oldest age category (Table 3). Additional adding of potential confounders (myocardial infarction, prevalent type 2 diabetes mellitus, dementia, prevalent cancer and lower limb disability; systemic corticosteroids use; risk of falling) did not essentially change the results (HR=1.06; 95% CI: 1.01-1.11; P=0.018; P=0.03; HR=1.07; 95% CI: 1.02-1.12; P=0.003). The skeletal site-specific fracture risk analyses showed that the effect was particularly strong at the hip (HR=1.09; 95% CI: 1.02-1.17; P=0.008) and at the spine (OR = 1.34; 95% CI: 1.14-1.58; P=0.0004), but not clearly present with wrist fractures (HR=1.06; 95% CI: 0.97-1.16; P=0.21). As observed for any type of fracture, correction of the associations with hip and vertebral fracture were not affected by the inclusion of covariates in the models. Analyses adjusting for smoking status (HR=1.06; 95% CI: 1.01-1.11; P=0.01) or stratifying according to smoking status did not display strong differences between groups ("never smokers": HR=1.07; 95% CI: 1.01-1.14; P=0.03; "past smokers": HR=1.09; 95% CI: 0.99-1.19; P=0.08; "current smokers": HR=1.07; 95% CI: 0.90-1.27; P=0.47). The interaction term CRP*BMI was not significant; stratification by BMI tertiles did not show any differences in the analysis with log-transformed CRP (Supplementary) but there seemed a trend towards greater effects in the higher BMI tertiles in the analyses per standard deviation of CRP (Table 3). Neither was the association between CRP and fracture free survival attenuated by adjustment of analyses for FN-BMD or LS-BMD (HR=1.08; 95% CI: 1.03-1.13; P=0.003; HR=1.07; 95% CI: 1.02-1.13; P=0.003). However, CRP and FN-BMD were weakly interacting with each other to affect the risk of fracture (beta-coefficient: -0.46; P=0.08), which was more obvious in the analyses per log-transformed unit of CRP (Supplementary). Indeed, the association with fracture was strongest in the lowest FN-BMD tertile (Table 3). The

Table 3. Association analyses between serum CRP and any-type of fracture per tertile of age, bone mineral density or body mass index.

Stratification category	First tertile	Second tertile	Third tertile
Age	<64 years: 0.95 [0.75–1.20]	64-73 years: 1.05 [0.99–1.12]	>73 years: 1.08 [1.02–1.15]
Body mass index	<24.5 kg/m^2: 1.05 [0.96–1.14]	24.5-27.5 kg/m^2: 1.07 [1.00–1.13]	>27.5 kg/m^2: 1.12 [0.99–1.27]
Femoral neck bone mineral density	<0.80 g/cm^2: 1.10 [1.01–1.20]	0.80-0.92 g/cm^2: 1.07 [1.00–1.14]	>0.92 g/cm^2: 1.05; [0.87–1.26]
Age-standardized femoral neck bone mineral density	1.12 [1.00–1.26]	1.06 [0.98–1.14]	1.09 [0.93–1.27]
Lumbar spine bone mineral density	<1.00 g/cm^2: 1.07 [1.02–1.13]	1.00-1.16 g/cm^2: 1.08 [0.95–1.23]	>1.16 g/cm^2: 1.06 [0.91–1. 24]
Age-standardized lumbar spine bone mineral density	1.06 [1.01–1.13]	1.07 [0.94–1.21]	1.03 [0.89–1.21]

Hazard ratio per unit increase of log C-reactive protein with corresponding 95% confidence intervals between brackets.

interaction term CRP*LS-BMD did not reach statistical significance (beta-coefficient: -0.02; P=0.92), yet, the association seemed strongest again in the lower ranges of LS-BMD (Table 3).

Association between serum CRP and fracture risk in relation to BMD and hip bone geometry

Next, we analyzed the relationship between serum CRP and hip bone geometry, correcting for potential confounding by sex, age and BMI. Serum CRP was neither statistically significantly associated with FN-BMD (-0.003 g/cm^2; per unit log-CRP increase; 95% CI: -0.006–0.001 g/cm^2; P=0.16) nor with LS-BMD (-0.002 g/cm^2; -0.008–0.003 g/cm^2; P=0.37) in our study sample. Hip bone geometry data was available for a subset of 4,528 participants. There was a significant inverse relationship between serum CRP levels and section modulus (-0.011 cm^3 per standard deviation increase; -0.020 to -0.003 cm^3; P=0.009). For narrow neck width we found a significant inverse association in the analysis per log-transformed unit of CRP (Supplementary) but this could not be replicated in the analysis per standard deviation increase of CRP (-0.05 mm; 95% CI: -0.10–0.002 mm; P=0.21). In line with no association with BMD levels there was also no significant association with cortical thickness (-0.0005 mm; -0.001–0.0002 mm; P=0.18). Increased CRP levels were associated with a weak increase of the buckling ratio instability index though not achieving statistical significance (+0.08 mm; -0.02 mm–0.18 mm; P=0.13); the buckling ratio instability index is the composite of narrow neck width and cortical thickness. When re-running the association analysis between CRP and hip fracture for the subset of participants with hip bone geometry data available and entering the hip bone geometry parameters analyzed the risk estimate did not change (HR=1.11; 95% CI: 1.03–1.20; P=0.007 to HR=1.12; 95% CI: 1.04–1.22; P=0.005).

Mendelian Randomization approach

The combined weighted genetic risk score for CRP was highly significantly associated with serum CRP levels in our study ($\beta = 0.011$, $P=2\times10^{-13}$) and explained 3% of the variance in serum CRP levels. However, this score did not show a significant association with fracture risk (HR=1.00; 95% CI: 0.99–1.00; P=0.23). Clearly, the requirement of $\beta_{\text{CRP gene score for serum CRP}} \times \beta_{\text{serum CRP for fracture}} \approx \beta_{\text{CRP gene score for fracture}}$ was not met as $0.011\times0.063=0.0007\neq-0.004$. Neither did association testing for each of the individual 29 SNPs demonstrate any significant effect for fracture (Table 4).

Table 4. Association statistics of individual CRP SNPs with any-type of fracture risk.

SNP	Beta-coefficient for fracture	99.8% confidence interval
rs12037222	0.03	-0.11 – 0.17
rs4420065	0.06	-0.06 – 0.19
rs4129267	0.00	-0.12 – 0.12
rs13962	-0.09	-0.28 – 0.09
rs2794520	0.05	-0.09 – 0.18
rs12239046	-0.02	-0.14 – 0.11
rs1260326	0.01	-0.12 – 0.14
rs1030023	0.01	-0.12 – 0.13
rs6734238	0.10	-0.02 – 0.22
rs4705952	0.00	-0.16 – 0.16
rs16889362	-0.09	-0.31 – 0.13
rs195522	0.04	-0.09 – 0.17
rs6901250	0.00	-0.13 – 0.13
rs13233571	0.02	-0.16 – 0.21
rs9987289	0.02	-0.19 – 0.23
rs4410870	-0.02	-0.15 – 0.10
rs10086637	-0.19	-0.45 – 0.07
rs6486122	0.00	-0.13 – 0.13
rs10745954	-0.01	-0.13 – 0.11
rs1183910	0.03	-0.10 – 0.16
rs4903031	-0.02	-0.17 – 0.12
rs340029	0.07	-0.05 – 0.20
rs10521222	-0.02	-0.33 – 0.29
rs1558902	-0.03	-0.15 – 0.10
rs2847281	0.02	-0.11 – 0.14
rs10460119	-0.06	-0.19 – 0.07
rs4420638	0.10	-0.10 – 0.31
rs1800961	0.02	-0.31 – 0.35
rs2836878	0.07	-0.06 – 0.20

A 99.8% confidence interval corresponds to a correction for 29 independent tests.

DISCUSSION

In this study we report an association between serum CRP levels and any-type of fracture risk for both men and women. Our skeletal-site specific analyses showed effects on at least hip fractures and radiographic vertebral fractures. Using a Mendelian Randomization approach we did not find any evidence for a causal relation. Moreover, even though the association is independent of BMD, it appears to be strongest for individuals with the lowest levels of BMD. Further, we show that higher CRP levels are associated with a lower bending strength (section modulus) of the hip.

Our results from the CRP gene weighted score for fracture risk are in line with the previously proposed hypothesis that complex interactions between inflammation and other factors modulating bone metabolism are likely.[2] Circulating high-sensitivity CRP levels may be a surrogate for unrecognized confounders that affect fracture risk, but it remains difficult to pinpoint which these unrecognized confounders are. CRP levels are known to be increased in multiple conditions, including infections, autoimmune diseases,

some malignancies,[4, 29] and chronic conditions such as obesity and atherosclerosis and therefore predict cardiovascular disease[30] and type 2 diabetes mellitus[19, 31]. Our association analyses between serum CRP and fracture risk have been corrected, to the best of our means, for potential confounders but these corrections did not essentially modify the associations. This lack of a causal relationship of CRP with fracture is similar to the outcome of a large-scale study scrutinizing the association between CRP and clinical cardiovascular events, i.e., incident cases of myocardial infarction and coronary heart disease. After applying the same methodology we used in our study, this well-powered effort also found that neither the individual CRP SNPs nor the combined weighted genetic risk score show consistent or genome-wide significant associations with disease risk.[16] Another group has investigated three tagging SNPs associated with CRP levels in relation to insulin resistance, serum glucose, and diabetes, where the researchers also did not find any conclusive evidence for causality.[32]

As stated before, CRP is elevated in inflammatory diseases. Rheumatoid arthritis,[33, 34] inflammatory bowel disease[35] and spondylarthropathies[36-38] have been found associated with increased risk of fracture and decreased BMD. In systemic lupus erythematosus BMD has been found to be decreased as well, and on top of that, hip bone geometry differences have been detected including an increased buckling ratio and decreases in bending (section modulus) and axial (cross-sectional areas).[39] Our present study also found decreases in section modulus and possibly femoral neck width, but not BMD. These differences did not explain the increase in hip fracture risk. For type 2 diabetes mellitus we have found earlier in the same Rotterdam Study cohort that patients have increased fracture risk, but increases in BMD with a decreased cortical buckling ratio.[40, 41] More specifically, recent work in the Study of Women's Health Across the Nation (SWAN) showed that CRP levels were inversely associated with hip geometry strength indices (a finding in line with those of our study) which partially explained the increased fracture risk observed in SWAN.[42]

The association of these inflammatory diseases with increased fracture risk has frequently been reported to be independent of BMD.[34, 36] On the other hand, the relationship between CRP levels and BMD is less clear. Several studies have reported an inverse relationship between serum CRP and BMD,[5-7, 9] while others have not clearly observed the same relation[2, 8]. In our study, the effect estimates between serum CRP and BMD were negative but not statistically significant. We found that the increased fracture risk in relation to CRP remains after accounting for BMD, which is supported by previous results from the STRAMBO study.[8] In addition, in the same study higher hs-CRP levels were associated with a poor trabecular, but not cortical, microarchitecture at the distal radius as assessed by pQCT in men aged 72 years an older but not in younger men. However, this again was shown not to fully explain the positive association between CRP level and fracture risk. These heterogeneous and multi-factorial observations are in line with the complex nature of bone metabolism and fracture risk.

Our findings suggest that cumulative effects of inflammation may eventually manifest predominantly in those elderly individuals with the frailest bones. The decline in femoral neck BMD with age, involves cortical thinning and widening of the bone width.[23] This is a homeostatic process preserving bone strength (section modulus) through subperiosteal apposition. Our results and those of others postulate that chronic inflammation undermines this compensatory physiological process.[39, 40] The underlying mechanisms underlying the geometrical differences observed remain to be elucidated. Further, experimental studies have demonstrated that inflammatory cytokines stimulate osteoclastic bone resorption[43] and suppress osteoblast function (bone formation).[44] Another very important (potentially) contributing factor is glucocorticoid use,[45] which is frequently indicated for the treatment of inflammatory diseases. Negative effects of steroids include both direct and indirect skeletal effects, displayed in decreases in

BMD and altered bone geometry.[46, 47] Our correction for glucocorticoid use indicates they are responsible for a fraction but not all of the effect of CRP levels on fracture risk. Finally, skeletal loading might be altered in patients with inflammation, because of pain, fatigue, metabolic wasting and steroid-induced myopathy[39] for which CRP levels can be a marker of such statuses.

Strengths of our study are the long follow-up duration and availability of various outcomes and covariables in a large prospective population-based study which includes both men and women. Serum CRP at baseline was measured by the high-sensitivity assay increasing the accuracy of the assessment. Additionally, this is to our knowledge the first study with to investigate the relationship of CRP with hip bone geometry. Yet, our study has some limitations. Causal inference is largely dependent on study power. Even though the instrumental variables (genetic markers) are strongly associated with CRP levels, the compound effect of the included CRP SNPs tested explain ~3-5% of the variance in serum CRP levels; this was the best we could achieve based on our current knowledge of genetics of CRP.[16] A post-hoc power calculation based on data from a simulation-based study by Pierce et al.[48] indicates that the power of our study is limited ranging between 0.11–0.36. Therefore, even though the effect estimates of the relation between the genetic score for CRP-levels and fracture risk is convincingly supports the null (HR=1.0; 95% CI: 0.99–1.00) hypothesis, lack of causality cannot be definitely concluded considering power limitations.

If the relationship between elevated CRP and increased fracture risk is indeed not causal, then interventions focused only on lowering CRP levels are not expected to lower fracture risk.

Future studies might be able to reveal which (set) of the conditions that are associated with elevated CRP can explain the relationship between CRP and fracture risk. In our study the effect seems to be strongest, or perhaps even restricted, to individuals with the lowest ranges of BMD, probably an indication of co-morbidity. Nevertheless, we cannot exclude inflammation can turn out to be more detrimental to bones that are already compromised. Finally, DXA-based bone geometry represents 2D assessments of a 3D structure, whose measurement relies on several assumptions and several of the parameters are not independent of BMD. Unfortunately, we currently do not have more optimal measurements like QCT or MRI available, which accurately measure the underlying bone geometry.

In conclusion, serum high-sensitivity CRP levels are associated with increased incident fracture risk. Nevertheless, there is not enough evidence for this relationship being causal. Finally, it is suggested that high serum CRP may also be associated with lower bending strength at the hip.

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REFERENCES

1. Krieckaert CL, Lems WF. Biologicals and bone loss. Ther Adv Musculoskelet Dis. 2012 Aug;4(4):245-7.
2. Pasco JA, Kotowicz MA, Henry MJ, Nicholson GC, Spilsbury HJ, Box JD, et al. High-sensitivity C-reactive protein and fracture risk in elderly women. JAMA. 2006 Sep 20;296(11):1353-5.
3. Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol. 2009 Jun;201(3):309-20.
4. Watson J, Round A, Hamilton W. Raised inflammatory markers. BMJ. 2012;344:e454.
5. de Pablo P, Cooper MS, Buckley CD. Association between bone mineral density and C-reactive protein in a large population-based sample. Arthritis Rheum. 2012 Aug;64(8):2624-31.
6. Ding C, Parameswaran V, Udayan R, Burgess J, Jones G. Circulating levels of inflammatory markers predict change in bone mineral density and resorption in older adults: a longitudinal study. J Clin Endocrinol Metab. 2008 May;93(5):1952-8.
7. Koh JM, Khang YH, Jung CH, Bae S, Kim DJ, Chung YE, et al. Higher circulating hsCRP levels are associated with lower bone mineral density in healthy pre- and postmenopausal women: evidence for a link between systemic inflammation and osteoporosis. Osteoporos Int. 2005 Oct;16(10):1263-71.
8. Rolland T, Boutroy S, Vilayphiou N, Blaizot S, Chapurlat R, Szulc P. Poor trabecular microarchitecture at the distal radius in older men with increased concentration of high-sensitivity C-reactive protein--the STRAMBO study. Calcif Tissue Int. 2012 Jun;90(6):496-506.
9. Ganesan K, Teklehaimanot S, Tran TH, Asuncion M, Norris K. Relationship of C-reactive protein and bone mineral density in community-dwelling elderly females. J Natl Med Assoc. 2005 Mar;97(3):329-33.
10. Ebrahim S, Davey Smith G. Mendelian randomization: can genetic epidemiology help redress the failures of observational epidemiology? Hum Genet. 2008 Feb;123(1):15-33.
11. Didelez V, Sheehan N. Mendelian randomization as an instrumental variable approach to causal inference. Statistical Methods in Medical Research. 2007;16(4):309-30.
12. Gray R, Wheatley K. How to Avoid Bias When Comparing Bone-Marrow Transplantation with Chemotherapy. Bone Marrow Transplantation. 1991;7:9-12.
13. Dupuis J, Larson MG, Vasan RS, Massaro JM, Wilson PW, Lipinska I, et al. Genome scan of systemic biomarkers of vascular inflammation in the Framingham Heart Study: evidence for susceptibility loci on 1q. Atherosclerosis. 2005 Oct;182(2):307-14.
14. Pankow JS, Folsom AR, Cushman M, Borecki IB, Hopkins PN, Eckfeldt JH, et al. Familial and genetic determinants of systemic markers of inflammation: the NHLBI family heart study. Atherosclerosis. 2001 Feb 15;154(3):681-9.
15. Retterstol L, Eikvar L, Berg K. A twin study of C-Reactive Protein compared to other risk factors for coronary heart disease. Atherosclerosis. 2003 Aug;169(2):279-82.
16. Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, et al. Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. Circulation. 2011 Feb 22;123(7):731-8.
17. Zacho J, Tybjaerg-Hansen A, Jensen JS, Grande P, Sillesen H, Nordestgaard BG. Genetically elevated C-reactive protein and ischemic vascular disease. N Engl J Med. 2008 Oct 30;359(18):1897-908.
18. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
19. Dehghan A, Kardys I, de Maat MP, Uitterlinden AG, Sijbrands EJ, Bootsma AH, et al. Genetic variation, C-reactive protein levels, and incidence of diabetes. Diabetes. 2007 Mar;56(3):872-8.
20. McCloskey EV, Spector TD, Eyres KS, Fern ED, O'Rourke N, Vasikaran S, et al. The assessment of vertebral deformity: a method for use in population studies and clinical trials. Osteoporos Int. 1993 May;3(3):138-47.
21. Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2002 Jun;17(6):1051-6.
22. Burger H, de Laet CE, van Daele PL, Weel AE, Witteman JC, Hofman A, et al. Risk factors for increased bone loss in an elderly population: the Rotterdam Study. Am J Epidemiol. 1998 May 1;147(9):871-9.
23. Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW. Structural trends in the aging femoral neck and proximal shaft: analysis of the Third National Health and Nutrition Examination Survey dual-energy X-ray absorptiometry data. J Bone Miner Res. 2000 Dec;15(12):2297-304.

24. Rivadeneira F, Zillikens MC, De Laet CE, Hofman A, Uitterlinden AG, Beck TJ, et al. Femoral neck BMD is a strong predictor of hip fracture susceptibility in elderly men and women because it detects cortical bone instability: the Rotterdam Study. J Bone Miner Res. 2007 Nov;22(11):1781-90.
25. Pincus T, Summey JA, Soraci SA, Jr., Wallston KA, Hummon NP. Assessment of patient satisfaction in activities of daily living using a modified Stanford Health Assessment Questionnaire. Arthritis Rheum. 1983 Nov;26(11):1346-53.
26. Odding E, Valkenburg HA, Algra D, Vandenouweland FA, Grobbee DE, Hofman A. Association of locomotor complaints and disability in the Rotterdam study. Ann Rheum Dis. 1995 Sep;54(9):721-5.
27. The International HapMap Project. Nature. 2003 Dec 18;426(6968):789-96.
28. Altshuler DM, Gibbs RA, Peltonen L, Dermitzakis E, Schaffner SF, Yu F, et al. Integrating common and rare genetic variation in diverse human populations. Nature. 2010 Sep 2;467(7311):52-8.
29. Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C-reactive protein and the risk of incident colorectal cancer. JAMA. 2004 Feb 4;291(5):585-90.
30. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med. 2004 Apr 1;350(14):1387-97.
31. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA. 2001 Jul 18;286(3):327-34.
32. Brunner EJ, Kivimaki M, Witte DR, Lawlor DA, Davey Smith G, Cooper JA, et al. Inflammation, insulin resistance, and diabetes--Mendelian randomization using CRP haplotypes points upstream. PLoS Med. 2008 Aug 12;5(8):e155.
33. Dirven L, van den Broek M, van Groenendael JH, de Beus WM, Kerstens PJ, Huizinga TW, et al. Prevalence of vertebral fractures in a disease activity steered cohort of patients with early active rheumatoid arthritis. BMC Musculoskelet Disord. 2012;13:125.
34. Orstavik RE, Haugeberg G, Mowinckel P, Hoiseth A, Uhlig T, Falch JA, et al. Vertebral deformities in rheumatoid arthritis: a comparison with population-based controls. Arch Intern Med. 2004 Feb 23;164(4):420-5.
35. van Staa TP, Cooper C, Brusse LS, Leufkens H, Javaid MK, Arden NK. Inflammatory bowel disease and the risk of fracture. Gastroenterology. 2003 Dec;125(6):1591-7.
36. Mitra D, Elvins DM, Speden DJ, Collins AJ. The prevalence of vertebral fractures in mild ankylosing spondylitis and their relationship to bone mineral density. Rheumatology (Oxford). 2000 Jan;39(1):85-9.
37. van der Weijden MA, van der Horst-Bruinsma IE, van Denderen JC, Dijkmans BA, Heymans MW, Lems WF. High frequency of vertebral fractures in early spondylarthropathies. Osteoporos Int. 2012 Jun;23(6):1683-90.
38. van der Weijden MA, Claushuis TA, Nazari T, Lems WF, Dijkmans BA, van der Horst-Bruinsma IE. High prevalence of low bone mineral density in patients within 10 years of onset of ankylosing spondylitis: a systematic review. Clin Rheumatol. 2012 Nov;31(11):1529-35.
39. Alele JD, Kamen DL, Hunt KJ, Ramsey-Goldman R. Bone geometry profiles in women with and without SLE. J Bone Miner Res. 2011 Nov;26(11):2719-26.
40. Oei L, Zillikens MC, Dehghan A, Buitendijk GH, Castano-Betancourt MC, Estrada K, et al. High Bone Mineral Density and Fracture Risk in Type 2 Diabetes as Skeletal Complications of Inadequate Glucose Control: The Rotterdam Study. Diabetes Care. 2013 Jan 11.
41. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol. 2012 May;27(5):319-32.
42. Ishii S, Cauley JA, Greendale GA, Crandall CJ, Danielson ME, Ouchi Y, et al. C-reactive protein, bone strength, and nine-year fracture risk: data from the Study of Women's Health Across the Nation (SWAN). J Bone Miner Res. 2013 Jul;28(7):1688-98.
43. Quinn JM, Gillespie MT. Modulation of osteoclast formation. Biochem Biophys Res Commun. 2005 Mar 18;328(3):739-45.
44. Tanabe N, Ito-Kato E, Suzuki N, Nakayama A, Ogiso B, Maeno M, et al. IL-1alpha affects mineralized nodule formation by rat osteoblasts. Life Sci. 2004 Sep 24;75(19):2317-27.

45. den Uyl D, Bultink IE, Lems WF. Glucocorticoid-induced osteoporosis. Clin Exp Rheumatol. 2011 Sep-Oct;29(5 Suppl 68):S93-8.
46. Canalis E, Mazziotti G, Giustina A, Bilezikian JP. Glucocorticoid-induced osteoporosis: pathophysiology and therapy. Osteoporos Int. 2007 Oct;18(10):1319-28.
47. Kaji H, Yamauchi M, Chihara K, Sugimoto T. Glucocorticoid excess affects cortical bone geometry in premenopausal, but not postmenopausal, women. Calcif Tissue Int. 2008 Mar;82(3):182-90.
48. Pierce BL, Ahsan H, Vanderweele TJ. Power and instrument strength requirements for Mendelian randomization studies using multiple genetic variants. Int J Epidemiol. 2011 Jun;40(3):740-52.

Chapter 2.4

Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies

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ABSTRACT

Type 2 diabetes mellitus (T2DM) influences bone metabolism, but the relation of T2DM with bone mineral density (BMD) remains inconsistent across studies. The objective of this study was to perform a meta-analysis and meta-regression of the literature to estimate the difference in BMD (g/cm^2) between diabetic and non-diabetic populations, and to investigate potential underlying mechanisms. A literature search was performed in PubMed and Ovid extracting data from articles prior to May 2010. Eligible studies were those where the association between T2DM and BMD measured by dual energy X-ray absorptiometry was evaluated using a cross-sectional, cohort or case–control design, including both healthy controls and subjects with T2DM. The analysis was done on 15 observational studies (3,437 diabetics and 19,139 controls). Meta-analysis showed that BMD in diabetics was significantly higher, with pooled mean differences of 0.04 (95% CI: 0.02–0.05) at the femoral neck, 0.06 (95% CI: 0.04–0.08) at the hip and 0.06 (95% CI: 0.04–0.07) at the spine. The differences for forearm BMD were not significantly different between diabetics and non-diabetics. Sex-stratified analyses showed similar results in both genders. Substantial heterogeneity was found to originate from differences in study design and possibly diabetes definition. Also, by applying meta-regression we could establish that younger age, male gender, higher body mass index and higher HbA_{1C} were positively associated with higher BMD levels in diabetic individuals. We conclude that individuals with T2DM from both genders have higher BMD levels, but that multiple factors influence BMD in individuals with T2DM.

INTRODUCTION

Osteoporosis and diabetes are both common human diseases. Albright and Reifenstein reported their coexistence in 1948,[1] but hitherto the association between them remains unclear. Due to the different pathogenesis of type 1 and type 2 diabetes mellitus (T2DM), it is not surprising that there is no uniform entity of diabetic bone disease as such. While decreased bone mineral density (BMD) has consistently been observed in type 1 diabetes mellitus patients,[2, 3] studies on BMD investigated in T2DM showed contradictory results with higher, lower or similar values in comparison with healthy control subjects.[4-7] These inconsistent findings may be related to vast differences in study design, BMD measurement technology, differences in site of BMD examination, selection of patients, and presence or absence of complications.

It is well known that advanced age is a risk factor for bone loss and osteoporosis.[8, 9] Some of the attributed mechanisms include increased production of inflammatory cytokines and cellular components, incremental osteoclast precursors generation and decreased bone preservation due to gonadal failure resulting in lower tissue production of sex steroids.[10] Advanced age is also associated with increased fall frequency, lack of exercise, use of drugs that negatively influence bone metabolism and renal function such as drugs prescribed for diabetes and hypertension.

Gender also appears to have an important effect on the relation between BMD and T2DM. Barrett-Connor[11] found that older women with T2DM had higher BMD levels at all sites compared to those with normal glucose tolerance, but this effect was not observed in men. It has also been suggested that obesity and hyperinsulinemia can lead to lower bone turnover in diabetic women,[7, 12] so that the adverse effects of estrogen deficiency on bone mass are attenuated and delayed after menopause.

Many studies have shown a difference in population characteristics between type 2 diabetic patients and healthy controls.[6, 11, 13, 14] Diabetic study participants tend to have a higher body mass index (BMI) or weight, increased insulin levels, less physical exercise, higher alcohol consumption and they usually smoke more. The use of diuretics is more common in diabetes. These characteristics might influence bone metabolism independently of diabetes. Paradoxically, an increased risk of osteoporotic fracture in T2DM has been repeatedly demonstrated and this was independent of BMD.[13, 15] This association with fracture adds uncertainty around the actual association between diabetes mellitus and BMD.

The aim of our study was to perform meta-analysis of published articles exploring differences between type 2 diabetics and healthy individuals in BMD levels measured at four anatomical sites. In addition, we evaluated factors influencing BMD variation like sex, age, BMI and glucose control (HbA_{1C} levels) for which a meta-regression was performed to evaluate potential mechanisms by which T2DM influences BMD variation.

METHODS

Search strategy

A systemic search for all literature that was published in May 2010 or earlier was performed using Pubmed and Ovid online (1950 to present with daily update). The search used MeSH terms "diabetes mellitus" and ("osteoporosis" OR "bone density" or "bone mass").

Study selection

Studies were considered eligible for the meta-analysis if (1) they evaluated the association between T2DM and BMD, (2) they were of a cross-sectional, cohort or case–control design, (3) they included

healthy subjects without DM as controls, (4) they reported gender-stratified statistics on both individuals with and without T2DM, (5) BMD was measured by dual energy X-ray absorptiometry (DXA) and (6) BMD measurements were expressed as an absolute value in g/cm^2. In the cases that more than one article presented data from the same study population, the study with more complete reporting of data was selected.

Studies in nonhuman populations, review articles, experimental studies, case reports or studies that lacked controls, studies on type 1 or other types of DM, studies that had no clear definition of T2DM, studies that measured BMD measured by computed tomography, ultrasound or single X-ray absorptiometry were all regarded as ineligible.

Only published results were used and papers in all languages were considered. We supplemented electronic searches by hand-searching reference lists of relevant articles and reviews. The abstracts and titles of primitive collections were initially browsed and all observational studies were extracted. Potentially relevant articles were then considered by double checkout. Disagreements were resolved by discussion between at least two reviewers.

Data

Quality-scoring varies in meta-analyses of observational studies and no criteria have been internationally accepted to date. Consequently, we appraised each article included in this analysis with the guidelines of the MOOSE group[16]. Some key points were: clear definition of study population, clear and internationally accepted criteria of diagnosing diabetes, description of the coefficient of variation for BMD measurements, consecutive selection of cases, random selection of controls and identification of important confounders. We required that at least 2 studies per site-specific BMD outcome should be available to perform a meta-analysis.

Mean and its standard deviation (SD) of BMD measurements at the calcaneus, femoral neck, total hip, spine and forearm in both diabetics and non-diabetics were extracted to explore the pooled mean difference estimation. If repeated measurements were available in cohort studies we extracted only the measurements at baseline (or the earliest available measurement) as being a cross-sectional study. The mean and standard deviation had to be unadjusted due to large variance of adjusted factors between different studies. If there were statistically significant age differences between patients and controls and the age-adjusted mean and deviation could be found, these data were used; if these were not found the study was excluded. In addition, we performed meta-analysis including the maximally adjusted estimates from studies where available. If sample size of either group in comparison was less than 30, it was not used in our analysis. Gender was considered to be a determinant for subgroup analysis.

If studies lacked SD estimates but provided P value, standard error (SE), confidence interval (CI) that related to the mean difference, we estimated SDs using the following methods[17]:

1. From SE to SD: the following formula was used:

$$SD = \frac{SE}{\sqrt{\frac{1}{Ncase} + \frac{1}{Ncontrol}}}$$

2. From CI to SD: SE=(upper limit - lower limit)/3.92 (if 95% CI), then replaced in formula.

3. From P value to SD: the corresponding t-value according to P value was obtained from a table of the t-distribution with the degrees of freedom given by $N_{case}+N_{control}-2$ (where N_{case}, $N_{control}$ are the sample sizes); then, assuming

$$SE = \frac{MD}{t}$$

(where MD is mean difference between case and control); we finally replaced SE in the formula:

$$SD = \frac{SE}{\sqrt{\frac{1}{Ncase} + \frac{1}{Ncontrol}}}$$

(where SD is the average of the SDs of the case and control arms);

Analyses

The weighted mean difference estimates of BMD in g/cm^2 comparing diabetes with controls were calculated as Der-Simonian and Laird estimators using random effects models. As secondary analyses inverse variance fixed effect models were applied. Publication bias was tested using funnel plots. Tests for heterogeneity were performed by applying the Cochran Q test and estimating the degree of inconsistency index (I^2)[18]. Sources of heterogeneity were investigated by sensitivity analyses stratifying on study design, by excluding studies: on Asian populations, presenting large differences in BMI between cases and controls, and/or having BMD measurements assessed by different densitometers. All analyses were conducted with the use of Review Manager, version 5.0 (Revman, The Cochrane Collaboration; Oxford, UK) and Comprehensive Meta-analysis version 2 (Biostat, Inc., Englewood, USA). To estimate the effects of gender, age, BMI and HbA_{1C} on the BMD measured at the different sites a meta-regression analysis was performed using STATA 11.0 (StataCorp LP, USA).

RESULTS

Figure 1 shows a flow diagram describing the study selection process. The initial search yielded 1,161 research reports, of which 222 were excluded for having the same title or authors; 788 were excluded due to not eligible study design (including non-human studies, review articles, case reports, comment, letter, experimental study, and/or fracture-only outcome). Additional 109 studies were found irrelevant to the original research question and excluded because the disease of interest was either type 1 or gestational DM (81 studies); or for not measuring bone mass using DXA, i.e. by single X-ray absorptiometry, CT or ultrasound (28 studies). Of the 42 remaining studies, 11 either lacked non-diabetic controls at all or did not report means and standard deviations in non-diabetic controls[19-29]. In addition, six studies had small sample sizes (N<30) in either group of comparison[30-35]. The study population of two studies was used in follow-up reports.[4, 36] In three studies there was a big age difference between individuals with diabetes and those without diabetes, but the investigators did not adjust for it.[37-39] One study matched cases and controls by age and BMI and presented data only on post-matching.[40] The original articles of four articles could not be retrieved.[41-44] All of these aforementioned studies were excluded. One study cited as reference in one of the research reports was traced and satisfied the inclusion criteria.[45] In one research report the results of gender-specific BMD analyses was mentioned, but not listed in detail.[14] We contacted the researchers and were able to retrieve this information. The study of Perez et al. found a significantly increased calcaneal BMD in female but not in males subjects with diabetes.[46] No meta-

analysis was attempted for this site since this was the only study that evaluated BMD at the calcaneus. Since no SD's for male comparison groups could be retrieved for the paper by Barrett-Connor et al. we were not able to include these results for men. As we extracted only a single measure and didn't examine repeated measurements, cohort studies were analyzed as cross-sectional using the baseline or earliest available measurement. A total of 15 observational studies (9 case–control, 6 cross-sectional) were included in our meta-analysis (3,437 diabetics and 19,139 controls).[5-7, 11, 12, 14, 45, 47-54] Table 1 indicates the quality evaluation of all studies. We did not observe indication of publication bias on the Funnel Plots (data not shown), with the effect magnitude of larger studies being closer to and smaller studies largely equally distributed at both sides of the summary estimate.

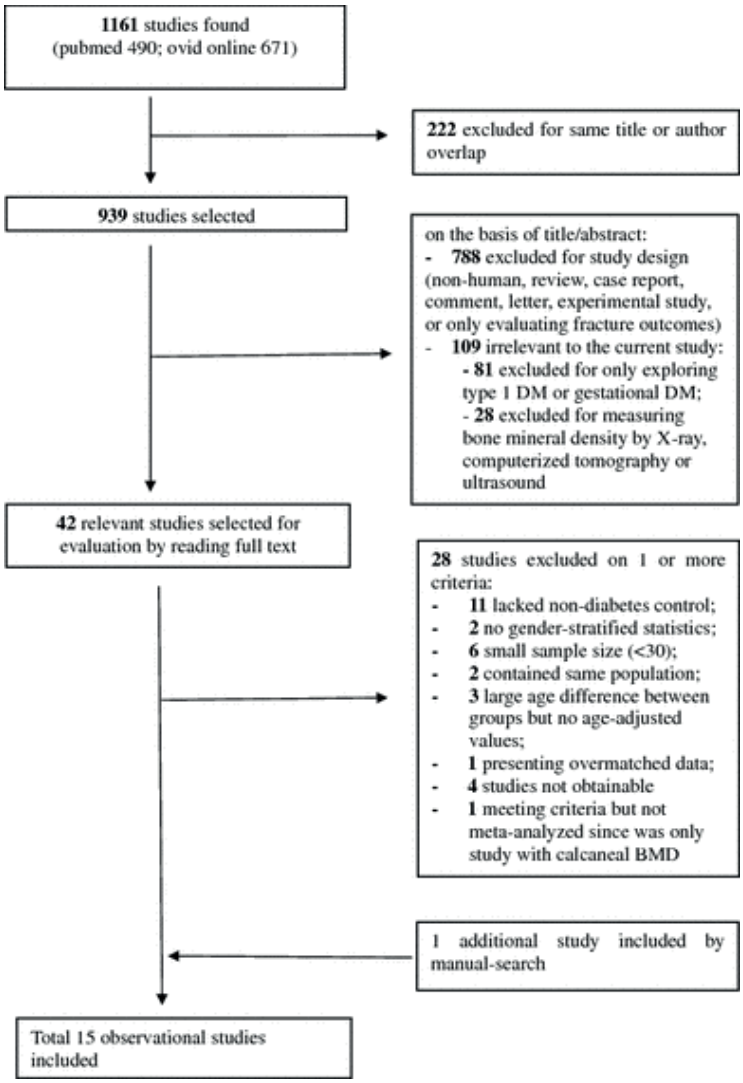

Figure 1. Flow diagram of the study-selection process. DM diabetes mellitus, CT computed tomography, US ultrasound.

Table 1. Aspects of quality and design of the included articles.

Reference	Study design	Clear definition of study population	Clear criteria of diagnosing diabetes	Precise control (CV) for BMD measurement	Consecutive selection of cases	Random selection of controls	Identification of important confounders
Barrett-Connor[11]	Cross-sectional	Yes	WHO criteria	NA	Yes	Yes	Yes
Sosa[47]	Case–control	Yes	NDDG criteria (Canada)	Yes	No	No (age-matched)	Yes
Tuominen[48]	Case–control	Yes	NA (hospital database)	NA	Yes	Yes	Yes
Kao[6]	Cross-sectional	Yes	WHO criteria, self-reported	Yes	Yes	Yes	Yes
Dennison[49]	Cross-sectional	Yes	OGTT	Yes	Yes	Yes	Yes
Bridges[50]	Case–control	Yes	NA (hospital database)	NA	No	Yes	Yes
Gerdhem[12]	Cross-sectional	Yes	Self-reported	Yes	Yes	Yes	Yes
de Liefde[14]	Cross-sectional	Yes	Screening (OGTT), drug use	Yes	Yes	Yes	Yes
Majima[7]	Case–control	Yes	OGTT	NA	Yes	Yes	Yes
Schwartz[51]	Case–control	Yes	FPG, OGTT, self-reported	Yes	Yes	Yes	Yes
Bonds[45]	Cross-sectional	Yes	Self-reported, drug use	NA	Yes	Yes	No
Rakic[52]	Case–control	Yes	WHO criteria	Yes	Yes	No (age-, sex-matched)	Yes
Hadzibegovic[53]	Case–control	Yes	NA	NA	Yes	Yes	Yes
Anaforoglu[54]	Case–control	Yes	NA (hospital database)	NA	Yes	No (age-matched)	Yes
Yaturu[5]	Case–control	NA	NA	Yes	Yes	Yes	Yes

Table 2. Characteristics of the study population and the effects of covariates on BMD.

Reference	Ethnicity/ nation	Gender (%women)	Age (y)	Covariates: comparison diabetes and non-diabetes (P value)	Findings
Barrett-Connor[11]	USA	61	55–88	NS: BMI, cigarette smoking, alcohol use (men), regular exercise, diuretic use (women), estrogen use <0.01: alcohol use (women), diuretic use (men)	No change of statistical significance of mean difference when adjusted for covariates
Sosa[47]	Spain	100	61.3/58.8	<0.05: weight	Analysis of variance (ANOVA) was used to examine the effects of diabetes and weight in bone mass. There were no statistical differences.
Tuominen[48]	Finland	52	45–64	NS: BMI <0.01: use of loop diuretics	No change of statistical significance of mean difference when adjusted for covariates
Kao[6]	USA	64	30–96	NS: diuretics (women), smoking (men), physical activity, calcium intake, estrogen use, menopause status <0.05: diuretics (men), smoking (women), alcohol, BMI	After adjusted for covariates, the increase of BMD attenuated but the decrease expanded No significant difference between newly diagnosed and previously diagnosed diabetes Positive correlation (hip, forearm): insulin level
Dennison[49]	UK	45	59–72	NA	After adjustment for BMI, all relationship were diminished, even femoral neck and total femur lose significance
Bridges[50]	UK	0	≥25	<0.01: BMI	Positive correlation: BMI No significant correlation: HbA_{1C}, disease duration, diabetic complication
Gerdhem[12]	Sweden	100	75	<0.001: body weight	Adjustment for body weight, significance remained but the mean difference attenuated
de Liefde[14]	Netherlands	61	≥55	<0.05: BMI, lower limb disability, smoking, baseline use of thiazides, baseline use of loop diuretics	No change of statistical significance of mean difference when adjusted covariates
Majima[7]	Japan	56	≥32	NS: BMI, Scr <0.01: FPG	Positive correlation: BMI, insulin level, HbA_{1C} No significant correlation: FPG
Schwartz[51]	USA	50	70–79	NS: IL-6 (black, white men), current smoker, walking speed (black), statin use, oral estrogen use, renal insufficiency(black), vitamin D supplement use <0.05: weight, weight change, IL-6 (white women), walking speed (white), renal insufficiency (white)	After adjusting for covariates, white women with DM lost more BMD per year on average than those without DM Adjustment for weight loss resulted in the largest attenuation in the association between DM and bone loss
Bonds[45]	USA	100	64.9/63.5	NA	NA

Table 2. Characteristics of the study population and the effects of covariates on BMD. (continued)

Reference	Ethnicity/ nation	Gender (%women)	Age (y)	Covariates: comparison diabetes and non-diabetes (P value)	Findings
Rakic[52]	Australia	44	Female: 65.5/64.8 Male: 66.0/66.3	NA	Adjustment for BMI, statistical significance of the mean differences was lost at the spine (women) and hip (men) Negative correlation: serum triglycerides, HbA_{1C}
Hadzibegovic[53]	Croatia	100	41–84	NS: BMI, menarche age, alkaline phosphatase	Positive correlation: BMI, menarche age Negative correlation: alkaline phosphatase
Anaforoglu[54]	Turkey	100	61.9/60.1	<0.05: BMI, calcium intake	Adjustment for BMI and calcium intake, no statistical significant change
Yaturu[5]	USA	0	67.5/66.2	<0.05: BMI, smoking, alcohol	Matched covariates, statistical significance of mean difference at the spine was lost and at the hip was cut down

Table 2 shows study population characteristics and the reported effect of covariates on the association between BMD and T2DM. Out of five studies performed in the US, one had included Mexican–American women[6] and one had white and black participants[51]. One study was done in Eastern Asia[7] and another two in Eastern Europe[53, 54]. The remaining eight studies collected data in Western Europe and Oceania. Participants in all study populations were aged 25 years and over and approximately 70 % were middle-aged or older. In addition, Table 2 shows that the most common covariates considered by the studies were BMI or weight, cigarette smoking, alcohol use, physical activity, diuretic use, calcium intake, estrogen use (women), menopause status (women), age at menarche (women), insulin level, HbA_{1C} and alkaline phosphatase. Table 3 shows the population characteristics of the source studies by gender.

Table 4 presents BMD levels in diabetics and non-diabetics at four skeletal sites across the different studies, also including subgroup analysis by gender. At the femoral neck, all studies except for Yaturu et al.[5] and Majima[7] found a higher BMD in subjects with diabetes. At the total hip, all referred studies showed significantly higher BMD in diabetics. At the lumbar spine, almost all of the studies reported a higher BMD in diabetics. These differences were statistically significant in the vast majority. At the forearm there were no significant differences between diabetics and non-diabetics in all analyses. No major differences between genders were found.

Table 3. Population characteristics of the source studies by gender.

	Female					Male				
Study	Age (years)	BMI (kg/m^2)	HbA_{1C} (%)	Serum creatine (µmol/L)	Disease duration (years)	Age (years)	BMI (kg/m^2)	HbA_{1C} (%)	Serum creatine (µmol/L)	Disease duration (years)
Barrett-Connor[11]	76.0	26.3	6.7	99.7	NA	76.0	26.3	6.7	99.7	NA
Tuominen[48]	63.3	25.3	9.8	NA	NA	63.3	25.3	9.8	NA	NA
Kao[6]	54.3	33.0	NA	NA	NA	54.3	33.0	NA	NA	NA
Dennison[49]	64.8	26.6	NA	NA	NA	64.8	26.6	NA	NA	NA
Bridges[50]	62.8	31.4	8.9	NA	10.1	62.8	31.4	8.9	NA	10.1
de Liefde[14]	69.6	25.8	NA	96.2	NA	69.6	25.8	NA	96.2	NA
Majima[7]	62.8	23.6	7.8	66.3	NA	62.8	23.6	7.8	66.3	NA
Schwartz[51] (white)	73.7	NA	7.2	NA	7.4	73.7	NA	7.2	NA	7.4
Schwartz[51] (black)	74.0	NA	8.2	NA	9.5	74.0	NA	8.2	NA	9.5
Rakic[52]	66.0	29.0	7.4	94.0	8.7	66.0	29.0	7.4	94.0	8.7
Yaturu[5]	67.5	30.1	NA	106.1	NA	67.5	30.1	NA	106.1	NA

Some reports concluded that the association remained significant despite the fact that the effect size decreased remarkably after correcting for aforementioned covariates.[6, 11, 12, 14, 48, 54] In others, the association disappeared or even shifted in the opposite direction after adjustment for covariates, particularly in the case of BMI or weight.[5, 49, 51, 52] We performed meta-analysis for maximally adjusted estimates where available, which did not significantly alter previously calculated mean differences. Nearly all studies found that BMI was positively correlated with BMD. There was some evidence suggesting that other factors such as insulin levels also had a positive correlation with BMD.[7] In contrast, HbA_{1C} levels had positive,[7] negative[51] or no correlation[50] with BMD. In a follow-up study, Schwartz[51] found that after adjustment for covariates white women with T2DM lost on average more BMD per year than those without DM.

Table 5 shows meta-analysis results of pooled mean differences and corresponding 95% confidence intervals of BMD values between diabetic and non-diabetic individuals. In the pooled meta-analyses the

Table 4. Unadjusted/age-adjusted, gender-specific BMD in patients with diabetes and controls per skeletal site (mean ± SD g/cm^2).

	Female				Male			
Reference	Sample size (case/control)	Diabetes	Non-diabetes	P value	Sample size (case/control)	Diabetes	Non-diabetes	P value
Skeletal site of BMD measurement: femoral neck								
Barrett-Connor[11]	37/237	0.664 ± 0.118[a]	0.610 ± 0.118[a]	<0.01	41/139	0.747 ± NA	0.744 ± NA[a]	NS
Sosa[47]	47/252	0.756 ± 0.146	0.737 ± 0.115	NS				
Tuominen[48]					34/240	0.881 ± 0.143	0.872 ± 0.131	NS
Dennison[49]	32/278	0.830 ± 0.120	0.740 ± 0.110	<0.0001	33/349	0.900 ± 0.130	0.840 ± 0.110	0.03
Gerdhem[12]	67/961	0.820 ± 0.120	0.740 ± 0.110	<0.0001				
de Liefde[14]	326/3,049	0.859 ± 0.148	0.826 ± 0.134	<0.0001	254/2,195	0.946 ± 0.149	0.914 ± 0.136	0.0003
Majima[7]	81/54	0.620 ± 0.153	0.660 ± 0.118	NS	64/41	0.759 ± 0.137	0.767 ± 0.108	NS
Schwartz[51] (white)	97/383	0.670 ± 0.110	0.640 ± 0.100	<0.05	153/395	0.800 ± 0.120	0.760 ± 0.130	<0.05
Schwartz[51] (black)	125/225	0.790 ± 0.130	0.730 ± 0.130	<0.05	105/169	0.890 ± 0.140	0.830 ± 0.120	<0.05
Rakic[52]	86/86	0.808 ± 0.153	0.722 ± 0.103	<0.001	108/108	0.851 ± 0.128	0.802 ± 0.129	0.01
Hadzibegovic[53]	130/166	0.870 ± 0.132	0.832 ± 0.134	<0.05				
Anaforoglu[54]	206/61	0.770 ± 0.110	0.730 ± 0.120	0.280				
Yaturu[5]					735/3,458	0.892 ± 0.244[b]	0.930 ± 0.176[b]	<0.0001
Skeletal site of BMD measurement: total hip								
Schwartz[51] (white)	97/383	0.790 ± 0.120	0.750 ± 0.120	<0.05	153/395	0.950 ± 0.130	0.930 ± 0.140	<0.05
Schwartz[51] (black)	125/225	0.910 ± 0.150	0.840 ± 0.150	<0.05	105/169	1.070 ± 0.150	1.000 ± 0.130	<0.05
Bonds[45]	469/5,916	0.900 ± 0.160	0.840 ± 0.140	<0.01				
Rakic[52]	86/86	0.993 ± 0.173	0.848 ± 0.118	<0.001	108/108	1.060 ± 0.156	1.013 ± 0.158	0.038
Skeletal site of BMD measurement: spine								
Barrett-Connor[11]	37/237	0.962 ± 0.225[a]	0.859 ± 0.225[a]	<0.01	41/136	1.081 ± NA[a]	1.069 ± NA[a]	NS
Sosa[47]	47/252	0.898 ± 0.137	0.892 ± 0.138	NS				
Kao[6]	98/285	1.071 ± 0.188[b]	1.011 ± 0.236[b]	<0.01	55/162	1.057 ± 0.222[b]	1.063 ± 0.255[b]	NS
Dennison[49]	32/278	1.070 ± 0.180	0.940 ± 0.180	0.0001	33/349	1.160 ± 0.120	1.070 ± 0.160	0.005
Gerdhem[12]	67/961	1.070 ± 0.230	0.990 ± 0.190	0.0001				
de Liefde[14]	327/3,052	1.084 ± 0.188	1.030 ± 0.179	<0.0001	255/2,205	1.196 ± 0.209	1.161 ± 0.196	0.007
Majima[7]	81/54	0.861 ± 0.193	0.831 ± 0.162	NS	64/41	0.972 ± 0.176	0.975 ± 0.108	NS
Bonds[45]	472/5,922	1.040 ± 0.190	0.970 ± 0.170	<0.01				

Table 4. Unadjusted/age-adjusted, gender-specific BMD in patients with diabetes and controls per skeletal site (mean ± SD g/cm^2). (continued)

	Female				Male			
Reference	Sample size (case/control)	Diabetes	Non-diabetes	P value	Sample size (case/control)	Diabetes	Non-diabetes	P value
Rakic[52]	86/86	1.031 ± 0.171	0.948 ± 0.152	<0.001	108/108	1.117 ± 0.176	1.102 ± 0.191	0.55
Hadzibegovic[53]	130/166	0.903 ± 0.165	0.824 ± 0.199	<0.001				
Anaforoglu[54]	206/61	0.900 ± 0.160	0.870 ± 0.150	0.264				
Yaturu[5]					735/3,458	1.223 ± 0.217[b]	1.149 ± 0.176[b]	<0.0001
Skeletal site of BMD measurement: forearm								
Kao[6]	98/285	0.477 ± 0.079[b]	0.463 ± 0.101[b]	NS	55/162	0.535 ± 0.096[b]	0.547 ± 0.102[b]	NS
Bridges[50]					90/50	0.560 ± 0.097[c]	0.560 ± 0.090[c]	NS
Majima[7]	81/54	0.493 ± 0.109	0.547 ± 0.095	<0.01	64/41	0.665 ± 0.092	0.721 ± 0.080	<0.05
Rakic[52]	86/86	0.540 ± 0.066	0.481 ± 0.068	<0.001	108/108	0.641 ± 0.062	0.627 ± 0.063	0.09
Hadzibegovic[53]	130/166	0.496 ± 0.065	0.485 ± 0.081	NS				
Anaforoglu[54]	206/61	0.48 ± 0.050	0.49 ± 0.010	0.696				

differences were 0.04 (95% CI: 0.02–0.05) at the femoral neck, 0.06 (95% CI: 0.04–0.08) at the hip, 0.06 (95% CI: 0.04–0.07) at the spine, and -0.003 (95% CI: -0.02–0.02) at the forearm, respectively. In the sex-stratified analysis these differences were most pronounced for females, being 0.04 (95% CI: 0.03–0.06), 0.07 (95% CI: 0.04–0.11), 0.07 (95% CI: 0.05–0.09), 0.01 (95% CI: -0.02–0.03) at the femoral neck, hip, spine, and forearm, respectively. In males these differences were statistically significant at the hip 0.04 (95% CI: 0.01–0.08) and spine 0.05 (95% CI: 0.02–0.07). The meta-analysis result in males was non-significant at the femoral neck 0.03 (95% CI: 0.00–0.05) and forearm -0.01 (95% CI: -0.04–0.02). This information is displayed in more detail in the forest plots of Figures 2, 3, 4, and 5.

Table 5. Pooled mean differences of BMD comparing diabetes with non-diabetes.

Site	Groups	Number of studies	Sample size (case/control)	Mean difference of BMD (g/cm^2)	P value	Heterogeneity	
						I^2 (%)	Q test P value
Femoral neck	Total	12	2,720/12,707	0.04 [0.02, 0.05]	<0.00001	83	<0.0001
	Female	10	1,234/5,752	0.04 [0.03, 0.06]	<0.00001	71	0.0002
	Male	7	1,486/6,955	0.03 [0.00, 0.05]	0.09	87	<0.0001
Hip	Total	3	1,143/7,282	0.06 [0.04, 0.08]	<0.00001	78	0.0002
	Female	3	777/6,610	0.07 [0.04, 0.11]	<0.00001	82	0.001
	Male	2	366/672	0.04 [0.01, 0.08]	0.007	63	0.07
Spine	Total	12	2,833/17,677	0.06 [0.04, 0.07]	<0.00001	66	<0.0001
	Female	11	1,583/11,354	0.07 [0.05, 0.09]	<0.00001	62	0.003
	Male	6	1,250/6,323	0.05 [0.01, 0.07]	0.008	74	0.002
Forearm	Total	6	918/1,013	−0.003 [−0.02, 0.02]	0.90	88	<0.0001
	Female	5	601/652	0.01 [−0.02, 0.03]	0.68	93	<0.0001
	Male	4	317/361	−0.01 [−0.04, 0.02]	0.44	79	0.003

The weighted mean difference estimates of BMD were calculated as DerSimonian and Laird estimators using random effects models Tests for heterogeneity were performed by applying the Cochran Q test.

The heterogeneity (Q) tests showed significant differences between individual studies (P<0.01) at all sites in the total group and sex-specific analyses (Table 5). Still, point estimates and statistical significance from fixed effects models were very similar to those derived from random effects models. We further performed sensitivity analyses to identify potential sources of the observed heterogeneity. Subgroup analyses per study design (case–control/cross-sectional) showed that case–control studies had effect estimates with larger variation around the pooled estimate thereby increasing the heterogeneity. For the femoral neck BMD analysis the largest source of heterogeneity was traced back to one study by Yaturu et al.[5] This study include only men and observed a positive relation with lumbar spine and a negative one for femoral neck; after removing this study the I^2 statistic dropped from 81 to 57%. Another study in Asians also displayed estimates in the opposite direction for different outcomes though not significant[7]. Removing seven studies with significantly different BMI between diabetes and non-diabetes[5, 12, 14, 47, 50, 51, 54] or six studies that did not use a densitometer manufactured by Hologic incorporation (USA)[5, 12, 14, 48, 50] from the analyses showed no significant influence on the observed heterogeneity, except for the femoral neck BMD analysis, but this was largely attributable to the large heterogeneity brought in by the Yaturu et al. study[5].

The results of a meta-regression on BMD by sex, age, BMI and glucose control (HbA_{1C} levels) is presented in Table 6 for individuals from the diabetic group of the studies. Being a woman was associated

with significantly lower BMD levels at all four anatomical sites, as compared to men. Age was negatively associated with BMD at hip but positively at the lumbar spine. Higher BMI was a strong determinant of higher BMD at the femoral neck and lumbar spine, with no apparent effect on forearm BMD. Higher HbA_{1C} levels (reflecting lesser glucose control) resulted in higher BMD at the femoral neck and total hip.

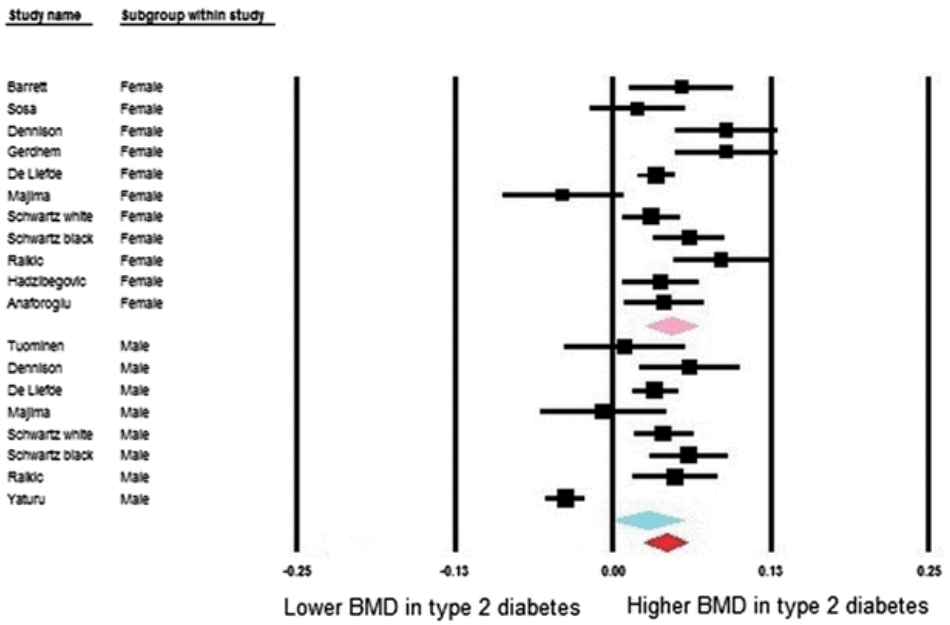

Figure 2. Forest plot for mean femoral neck bone mineral density. Difference in means (g/cm^2) and 95% confidence interval for femoral neck bone mineral density between comparison groups with and without type 2 diabetes mellitus, stratified per study and gender. Diamonds represent joint estimate for subgroups of available studies for women (upper) and men (middle), respectively. Pooled estimate for all studies displayed with the diamond at the bottom.

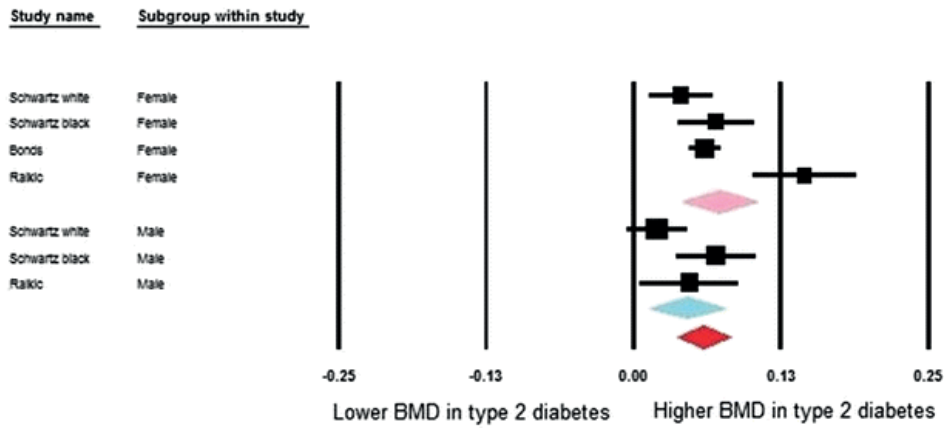

Figure 3. Forest plot for mean hip bone mineral density. Difference in means (g/cm^2) and 95% confidence interval for hip bone mineral density between comparison groups with and without type 2 diabetes mellitus, stratified per study and gender. Diamonds represent joint estimate for subgroups of available studies for women (upper) and men (middle), respectively. Pooled estimate for all studies displayed with the diamond at the bottom.

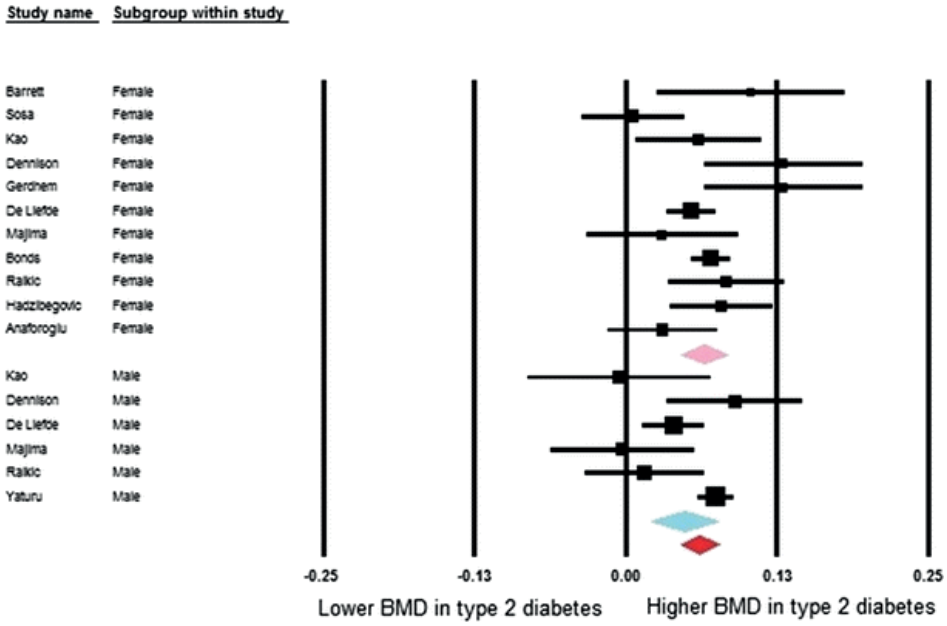

Figure 4. Forest plot for mean spine bone mineral density. Difference in means (g/cm^2) and 95% confidence interval for spine bone mineral density between comparison groups with and without type 2 diabetes mellitus, stratified per study and gender. Diamonds represent joint estimate for subgroups of available studies for women (upper) and men (middle), respectively. Pooled estimate for all studies displayed with the diamond at the bottom.

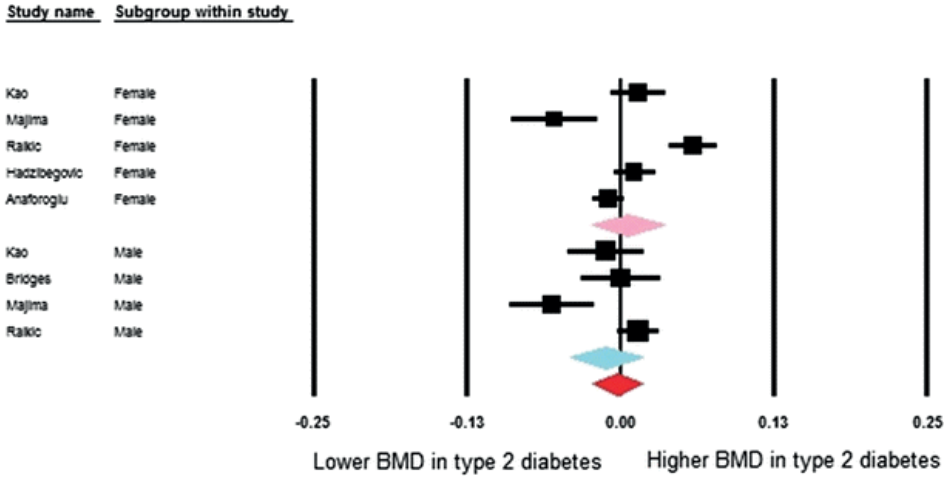

Figure 5. Forest plot for mean forearm bone mineral density. Difference in means (g/cm^2) and 95% confidence interval for forearm bone mineral density between comparison groups with and without type 2 diabetes mellitus, stratified per study and gender. Diamonds represent joint estimate for subgroups of available studies for women (upper) and men (middle), respectively. Pooled estimate for all studies displayed with the diamond at the bottom.

Table 6. Meta-regression results for BMD for individuals from the diabetic group of the studies.

Site	Gender (female–male)	Age (years)	BMI (kg/m^2)	HbA_{1C} (%)
Femoral neck	−0.114 ± 0.012*	0.002 ± 0.002	0.022 ± 0.002*	0.045 ± 0.013*
Hip	−0.119 ± 0.021*	−0.015 ± 0.003*	–	0.117 ± 0.024*
Spine	−0.164 ± 0.018*	0.030 ± 0.006*	0.029 ± 0.004*	0.241 ± 0.090*
Forearm	−0.150 ± 0.050*	0.001 ± 0.013	−0.001 ± 0.006	−0.062 ± 0.052

Values are regression coefficients ± SEM, *P value <0.05

DISCUSSION

Our study provides insights into the inconsistently reported relationship between T2DM and BMD. In line with what is suggested by the majority of reviewed studies our meta-analysis concluded that overall individuals with T2DM have about 25–50% SD higher BMD compared to nondiabetic control subjects.

In this study we found no strong evidence for skeletal site specificity of this association. Subjects with T2DM had elevated BMD at the femoral neck, hip, and spine. No major differences in BMD at the forearm were seen but there are no obvious biological reasons we can attribute to them. This lack of association with forearm BMD may be the consequence of limited sample size. We also found no strong evidence suggesting there is sex-specificity in the observed BMD differences between diabetics and nondiabetics. BMD differences seem larger in women than in men but power limitations can also play a role. We did find considerable heterogeneity influencing the association as reflected by a high I^2 statistic. This large heterogeneity could most probably stem from a large variation in types of study design, diagnostic definitions and individual characteristics that were not considered by each study. We did sensitivity analyses trying to find sources of heterogeneity and concluded that study design and Asian ethnicity are a likely, but not sufficient sources to explain the observed heterogeneity. In contrast, differences in DXA manufacturers and levels or correction for BMI do not seem to be an important source of heterogeneity.

Our study has limitations. We procured including all eligible studies to the best of our capacities but at least four studies were not able to be traced back. Sensitivity analyses considering such studies did not essentially change our results or conclusions. Variation in the definition of T2DM was present across studies with some combining selfreports and blood glucose tests, while others only used blood glucose tests. Studies which relied either on selfreports, population screening or which used register data will be subject to potential disease misclassification bias. Similarly, differences in mode of diagnosis can affect the prevalence of disease across studies and, hence, influence the power for detecting BMD differences. Disease duration can also be an important confounder, but uniform assessment for this co-variable was not possible across studies. Another drawback is that not all studies reported on or adjusted for covariates. Yet another potential source for heterogeneity that we could not control for are differences in glucose control and prevalence of diabetic complications. Nevertheless, the meta-regression done for BMD on the group of diabetic individuals across studies shows that in addition to BMI, HbA_{1C} levels also has a significant positive effect on BMD measured at any site.

Since May 2010 about 134 articles have been published on the topic of which we could identify two that would have met our inclusion criteria[55, 56]. These were studies based on Chinese populations showing opposite results with one concluding type 2 diabetics had higher BMD[55] while the other[56] concluded diabetics had lower BMD and higher risk of osteoporosis.

Mechanisms that might account for an association between T2DM and increasing BMD are plentiful and largely unclear. We discuss below from a clinical perspective the most important factors which can influence the relationship between T2DM and BMD.

Obesity

Historically, overweight and hyperinsulinemia have been postulated as two important features of T2DM which are positively correlated with BMD. Yet, we saw that in a considerable number of the included studies the correction for BMI did not essentially modify the association. There are several complex pathways by which obesity may influence the relation between diabetes and BMD. Body fatness may have an impact on the accuracy of DXA-based BMD measures as demonstrated in obese diabetic patients.[57] Yet, such measurement error should be negligible considering that this phenomenon can either under or overestimate the values and have been shown to have low impact on the accuracy of the BMD measurement.[58] On the other hand, adipose tissue releases a wide variety of adipokines that have been implicated either directly or indirectly in the regulation of bone remodeling.[59] Plasma leptin concentrations have been shown to be higher in diabetic men than in healthy controls.[60] Leptin induces bone growth by stimulating osteoblast proliferation and differentiation in vitro[61-63] and it has also been shown to inhibit osteoclastogenesis through reducing RANK/RANKligand production and increasing osteoprotegerin[64, 65]. Other adipokines such as adiponectin and resistin are also expressed in osteoblasts and osteoclasts.[66, 67] The effects of these adipokines on bone metabolism remain largely ambiguous but differentiation from mesenchymal progenitor cells to osteo- or adipocytes may play a role.[67-70] Some reports indicate that circulating adiponectin[71] and resistin levels[72] are reduced in diabetes in line with a recent report demonstrating that higher adiponectin levels are associated with lower BMD[73].

Hyperinsulinemia

Some of the reviewed studies indicated that insulin levels could mediate in part a positive association between T2DM and elevated BMD. Individuals with T2DM usually have an excess of insulin. Physiologically, insulin has an anabolic effect on bone due to its structural homology to IGF-1 by interacting with the IGF-1 receptor which is present on osteoblasts[74]. The IGF-1 signaling pathway is crucial for bone acquisition[75]: both human and mouse studies have demonstrated a significant positive association between IGF-1 and BMD[76, 77]. From this perspective it can be hypothesized that hyperinsulinemia could have a mitogenic effect on osteoblasts and their differentiation by stimulating the IGF-1 signaling pathway. Some indirect influences of insulin on bone formation could possibly be mediated by osteogenic factors such as amylin, osteoprotegerin, sex steroids and sex hormone-binding globulin (SHBG).

Medication use

Thiazide use which is expected to be higher in diabetic individuals has also been associated with higher BMD at different skeletal sites.[78, 79] Similarly, statin use (also more prevalent in diabetics) is also associated with higher BMD.[80, 81] Nevertheless, several of the included studies controlled for medication use, and thus it is unlikely that this alone can explain the observed associations. On the other hand medication use can well be a source of the large heterogeneity observed in the meta-analysis.

Paradoxically increased fracture risk

For many of the aforementioned mechanisms resulting in higher BMD it is rather difficult to fit their role in the paradoxically increased fracture risk. It has been well established that diabetic patients have impaired bone healing after fracture.[82] This probably indicates a compromise of both osteoclastic[82] and osteoblastic cell lineages,[83] and possibly also on bone remodeling. Indeed, a recent study by Burghardt et al. using high-resolution peripheral quantitative computed tomography (HR-pQCT) reported up to twice the cortical porosity observed in type 2 diabetes patients as compared to controls.[84] The results

of this pilot investigation provide a potential explanation for the inability of standard BMD measures to explain the elevated fracture incidence in patients with T2DM presenting with higher BMD levels. Specifically, the findings suggest that T2DM may be associated with an inefficient redistribution of bone mass and insufficient compensation for increased body mass, which may result in impaired bending strength. In addition, bone strength might be compromised through different mechanisms, such as increased production of non-enzymatic cross-links within collagen fibers, accumulation of advanced glycation end products,[85] higher serum glucose levels that can negatively influence bone matrix properties[86] or indirectly as a consequence of sarcopenia[87]. Finally, patients with diabetes have increased fall risk, which can arise as a consequence of sarcopenia, retinopathy and/or neuropathy. Very recently, it has been shown how T2DM underestimates the risk of fracture at a given BMD level,[88] reason why the diabetic status is needed to be considered in risk fracture algorithms[89, 90].

CONCLUSION

Our meta-analysis showed that diabetic individuals have higher BMD levels than non-diabetics independent of the skeletal site of measurement, gender, age, BMI or medication use. In addition, by applying a meta-regression we could establish that younger age, male gender, higher BMI and higher HbA_{1C} are positively associated with higher BMD levels in diabetic individuals. The potential mechanisms underlying these associations remain complex suggesting that several influential factors need to be considered while interpreting the association between T2DM and BMD. Large prospective studies are needed to establish the mechanisms underlying this association, and most importantly the relationship with fracture risk, the most adverse consequence of osteoporosis.

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REFERENCES

1. Albright F, Reifenstein EC. The parathyroid glands and metabolic bone disease: selected studies. Baltimore: Williams and Wilkins; 1948.
2. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int. 2007 Apr;18(4):427-44.
3. Saller A, Maggi S, Romanato G, Tonin P, Crepaldi G. Diabetes and osteoporosis. Aging Clin Exp Res. 2008 Aug;20(4):280-9.
4. van Daele PL, Stolk RP, Burger H, Algra D, Grobbee DE, Hofman A, et al. Bone density in non-insulin-dependent diabetes mellitus. The Rotterdam Study. Ann Intern Med. 1995 Mar 15;122(6):409-14.
5. Yaturu S, Humphrey S, Landry C, Jain SK. Decreased bone mineral density in men with metabolic syndrome alone and with type 2 diabetes. Med Sci Monit. 2009 Jan;15(1):CR5-9.
6. Kao WH, Kammerer CM, Schneider JL, Bauer RL, Mitchell BD. Type 2 diabetes is associated with increased bone mineral density in Mexican-American women. Arch Med Res. 2003 Sep-Oct;34(5):399-406.
7. Majima T, Komatsu Y, Yamada T, Koike Y, Shigemoto M, Takagi C, et al. Decreased bone mineral density at the distal radius, but not at the lumbar spine or the femoral neck, in Japanese type 2 diabetic patients. Osteoporos Int. 2005 Aug;16(8):907-13.

8. Kahn A, Gibbons R, Perkins S, Gazit D. Age-related bone loss. A hypothesis and initial assessment in mice. Clin Orthop Relat Res. 1995 Apr(313):69-75.
9. Min JY, Min KB, Paek D, Kang D, Cho SI. Age curves of bone mineral density at the distal radius and calcaneus in Koreans. J Bone Miner Metab. 2010;28(1):94-100.
10. Tung S, Iqbal J. Evolution, aging, and osteoporosis. Ann N Y Acad Sci. 2007 Nov;1116:499-506.
11. Barrett-Connor E, Holbrook TL. Sex differences in osteoporosis in older adults with non-insulin-dependent diabetes mellitus. JAMA. 1992 Dec 16;268(23):3333-7.
12. Gerdhem P, Isaksson A, Akesson K, Obrant KJ. Increased bone density and decreased bone turnover, but no evident alteration of fracture susceptibility in elderly women with diabetes mellitus. Osteoporos Int. 2005 Dec;16(12):1506-12.
13. Schwartz AV, Sellmeyer DE, Ensrud KE, Cauley JA, Tabor HK, Schreiner PJ, et al. Older women with diabetes have an increased risk of fracture: a prospective study. J Clin Endocrinol Metab. 2001 Jan;86(1):32-8.
14. de Liefde I, van der Klift M, de Laet CE, van Daele PL, Hofman A, Pols HA. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporos Int. 2005 Dec;16(12):1713-20.
15. Strotmeyer ES, Cauley JA, Schwartz AV, Nevitt MC, Resnick HE, Bauer DC, et al. Nontraumatic fracture risk with diabetes mellitus and impaired fasting glucose in older white and black adults: the health, aging, and body composition study. Arch Intern Med. 2005 Jul 25;165(14):1612-7.
16. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. JAMA. 2000 Apr 19;283(15):2008-12.
17. Higgins JPT. Cochrane handbook for systematic reviews of interventions. Version 5.0.2 [updated September 2009]: The Cochrane Collaboration; 2009.
18. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med. 2002 Jun 15;21(11):1539-58.
19. Al-Elq AH, Sadat-Ali M. Diabetes mellitus and male osteoporosis. Is there a relationship? Saudi Med J. 2006 Nov;27(11):1729-33.
20. Al-Maatouq MA, El-Desouki MI, Othman SA, Mattar EH, Babay ZA, Addar M. Prevalence of osteoporosis among postmenopausal females with diabetes mellitus. Saudi Med J. 2004 Oct;25(10):1423-7.
21. Buysschaert M, Cauwe F, Jamart J, Brichant C, De Coster P, Magnan A, et al. Proximal femur density in type 1 and 2 diabetic patients. Diabete Metab. 1992 Jan-Feb;18(1):32-7.
22. Christensen JO, Svendsen OL. Bone mineral in pre- and postmenopausal women with insulin-dependent and non-insulin-dependent diabetes mellitus. Osteoporos Int. 1999;10(4):307-11.
23. Cutrim DM, Pereira FA, de Paula FJ, Foss MC. Lack of relationship between glycemic control and bone mineral density in type 2 diabetes mellitus. Braz J Med Biol Res. 2007 Feb;40(2):221-7.
24. Hadjidakis DJ, Mylonakis AM, Sfakianakis ME, Raptis AE, Raptis SA. Diabetes and premature menopause: is their co-existence detrimental to the skeleton? Eur J Endocrinol. 2005 Mar;152(3):437-42.
25. Hanley DA, Brown JP, Tenenhouse A, Olszynski WP, Ioannidis G, Berger C, et al. Associations among disease conditions, bone mineral density, and prevalent vertebral deformities in men and women 50 years of age and older: cross-sectional results from the Canadian Multicentre Osteoporosis Study. J Bone Miner Res. 2003 Apr;18(4):784-90.
26. Kwon DJ, Kim JH, Chung KW, Lee JW, Kim SP, Lee HY. Bone mineral density of the spine using dual energy X-ray absorptiometry in patients with non-insulin-dependent diabetes mellitus. J Obstet Gynaecol Res. 1996 Apr;22(2):157-62.
27. Nishitani H, Miki T, Morii H, Nishizawa Y, Ishimura E, Hagiwara S, et al. Decreased bone mineral density in diabetic patients on hemodialysis. Contrib Nephrol. 1991;90:223-7.
28. Okuno Y, Nishizawa Y, Sekiya K, Hagiwara S, Miki T, Morii H. Total and regional bone mineral content in patients with non-insulin dependent diabetes mellitus. J Nutr Sci Vitaminol (Tokyo). 1991 Dec;37 Suppl:S43-9.
29. Yamaguchi T, Kanazawa I, Yamamoto M, Kurioka S, Yamauchi M, Yano S, et al. Associations between components of the metabolic syndrome versus bone mineral density and vertebral fractures in patients with type 2 diabetes. Bone. 2009 Aug;45(2):174-9.

30. Akin O, Gol K, Akturk M, Erkaya S. Evaluation of bone turnover in postmenopausal patients with type 2 diabetes mellitus using biochemical markers and bone mineral density measurements. Gynecol Endocrinol. 2003 Feb;17(1):19-29.
31. Hampson G, Evans C, Petitt RJ, Evans WD, Woodhead SJ, Peters JR, et al. Bone mineral density, collagen type 1 alpha 1 genotypes and bone turnover in premenopausal women with diabetes mellitus. Diabetologia. 1998 Nov;41(11):1314-20.
32. Krakauer JC, McKenna MJ, Buderer NF, Rao DS, Whitehouse FW, Parfitt AM. Bone loss and bone turnover in diabetes. Diabetes. 1995 Jul;44(7):775-82.
33. Oz SG, Guven GS, Kilicarslan A, Calik N, Beyazit Y, Sozen T. Evaluation of bone metabolism and bone mass in patients with type-2 diabetes mellitus. J Natl Med Assoc. 2006 Oct;98(10):1598-604.
34. Rishaug U, Birkeland KI, Falch JA, Vaaler S. Bone mass in non-insulin-dependent diabetes mellitus. Scand J Clin Lab Invest. 1995 May;55(3):257-62.
35. Sert M, Tetiker T, Kirim S, Soyupak S, Canataroglu A, Kocak M. Type 2 diabetes mellitus and osteopenia: is there an association? Acta Diabetol. 2003 Jun;40(2):105-8.
36. Strotmeyer ES, Cauley JA, Schwartz AV, Nevitt MC, Resnick HE, Zmuda JM, et al. Diabetes is associated independently of body composition with BMD and bone volume in older white and black men and women: The Health, Aging, and Body Composition Study. J Bone Miner Res. 2004 Jul;19(7):1084-91.
37. Shan PF, Wu XP, Zhang H, Cao XZ, Gu W, Deng XG, et al. Bone mineral density and its relationship with body mass index in postmenopausal women with type 2 diabetes mellitus in mainland China. J Bone Miner Metab. 2009;27(2):190-7.
38. Yamamoto M, Yamaguchi T, Yamauchi M, Kaji H, Sugimoto T. Diabetic patients have an increased risk of vertebral fractures independent of BMD or diabetic complications. J Bone Miner Res. 2009 Apr;24(4):702-9.
39. Schneider S, Schmitt G, Mau H, Schmitt H, Sabo D, Richter W. [Prevalence and correlates of osteoarthritis in Germany. Representative data from the First National Health Survey]. Orthopade. 2005 Aug;34(8):782-90.
40. Isaia G, Bodrato L, Carlevatto V, Mussetta M, Salamano G, Molinatti GM. Osteoporosis in type II diabetes. Acta Diabetol Lat. 1987 Oct-Dec;24(4):305-10.
41. Buday B, Horvath T, Kulcsar E, Salamon C, Literati Nagy B, Barta K, et al. [Effect of progressive insulin resistance on the correlation of glucose metabolism and bone status]. Orv Hetil. 2007 Jun 17;148(24):1127-33.
42. Horiuchi T, Nakamura T, Miyao M, Inoue J, Hattori A, Araki A, et al. [Bone mineral density in postmenopausal elderly women with type 2 diabetes]. Nihon Ronen Igakkai Zasshi. 1995 Nov;32(11):756-60.
43. Komatsu Y, Majima T. [Negative correlation between BMD and HbA1C in patients with type 2 diabetes]. Clin Calcium. 2006 Aug;16(8):1327-31.
44. Sahin G, Bagis S, Cimen OB, Ozisik S, Guler H, Erdogan C. Lumbar and femoral bone mineral density in type 2 Turkish diabetic patients. Acta Medica (Hradec Kralove). 2001;44(4):141-3.
45. Bonds DE, Larson JC, Schwartz AV, Strotmeyer ES, Robbins J, Rodriguez BL, et al. Risk of fracture in women with type 2 diabetes: the Women's Health Initiative Observational Study. J Clin Endocrinol Metab. 2006 Sep;91(9):3404-10.
46. Perez-Castrillon JL, De Luis D, Martin-Escudero JC, Asensio T, del Amo R, Izaola O. Non-insulin-dependent diabetes, bone mineral density, and cardiovascular risk factors. J Diabetes Complications. 2004 Nov-Dec;18(6):317-21.
47. Sosa M, Dominguez M, Navarro MC, Segarra MC, Hernandez D, de Pablos P, et al. Bone mineral metabolism is normal in non-insulin-dependent diabetes mellitus. J Diabetes Complications. 1996 Jul-Aug;10(4):201-5.
48. Tuominen JT, Impivaara O, Puukka P, Ronnemaa T. Bone mineral density in patients with type 1 and type 2 diabetes. Diabetes Care. 1999 Jul;22(7):1196-200.
49. Dennison EM, Syddall HE, Aihie Sayer A, Craighead S, Phillips DI, Cooper C. Type 2 diabetes mellitus is associated with increased axial bone density in men and women from the Hertfordshire Cohort Study: evidence for an indirect effect of insulin resistance? Diabetologia. 2004 Nov;47(11):1963-8.
50. Bridges MJ, Moochhala SH, Barbour J, Kelly CA. Influence of diabetes on peripheral bone mineral density in men: a controlled study. Acta Diabetol. 2005 Jun;42(2):82-6.

51. Schwartz AV, Sellmeyer DE, Strotmeyer ES, Tylavsky FA, Feingold KR, Resnick HE, et al. Diabetes and bone loss at the hip in older black and white adults. J Bone Miner Res. 2005 Apr;20(4):596-603.
52. Rakic V, Davis WA, Chubb SA, Islam FM, Prince RL, Davis TM. Bone mineral density and its determinants in diabetes: the Fremantle Diabetes Study. Diabetologia. 2006 May;49(5):863-71.
53. Hadzibegovic I, Miskic B, Cosic V, Prvulovic D, Bistrovic D. Increased bone mineral density in postmenopausal women with type 2 diabetes mellitus. Ann Saudi Med. 2008 Mar-Apr;28(2):102-4.
54. Anaforoglu I, Nar-Demirer A, Bascil-Tutuncu N, Ertorer ME. Prevalence of osteoporosis and factors affecting bone mineral density among postmenopausal Turkish women with type 2 diabetes. J Diabetes Complications. 2009 Jan-Feb;23(1):12-7.
55. Shan PF, Wu XP, Zhang H, Cao XZ, Yuan LQ, Liao EY. Age-related bone mineral density, osteoporosis rate and risk of vertebral fracture in mainland Chinese women with type 2 diabetes mellitus. J Endocrinol Invest. 2011 Mar;34(3):190-6.
56. Zhou Y, Li Y, Zhang D, Wang J, Yang H. Prevalence and predictors of osteopenia and osteoporosis in postmenopausal Chinese women with type 2 diabetes. Diabetes Res Clin Pract. 2010 Dec;90(3):261-9.
57. Javed F, Yu W, Thornton J, Colt E. Effect of fat on measurement of bone mineral density. Int J Body Compos Res. 2009 Jul 1;7(1):37-40.
58. Evans EM, Mojtahedi MC, Kessinger RB, Misic MM. Simulated change in body fatness affects Hologic QDR 4500A whole body and central DXA bone measures. J Clin Densitom. 2006 Jul-Sep;9(3):315-22.
59. Reid IR. Relationships between fat and bone. Osteoporos Int. 2008 May;19(5):595-606.
60. Kanabrocki EL, Hermida RC, Wright M, Young RM, Bremner FW, Third JL, et al. Circadian variation of serum leptin in healthy and diabetic men. Chronobiol Int. 2001 Mar;18(2):273-83.
61. Hamrick MW, Della-Fera MA, Choi YH, Pennington C, Hartzell D, Baile CA. Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. J Bone Miner Res. 2005 Jun;20(6):994-1001.
62. Steppan CM, Crawford DT, Chidsey-Frink KL, Ke H, Swick AG. Leptin is a potent stimulator of bone growth in ob/ob mice. Regul Pept. 2000 Aug 25;92(1-3):73-8.
63. Gordeladze JO, Drevon CA, Syversen U, Reseland JE. Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: Impact on differentiation markers, apoptosis, and osteoclastic signaling. J Cell Biochem. 2002;85(4):825-36.
64. Cornish J, Callon KE, Bava U, Lin C, Naot D, Hill BL, et al. Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. J Endocrinol. 2002 Nov;175(2):405-15.
65. Holloway WR, Collier FM, Aitken CJ, Myers DE, Hodge JM, Malakellis M, et al. Leptin inhibits osteoclast generation. J Bone Miner Res. 2002 Feb;17(2):200-9.
66. Berner HS, Lyngstadaas SP, Spahr A, Monjo M, Thommesen L, Drevon CA, et al. Adiponectin and its receptors are expressed in bone-forming cells. Bone. 2004 Oct;35(4):842-9.
67. Thommesen L, Stunes AK, Monjo M, Grosvik K, Tamburstuen MV, Kjobli E, et al. Expression and regulation of resistin in osteoblasts and osteoclasts indicate a role in bone metabolism. J Cell Biochem. 2006 Oct 15;99(3):824-34.
68. Cornish J, Palmano K, Callon KE, Watson M, Lin JM, Valenti P, et al. Lactoferrin and bone; structure-activity relationships. Biochem Cell Biol. 2006 Jun;84(3):297-302.
69. Luo XH, Guo LJ, Xie H, Yuan LQ, Wu XP, Zhou HD, et al. Adiponectin stimulates RANKL and inhibits OPG expression in human osteoblasts through the MAPK signaling pathway. J Bone Miner Res. 2006 Oct;21(10):1648-56.
70. Oshima K, Nampei A, Matsuda M, Iwaki M, Fukuhara A, Hashimoto J, et al. Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. Biochem Biophys Res Commun. 2005 Jun 3;331(2):520-6.
71. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, et al. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab. 2001 May;86(5):1930-5.
72. Gomez-Ambrosi J, Fruhbeck G. Evidence for the involvement of resistin in inflammation and cardiovascular disease. Curr Diabetes Rev. 2005 Aug;1(3):227-34.

73. Richards JB, Valdes AM, Burling K, Perks UC, Spector TD. Serum adiponectin and bone mineral density in women. J Clin Endocrinol Metab. 2007 Apr;92(4):1517-23.
74. Pun KK, Lau P, Ho PW. The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line. J Bone Miner Res. 1989 Dec;4(6):853-62.
75. Kawai M, Rosen CJ. Insulin-like growth factor-I and bone: lessons from mice and men. Pediatr Nephrol. 2009 Jul;24(7):1277-85.
76. Langlois JA, Rosen CJ, Visser M, Hannan MT, Harris T, Wilson PW, et al. Association between insulin-like growth factor I and bone mineral density in older women and men: the Framingham Heart Study. J Clin Endocrinol Metab. 1998 Dec;83(12):4257-62.
77. Zhao G, Monier-Faugere MC, Langub MC, Geng Z, Nakayama T, Pike JW, et al. Targeted overexpression of insulin-like growth factor I to osteoblasts of transgenic mice: increased trabecular bone volume without increased osteoblast proliferation. Endocrinology. 2000 Jul;141(7):2674-82.
78. Wasnich RD, Benfante RJ, Yano K, Heilbrun L, Vogel JM. Thiazide effect on the mineral content of bone. N Engl J Med. 1983 Aug 11;309(6):344-7.
79. Schoofs MW, van der Klift M, Hofman A, de Laet CE, Herings RM, Stijnen T, et al. Thiazide diuretics and the risk for hip fracture. Ann Intern Med. 2003 Sep 16;139(6):476-82.
80. Pasco JA, Kotowicz MA, Henry MJ, Sanders KM, Nicholson GC. Statin use, bone mineral density, and fracture risk: Geelong Osteoporosis Study. Arch Intern Med. 2002 Mar 11;162(5):537-40.
81. Schoofs MW, Sturkenboom MC, van der Klift M, Hofman A, Pols HA, Stricker BH. HMG-CoA reductase inhibitors and the risk of vertebral fracture. J Bone Miner Res. 2004 Sep;19(9):1525-30.
82. Schurman L, McCarthy AD, Sedlinsky C, Gangoiti MV, Arnol V, Bruzzone L, et al. Metformin reverts deleterious effects of advanced glycation end-products (AGEs) on osteoblastic cells. Exp Clin Endocrinol Diabetes. 2008 Jun;116(6):333-40.
83. Zhou Z, Immel D, Xi CX, Bierhaus A, Feng X, Mei L, et al. Regulation of osteoclast function and bone mass by RAGE. J Exp Med. 2006 Apr 17;203(4):1067-80.
84. Burghardt AJ, Issever AS, Schwartz AV, Davis KA, Masharani U, Majumdar S, et al. High-resolution peripheral quantitative computed tomographic imaging of cortical and trabecular bone microarchitecture in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Nov;95(11):5045-55.
85. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005 Jun;54(6):1615-25.
86. Yamaguchi T, Sugimoto T. Bone metabolism and fracture risk in type 2 diabetes mellitus [Review]. Endocr J. 2011;58(8):613-24.
87. Evans WJ, Campbell WW. Sarcopenia and age-related changes in body composition and functional capacity. J Nutr. 1993 Feb;123(2 Suppl):465-8.
88. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.
89. Giangregorio LM, Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, et al. FRAX underestimates fracture risk in patients with diabetes. J Bone Miner Res. 2012 Feb;27(2):301-8.
90. Hippisley-Cox J, Coupland C. Predicting risk of osteoporotic fracture in men and women in England and Wales: prospective derivation and validation of QFractureScores. BMJ. 2009;339:b4229.

Chapter 2.5

High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study

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ABSTRACT

Objective Individuals with type 2 diabetes have increased fracture risk despite higher bone mineral density (BMD). Our aim was to examine the influence of glucose control on skeletal complications.

Research design and methods Data of 4,135 participants of the Rotterdam Study, a prospective population-based cohort, were available (mean follow-up 12.2 years). At baseline, 420 participants with type 2 diabetes were classified by glucose control (according to HbA_{1C} calculated from fructosamine) resulting in three comparison groups: adequately controlled diabetes (ACD; N=203; HbA_{1c}<7.5%), inadequately controlled diabetes (ICD; N=217; HbA_{1C}≥7.5%) and no diabetes (ND; N=3,715). Models adjusted for sex, age, height, and weight (and femoral neck BMD) were used to test for differences in bone parameters and fracture risk (hazard ratios [HR]; [95% CI]).

Results The ICD group had 1.1–5.6% higher BMD, 4.6–5.6% thicker cortices and -1.2 to to -1.8% narrower femoral necks than ACD and ND, respectively. Participants with ICD had 47–62% higher fracture risk than individuals without diabetes (HR 1.47; [1.12–1.92]) and ACD (HR 1.62; [1.09–2.40]); whereas those with ACD had a risk similar to those without diabetes (HR 0.91; [0.67–1.23]).

Conclusions Poor glycemic control in type 2 diabetes is associated with fracture risk, high BMD, and thicker femoral cortices in narrower bones. We postulate that fragility in apparently "strong" bones in ICD can result from micro-cracks accumulation and/or cortical porosity reflecting impaired bone repair.

INTRODUCTION

Type 2 diabetes and osteoporosis are common diseases with increasing prevalence in the aging population. Due to their associated morbidity and mortality the conditions cause a high health burden to Western societies.[1-3]

There is increasing evidence supporting an association between type 2 diabetes and increased fracture risk, even though individuals with type 2 diabetes have high bone mineral density (BMD).[4-6] One of these studies was based on the Rotterdam Study, where de Liefde et al. showed that individuals with type 2 diabetes had 69% increased fracture risk than those without diabetes despite having higher BMD at the femoral neck and lumbar spine.[7] Results from a joint effort by three large prospective observational studies indicated that the fracture risk for any given femoral neck BMD T score and age is increased in type 2 diabetes patients compared to those without diabetes.[5] Recently, the World Health Organization's fracture risk assessment tool (FRAX) has been shown to underestimate osteoporotic fracture risk in individuals with diabetes, reason why diabetes as a risk factor will be considered for inclusion in future iterations of FRAX.[8] These findings suggest that factors other than BMD may be underlying the higher fracture risk observed in diabetes patients, and that in fact the BMD measurement does not reflect the actual tendency of patients with type 2 diabetes to develop bone fragility. We recently meta-analyzed published studies that compared BMD in type 2 diabetes to no diabetes, where by meta-regression we established that higher HbA_{1C} is associated with higher BMD across type 2 diabetes groups.[6]

Our aim was to investigate if the intricate relationship between BMD, bone geometry and fractures in type 2 diabetes is influenced by glucose control. Using data from the Rotterdam Study, a large prospective population-based study in elderly Dutch individuals, we examined bone parameters and incident fracture risk across groups of diabetes with adequate and inadequate glucose control as compared to the rest of the population without diabetes.

METHODS

Ethics statement

The Medical Ethics Committee of the Erasmus Medical Centre has approved the Rotterdam Study, and informed consent was obtained from all participants.

The Rotterdam Study

The Rotterdam Study is a prospective population-based cohort studying the determinants of chronic diseases and disability in Dutch men and women. Both the objectives and the study design have been described previously.[9] The study targets investigations on endocrine diseases like osteoporosis and diabetes amongst others. In short, all inhabitants aged 55 years and over of the Ommoord district in the city of Rotterdam in The Netherlands were invited to participate from January 1990 onwards (response rate 78%). Between 1990 and 1993, a baseline home interview on medical history, risk factors for chronic diseases and medication use and information on age at menopause was taken by trained interviewers. Falling was assessed using structured personal interviews by trained medical research nurses. A faller was defined as an individual with a history of one, two or more falls without precipitating trauma (e.g., car accident or sport injury) in the 12 months preceding the baseline interview. Falling frequency at baseline was recorded as "never", "less than once a month", and "more than once a month". Follow up data was collected using different questionnaire at second and third follow-up. Smoking habits were coded as "current", "former", and "never". A trained dietician used an extensive, validated semi-quantitative

food-frequency questionnaire to assess alcohol intake, which was reported in standard alcoholic drinks (9.8625g/12.5 cc of alcohol) per day. Subsequently, participants were invited to the research center for clinical examination. During the baseline visit height and weight were measured with indoor clothing and no shoes. Body mass index (BMI) was calculated as weight (in kg)/height (in m^2). Information on medication use included use of anti-diabetic medication, diuretics, hormonal replacement therapy and systemic corticosteroids.

Laboratory investigations

Creatinine was measured using standard laboratory methods. Serum insulin and sex steroids (including testosterone, E1, E2, SHBG, DHEAS) levels were determined in plasma samples using radioimmunoassay's purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX) and Medgenix diagnostics (Brussels, Belgium), respectively. Fasting serum insulin levels were measured only in those individuals using anti-diabetic medication and the small fraction of exogenous insulin users were not excluded from the analysis. Fructosamine serum levels were measured by colorimetry and reported in µmol/l; fructosamine measurements in the Rotterdam Study had an interassay coefficient of variation (CV) of 3.0.[10] HbA_{1C} was computed at baseline from fructosamine levels using the following formula as described previously[11]: HbA_{1c}=0.017*Fructosamine [µmol/L]+1.61.

Assessment of type 2 diabetes

All participants, except those on anti-diabetic medication, underwent an oral glucose tolerance test (OGTT) with a 37.5% oral glucose solution (75 g of glucose) in a non-fasting state. Blood samples were drawn by venipuncture before and 2 hours after the OGTT. Serum glucose levels were measured using glucose hexokinase. Diabetes was defined as anti-diabetic medication use or a pre-load or post load serum glucose levels above 11.1 mmol/l. Medical profiles were checked to exclude type 1 diabetes cases (e.g., restriction to those who reported having diabetes at or after the age of 30) [7]. From the 4,135 included participants 420 (10.2%) were classified as having type 2 diabetes at baseline according to both OGTT and anti-diabetic medication use. Inadequate glucose control in diabetes was defined as a serum HbA_{1C} level≥7.5% (58 mmol/mol) measured at baseline. This way, three comparison groups were defined including no diabetes (N=3,715), adequately controlled diabetes with serum HbA_{1C} level <7.5% (N=203) and inadequately controlled diabetes with serum HbA_{1C} level≥7.5% (N=217).

Ophthalmic examinations

Visual acuity was measured at 3-m distance using Lighthouse Distance Visual Acuity Test which is a modified Early Treatment Diabetic Retinopathy Study chart.[12] For best-corrected visual acuity (BCVA), optimal refraction was obtained subjectively after objective autorefraction (Topcon RM-A2000, Topcon Optical Company, Tokyo, Japan). After pharmacologic mydriasis participants underwent fundus photography, covering a 35-degree field centered on the macula of both eyes. For the assessment of Visual impairment two sets of commonly used criteria for categorization of blindness and low-vision were applied based on: 1) World Health Organization (WHO) criteria [13]: with blindness defined as BCVA <0.05 (Snellen, 20/400) in the better eye and low vision defined as 0.05 (20/400) ≤ BCVA < 0.3 (20/60) in the better eye; and 2) the most commonly used criteria in the United States (US) defining blindness as BCVA<0.1 (20/200) in the better eye and low vision as 0.1 (20/200) ≤ BCVA < 0.5 (20/40) in the better eye. Retinopathy at baseline, was defined as the presence of cotton wool spots, evidence of laser treatment for retinopathy or the presence of one or more dot/blot hemorrhages or microaneurysms.

Bone mineral density and hip structural analysis

Femoral neck and lumbar spine BMD was measured by dual-energy X-ray absorptiometry (DXA), using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA).[14] Hip structural analysis[15] was used to measure hip bone geometry from the DXA scans of the femur narrow neck region. BMD, and bone width (outer diameter) was measured directly from mineral mass distributions[15] Estimates of mean cortical thickness and endocortical diameter were obtained by modeling the narrow neck region as a circular annulus, which assumes a proportion of cortical/trabecular bone of 60/40. Section modulus was calculated as CSMI/d_{max}, where d_{max} is the maximum distance from the center of mass to the medial or lateral surface. Buckling ratios were computed as d_{max} divided by estimated mean cortical thickness. Data on bone geometry and glucose controls were available for 3,339 individuals including those without diabetes (N=2,995), with adequately controlled diabetes with serum HbA_{1C} level<7.5% (N=157) and with inadequately controlled diabetes with serum HbA_{1C} level≥7.5% (N=187).

Incident fracture assessment

All events, including fractures and death were reported by general practitioners (GPs) in the research area (covering 80% of the cohort) by means of a computerized system. All reported events were verified by two trained research physicians, who independently reviewed and coded the information. Subsequently, all coded events were reviewed by a medical expert for final classification. Subjects were followed from their baseline visit until January 1, 2007 or until a first fracture or death occurred resulting in a mean fracture follow-up of 12.2 years (SD=4.2 years).

Statistical analysis

Mean differences in continuous baseline characteristics, BMD and geometry parameters were tested adjusted for age, sex, height and weight using analysis of variance (ANOVA) and (post-hoc) independent-samples T-test of subgroups. Baseline characteristics that were counts were analyzed with Pearson's chi-squared and Fisher's exact tests. Cox proportional hazard regression models were used to estimate the risk of fracture in a model adjusted for sex, age, height and weight. In addition, the fracture analyses were adjusted for femoral neck BMD. Differences in beta-coefficients between groups were tested with a Z-test. Potential confounders were tested by adding them to the models, including: serum creatinine, serum insulin, use of diuretics, systemic corticosteroid use, alcohol intake, smoking status and falling frequency in the year preceding baseline visit. We evaluated if the change in effect estimate was 10% or more and if statistical significance was lost. The role of anti-diabetic medication use was evaluated in a sensitivity analysis by running the regression model after excluding individuals who were using anti-diabetic medication. S-plus software was used to generate Kaplan-Meier curves and to test for proportionality of hazards. If not stated otherwise SPSS 15 was used for the analyses.

RESULTS

Baseline characteristics by status of glycemic control

The baseline characteristics of the three comparison groups are shown in Table 1. On average, individuals with diabetes were older, had a higher BMI, higher serum insulin and creatinine levels, and used diuretics more frequently than ND. Individuals classified as ICD had the highest insulin levels, highest frequency of retinopathy and used anti-diabetic medication more frequently. In female participants, age

at menopause, frequency of hormone-replacement therapy, and serum sex steroid levels did not differ significantly between women in any of the comparison groups (data not shown).

Table 1. Baseline characteristics of study participants stratified by comparison group.

	Comparison groups			
Mean (SD)	ND (N=3,715)	ACD (N=203)	ICD (N=217)	ANOVA P value
Age (years)[a]	68.2 (7.7)	71.9 (7.6)	68.5 (7.8)	<0.05
Height (meter) [a]	1.67 (0.09)	1.65 (0.09)	1.67 (0.10)	NS
Weight (kg) [a]	73.1 (11.6)	73.8 (12.5)	74.2 (11.4)	NS
Body mass index (kg/m^2) [a]	26.3 (3.7)	27.0 (4.2)	26.9 (4.0)	<0.05
Serum insulin (pmol/l) [a]	85.9 (99.0)	109.5 (109.5)	199.9 (266.0)	<0.05
Serum creatinine (µmol/l) [a]	82.1 (20.3)	85.7 (22.5)	89.2 (21.6)	<0.05
Females (%)[b]	59.7	61.6	53.0	NS
Use of diuretics (%)[b]	13.1	22.8	24.4	<0.05
Use of corticosteroids (%)[b]	1.86	3.47	2.76	NS
Use of anti-diabetic drugs (%)[b]	0.0	20.8	34.8	<0.05
Current smoking (%)[b]	24.8	31.0	22.7	NS
Ever smoking (%)[b]	42.3	38.0	46.5	NS
Recent fall (%)[b]	17.2	19.5	18.1	NS
History of myocardial infarction (%)[b]	13.6	13.6	17.2	NS
Retinopathy (%)[b]	6.5	11.0	19.5	<0.05
Visual impairment WHO/U.S. (%)[b]	2.3/1.1	4.0/1.5	4.7/1.4	NS/NS
Lumbar spine BMD (g/cm^2)[c]	1.08 (0.003)	1.10 (0.01)	1.14 (0.01)	<0.05
Femoral neck BMD (g/cm^2) [c]	0.86 (0.002)	0.88 (0.009)	0.89 (0.008)	<0.05

Data presented as mean (SD). WHO, World Health Organization. [a]Unadjusted means with SDs. [b]Percentages from total assessed at baseline. [c]Sex-, age-, height- and weight- adjusted means with SEs.

Association with BMD and hip bone geometry

Overall participants with diabetes had higher BMD than those without diabetes at the lumbar spine and femoral neck (Table 1). ICD had between 1.1% to 5.6% higher BMD (g/cm^2) at both the femoral neck (0.89) and lumbar spine (1.14) as compared to ACD (femoral neck: 0.88, P=0.26; lumbar spine: 1.10, P=0.02) and ND (femoral neck: 0.86, P=0.00006; lumbar spine: 1.08, P=0.00003). In addition, bone geometry parameters of the narrow neck region assessed in a subset of the sample (N=3,319) were studied across glucose-control comparison groups (Table 2). As expected from the results from the lumbar spine and the femoral neck region, the mean narrow neck BMD (g/cm^2) was also the highest in individuals with ICD. As compared to ND, individuals with ICD had 5.6% thicker cortices than ND (P=0.00002) and 4.6% thicker cortices than ACD (P=0.02). No significant difference in cortical thickness was observed between ND and ACD (P=0.48). Differential effects were also seen for neck width, where individuals with ICD had -1.8% narrower femoral necks than ND (P=0.0004) and -1.2% narrower femoral necks than ACD, though this difference did not achieve statistical significance (P=0.10). The narrow neck width in individuals with ACD showed no significant differences from those observed in ND. No significant differences in bending strength (section modulus) were observed across comparison groups. In contrast, shorter necks with thicker cortices suggest higher cortical bone stability (lower buckling ratios), and ICD individuals had -6.8% significantly lower buckling ratios (higher cortical bone stability) than those observed in individuals from ACD (P=0.005) and ND groups (P=0.0001). To further evaluate

the relationship between cortical thickness, femoral neck width and glucose control, we examined the relationship across age tertiles (Figure 1). The observed differences were particularly prominent in the oldest tertile where individuals with ICD had 8.1% thicker cortices than ND (P=0.001) and 9.3% thicker than ACD (P=0.08). Similarly, neck width of ICD individuals in this older tertile had -2.5% narrower necks than ND (P=0.003) and, though not statistically significant, -1.2% narrower necks than ACD (P=0.31).

Table 2. Hip structural analysis (bone geometry) parameters stratified by glucose control groups.

	Comparison groups			ANOVA
Measurement	ND (N=3,715)	ACD (N=125)	ICD (N=115)	P value
Narrow neck BMD (g/cm^2)	0.69 ± 0.002 (0.68–0.69)	0.69 ± 0.009 (0.68–0.71)	0.72 ± 0.008 (0.71–0.74)	0.0002
Cortical thickness (mm)	1.31 ± 0.004 (1.30–1.32)	1.32 ± 0.02 (1.28–1.35)	1.38 ± 0.02 (1.35–1.41)	0.0001
Neck width (cm)	3.20 ± 0.004 (3.19–3.21)	3.18 ± 0.02 (3.15–3.22)	3.14 ± 0.02 (3.11–3.17)	0.001
Section modulus (cm^3)	1.118 ± 0.004 (1.111–1.126)	1.124 ± 0.018 (1.089–1.158)	1.125 ± 0.016 (1.094–1.157)	0.89
Cortical buckling ratio	13.99 ± 0.06 (13.88–14.11)	14.00 ± 0.3 (13.51–14.49)	13.04 ± 0.2 (12.59–13.49)	0.0003

Values are mean ± SEM (95% CIs) adjusted for age, sex, height and weight.

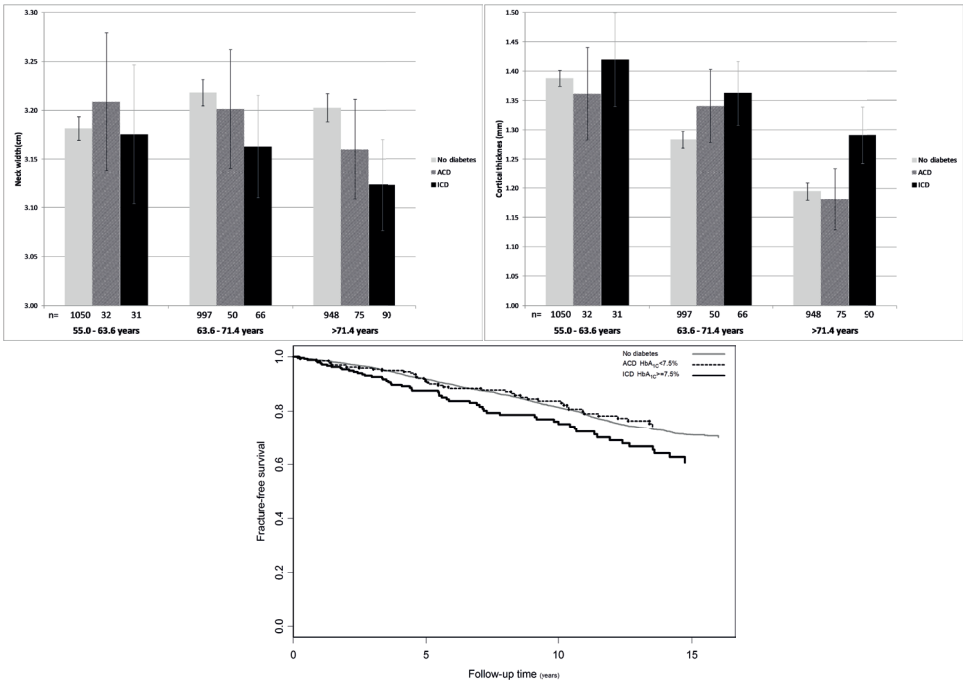

Figure 1. Adjusted means of narrow neck width (top bar chart) and cortical thickness (bottom bar chart) in relation to glucose control by age tertiles: youngest 55.0–63.6 years of age; middle 63.6–71.4 years of age; oldest >71.4 years of age. Kaplan-Meier curve per comparison group showing the adjusted cumulative hazards for fracture using follow-up time as timescale. Cox proportional hazard model: ICD vs. no diabetes HR 1.47 (95%CI 1.12–1.92); P=0.005, ACD vs. no diabetes HR 0.91 (0.67–1.23); P=0.54. Cumulative hazard ratio adjusted for femoral neck BMD, age, sex, height and weight. Light gray, ND; dark grey or dashed, ACD; black, ICD.

Fracture-free survival analysis

Tables 3 and 4 shows the site-specific fracture incidence rates and hazard ratios stratified by glucose control. During follow-up, 1,068 subjects experienced at least one incident fracture, including 253 individuals presenting with a hip fracture and 257 individuals with a wrist fracture. Individuals in the ICD group had an increased fracture risk compared to ACD (HR: 1.62; 95% CI: 1.09–2.40) and ND (HR: 1.47; 1.12–1.92); while those with ACD had a HR of 0.91 (0.67–1.23) as compared to ND. Kaplan-Meier fracture-free survival curves are shown in Figure 1. The analysis of fracture subtypes showed a similar trend for wrist (Colles' distal forearm) fracture as that observed for all-types of fracture; while the pattern for hip fracture risk was inconsistent (Tables 3 and 4).

Table 3. Site-specific fracture incidence rates stratified by glucose control groups.

	Comparison groups					
	ND		ACD		ICD	
	(3,715 subjects; 46,130 person-years)		(203 subjects; 2,165 person-years)		(217 subjects; 2,134 person-years)	
Type of fracture	Cases	Incidence	Cases	Incidence	Cases	Incidence
All types	967	0.0241	44	0.0230	57	0.0311
Hip	227	0.0050	15	0.0072	11	0.0052
Wrist	232	0.0052	9	0.0042	16	0.0078

Table 4. HRs stratified by glucose control groups.

		Cox analysis of fracture-free survival								
		ICD vs. ACD			ICD vs. ND			ACD vs. ND		
Type of fracture		HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
All types	Basic model	1.54	(1.04–2.29)	0.03	1.31	(1.00–1.71)	0.05	0.85	(0.63–1.15)	0.29
	BMD adjusted	1.62	(1.09–2.40)	0.02	1.47	(1.12–1.92)	0.005	0.91	(0.67–1.23)	0.54
Hip	Basic model	0.83	(0.38–1.81)	0.64	0.96	(0.52–1.75)	0.89	1.15	(0.68–1.94)	0.60
	BMD adjusted	0.93	(0.42–2.02)	0.85	1.16	(0.63–2.13)	0.63	1.25	(0.74–2.12)	0.40
Wrist	Basic model	2.12	(0.94–4.80)	0.07	1.59	(0.95–2.64)	0.07	0.75	(0.38–1.46)	0.40
	BMD adjusted	2.23	(0.99–5.06)	0.05	1.71	(1.03–2.86)	0.04	0.77	(0.39–1.50)	0.44

Basic model, adjusted for age, sex, height and weight; BMD-adjusted model, adjusted for age, sex, height, weight and femoral neck BMD.

Even though mean BMD was higher in individuals with diabetes, being the highest in the ICD group, lower femoral neck BMD was significantly associated with increased fracture risk across all study groups: ND (HR: 1.60 per SD decrease; 95% CI: 1.46–1.75), ACD (HR: 2.72 per SD decrease; 95% CI: 1.76–4.20) and ICD (1.54; 1.11–2.14). The association was less strong in ICD individuals suggesting that they fracture at a higher BMD threshold. We tested for sex interaction finding that the increase in fracture risk was significantly stronger in women (P=0.02). ICD vs. ACD (HR women: 2.08; 1.31–3.30; HR men: 0.72; 0.33–1.56); ICD vs. ND (HR women: 1.64; 1.20–2.22; HR men 1.09; 0.62–1.92).

The effect of inadequate glucose control on fracture risk, BMD or bone geometry was not essentially changed by any of the confounders tested. For example, falling more than once a month was independently and highly significantly associated with fracture risk (HR: 1.80; 1.18–2.74) as expected, but when added to the model the risk estimates for the diabetes comparison groups remained similar (ICD vs. ACD effect estimate -0.9% and ICD vs. ND effect estimate -1.4%). Also, serum creatinine as a

measure of kidney function was independently significantly associated with fracture risk (HR: 0.89 per SD increase in serum creatinine; 95% CI: 0.81–0.98), but when added to the model the estimates for the diabetes comparison groups remained statistically significant and essentially the same magnitude (ICD vs. ACD effect estimate +2.0% and ICD vs. ND effect estimate -6.4%). The elevated HR for fracture risk in the inadequately controlled group remained after excluding individuals who were using anti-diabetic medication. In addition, further adjustment of the analyses for serum insulin levels in a subset of the study population did not essentially alter the effect estimates. Finally, following the classification used in de Liefde et al.[7] we examined the group without diabetes classified by presence of impaired glucose tolerance (IGT, pre-load or post-load OGTT serum glucose from 7.8 to 11.1 mmol/L) and observed no differences in BMD or bone geometry parameters. In contrast, individuals without diabetes and an impaired glucose tolerance test had 0.80 (95% CI: 0.66–0.97) decreased risk for any-type of fracture as compared to individuals without diabetes and no impaired glucose tolerance, a finding that requires further evaluation in future studies.

DISCUSSION

To our knowledge, this is the first study examining glucose control in subjects with type 2 diabetes in relation to BMD, bone geometry parameters and fracture risk. ICD individuals have higher BMD at the lumbar spine and femoral neck, with thicker cortices and smaller bone diameter at the femoral neck. This hip bone geometry configuration results in lower estimates of femoral narrow neck instability and no differences in bending strength. ICD individuals present stronger geometry associated with a lower risk of fracture.[16, 17] However, we found that ICD individuals have an increased fracture risk compared to individuals with ACD and individuals without diabetes. This association did not seem to be influenced by potential confounders or arising from diabetes complications (extra-skeletal risk factors) like risk of falling at baseline, decline in renal function, nor by the use of systemic corticosteroids or diuretics. The discrepancy between BMD and geometrical findings with fracture incidence observed here could be attributed to weaker material causing failure at lower stress or biomechanical skeletal properties, which cannot be detected by DXA assessments.

Our study has several strengths. First, this is a large prospective population-based study including 4,135 participants with long and comprehensive follow-up of more than 12 years on average. Second, we had various co-variables available for analyses, including the fracture incidence, bone geometry parameters at baseline and various other determinants of fractures. Third, classification of type 2 diabetes was robustly determined taking into account OGTT and anti-diabetic medication use. The broad availability of assessments in our study enabled extensive analyses. Yet, our study has limitations. The age of onset of diabetes was unknown, nor can we be sure about the duration of the glucose control assessment beyond the three to four months around the fructosamine measurement. Similarly, deriving HbA_{1C} from fructosamine may result in a somewhat different classification of glycemic control. Yet, it has been shown that fructosamine is as or even more strongly associated with microvascular conditions than HbA_{1C}, with excellent assay reliability.[10, 18] In addition, hip structural properties and risk of falling during follow-up were assessed with different methods than at baseline, so we were less able to infer relationships with incident fractures that occurred many years after the baseline visit. Falling risk is a potential confounder, because patients with diabetes have increased risk of falling.[19] We showed that risk of falling at baseline does not explain the association with increased fracture risk. Nevertheless, we cannot exclude that during follow-up falling frequency and subsequent fracture risk can increase as a

consequence of diabetes complications (i.e., retinopathy, neuropathy), which we show is higher in the inadequately controlled group of individuals with diabetes. Alternatively, insulin users with low HbA_{1C} levels are reported to fall more, likely as a consequence of hypoglycemia.[20] We propose that even with a similar risk for falling, individuals in the group of ICD would have (when falling) higher propensity to fracture given their unfavorable skeletal properties.

Interestingly, ICD individuals actually seem to have a stronger bone geometry, which would protect against fractures. Unfortunately, no bone geometry parameters for sites other than the femoral neck were available in our study; neither did we have access to techniques such as peripheral quantitative computed tomography (pQCT) scanning allowing a three-dimensional assessment of bone structure and microarchitecture. Others have shown before that bone strength in patients with type 2 diabetes may be compromised despite a higher BMD[21-23] and as a result of altered adaptation to loading.[21] Sex-specific differences may exist reflecting differential patterns of bone apposition between sexes (bone dimorphism). A direct interaction between estradiol and insulin-like growth factor-I (IGF-I) in the determination of periosteal apposition has been proposed[24] and serum IGF-I levels are negatively associated with increased risk for prevalent vertebral fractures in postmenopausal women but not in men with type 2 diabetes[25]. Yet, our sex-specific analyses are restricted because of a lower power setting in men, partly due to survival bias and lower incidence of fractures. A final caveat to bear in mind in relation to the applicability of our findings is that our study population consisted of Dutch individuals of Northeastern European background. Additional studies in multiple settings with sufficiently large sample sizes are required.

Some studies evaluated the relationship between glycemic control and fracture risk finding conflicting results.[26-30] A study by Ivers et al. in 3,654 Australian middle-aged subjects found that fasting blood glucose >7mmol/l, disease duration >10 years, insulin treatment, and the presence of diabetic retinopathy were associated with increased risk of all fractures.[27] On the other hand, Melton et al. found no association of fracture risk with baseline fasting plasma glucose level, yet the follow-up time was limited [29]. None of these studies measured HbA_{1C} (a better indicator of diabetes control) which correlates strongly with disease severity. In a study in Japanese men[28] Kanazawa et al. found that obese individuals with HbA_{1C}>9 and higher BMD had 3 times increased risk of vertebral fracture than non-obese men with diabetes. In another study, Forsen et al.[26] used a similarly high cut-off of HbA_{1C} >9.5 for diabetic subjects from a large Norwegian population (N=35,444) and found no association. Yet, they did find that fracture risk was higher in subjects with disease duration greater than 5 years and being treated with insulin. A threshold of HbA_{1C} >7% was used by Strotmeyer et al. to define poor glycemic control for diabetics, in a study in 3,075 older white and black adults from the Health ABC study.[30] Individuals with diabetes had 1.6 increased risk of fracture (after correction for BMD) but, longer disease duration, and insulin use were not significantly different. Therefore, different HbA_{1C} thresholds can make a difference in the definition of glucose control and the relationship with fracture. In our study, we used a 7.5% cut-off (closest to the median/mean HbA_{1C} in our data) which has been proposed for patients of old age (mean age in our study was 69 years), those with co-morbidities and in those with established cardiovascular complications,[31] in line with the established relationship between diabetes control, (cardiovascular) complications and mortality[32].

Our data on hip bone geometry shows how individuals with ICD have persistently thicker cortices than ND and ACD (Figure 2). In addition, a lesser tendency to undergo physiological bone expansion (periosteal apposition) is also inferred from narrower bone diameters in individuals with ICD. A recent study by Burghardt et al.,[33] using high-resolution pQCT (HR-pQCT), reported that the cortical porosity

in type 2 diabetes patients was up to twice that of controls. Our findings are compatible with those described by Ahlborg et al.,[34] where impaired bone remodeling is suggested by a lack of cortical thinning, with consequent lack of compensatory bone expansion. Since such differences in geometry are accentuated at older ages, we postulate that an accumulation of micro cracks and/or cortical porosity in time may well be the consequence of impaired bone repair or decreased bone remodeling. Taken together, these results suggest an inefficient redistribution of bone in ICD. This configuration can predispose individuals with ICD to increased bone fragility as a result of increased micro-cracks and/or cortical porosity. Additional studies using HR-pQCT to evaluate bone properties in type 2 diabetes while considering glucose control are thus warranted.

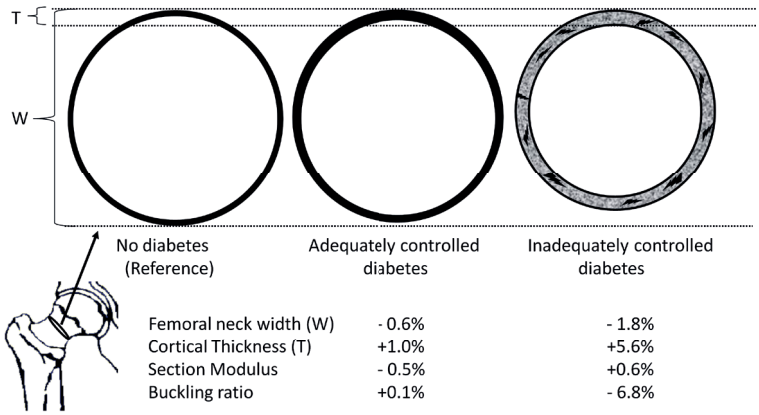

Figure 2. Cartoon depicting the differences in bone geometry across glucose control groups for a cross-section of the femoral neck. Individuals with ICD have thicker cortices and narrower neck width than those without diabetes and ACD. With lower instability of cortical bone (lower buckling ratios) the accumulation of micro-cracks and cortical porosity become a possibility to explain bone fragility and fracture susceptibility. Drawing is not to scale.

The exact mechanisms underlying these bone parameters in ICD remain to be elucidated. Nevertheless, it can be hypothesized that the following factors may play a role: the accumulation of advanced glycation end (AGE) products,[35] impaired bone healing,[36] altered body composition (e.g., sarcopenia[37]), increased production of non-enzymatic cross-links within collagen fibers negatively influencing bone matrix properties,[38] etc. Probably both osteoclastic[39] and osteoblastic[40] cell lineages are compromised, knowing that bone remodeling involves both bone resorption and formation. From this perspective, the narrower neck width observed in ICD may well reflect alterations in the differentiation and/or function of the osteoblastic lineage. Considering the known anabolic effects of insulin-like growth factor-I (IGF-I) and insulin on bone and periosteal expansion,[41-43] it can be expected that the altered insulin-IGF-I-growth hormone axis (lower bio-availability of IGF-I) present in ICD[44-46] may also contribute to the observed geometrical alterations we observed. Also, follow-up studies focusing on the actual metabolic pathways involved in such mechanisms are thus also needed.

Our findings indicate that the detrimental effects of chronically elevated glucose levels on bone should be added to the more well-known complications of inadequately regulated diabetes, such as retinopathy, nephropathy, micro- and macro- cardiovascular disease. Furthermore, a high BMD in inadequately controlled diabetes may in fact reflect a skeletal complication of the disease. If so, evaluation of BMD and the most commonly used clinical risk factors might be inadequate for predicting fracture risk

in ICD, who (due to their high BMD) are unlikely to be diagnosed with osteoporosis and increased risk of fracture. Similarly, our data showed that individuals with type 2 diabetes who are adequately controlled have a similar fracture risk as ND. This indicates that the first line of action for fracture prevention in diabetes is targeting adequate glycemic control. However, results from a randomized trial published very recently did not find changes in fracture or fall risk between standard glycemia and intensive glycemia[47]. Nevertheless, average follow-up until now was merely 3.8 (SD 1.3) years so inference of long-term effects, i.e., from long-standing control and diminishing carry-over of pre-treatment glycemic exposure, is not yet possible. Type 2 diabetes can seriously affect patients' quality of life, especially in the presence of diabetes-related complications.[48] Bone fractures occurring on top of these altered conditions might further increase the health burden already observed in individuals with inadequate glucose control of their diabetes. Randomized controlled trials could reveal if certain anti-diabetic drugs associated with increased risk of fracture (i.e., thiazolidinediones) are a case of confounding by indication (inadequate glucose control);[49] or alternatively, if skeletal specific interventions to activate remodeling (e.g., vibration plate) could indeed benefit the bone health of individuals with diabetes.

CONCLUSION

Increased fracture risk in type 2 diabetes is driven by poor glycemic control and occurs in the presence of higher BMD, and thicker femoral cortices in narrower bones. We postulate that fragility in the apparently strong bones of ICD is the consequence of an accumulation of micro-cracks (cortical porosity) that reflects sustained impairment of bone repair. This should be investigated in future research. We recommend that fracture risk assessments in ICD should not be based on BMD alone, since high BMD could actually reflect a complication of inadequate glycemic control. Reassessment of risk factors (particularly BMD) is needed for the prevention of this skeletal complication in ICD. Finally, maintaining more stringent parameters of glycemic control can emerge as the first line of action to prevent fractures and their subsequent deleterious consequences on the quality of life of individuals with diabetes.

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REFERENCES

1. National Diabetes Statistics. National Diabetes Information Clearinghouse (NDIC); 2011 [July 1 2011]; Available from: http://diabetes.niddk.nih.gov/dm/pubs/statistics/.
2. Age- and sex-specific prevalences of diabetes and impaired glucose regulation in 13 European cohorts. Diabetes Care. 2002/12/28 ed. DECODE Study Group2003. p. 61-9.
3. Ström O, Borgström F, Kanis J, Compston J, Cooper C, McCloskey E, et al. Osteoporosis: burden, health care provision and opportunities in the EU. Arch Osteoporos. 2011;DOI 10.1007/s11657-011-0060-1.
4. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007 Sep 1;166(5):495-505.
5. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.
6. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol. 2012 May;27(5):319-32.
7. de Liefde I, van der Klift M, de Laet CE, van Daele PL, Hofman A, Pols HA. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporos Int. 2005 Dec;16(12):1713-20.
8. Giangregorio LM, Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, et al. FRAX underestimates fracture risk in patients with diabetes. J Bone Miner Res. 2011 Nov 2.
9. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
10. Stolk RP, Pols HA, Lamberts SW, de Jong PT, Hofman A, Grobbee DE. Diabetes mellitus, impaired glucose tolerance, and hyperinsulinemia in an elderly population. The Rotterdam Study. Am J Epidemiol. 1997 Jan 1;145(1):24-32.
11. Cohen RM, Holmes YR, Chenier TC, Joiner CH. Discordance between HbA1c and fructosamine: evidence for a glycosylation gap and its relation to diabetic nephropathy. Diabetes Care. 2003 Jan;26(1):163-7.
12. Early Treatment Diabetic Retinopathy Study (ETDRS). Manual of Operations. ETDRS Coordinating Center UoM, Dept of Epidemiology and Preventive Medicine, editor. Baltimore1980.
13. World Health Organization. International Statistical Classification of Diseases and Related Health problems, Tenth Revision. Tenth Revision. Vol 1 ed. Geneva, Switzerland1992.
14. Burger H, de Laet CE, van Daele PL, Weel AE, Witteman JC, Hofman A, et al. Risk factors for increased bone loss in an elderly population: the Rotterdam Study. Am J Epidemiol. 1998 May 1;147(9):871-9.
15. Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW. Structural trends in the aging femoral neck and proximal shaft: analysis of the Third National Health and Nutrition Examination Survey dual-energy X-ray absorptiometry data. J Bone Miner Res. 2000 Dec;15(12):2297-304.
16. Melton LJ, 3rd, Beck TJ, Amin S, Khosla S, Achenbach SJ, Oberg AL, et al. Contributions of bone density and structure to fracture risk assessment in men and women. Osteoporos Int. 2005 May;16(5):460-7.
17. Rivadeneira F, Zillikens MC, De Laet CE, Hofman A, Uitterlinden AG, Beck TJ, et al. Femoral neck BMD is a strong predictor of hip fracture susceptibility in elderly men and women because it detects cortical bone instability: the Rotterdam Study. J Bone Miner Res. 2007 Nov;22(11):1781-90.
18. Selvin E, Francis LM, Ballantyne CM, Hoogeveen RC, Coresh J, Brancati FL, et al. Nontraditional markers of glycemia: associations with microvascular conditions. Diabetes Care. 2011 Apr;34(4):960-7.
19. Schwartz AV, Hillier TA, Sellmeyer DE, Resnick HE, Gregg E, Ensrud KE, et al. Older women with diabetes have a higher risk of falls: a prospective study. Diabetes Care. 2002 Oct;25(10):1749-54.
20. Schwartz AV, Vittinghoff E, Sellmeyer DE, Feingold KR, de Rekeneire N, Strotmeyer ES, et al. Diabetes-related complications, glycemic control, and falls in older adults. Diabetes Care. 2008 Mar;31(3):391-6.
21. Garg R, Chen Z, Beck T, Cauley JA, Wu G, Nelson D, et al. Hip geometry in diabetic women: Implications for fracture risk. Metabolism. 2012 Jun 20.
22. Melton LJ, 3rd, Riggs BL, Leibson CL, Achenbach SJ, Camp JJ, Bouxsein ML, et al. A bone structural basis for fracture risk in diabetes. J Clin Endocrinol Metab. 2008 Dec;93(12):4804-9.
23. Petit MA, Paudel ML, Taylor BC, Hughes JM, Strotmeyer ES, Schwartz AV, et al. Bone mass and strength in older men with type 2 diabetes: the Osteoporotic Fractures in Men Study. J Bone Miner Res. 2010 Feb;25(2):285-91.

24. Seeman E. Clinical review 137: Sexual dimorphism in skeletal size, density, and strength. J Clin Endocrinol Metab. 2001 Oct;86(10):4576-84.
25. Kanazawa I, Yamaguchi T, Sugimoto T. Serum insulin-like growth factor-I is negatively associated with serum adiponectin in type 2 diabetes mellitus. Growth Horm IGF Res. 2011 Oct;21(5):268-71.
26. Forsen L, Meyer HE, Midthjell K, Edna TH. Diabetes mellitus and the incidence of hip fracture: results from the Nord-Trondelag Health Survey. Diabetologia. 1999 Aug;42(8):920-5.
27. Ivers RQ, Cumming RG, Mitchell P, Peduto AJ. Diabetes and risk of fracture: The Blue Mountains Eye Study. Diabetes Care. 2001 Jul;24(7):1198-203.
28. Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Yano S, Sugimoto T. Combination of obesity with hyperglycemia is a risk factor for the presence of vertebral fractures in type 2 diabetic men. Calcif Tissue Int. 2008 Nov;83(5):324-31.
29. Melton LJ, 3rd, Leibson CL, Achenbach SJ, Therneau TM, Khosla S. Fracture risk in type 2 diabetes: update of a population-based study. J Bone Miner Res. 2008 Aug;23(8):1334-42.
30. Strotmeyer ES, Cauley JA, Schwartz AV, Nevitt MC, Resnick HE, Bauer DC, et al. Nontraumatic fracture risk with diabetes mellitus and impaired fasting glucose in older white and black adults: the health, aging, and body composition study. Arch Intern Med. 2005 Jul 25;165(14):1612-7.
31. Benhalima K, Standl E, Mathieu C. The importance of glycemic control: how low should we go with HbA1c? Start early, go safe, go low. J Diabetes Complications. 2011 May-Jun;25(3):202-7.
32. Currie CJ, Peters JR, Tynan A, Evans M, Heine RJ, Bracco OL, et al. Survival as a function of HbA(1c) in people with type 2 diabetes: a retrospective cohort study. Lancet. 2010 Feb 6;375(9713):481-9.
33. Burghardt AJ, Issever AS, Schwartz AV, Davis KA, Masharani U, Majumdar S, et al. High-resolution peripheral quantitative computed tomographic imaging of cortical and trabecular bone microarchitecture in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Nov;95(11):5045-55.
34. Ahlborg HG, Johnell O, Turner CH, Rannevik G, Karlsson MK. Bone loss and bone size after menopause. N Engl J Med. 2003 Jul 24;349(4):327-34.
35. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005 Jun;54(6):1615-25.
36. Blakytny R, Spraul M, Jude EB. Review: The diabetic bone: a cellular and molecular perspective. Int J Low Extrem Wounds. 2011 Mar;10(1):16-32.
37. Evans WJ, Campbell WW. Sarcopenia and age-related changes in body composition and functional capacity. J Nutr. 1993 Feb;123(2 Suppl):465-8.
38. Yamaguchi T, Sugimoto T. Bone metabolism and fracture risk in type 2 diabetes mellitus [Review]. Endocr J. 2011 Jul 20;58(8):613-24.
39. Schurman L, McCarthy AD, Sedlinsky C, Gangoiti MV, Arnol V, Bruzzone L, et al. Metformin reverts deleterious effects of advanced glycation end-products (AGEs) on osteoblastic cells. Exp Clin Endocrinol Diabetes. 2008 Jun;116(6):333-40.
40. Zhou Z, Immel D, Xi CX, Bierhaus A, Feng X, Mei L, et al. Regulation of osteoclast function and bone mass by RAGE. J Exp Med. 2006 Apr 17;203(4):1067-80.
41. Mohan S, Richman C, Guo R, Amaar Y, Donahue LR, Wergedal J, et al. Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms. Endocrinology. 2003 Mar;144(3):929-36.
42. Libanati C, Baylink DJ, Lois-Wenzel E, Srinvasan N, Mohan S. Studies on the potential mediators of skeletal changes occurring during puberty in girls. J Clin Endocrinol Metab. 1999 Aug;84(8):2807-14.
43. Vanderschueren D, Venken K, Ophoff J, Bouillon R, Boonen S. Clinical Review: Sex steroids and the periosteum--reconsidering the roles of androgens and estrogens in periosteal expansion. J Clin Endocrinol Metab. 2006 Feb;91(2):378-82.
44. Chestnut RE, Quarmby V. Evaluation of total IGF-I assay methods using samples from Type I and Type II diabetic patients. J Immunol Methods. 2002 Jan 1;259(1-2):11-24.

45. Clauson PG, Brismar K, Hall K, Linnarsson R, Grill V. Insulin-like growth factor-I and insulin-like growth factor binding protein-1 in a representative population of type 2 diabetic patients in Sweden. Scand J Clin Lab Invest. 1998 Jul;58(4):353-60.
46. Sesti G, Sciacqua A, Cardellini M, Marini MA, Maio R, Vatrano M, et al. Plasma concentration of IGF-I is independently associated with insulin sensitivity in subjects with different degrees of glucose tolerance. Diabetes Care. 2005 Jan;28(1):120-5.
47. Schwartz AV, Margolis KL, Sellmeyer DE, Vittinghoff E, Ambrosius WT, Bonds DE, et al. Intensive Glycemic Control Is Not Associated With Fractures or Falls in the ACCORD Randomized Trial. Diabetes Care. 2012 Jul;35(7):1525-31.
48. Vadstrup ES, Frolich A, Perrild H, Borg E, Roder M. Health-related quality of life and self-related health in patients with type 2 diabetes: Effects of group-based rehabilitation versus individual counselling. Health Qual Life Outcomes. 2011 Dec 7;9(1):110.
49. Habib ZA, Havstad SL, Wells K, Divine G, Pladevall M, Williams LK. Thiazolidinedione use and the longitudinal risk of fractures in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Feb;95(2):592-600.

Chapter 2.6

Diabetes, diabetic complications, and fracture risk

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ABSTRACT

Diabetes and osteoporosis are both common diseases with increasing prevalences in the aging population. There is increasing evidence corroborating an association between diabetes mellitus and bone. This review will discuss the disease complications of diabetes on the skeleton, highlighting findings from epidemiological, molecular, and imaging studies in animal models and humans. Compared to control subjects, decreased bone mineral density (BMD) has been observed in type 1 diabetes mellitus, while on average, higher BMD has been found in type 2 diabetes; nonetheless, patients with both types of diabetes are seemingly at increased risk of fractures. Conventional diagnostics such as DXA measurements and the current fracture risk assessment tool (FRAX) risk prediction algorithm for estimating risk of osteoporotic fractures are not sufficient in the case of diabetes. A deterioration in bone microarchitecture and an inefficient distribution of bone mass with insufficiency of repair and adaptation mechanisms appear to be factors of relevance. A highly complex and heterogeneous molecular pathophysiology underlies diabetes-related bone disease, involving hormonal, immune, and perhaps genetic pathways. The detrimental effects of chronically elevated glucose levels on bone should be added to the more well-known complications of diabetes.

INTRODUCTION

Diabetes and osteoporosis are common diseases with increasing prevalences in the aging population. There is growing evidence corroborating that diabetes mellitus influences the skeletal metabolism. Decreased bone mineral density (BMD) and increased fracture risk have fairly consistently been observed in type 1 diabetes mellitus patients.[1] This review will primarily focus on type 2 diabetes. Contradictory results with higher, lower or similar values for BMD observed in persons with type 2 diabetes compared to control subjects have been reported across individual and relatively small studies with diverse designs.[2-5] Nevertheless, several lines of evidence arising from meta-analytical efforts suggest that individuals with type 2 diabetes have generally higher BMD levels at the femoral neck, hip and spine than persons without diabetes, independently of gender or body mass index (which is usually higher in subjects with type 2 diabetes and discussed in further detail below).[1, 6] The between-study heterogeneity was very high and originated at least in part from differences in design and possibly diabetes definition across studies. Nonetheless, meta-regression of the results across studies showed that younger age, male gender, higher body mass index and higher hemoglobin A1c (HbA_{1C}) were positively associated with higher BMD levels in individuals with type 2 diabetes.

Higher fracture risk despite a higher bone mineral density in type 2 diabetes

Based on evidence in non-diabetics, higher levels of BMD should be protective against fracture; this association seems somewhat different in type 2 diabetes.[2, 7-9] Using data from the prospective Rotterdam Study cohort, De Liefde et al. were among the first to show that individuals with type 2 diabetes have 69% higher risk of non-vertebral fractures than those without diabetes despite having higher BMD at the femoral neck and lumbar spine.[9] The aforementioned meta-analysis by Vestergaard et al. found summary estimates for hip fracture risk of 6.9 in type 1 and 1.4 in type 2 diabetes compared to subjects without diabetes, respectively.[1] Schwartz and colleagues established in a meta-analysis based on three prospective observational studies with adjudicated fracture outcomes (Study of Osteoporotic Fractures; Osteoporotic Fractures in Men Study; and Health, Aging, and Body Composition study) that in type 2 diabetes patients the fracture risk was higher for a given BMD and age as compared with participants without diabetes, and most importantly, that the World Health Organization's fracture risk assessment tool (FRAX) underestimates osteoporotic fracture risk in individuals with diabetes;[8] similar work by Giangregorio et al. in the Canadian Manitoba Bone Density Program illustrated how diabetes as a risk factor is necessary to be considered for inclusion in future iterations of FRAX[10]. Even though most of the work has been done in populations of European background, similar relationships have been observed across different ethnicities, particularly in relation to increased risk of vertebral fractures.[11-13]

Many studies have shown a difference in population characteristics between type 2 diabetic patients and healthy controls.[3, 9, 14, 15] In these studies, diabetic study participants tend to be older, have a higher body mass index (BMI) or weight, increased insulin levels, less physical exercise, higher alcohol consumption and they usually smoke more and more often. Also, the use of diuretics is more common in diabetes, and particularly loop diuretics (e.g. furosemide) may be associated with decreased BMD and increased risk of fractures through increasing urinary calcium excretion and osteoclastic bone resorption,[16] while thiazides are associated with higher BMD and lower fracture risk.[17, 18] Further, use of anti-diabetic thiazolidinediones has been reported to increase fracture risk.[19] Patients with diabetes fall more often, which can be a consequence from suffering from suboptimal physical fitness, neuropathy, retinopathy or sarcopenia.[20] Alternatively, insulin users with low HbA_{1C} levels are reported to fall more, likely as a consequence of hypoglycemia.[21] These characteristics might influence bone metabolism

and fracture risk, nevertheless, statistical analyses with corrections in aforementioned studies suggest independence of the differences in BMD and fracture risk from these measured confounders[3, 9, 14, 15] such as risk of falling[9, 14].

Relation of diabetes regulation with fracture risk

Some studies evaluating the relationship between glycemic control based on fasting blood glucose and fracture risk have found conflicting results. Other factors that do seem to matter are use of insulin and disease duration. Among these studies is an investigation by Ivers et al.[22] which found that fasting blood glucose greater than 7 mmol/L, disease duration longer than 10 years, insulin treatment, and the presence of diabetic retinopathy were associated with increased risk of all-type of fractures. The oral glucose tolerance test (OGTT) remains the gold standard for distinguishing diabetes mellitus (pre-glucose load or post-glucose load challenge serum glucose level of 11.1 mmol/l or higher) and impaired glucose tolerance (pre-glucose load or post-glucose load challenge serum glucose level from 7.8 mmol/l to 11.1 mmol/l).[23] In the Rotterdam Study subjects with type 2 diabetes and impaired glucose tolerance were both found to have higher BMD, whereas contrary to those with impaired glucose tolerance, patients with type 2 diabetes had higher fracture risk, particularly those on anti-diabetic medication.[9] Nevertheless, HbA_{1C} is a better indicator than serum glucose for long-term diabetes control and is therefore considered the main parameter in clinical practice.

Higher HbA_{1C} reflects a higher average plasma glucose concentration over a prolonged period, in the order of weeks. We observed in Rotterdam Study data that poor glycemic control based on an HbA_{1C} cut-off of 7.5% (58 mmol/l) in type 2 diabetes is associated with higher all-type of fracture risk, higher BMD, and thicker femoral cortices in narrower bones.[24] Intriguingly, different HbA_{1C} thresholds were applied in various studies, possibly due to heterogeneity in effects and study population. Similar to our observations, the Atherosclerosis Risk in Communities (ARIC) Study found that type 2 diabetes was significantly and independently associated with increased risk of fracture. In this study, an increased risk of fracture of 1.87 times was observed among persons treated with insulin and an increased risk of 1.63 times among persons with diagnosed diabetes with HbA_{1C} ≥8% (64 mmol/l) as compared to those individuals with HbA_{1C} below 8%.[25] Kanazawa et al. found that obese Japanese men with type 2 diabetes and HbA_{1C} of 9% and above had three times increased risk of vertebral fracture than men with diabetes but normal BMI, despite equal or higher BMD.[26] Strotmeyer et al. found that older white and black adults with type 2 diabetes in the Health ABC study had 1.6 increased risk of fracture.[13] However, when comparing diabetes patients with and without fractures, poor glycemic control (threshold of HbA_{1C} 7% (53 mmol/l)), longer disease duration, and insulin use were not significantly different. Forsén et al.(26) found that fracture risk was higher in Norwegian subjects with disease duration longer than 5 years and insulin use, but failed to demonstrate any effect on fractures using a high cutoff of HbA_{1C} 9.5% (80 mmol/l). Yet, this cut-off was very high and a consequent lack of study power cannot be ruled out.

Pathophysiology

A highly complex and heterogeneous molecular pathophysiology seems to underlie fracture risk in diabetes-related bone disease. One of the factors that have been found detrimental are advanced glycation endproducts (AGEs). AGEs are generated by the sequential non-enzymatic addition of carbohydrate molecules to protein amino groups.[27] AGEs accumulate in various tissues including bone,[28, 29] kidney and coronary arteries.[30] This may result in development of diabetic complications through increased inflammation, interference with normal tissue function and cellular damage. Pentosidine is one of the

well-known AGEs, and accumulation of pentosidine in cortical and trabecular bone is negatively associated with bone strength.[28, 29, 31] Histopathological analyses comparing bone samples from femoral neck fracture cases with post-mortem controls revealed a higher extent of hydroxylation and higher pentosidine content.[32, 33] Furthermore, Yamamoto et al. showed that individuals with type 2 diabetes suffering from vertebral fractures have increased serum levels of pentosidine,[34] while higher levels of the endogenous secretory receptor for AGEs (esRAGE), acting as a decoy receptor binding AGEs, have protective effects on fracture risk in diabetes[35]. esRAGE is the most prevalent splice variant of RAGE, while the most common form is full-length RAGE,[36] which possesses a transmembrane domain and is therefore able to transduce signals as a membrane-bound receptor.[37] Seemingly, full-length RAGE has a role in bone remodeling by regulating osteoclast function possibly through integrin signaling and bone mass given that mice lacking RAGE have increased bone mass and BMD and decreased bone resorptive activity in vivo.[38]

Insulin levels could mediate in part a positive association between type 2 diabetes and elevated BMD. Individuals with type 2 diabetes usually have an excess of insulin and those with worse glucose control have the highest serum levels.[24] Physiologically, insulin has an anabolic effect on bone due to its structural homology to insulin-like growth factor-I (IGF-I) by interacting with the IGF-I receptor present on osteoblasts.[39] The IGF-I signaling pathway is crucial for bone acquisition and bone remodeling.[40] Lower concentrations of serum IGF-I levels are associated with the presence of and a higher number of prevalent vertebral fractures in postmenopausal women with type 2 diabetes.[41, 42] Additionally, novel data from a mouse study with osteoprogenitor-selective ablation of the insulin receptor suggest that insulin receptor malfunction itself may directly lead to biomechanical microarchitecture alterations in both cortical and trabecular bone.[43] Furthermore, there is evidence that insulin receptor signaling promotes the differentiation of osteoblasts and enhances production and activation of osteocalcin.[44, 45]

Osteocalcin is an osteoblast-specific secreted protein that regulates hydroxyapatite size and shape through its vitamin-K-dependent, gamma-carboxylated form, thereby reflecting bone remodeling and, in particular, bone formation.[46] Metabolic roles of osteocalcin have been identified in animal studies, including increasing insulin secretion and sensitivity.[47] The regulation of insulin sensitivity by osteocalcin may be either direct or indirect, via the adipocyte-derived hormone adiponectin (discussed below).[46] Osteocalcin has also been found to be negatively correlated with HbA_{1C} as a marker of glycemic control in type 1 and type 2 diabetes.[26] Osteocalcin knock-out mice display glucose intolerance and insulin resistance with a concomittant slight increase in bone density.[48] In bone and serum, osteocalcin is incompletely carboxylated (undercarboxylated osteocalcin) and it is this uncarboxylated form that has been negatively implicated in energy metabolism and glucose control in both mice and humans.[45, 47] Higher undercarboxylated osteocalcin may be linked to increased risk of hip fracture,[49] where calcium and vitamin D2 suppletion was able to normalize the undercarboxylated osteocalcin levels.[50] The underlying mechanism is largely unknown; It is known that 1,25-dihydroxyvitamin D enhances the transcription of osteocalcin by means of the gene possessing a vitamin D-responsive element[51] but whether vitamin D might directly influence the γ-carboxylation reaction of osteocalcin remains unclear.[52, 53] Cardiovascular disease including atherosclerosis is more common in type 2 diabetes mellitus; studies carried out so far suggest that abdominal aortic calcification is more common in diabetics.[54] In Asian women it has been observed that osteocalcin significantly correlated with aortic calcification, which again is associated with a threefold increased risk of vertebral fractures.[55]

Adipokines are cell signaling proteins secreted by adipose tissue and include for instance leptin, adiponectin and resistin. The release of these adipokines leads to a chronic subinflammatory state that

could play a central role in the development of insulin resistance and type 2 diabetes.[56] It has been observed that plasma leptin concentrations are higher in obese persons with diabetes than in healthy controls.[57] Leptin induces bone growth by stimulating osteoblast proliferation and differentiation[58-60] and it has also been shown to inhibit osteoclastogenesis through reducing RANK/RANK-ligand production and increasing osteoprotegerin.[61, 62] Plasma leptin concentrations have been found inversely related with BMD in cross-sectional studies.[63-65] Further, higher leptin levels were associated with a lower prevalence of fracture in some cohorts,[66] though the effect may not be as clear in individuals aged 70 to 79 years from the Health Aging and Body Composition Study.[67] Some reports indicate that circulating adiponectin and resistin levels are reduced in diabetes.[68] Adiponectin is expressed in osteoblasts and osteoclasts[69] and adiponectin seems to influence differentiation from mesenchymal progenitor cells into osteocytes or adipocytes, yet the effects on bone metabolism remain unclear.[70, 71] After adjustments of measures of body fat, each doubling of adiponectin is associated with a 2-3% decrease in BMD,[72] and higher adiponectin levels may be a risk factor for increased fracture risk.[67] The gut-derived peptide hormone ghrelin has been shown to modulate osteoblast differentiation and function, both directly and perhaps also through regulation of the growth hormone–insulin-like growth factor axis, and through interaction with leptin ghrelin has a role in modulating bone structure.[73] A systematic review and meta-analysis by Biver et al. concluded that the most relevant adipokines influencing BMD and fracture risk are indeed leptin and adiponectin, whereas no convincing data are available for resistin, visfatin or gut-derived ghrelin.[74]

The role of inflammation in the pathogenesis of type 2 diabetes, as touched upon before, and associated complications is now well established.[75] C-reactive protein (CRP) is an extremely sensitive marker of systemic inflammation produced mainly by the liver under the stimulation of macrophage- and adipocyte-derived proinflammatory cytokines, principally interleukin-6 (IL-6).[76] Elevated levels of CRP are described in persons with type 2 diabetes; however, it is not clear if they are related to the presence of obesity, diabetes, or both.[77] Studies in general populations have found lower BMD,[78, 79] lower hip geometrical bending strength[80] and an increased risk of fracture[80, 81] for higher CRP levels, which intriguingly appeared to be independent of BMD or trabecular microarchitecture.[82] Some studies explicitly indicate a relationship between CRP and complications of diabetes,[83-86] nonetheless, evidence is lacking for a direct mechanism and CRP may very well merely be a marker of the ongoing inflammation.[80, 87-89]

Shared genetic factors between diabetes and bone disease

A genome-wide association study (GWAS) meta-analysis for gene expression levels in relation to type 2 diabetes as the phenotype of interest including 1,175 case-control microarrays showed a significantly differential gene expression of osteopontin (OPN), also known as phosphoprotein 1 (SPP1) or bone sialoprotein I (BSP-I).[90] This same investigation brought forward that osteopontin is a ligand for the most prominent top hit of this genome-wide screening being the immune-cell receptor CD44; and that the expression profiles of CD44 and osteopontin are frequently coordinately dysregulated, especially in adipose tissue. The gene encoding osteopontin maps to the 4q22.1 locus, which has frequently appeared as a femoral neck-BMD and lumbar spine-BMD locus in large-scale meta-analyses and contains many bone-active genes.[91-94] Osteopontin is an extracellular structural protein in bone able to bind strongly to calcium crystals.[95] It has been proposed that osteopontin is an important factor in bone remodeling,[96] which may be by anchoring osteoclasts to the mineral matrix of bones.[97] In addition, osteopontin enhances B lymphocyte proliferation and immunoglobulin production, and is chemotactic for many immune cell types including macrophages, dendritic cells, and T cells.[98] Osteopontin null

mice of all ages display a bone phenotype probably mediated by altered osteoclast activity, protecting them from developing osteoporosis.[99] Fascinatingly, wild type mice exposed to a high-fat diet exhibit increased plasma osteopontin levels with elevated expression in macrophages recruited into adipose tissue, while on the other hand, obese osteopontin null mice exhibit decreased markers of inflammation with less macrophage infiltration into adipose tissue, display improved insulin sensitivity and are seemingly protected from the effects of diet-induced obesity on body composition or energy expenditure.[100] Altogether this suggests a key role for osteopontin in the development of age-related osteoporosis and the link of obesity to the development of insulin resistance and possibly type 2 diabetes.

A GWAS meta-analysis targeting copy number variations (CNV), which are a type of structural variants of the genome in which large (>1 kb) segments of the genome are either lost or duplicated, found evidence that a deletion in the 6p25.1 locus predisposes to risk of all-type of fracture.[101] The deletion is located in an intergenic region in the subtelomeric region of chromosome 6p in the proximity of the Peroxisomal D3,D2-EnoylCoA Isomerase (*PECI*) gene which codes for an enzyme relevant for the metabolism of fatty acids. *PECI* was first cloned by using pooled antisera from autoimmune diabetes patients.[102] The increased risk seen with individuals with the 6p25del may be mediated by co-morbidity with diabetes, yet, more studies are needed to convincingly replicate the potential association of this copy number variant with fracture risk and elucidate the underlying functional mechanism.

The association between BMD, type 2 diabetes and glycemic traits[103] was also tested in the context of pleiotropic relations by members of the Genetic Factors of Osteoporosis (GEFOS) and Meta-Analyses of Glucose and Insulin-related traits (MAGIC) consortia. None of the BMD single nucleotide polymorphisms (SNPs) reached the a priori P-value threshold corrected for multiple testing, except a SNP at the *ITGA1* locus. This marker was found associated with type 2 diabetes, serum insulin levels, β-cell function and glucose tolerance. Null *ITGA1* mice have impaired fracture healing and cartilage remodeling,[104] although it is not yet clear what role this gene product has on BMD or bone structure.

Bone geometry

Our data on hip bone geometry in the Rotterdam Study showed that individuals with inadequately controlled diabetes have persistently thicker cortices in narrower femoral necks than those with adequately controlled diabetes or those without diabetes.[24] A lesser tendency to undergo physiological bone expansion (periosteal apposition), i.e. a process in which a limited amount of bone mass is efficiently redistributed, could be inferred from narrower bone diameters in these individuals. This led us to propose that changes in microarchitecture (i.e. microcracks and cortical porosity) could be underlying the increased risk of fractures observed in inadequately controlled diabetics. A peripheral quantitative computed tomography (pQCT) investigation in the Osteoporotic Fractures in Men study found that participants with type 2 diabetes displayed greater volumetric bone mineral density (vBMD) but a smaller bone area at both the distal tibia and radius, which resulted in a bone strength which was particularly low relative to body weight[105]. As described by Ahlborg et al.,[106] a process of rapid physiological bone expansion occurs in women after menopause, highlighting a complex interplay of hormones such as estradiol, IGF-I and insulin.[107, 108] Considering the known anabolic effects of IGF-I and insulin on bone and periosteal expansion, it can be expected that the altered insulin–IGF-I–growth hormone axis (lower bioavailability of IGF-I) may also contribute to the observed geometrical alterations observed in inadequately controlled diabetes, as a lack of periosteal apposition and bone repair. Since such differences in geometry are accentuated at older ages, we previously postulated that an accumulation of microcracks with time may well be an skeletal complication of inadequately controlled diabetes resulting in impaired

bone repair, decreased bone remodeling, high BMD and increased risk of fracture.[24] There is a growing body of evidence for deterioration of bone microarchitecture in type 2 diabetes leading to a porous skeleton susceptible to fracture. Burghardt et al. applied a novel derivative of cortical porosity for high-resolution peripheral quantitative computed tomography (HR-pQCT) and reported that the cortical porosity in type 2 diabetic patients is up to twice that of controls at the radius.[109] Subsequently, Patsch et al. compared type 2 diabetes patients with fragility fractures to patients diabetes with diabetes without fractures and controls with and without fractures.[110] The investigators showed nicely that the cortical porosity is specific to those type 2 diabetes patients that fracture. Similarly, The trabecular bone score (TBS) is a measure of bone texture that can be derived from DXA, which correlates with 3D parameters of bone microarchitecture.[111] One of the first studies utilizing this invention demonstrated that TBS is lower at the lumbar spine in diabetes-related bone disease.[112] The results of these investigations provide a potential explanation for the inability of standard DXA measures to explain the elevated fracture incidence in patients with diabetes presenting with higher BMD and apparently stronger bone geometry.

Recently researchers have started to examine bone marrow fat composition, regarding presence and types of hydrogen bonds, where unsaturated fats contain at least one double bond and saturated fats have the maximum number of hydrogens bonded to carbons. The radiological research group of Dr. Link has demonstrated in their combined quantitative computed tomography (QCT) and magnetic resonance (MR) spectroscopy studies that the prevalence of fragility fractures is associated with lower unsaturation levels and higher saturation levels of bone marrow fat, in which the participants with diabetes with fractures have the lowest marrow unsaturation and highest saturation.[113] In contrast to controls without diabetes, higher mean vertebral bone marrow fat content is significantly correlated with visceral adipose tissue and Hb_{A1C} in persons with type 2 diabetes, representing worse metabolic profiles.[114] The concept of high-saturated fat-associated adipose inflammation and insulin resistance have been proposed; however, underlying molecular mechanisms remain to be elucidated.

Reference point indentation[115, 116] allows minimally invasive measurements of bone material properties of human bone in vivo by microindentation, which is correlated with risk of osteoporotic fractures.[117, 118] Recently, Farr et al. showed that patients with type 2 diabetes have reduced serum markers of bone turnover and lower bone material strength at the tibia than age-matched, controls without diabetes.[119] Further, in this same study the average Hb_{A1C} level over the previous ten years was negatively correlated with bone material strength,[119] supporting the contention recognizing the skeleton as another important target tissue subject to diabetic complications.[24]

Therapeutic options

Not only are patients with diabetes at increased risk for fractures, they are prone to impaired bone healing after fracture as well.[120] In usual fracture healing serum concentrations of biomarkers such as alkaline phosphatase, IGF-I and osteocalcin peak in the first few weeks of recovery[121, 122] and decrease again thereafter, but possibly in disturbed consolidation these levels remain elevated for an even longer time.[123] An experimental study using the diabetic Zucker (fa/fa) rat model with creation of femoral defects demonstrated that administration of parathyroid hormone (PTH) could partially reverse the adverse skeletal effects of diabetes on bone defect.[124]

Systematic screening for complications and fall prevention efforts, along with calcium and vitamin D repletion and adequate physical activity, represents the mainstay of fracture prevention in patients with diabetes. Nonetheless, we should mention that the controversy regarding the anti-fracture efficacy versus the side-effect profile of calcium supplements in general is still unresolved.[125-127] A few meta-

analyses with different methodologies have been published on this topic to date yielding conflicting results,[128-131] of which the investigation by Bolland et al. suggested an increased risk of myocardial infarction (MI) and possibly stroke in men and women together for calcium supplements, particularly without co-administered vitamin D.[128] These specific potential side effects of calcium supplements may be of particular importance in patients with diabetes as they are already at increased risk of cardiovascular disease complications, however, no studies have been performed in this area yet. As discussed above, the current FRAX risk score underestimates fracture risk in patients with diabetes, which leads to undertreatment of the diabetic individuals that are actually at increased fracture risk. Anti-catabolic drugs (raloxifene, bisphosphonates, denosumab) might be effective, but on the basis of pathophysiological evidence that suggests low bone formation in the aforementioned research in model organisms,[124] osteo-anabolic therapies such as teriparatide might represent an important therapeutic option for diabetes-related bone disease.[132] More studies including randomized controlled trials in this area are needed.

CONCLUSION

The detrimental effects of diabetes on bone should be added to the more well-known complications of diabetes. A deterioration in bone microarchitecture and an inefficient distribution of bone mass with insufficiency of repair and adaptation mechanisms in combination with increased risk of falling all lead to an elevated fracture risk as skeletal complications of diabetes. Improved risk prediction with epidemiological determinants and integration of novel biochemical and imaging biomarkers will be necessary to correctly and timely diagnose those individuals at increased risk. More research is needed to unravel the pathophysiology underlying diabetes-related bone disease, which may eventually contribute to preventative and curative therapies.

Strength of Evidence

The evidence outlined in this review includes studies in humans and animals. Animal studies cited are mostly knockout mice experiments. Human studies include observational studies of varying sizes, meta-analyses summarizing these results, and a few randomized controlled trials of generally smaller sample sizes. At present, it may not be very well possible to grade the evidence; replication studies in this field are desirable.

REFERENCES

1. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int. 2007 Apr;18(4):427-44.
•• **This meta-analysis was among the first to distil the evidence that hip fracture risk is increased in both type 1 and type 2 diabetes mellitus, whereas bone mineral density is increased in type 2 diabetes mellitus and decreased in type 1 diabetes mellitus.**
2. van Daele PL, Stolk RP, Burger H, Algra D, Grobbee DE, Hofman A, et al. Bone density in non-insulin-dependent diabetes mellitus. The Rotterdam Study. Ann Intern Med. 1995 Mar 15;122(6):409-14.
3. Kao WH, Kammerer CM, Schneider JL, Bauer RL, Mitchell BD. Type 2 diabetes is associated with increased bone mineral density in Mexican-American women. Arch Med Res. 2003 Sep-Oct;34(5):399-406.

4. Majima T, Komatsu Y, Yamada T, Koike Y, Shigemoto M, Takagi C, et al. Decreased bone mineral density at the distal radius, but not at the lumbar spine or the femoral neck, in Japanese type 2 diabetic patients. Osteoporos Int. 2005 Aug;16(8):907-13.
5. Yaturu S, Humphrey S, Landry C, Jain SK. Decreased bone mineral density in men with metabolic syndrome alone and with type 2 diabetes. Med Sci Monit. 2009 Jan;15(1):CR5-9.
6. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol. 2012 May;27(5):319-32.

• **Systematic review containing an updated meta-analysis and meta-regression which showed that individuals with type 2 diabetes have generally higher bone mineral density throughout the skeleton, which is again under the influence of multiple factors.**

7. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007 Sep 1;166(5):495-505.
8. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.

•• **Data from three large prospective observational studies demonstrated that current risk prediction based on femoral neck bone mineral density and FRAX score underestimate fracture risk among older adults with type 2 diabetes mellitus.**

9. de Liefde II, van der Klift M, de Laet CE, van Daele PL, Hofman A, Pols HA. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporos Int. 2005 Dec;16(12):1713-20.
10. Giangregorio LM, Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, et al. FRAX underestimates fracture risk in patients with diabetes. J Bone Miner Res. 2012 Feb;27(2):301-8.
11. Yamamoto M, Yamaguchi T, Yamauchi M, Kaji H, Sugimoto T. Diabetic patients have an increased risk of vertebral fractures independent of BMD or diabetic complications. J Bone Miner Res. 2009 Apr;24(4):702-9.
12. Kilpadi KL, Eldabaje R, Schmitz JE, Ehler B, Thames TA, Joshi AP, et al. Type 2 diabetes is associated with vertebral fractures in a sample of clinic- and hospital-based Latinos. J Immigr Minor Health. 2014 Jun;16(3):440-9.
13. Strotmeyer ES, Cauley JA, Schwartz AV, Nevitt MC, Resnick HE, Bauer DC, et al. Nontraumatic fracture risk with diabetes mellitus and impaired fasting glucose in older white and black adults: the health, aging, and body composition study. Arch Intern Med. 2005 Jul 25;165(14):1612-7.
14. Schwartz AV, Sellmeyer DE, Ensrud KE, Cauley JA, Tabor HK, Schreiner PJ, et al. Older women with diabetes have an increased risk of fracture: a prospective study. J Clin Endocrinol Metab. 2001 Jan;86(1):32-8.
15. Barrett-Connor E, Holbrook TL. Sex differences in osteoporosis in older adults with non-insulin-dependent diabetes mellitus. JAMA. 1992 Dec 16;268(23):3333-7.
16. Ruiter R, Oei L, Visser LE, Peltenburg HG, Hofman A, Zillikens MC, et al. The effect of thiazide and loop diuretics on urinary levels of free deoxypyridinoline: an osteoclastic bone-resorption marker. J Clin Pharm Ther. 2013 Jun;38(3):225-9.
17. Aung K, Htay T. Thiazide diuretics and the risk of hip fracture. Cochrane Database Syst Rev. 2011(10):CD005185.
18. Schoofs MW, van der Klift M, Hofman A, de Laet CE, Herings RM, Stijnen T, et al. Thiazide diuretics and the risk for hip fracture. Ann Intern Med. 2003 Sep 16;139(6):476-82.
19. Habib ZA, Havstad SL, Wells K, Divine G, Pladevall M, Williams LK. Thiazolidinedione use and the longitudinal risk of fractures in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Feb;95(2):592-600.
20. Schwartz AV, Hillier TA, Sellmeyer DE, Resnick HE, Gregg E, Ensrud KE, et al. Older women with diabetes have a higher risk of falls: a prospective study. Diabetes Care. 2002 Oct;25(10):1749-54.
21. Schwartz AV, Vittinghoff E, Sellmeyer DE, Feingold KR, de Rekeneire N, Strotmeyer ES, et al. Diabetes-related complications, glycemic control, and falls in older adults. Diabetes Care. 2008 Mar;31(3):391-6.
22. Ivers RQ, Cumming RG, Mitchell P, Peduto AJ. Diabetes and risk of fracture: The Blue Mountains Eye Study. Diabetes Care. 2001 Jul;24(7):1198-203.
23. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014 Jan;37 Suppl 1:S81-90.
24. Oei L, Zillikens MC, Dehghan A, Buitendijk GH, Castano-Betancourt MC, Estrada K, et al. High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study. Diabetes Care. 2013 Jun;36(6):1619-28.

•• **Prospective population-based study showing that poor glycemic control is associated with higher fracture risk, higher bone mineral density and thicker femoral cortices in narrower bones.**

25. Schneider AL, Williams EK, Brancati FL, Blecker S, Coresh J, Selvin E. Diabetes and risk of fracture-related hospitalization: the Atherosclerosis Risk in Communities Study. Diabetes Care. 2013 May;36(5):1153-8.

•• **Participants with diabetes from the Atherosclerosis Risk in Communities (ARIC) study had an increased risk of fracture, where persons treated with insulin and those with higher hemoglobin A1c had the highest risk.**

26. Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Yano S, Sugimoto T. Combination of obesity with hyperglycemia is a risk factor for the presence of vertebral fractures in type 2 diabetic men. Calcif Tissue Int. 2008 Nov;83(5):324-31.
27. Brownlee M. Advanced protein glycosylation in diabetes and aging. Annu Rev Med. 1995;46:223-34.
28. Hernandez CJ, Tang SY, Baumbach BM, Hwu PB, Sakkee AN, van der Ham F, et al. Trabecular microfracture and the influence of pyridinium and non-enzymatic glycation-mediated collagen cross-links. Bone. 2005 Dec;37(6):825-32.
29. Wang X, Shen X, Li X, Agrawal CM. Age-related changes in the collagen network and toughness of bone. Bone. 2002 Jul;31(1):1-7.
30. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med. 1988 May 19;318(20):1315-21.
31. Viguet-Carrin S, Roux JP, Arlot ME, Merabet Z, Leeming DJ, Byrjalsen I, et al. Contribution of the advanced glycation end product pentosidine and of maturation of type I collagen to compressive biomechanical properties of human lumbar vertebrae. Bone. 2006 Nov;39(5):1073-9.
32. Saito M, Fujii K, Soshi S, Tanaka T. Reductions in degree of mineralization and enzymatic collagen cross-links and increases in glycation-induced pentosidine in the femoral neck cortex in cases of femoral neck fracture. Osteoporos Int. 2006;17(7):986-95.
33. Saito M, Fujii K, Marumo K. Degree of mineralization-related collagen crosslinking in the femoral neck cancellous bone in cases of hip fracture and controls. Calcif Tissue Int. 2006 Sep;79(3):160-8.
34. Yamamoto M, Yamaguchi T, Yamauchi M, Yano S, Sugimoto T. Serum pentosidine levels are positively associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes. J Clin Endocrinol Metab. 2008 Mar;93(3):1013-9.

• **Case-control study showing increased serum levels of pentosidine in women with vertebral fractures than in those without vertebral fractures Replication of these results and more understanding of the advanced glycation endproducts (AGEs) pathofysiology will be necessary.**

35. Yamamoto M, Yamaguchi T, Yamauchi M, Sugimoto T. Low serum level of the endogenous secretory receptor for advanced glycation end products (esRAGE) is a risk factor for prevalent vertebral fractures independent of bone mineral density in patients with type 2 diabetes. Diabetes Care. 2009 Dec;32(12):2263-8.
36. Hudson BI, Carter AM, Harja E, Kalea AZ, Arriero M, Yang H, et al. Identification, classification, and expression of RAGE gene splice variants. FASEB J. 2008 May;22(5):1572-80.
37. Kalea AZ, Reiniger N, Yang H, Arriero M, Schmidt AM, Hudson BI. Alternative splicing of the murine receptor for advanced glycation end-products (RAGE) gene. FASEB J. 2009 Jun;23(6):1766-74.
38. Zhou Z, Immel D, Xi CX, Bierhaus A, Feng X, Mei L, et al. Regulation of osteoclast function and bone mass by RAGE. J Exp Med. 2006 Apr 17;203(4):1067-80.
39. Pun KK, Lau P, Ho PW. The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line. J Bone Miner Res. 1989 Dec;4(6):853-62.
40. Kawai M, Rosen CJ. Insulin-like growth factor-I and bone: lessons from mice and men. Pediatr Nephrol. 2009 Jul;24(7):1277-85.
41. Kanazawa I, Yamaguchi T, Sugimoto T. Serum insulin-like growth factor-I is a marker for assessing the severity of vertebral fractures in postmenopausal women with type 2 diabetes mellitus. Osteoporos Int. 2011 Apr;22(4):1191-8.

42. Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Yano S, Sugimoto T. Serum insulin-like growth factor-I level is associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes mellitus. Osteoporos Int. 2007 Dec;18(12):1675-81.
43. Thrailkill K, Bunn RC, Lumpkin C, Jr., Wahl E, Cockrell G, Morris L, et al. Loss of insulin receptor in osteoprogenitor cells impairs structural strength of bone. J Diabetes Res. 2014;2014:703589.
• **Osteoprogenitor-selective (osterix-Cre) ablation of the insulin receptor in mice yields a skeletal phenotype with growth restriction, illustrating that insulin receptor expression in osteoblasts is critically important for bone development and maintenance of structural integrity.**
44. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. Cell. 2010 Jul 23;142(2):309-19.
45. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, et al. Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell. 2010 Jul 23;142(2):296-308.
46. Booth SL, Centi A, Smith SR, Gundberg C. The role of osteocalcin in human glucose metabolism: marker or mediator? Nat Rev Endocrinol. 2013 Jan;9(1):43-55.
47. Rached MT, Kode A, Silva BC, Jung DY, Gray S, Ong H, et al. FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice. J Clin Invest. 2010 Jan;120(1):357-68.
48. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, et al. Increased bone formation in osteocalcin-deficient mice. Nature. 1996 Aug 1;382(6590):448-52.
•• **Osteocalcin-deficient mice develop glucose intolerance and insulin resistance, but at the same time a higher bone mass and improved functional quality.**
49. Vergnaud P, Garnero P, Meunier PJ, Breart G, Kamihagi K, Delmas PD. Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: the EPIDOS Study. J Clin Endocrinol Metab. 1997 Mar;82(3):719-24.
• **Large prospective population-based cohort study in elderly women replicating the observation that higher levels of circulating undercarboxylated osteocalcin, but not total osteocalcin, predict hip fracture risk independently of femoral neck bone mineral density.**
50. Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. J Clin Invest. 1993 Apr;91(4):1769-74.
•• **Prospective cohort study in 195 institutionalized elderly women. The risk of hip fracture was increased in women with elevated undercarboxylated osteocalcin. The level of undercarboxylated osteocalcin was negatively correlated with 25OH-vitamin D, and one year of calcium/vitamin D2 treatment decreased undercarboxylated osteocalcin, yet, no effects on fracture end points were reported.**
51. Lian JB, Stein GS, Stein JL, van Wijnen AJ. Regulated expression of the bone-specific osteocalcin gene by vitamins and hormones. Vitam Horm. 1999;55:443-509.
52. Bolton-Smith C, McMurdo ME, Paterson CR, Mole PA, Harvey JM, Fenton ST, et al. Two-year randomized controlled trial of vitamin K1 (phylloquinone) and vitamin D3 plus calcium on the bone health of older women. J Bone Miner Res. 2007 Apr;22(4):509-19.
53. Deyl Z, Adam M. Evidence for vitamin D dependent gamma-carboxylation in osteocalcin related proteins. Biochem Biophys Res Commun. 1983 May 31;113(1):294-300.
54. Jayalath RW, Mangan SH, Golledge J. Aortic calcification. Eur J Vasc Endovasc Surg. 2005 Nov;30(5):476-88.
55. Kim KJ, Kim KM, Park KH, Choi HS, Rhee Y, Lee YH, et al. Aortic calcification and bone metabolism: the relationship between aortic calcification, BMD, vertebral fracture, 25-hydroxyvitamin D, and osteocalcin. Calcif Tissue Int. 2012 Dec;91(6):370-8.
56. Antuna-Puente B, Feve B, Fellahi S, Bastard JP. Adipokines: the missing link between insulin resistance and obesity. Diabetes Metab. 2008 Feb;34(1):2-11.
57. Kanabrocki EL, Hermida RC, Wright M, Young RM, Bremner FW, Third JL, et al. Circadian variation of serum leptin in healthy and diabetic men. Chronobiol Int. 2001 Mar;18(2):273-83.

58. Gordeladze JO, Drevon CA, Syversen U, Reseland JE. Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: Impact on differentiation markers, apoptosis, and osteoclastic signaling. J Cell Biochem. 2002;85(4):825-36.
59. Hamrick MW, Della-Fera MA, Choi YH, Pennington C, Hartzell D, Baile CA. Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. J Bone Miner Res. 2005 Jun;20(6):994-1001.
60. Steppan CM, Crawford DT, Chidsey-Frink KL, Ke H, Swick AG. Leptin is a potent stimulator of bone growth in ob/ob mice. Regul Pept. 2000 Aug 25;92(1-3):73-8.
61. Cornish J, Callon KE, Bava U, Lin C, Naot D, Hill BL, et al. Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. J Endocrinol. 2002 Nov;175(2):405-15.
62. Holloway WR, Collier FM, Aitken CJ, Myers DE, Hodge JM, Malakellis M, et al. Leptin inhibits osteoclast generation. J Bone Miner Res. 2002 Feb;17(2):200-9.
63. Di Monaco M, Vallero F, Di Monaco R, Mautino F, Cavanna A. Fat body mass, leptin and femur bone mineral density in hip-fractured women. J Endocrinol Invest. 2003 Dec;26(12):1180-5.
64. Zoico E, Zamboni M, Adami S, Vettor R, Mazzali G, Tosoni P, et al. Relationship between leptin levels and bone mineral density in the elderly. Clin Endocrinol (Oxf). 2003 Jul;59(1):97-103.
65. Jurimae J, Kums T, Jurimae T. Adipocytokine and ghrelin levels in relation to bone mineral density in physically active older women: longitudinal associations. Eur J Endocrinol. 2009 Mar;160(3):381-5.
66. Schett G, Kiechl S, Bonora E, Redlich K, Woloszczuk W, Oberhollenzer F, et al. Serum leptin level and the risk of nontraumatic fracture. Am J Med. 2004 Dec 15;117(12):952-6.
67. Barbour KE, Zmuda JM, Boudreau R, Strotmeyer ES, Horwitz MJ, Evans RW, et al. Adipokines and the risk of fracture in older adults. J Bone Miner Res. 2011 Jul;26(7):1568-76.
68. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, et al. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab. 2001 May;86(5):1930-5.
69. Berner HS, Lyngstadaas SP, Spahr A, Monjo M, Thommesen L, Drevon CA, et al. Adiponectin and its receptors are expressed in bone-forming cells. Bone. 2004 Oct;35(4):842-9.
70. Luo XH, Guo LJ, Xie H, Yuan LQ, Wu XP, Zhou HD, et al. Adiponectin stimulates RANKL and inhibits OPG expression in human osteoblasts through the MAPK signaling pathway. J Bone Miner Res. 2006 Oct;21(10):1648-56.
71. Oshima K, Nampei A, Matsuda M, Iwaki M, Fukuhara A, Hashimoto J, et al. Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. Biochem Biophys Res Commun. 2005 Jun 3;331(2):520-6.
72. Richards JB, Valdes AM, Burling K, Perks UC, Spector TD. Serum adiponectin and bone mineral density in women. J Clin Endocrinol Metab. 2007 Apr;92(4):1517-23.
73. Delhanty PJ, van der Eerden BC, van Leeuwen JP. Ghrelin and bone. Biofactors. 2014 Jan-Feb;40(1):41-8.
74. Biver E, Salliot C, Combescure C, Gossec L, Hardouin P, Legroux-Gerot I, et al. Influence of adipokines and ghrelin on bone mineral density and fracture risk: a systematic review and meta-analysis. J Clin Endocrinol Metab. 2011 Sep;96(9):2703-13.
75. Donath MY. Targeting inflammation in the treatment of type 2 diabetes: time to start. Nat Rev Drug Discov. 2014 Jun;13(6):465-76.
76. Bastard JP, Jardel C, Delattre J, Hainque B, Bruckert E, Oberlin F. Evidence for a link between adipose tissue interleukin-6 content and serum C-reactive protein concentrations in obese subjects. Circulation. 1999 Apr 27;99(16):2221-2.
77. Kahn SE, Zinman B, Haffner SM, O'Neill MC, Kravitz BG, Yu D, et al. Obesity is a major determinant of the association of C-reactive protein levels and the metabolic syndrome in type 2 diabetes. Diabetes. 2006 Aug;55(8):2357-64.
78. de Pablo P, Cooper MS, Buckley CD. Association between bone mineral density and C-reactive protein in a large population-based sample. Arthritis Rheum. 2012 Aug;64(8):2624-31.
79. Koh JM, Khang YH, Jung CH, Bae S, Kim DJ, Chung YE, et al. Higher circulating hsCRP levels are associated with lower bone mineral density in healthy pre- and postmenopausal women: evidence for a link between systemic inflammation and osteoporosis. Osteoporos Int. 2005 Oct;16(10):1263-71.

80. Oei L, Campos-Obando N, Dehghan A, Oei EH, Stolk L, van Meurs JB, et al. Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study. Osteoporos Int. 2014 Apr;25(4):1247-54.

- **Serum high-sensitivity C-reactive protein is associated with fracture risk and lower bending strength, yet, there is no evidence so far for this relationship being causal.**

81. Pasco JA, Kotowicz MA, Henry MJ, Nicholson GC, Spilsbury HJ, Box JD, et al. High-sensitivity C-reactive protein and fracture risk in elderly women. JAMA. 2006 Sep 20;296(11):1353-5.
82. Rolland T, Boutroy S, Vilayphiou N, Blaizot S, Chapurlat R, Szulc P. Poor trabecular microarchitecture at the distal radius in older men with increased concentration of high-sensitivity C-reactive protein--the STRAMBO study. Calcif Tissue Int. 2012 Jun;90(6):496-506.
83. Kanai A, Kawamura T, Umemura T, Nagashima M, Nakamura N, Nakayama M, et al. Association between future events of brain infarction and soluble levels of intercellular adhesion molecule-1 and C-reactive protein in patients with type 2 diabetes mellitus. Diabetes Res Clin Pract. 2008 Nov;82(2):157-64.
84. Pu LJ, Lu L, Xu XW, Zhang RY, Zhang Q, Zhang JS, et al. Value of serum glycated albumin and high-sensitivity C-reactive protein levels in the prediction of presence of coronary artery disease in patients with type 2 diabetes. Cardiovasc Diabetol. 2006;5:27.
85. Cox AJ, Agarwal S, D MH, Carr JJ, Freedman BI, Bowden DW. C-reactive protein concentration predicts mortality in type 2 diabetes: the Diabetes Heart Study. Diabet Med. 2012 Jun;29(6):767-70.
86. Jin C, Lu L, Zhang RY, Zhang Q, Ding FH, Chen QJ, et al. Association of serum glycated albumin, C-reactive protein and ICAM-1 levels with diffuse coronary artery disease in patients with type 2 diabetes mellitus. Clin Chim Acta. 2009 Oct;408(1-2):45-9.
87. Wensley F, Gao P, Burgess S, Kaptoge S, Di Angelantonio E, Shah T, et al. Association between C reactive protein and coronary heart disease: mendelian randomisation analysis based on individual participant data. BMJ. 2011;342:d548.
88. Sorisky A. Molecular links between obesity and cardiovascular disease. Am J Ther. 2002 Nov-Dec;9(6):516-21.
89. Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation. 2000 Oct 31;102(18):2165-8.
90. Kodama K, Horikoshi M, Toda K, Yamada S, Hara K, Irie J, et al. Expression-based genome-wide association study links the receptor CD44 in adipose tissue with type 2 diabetes. Proc Natl Acad Sci U S A. 2012 May 1;109(18):7049-54.
91. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.

- **Genome-wide association study meta-analysis that identified loci for bone mineral density and fracture risk, among which the 4q22.1 locus containing osteopontin.**

92. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
93. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
94. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008 May 29;358(22):2355-65.
95. Hunter GK, O'Young J, Grohe B, Karttunen M, Goldberg HA. The flexible polyelectrolyte hypothesis of protein-biomineral interaction. Langmuir. 2010 Dec 21;26(24):18639-46.
96. Choi ST, Kim JH, Kang EJ, Lee SW, Park MC, Park YB, et al. Osteopontin might be involved in bone remodelling rather than in inflammation in ankylosing spondylitis. Rheumatology (Oxford). 2008 Dec;47(12):1775-9.
97. Reinholt FP, Hultenby K, Oldberg A, Heinegard D. Osteopontin--a possible anchor of osteoclasts to bone. Proc Natl Acad Sci U S A. 1990 Jun;87(12):4473-5.

98. Wang KX, Denhardt DT. Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev. 2008 Oct-Dec;19(5-6):333-45.
99. Franzen A, Hultenby K, Reinholt FP, Onnerfjord P, Heinegard D. Altered osteoclast development and function in osteopontin deficient mice. J Orthop Res. 2008 May;26(5):721-8.
100. Nomiyama T, Perez-Tilve D, Ogawa D, Gizard F, Zhao Y, Heywood EB, et al. Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. J Clin Invest. 2007 Oct;117(10):2877-88.
101. Oei L, Hsu YH, Styrkarsdottir U, Eussen BH, de Klein A, Peters MJ, et al. A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus. J Med Genet. 2014 Feb;51(2):122-31.
• **Fracture associated deletions in the 6p25.1 locus may influence the regulatory region of peroxisomal D3,D2-enoylCoA isomerase (PECI) which has been suggested as an auto-antigen in diabetes.**
102. Mountz JD, Edwards CK, 3rd. Murine models of autoimmunity: T-cell and B-cell defects. Curr Opin Rheumatol. 1992 Oct;4(5):612-20.
103. Billings LK, Hsu YH, Ackerman RJ, Dupuis J, Voight BF, Rasmussen-Torvik LJ, et al. Impact of common variation in bone-related genes on type 2 diabetes and related traits. Diabetes. 2012 Aug;61(8):2176-86.
104. Ekholm E, Hankenson KD, Uusitalo H, Hiltunen A, Gardner H, Heino J, et al. Diminished callus size and cartilage synthesis in alpha 1 beta 1 integrin-deficient mice during bone fracture healing. Am J Pathol. 2002 May;160(5):1779-85.
105. Petit MA, Paudel ML, Taylor BC, Hughes JM, Strotmeyer ES, Schwartz AV, et al. Bone mass and strength in older men with type 2 diabetes: the Osteoporotic Fractures in Men Study. J Bone Miner Res. 2010 Feb;25(2):285-91.
106. Ahlborg HG, Johnell O, Turner CH, Rannevik G, Karlsson MK. Bone loss and bone size after menopause. N Engl J Med. 2003 Jul 24;349(4):327-34.
107. Seeman E. Clinical review 137: Sexual dimorphism in skeletal size, density, and strength. J Clin Endocrinol Metab. 2001 Oct;86(10):4576-84.
108. Sayers A, Lawlor DA, Sattar N, Tobias JH. The association between insulin levels and cortical bone: findings from a cross-sectional analysis of pQCT parameters in adolescents. J Bone Miner Res. 2012 Mar;27(3):610-8.
109. Burghardt AJ, Issever AS, Schwartz AV, Davis KA, Masharani U, Majumdar S, et al. High-resolution peripheral quantitative computed tomographic imaging of cortical and trabecular bone microarchitecture in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Nov;95(11):5045-55.
•• **A high-resolution peripheral quantitative computed tomography (HR-pQCT) pilot study demonstrating that type 2 diabetes mellitus patients have higher trabecular volumetric bone mineral density adjacent to the cortex, possibly reflecting an inefficient redistribution of bone mass, characterized by loss of intracortical bone offset by an elevation in trabecular bone density.**
110. Patsch JM, Burghardt AJ, Yap SP, Baum T, Schwartz AV, Joseph GB, et al. Increased cortical porosity in type 2 diabetic postmenopausal women with fragility fractures. J Bone Miner Res. 2013 Feb;28(2):313-24.
•• **A medium-size imaging study comparing peripheral bone microarchitecture and strength between postmenopausal women with type 2 diabetes with and without fragility fractures, implicating a part for deficits in cortical bone quality.**
111. Pothuaud L, Carceller P, Hans D. Correlations between grey-level variations in 2D projection images (TBS) and 3D microarchitecture: applications in the study of human trabecular bone microarchitecture. Bone. 2008 Apr;42(4):775-87.
112. Leslie WD, Aubry-Rozier B, Lamy O, Hans D. TBS (trabecular bone score) and diabetes-related fracture risk. J Clin Endocrinol Metab. 2013 Feb;98(2):602-9.
113. Patsch JM, Li X, Baum T, Yap SP, Karampinos DC, Schwartz AV, et al. Bone marrow fat composition as a novel imaging biomarker in postmenopausal women with prevalent fragility fractures. J Bone Miner Res. 2013 Aug;28(8):1721-8.
114. Baum T, Yap SP, Karampinos DC, Nardo L, Kuo D, Burghardt AJ, et al. Does vertebral bone marrow fat content correlate with abdominal adipose tissue, lumbar spine bone mineral density, and blood biomarkers in women with type 2 diabetes mellitus? J Magn Reson Imaging. 2012 Jan;35(1):117-24.

115. Bridges D, Randall C, Hansma PK. A new device for performing reference point indentation without a reference probe. Rev Sci Instrum. 2012 Apr;83(4):044301.
116. Randall C, Bridges D, Guerri R, Nogues X, Puig L, Torres E, et al. Applications of a New Handheld Reference Point Indentation Instrument Measuring Bone Material Strength. J Med Device. 2013 Dec;7(4):410051-6.
117. Diez-Perez A, Guerri R, Nogues X, Caceres E, Pena MJ, Mellibovsky L, et al. Microindentation for in vivo measurement of bone tissue mechanical properties in humans. J Bone Miner Res. 2010 Aug;25(8):1877-85.
118. Guerri-Fernandez RC, Nogues X, Quesada Gomez JM, Torres Del Pliego E, Puig L, Garcia-Giralt N, et al. Microindentation for in vivo measurement of bone tissue material properties in atypical femoral fracture patients and controls. J Bone Miner Res. 2013 Jan;28(1):162-8.
•• **In vivo microindentation testing of the tibia was applied in patients with type 2 diabetes finding reduced serum markers of bone turnover and lower bone material strength at the tibia than age-matched, controls without diabetes.**
119. Farr JN, Drake MT, Amin S, Melton LJ, 3rd, McCready LK, Khosla S. In Vivo assessment of bone quality in postmenopausal women with type 2 diabetes. J Bone Miner Res. 2013 Oct 1.
120. Blakytny R, Spraul M, Jude EB. Review: The diabetic bone: a cellular and molecular perspective. Int J Low Extrem Wounds. 2011 Mar;10(1):16-32.
121. Taniguchi T, Matsumoto T, Shindo H. Changes of serum levels of osteocalcin, alkaline phosphatase, IGF-I and IGF-binding protein-3 during fracture healing. Injury. 2003 Jul;34(7):477-9.
122. Akesson K, Vergnaud P, Delmas PD, Obrant KJ. Serum osteocalcin increases during fracture healing in elderly women with hip fracture. Bone. 1995 Apr;16(4):427-30.
123. Nyman MT, Paavolainen P, Forsius S, Lamberg-Allardt C. Clinical evaluation of fracture healing by serum osteocalcin and alkaline phosphatase. Ann Chir Gynaecol. 1991;80(3):289-93.
124. Hamann C, Picke AK, Campbell GM, Balyura M, Rauner M, Bernhardt R, et al. Effects of parathyroid hormone on bone mass, bone strength, and bone regeneration in male rats with type 2 diabetes mellitus. Endocrinology. 2014 Apr;155(4):1197-206.
125. Bauer DC. Calcium supplements and fracture prevention. N Engl J Med. 2014 Jan 23;370(4):387-8.
126. Bolland MJ, Grey A, Avenell A, Reid IR. Calcium Supplements Increase Risk of Myocardial Infarction. J Bone Miner Res. 2014 Sep 11.
127. Lewis JR, Radavelli-Bagatini S, Rejnmark L, Chen JS, Simpson JM, Lappe JM, et al. Response to "Calcium supplements increase risk of myocardial infarction". J Bone Miner Res. 2014 Nov 21.
128. Bolland MJ, Avenell A, Baron JA, Grey A, MacLennan GS, Gamble GD, et al. Effect of calcium supplements on risk of myocardial infarction and cardiovascular events: meta-analysis. BMJ. 2010;341:c3691.
129. Michaelsson K, Melhus H, Warensjo Lemming E, Wolk A, Byberg L. Long term calcium intake and rates of all cause and cardiovascular mortality: community based prospective longitudinal cohort study. BMJ. 2013;346:f228.
130. Prentice RL, Pettinger MB, Jackson RD, Wactawski-Wende J, Lacroix AZ, Anderson GL, et al. Health risks and benefits from calcium and vitamin D supplementation: Women's Health Initiative clinical trial and cohort study. Osteoporos Int. 2013 Feb;24(2):567-80.
131. Lewis JR, Radavelli-Bagatini S, Rejnmark L, Chen JS, Simpson JM, Lappe JM, et al. The effects of calcium supplementation on verified coronary heart disease hospitalization and death in postmenopausal women: a collaborative meta-analysis of randomized controlled trials. J Bone Miner Res. 2015 Jan;30(1):165-75.
132. Gonnelli S, Caffarelli C, Giordano N, Nuti R. The prevention of fragility fractures in diabetic patients. Aging Clin Exp Res. 2014 Jul 25.

Chapter 2.7

Osteoporotic vertebral fractures as part of systemic disease

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ABSTRACT

Our understanding of the genetic control of skeletogenesis and bone remodeling is expanding, and normally, bone resorption and bone formation are well balanced through regulation by hormones, growth factors and cytokines. Osteoporosis is considered a systemic disease characterized by low bone mass and microarchitectural deterioration of bone tissue. Consequent increased bone fragility results in higher fracture risk. The most common osteoporotic fractures are located in the spine and they form a significant health issue. A large variety of systemic diseases are associated with risk of osteoporotic vertebral fractures, illustrating its multi-factorial etiology. Prevalences of these conditions vary from common to extremely rare and incidence peaks differ according to etiology. This review appreciates different aspects of osteoporotic vertebral fractures as part of systemic disease, including genetic, immunologic, inflammatory, metabolic and endocrine pathways. It seems impossible to be all-comprehensive on this topic, nevertheless, we hope to provide a reasonably thorough overview. Plenty remains to be elucidated in this field, identifying even more associated diseases and further exposing pathophysiological mechanisms underlying osteoporotic vertebral fractures.

INTRODUCTION

Osteoporosis is considered a systemic disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. Our understanding of the genetic control of skeletogenesis and bone remodeling is expanding. Normally, bone resorption and bone formation are well balanced and regulated by hormones, growth factors and cytokines. Various internal and external factors are known to contribute to the risk of osteoporosis, illustrating the multi-factorial etiology of the condition. The most well-known clinical risk factors for osteoporosis and fractures include age, lower body mass index,[1] immobility,[2-4] smoking,[5] alcohol consumption,[6] and glucocorticoid use[7]. In addition, a positive family history confers an increased risk of fracture[8]. The term secondary osteoporosis refers to disorders that are strongly associated with osteoporosis;[9] these include diseases with systemic inflammation like rheumatoid arthritis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), but also diabetes, hypogonadism (including premature menopause), malnutrition and malabsorption. Medication use may also predispose to elevated fracture risk, but, this is beyond the scope of this review. This list is all-but comprehensive and, undoubtedly, many more risk factors and associated diseases are to be discovered.

Vertebral fractures are the most common osteoporotic fractures and they are often a first manifestation of osteoporosis. These fractures represent a significant health issue[10, 11] as they are associated with a high morbidity, including but not limited to acute and chronic pain, loss of independence, height loss, kyphosis, depression, higher risk of additional future vertebral and non-vertebral fractures,[12-17] and increased mortality[18, 19]. There may be skeletal-site specific effects of fracture determinants, meriting the study of vertebral fractures apart from non-vertebral fractures, as we will discuss later in this review. Risk factors that have been specifically validated for incident vertebral fractures include: prevalent vertebral fractures, older age, female gender, lower body height and weight, smoking history and use of a walking aid.[20, 21] Very recently, Schousboe et al. pioneered in prediction models for osteoporotic vertebral fractures in older men[22] and women[23], and although promising yielding area under the receiver operating curves of up to 0.69, validation in independent studies is needed and future research may identify additional risk factors that enhance prediction of incident osteoporotic vertebral fractures.

This review appreciates different aspects of osteoporotic vertebral fractures as part of systemic disease, touching on genetic, metabolic and inflammatory pathways and organ system dysfunction.

Structural vertebral deformities and fractures

Several methods for radiological assessment of vertebral fractures exist, but a gold standard is lacking.[24] Traditionally conventional radiography has been the imaging modality of choice. Yet, two advantages of dual-energy X-ray absorptiometry (DXA) over conventional radiography for vertebral fracture assessment are the lower radiation dose and capture of the whole spine in one image with virtually no divergent radiation beam issues, particularly because DXA imaging resolution has improved drastically with the introduction of state-of-the-art machinery. Another novel add-on to DXA is the trabecular bone score (TBS), a measure of bone texture, which correlates with 3D parameters of bone microarchitecture reflecting bone quality and which is partly independent from DXA-measured lumbar spine bone mineral density (LS-BMD).[25] In any case, a number of differential diagnoses remain that complicate the diagnosis of vertebral fractures, including degenerative diseases, anatomical variation and anomalies.[26] More is becoming clear about these conditions and the possible presence or absence of an interrelationship with osteoporosis and associated fractures, as discussed in the following section. Therefore, we start the

review by discussing the definition of osteoporotic vertebral fractures and mimickers of that should not be confused with vertebral fractures.

Non-fracture deformities represented by anatomical variation and developmental abnormalities have been reviewed extensively by Ferrar et al.[27] From a lateral view, the spine has a natural curvature. Vertebrae in the mid-thoracic region are more wedge-shaped, causing a mild kyphosis. Lumbar vertebrae have a relatively shorter posterior height and tend to be biconcave resulting in a normal lordotic curve. Some individuals have developmentally smaller or shorter vertebrae, particularly in anterior height found most commonly in the mid-thoracic region. This is thought to be due to congenital variation or as the result of inhibited growth of the vertebral body during childhood or adolescence, and it is also thought that these variants should not be regarded as fractures.[28] In so-called "step-like" or "step-off" endplates the central endplate is deeper with an abrupt transition to the more normal periphery. This is in contrast to the appearance of the fractured endplate in osteoporosis, in which, a smooth, concave depression extends from corner to corner of the vertebral body.[27] These "step-off" endplates seem to be the consequence of a growth retardation in the central portion of the endplate due to central circulatory stasis. In contrast, the periphery of the growth plate has a different blood supply through short arteries, in which vaso-occlusion and microinfarction may lead to avascular necrosis and further developmental disruption of the vertebral body.[29] Diseases that have been listed as associated with these observations are Gaucher's disease, hemolytic anemias including hereditary spherocytosis, sickle cell, and thalassemia hemoglobinopathies.[30] The cortical margins of the inferior endplates of predominantly lumbar vertebral bodies L3 to L5 frequently have paired parasagittal concavities when viewed in the frontal projection, resembling the curvature of an aimed bow.[31] When viewed in the lateral projection, the concavities are superimposed and lie in the posterior portion of the vertebral body, and could then be confused with fractures.[27] This aspect, called "Cupid's bow" is considered a normal anatomic variant. Histologic examination in cadavers showed thickened bone in the Cupid's bow endplate with annular fibers inserting into this region, which was detected at multiple lumbar and thoracic levels, with the highest frequency in the lower lumbar spine.[32] Furthermore, the endplates tend to become progressively deeper with lower vertebral level and another commonly seen normal variant is a deep inferior endplate. Another developmental variation is represented by balloon discs, where there is an occurrence of an unusually concave disc-vertebral border at multiple levels. A Japanese study has reported a prevalence up to 14% in the healthy population, with an association with male gender and height, but a lack of a relation with back pain or age;[33] yet, to our knowledge no replication and validation studies have been published.

A specific example of an anatomical anomaly of the vertebrae that could be confused with vertebral fractures is Scheuermann's disease. With reported prevalence rates of up to 10%, the disease is frequently mentioned in the differential diagnosis of osteoporotic vertebral fractures.[27] It is a form of osteochondrosis of the spine characterized by increased posterior rounding of the thoracic spine in association with structural deformity of the vertebral elements.[34, 35] Scheuermann's disease often first appears during adolescence at the time of puberty, resulting in permanent vertebral distortion and back pain in many cases. The etiology is unknown, but genetics most likely plays a significant role;[36] genetic surveys are underway. Scheuermann's disease is diagnosed on the basis of radiographic criteria of which those defined by Sørensen and Sachs are the most commonly applied: a thoracic kyphosis greater than 45°; at least three adjacent wedge-shaped vertebral bodies of 5° or more; endplate irregularities with possible vertebral elongation; disc space narrowing. In addition, Schmorl's nodes are thought to be a common but not obligate manifestation of Scheuermann's disease.[37-40] Although coexistence of Scheuermann's disease with osteoporotic vertebral fractures may occur, it is thought that the disorders

should be distinguished as clinical disease treatments differ.[41] Nonetheless, very little data exists on a possible connection between the two diseases. A few studies with small patient numbers have been conducted a long time ago, of which some suggest that patients with Scheuermann's disease have generalized lower bone mineral density (BMD).[42, 43] Some suggest this is a transient effect that resolves at adulthood,[44] whereas no effect at all was found by others[45]. However, no investigations have looked into osteoporotic vertebral fracture risk in Scheuermann's disease.

More generally speaking, the vertebral endplates are thought to contain the adjacent intervertebral discs and evenly distribute applied loads to these discs and the bony material of the vertebral bodies themselves. Consequently, sclerosis or ossification of the endplate may impact the nutritional supply and hydration of the intervertebral disc. Similar to Scheuermann's disease, also with aging, degenerative alterations may take place such as lumbar disc degeneration.[46] Lumbar disc degeneration and osteoporosis are two age-related skeletal diseases that are highly prevalent in the elderly.[47, 48] Intriguingly, it has been previously shown, that the presence of lumbar disc degeneration is associated with higher BMD.[49-51] In theory, the higher BMD found in subjects with lumbar disc degeneration should correspond to lower fracture risk compared to subjects without the condition. However, a systematic review that was published recently on this topic demonstrated that although subjects with lumbar disc degeneration have systematically higher BMD, at least at the lumbar spine, femoral neck, skull, and total body. In spite of this systematically higher BMD, persons with lumbar disc degeneration are at higher risk of osteoporotic fractures. This particularly applies to males in whom lumbar disc degeneration seems more severe. Possibly, these observations could be explained by direct and indirect effects. Loss of disc height and deterioration of its biomechanical properties produce high tensile strains in the vertebral endplate which may be causally related to "failure of the vertebra".[52, 53] Furthermore, individuals with lumbar disc degeneration have more stiffness in the trunk and lower legs, which could increase the reaction time during falling and other demanding occupational activities.[54, 55] The exact mechanisms to explain the associations merit further investigation.

From an antero-posterior view, a few percent of the general population has a sideways curvature of their spine, that is, scoliosis. A scoliosis is diagnosed with a Cobb angle of more than 10^0 in the frontal plain.[56, 57] Presence of scoliosis may impede DXA-BMD measurement and assessment for vertebral fractures.[27, 58] Scoliosis can be categorized into several groups according to the etiology. First, scoliosis may occasionally be of congenital origin, arising during embryonic development and oftentimes it is then part of a syndrome.[59] Second, adolescent idiopathic scoliosis embodies a substantial proportion of the cases and it is the most common pediatric skeletal disease. The etiology of adolescent idiopathic scoliosis remains largely unknown, but population and twin studies strongly suggest a contribution from genetic factors.[60] So far, findings from linkage and candidate gene association studies involved genes related to connective tissue structure, bone formation/metabolism, melatonin signaling pathways, puberty and growth, and axon guidance pathways, but these results remain to be replicated.[61] Two genome-wide association studies (GWAS) containing a replication phase have been published until now and described associations with loci containing the candidate genes ladybird homeobox 1 (*LBX1*) and G protein–coupled receptor 126 (*GPR126*), of which the functions remain to be elucidated further. Bone quality deterioration and lower bone mass have been recounted as a systemic phenomenon at the hip, spine and other peripheral sites in a significant percentage of adult idiopathic scoliosis patients.[58, 62-65]. Whether this decrease in bone mass is associated with an increased risk of osteoporotic spine fractures or not has not yet been investigated prospectively. Thirdly, the clinically most prominent groups in adult scoliosis are primary and secondary degenerative scoliosis.[66] In the so-called primary degenerative

scoliosis ("de novo" form) there is an asymmetric disc degeneration and facet joint degeneration, mostly located in the thoracolumbar or lumbar spine, which is associated with aging.[67] Secondary degenerative scoliosis may appear as a consequence of pelvic obliquity due to a leg length discrepancy, hip pathology or a neuromuscular problem. Finally, osteoporotic vertebral fractures may bring about an asymmetric configuration with the appearance of kyphosis, scoliosis, or both.[66]

Systems genetics of vertebral fractures

As mentioned previously, one of the most important risk factors for osteoporosis and fractures is a positive family history.[68] Previous studies have reported estimates of heritability of BMD and fractures of up to 66% and 46%, respectively.[69, 70] Furthermore, a recent report has formally quantified that there is a mixture of shared and specific genetic influences for distinct DXA-BMD traits, where the strength of genetic variants may differ in their association and magnitude of effect across different skeletal sites, or, moreover, some loci seem to act in certain skeletal locations, whereas they are irrelevant to others.[71] Such a difference in genetic basis had previously been proposed for LS-BMD versus femoral neck BMD (FN-BMD) in GWAS.[72] Although the occurrence of fracture is the most important clinical outcome in osteoporosis, identifying genetic determinants contributing to the risk of fracture has been difficult because of its multifactorial nature and occurrence late in life. For this reason, correlated intermediate phenotypes such as BMD measured by DXA have attracted the interest of researchers in the field of genetics of osteoporosis. The most recent GWAS meta-analysis concluded that the identified genetic factors related to LS-BMD and FN-BMD as measured by DXA cluster in three key biological pathways being mesenchymal stem cell differentiation, Wnt signaling and RANK–RANKL–OPG (receptor activator of nuclear factor kappa-B–receptor activator of nuclear factor kappa-B ligand–osteoprotegerin).[73] Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone-forming cells), chondrocytes (cartilage cells), and adipocytes (fat cells). Wnt signaling consists of a group of signal transduction pathways made of proteins that pass signals from extracellularly through cell surface receptors to the inside of the cell. It plays an essential role in development and maintenance of a multitude of organs and tissues including bone, contributing fundamentally in osteoblastogenesis.[74] The RANK–RANKL–OPG system is crucial for well-balanced bone remodeling through well-orchestrated activation of osteoclasts by osteoblasts.[75] Another critical process highlighted in this study was endochondral ossification, the accumulation of intracellular calcium by chondrocytes to form calcified bone tissue in the developing skeleton.

A growing number of GWAS have revealed genetic loci for fracture risk of any type.[73, 76, 77] A study by Liu et al. has obtained heritability estimates of prevalent moderate and severe vertebral fractures (semi-quantitative (SQ) grade ≥2) as assessed on computed tomography (CT) ranging between 43% and 53%, and increasing to approximately 69% when adjusting for volumetric BMD (vBMD) and cross-sectional area (CSA).[78] The very first GWAS for radiographic vertebral fractures discovered a single nucleotide polymorphism (SNP) on chromosome 16q24 at genome-wide significant level.[79]. The SNP maps to a region previously found associated with DXA LS-BMD in two large meta-analyses.[72, 73]. From a biomedical perspective, deletions/mutations in this 16q24 locus are implicated in a combination of birth defects that includes vertebral defects in both humans and mice.[80] Furthermore, the neighboring forkhead box C2 (*FOXC2*) candidate gene encodes a transcription factor essential for axial skeletogenesis in mice.[81] However, this GWAS association could not be convincingly replicated by de novo genotyping the specific marker in a large-scale global replication effort, displaying a high degree of heterogeneity of effects.[79] It was speculated that apart from the possibility of the signal being a false positive associa-

tion in the discovery, phenotype definition issues as discussed in the previous subchapter may have undermined establishment of a firm correlation. For instance, it is thought that many mild vertebral deformities represent non-fracture deformities such as degenerative changes. Indeed, in the study by Liu et al., after adding mild grade 1 vertebral deformities into the analyses, the heritability was much lower (aforementioned 43% to 69% dropping to 19% to 27%) with a lower intra-reader agreement (kappa =0.56–0.59 versus kappa=0.68–0.72 for grade ≥2).[78]

There are a multitude of additional candidate genes that could be screened for in unusual clinical cases presenting with vertebral fractures, for instance genetic mutations that are known to cause monogenic forms of osteoporosis or osteogenesis imperfecta, for example: *COL1A1*,[82]*COL1A2, LRP5*,[83]*WNT1*,[84]*LGR4*,[85], *PLS3*[86], *CRTAP, FKBP10, LEPRE1, PLOD2, PPIB, SERPINF1, SERPINH1* and *SP7*[87].

Immunologic, metabolic and endocrine factors with respect to vertebral fractures

Chronic inflammation is associated with bone loss, and even low grade sub-clinical inflammation has been reported to increase fracture risk.[88] Bone loss in patients with inflammatory diseases is thought to be due to direct effects of inflammation, poor nutrition, reduced lean body mass, immobility and the side-effects of treatments, especially glucocorticoids.[89] Inflammatory diseases can increase bone resorption, decrease bone formation, but most commonly affect both these processes resulting in an uncoupling of bone formation from resorption resulting in bone loss. This will be detrimental to the total quantity of bone mass and the spatial distribution at macro- and micro-architectural levels[90]. The most commonly measured inflammatory serum bio-marker is C-reactive protein (CRP), a protein found in the blood which levels rise in response to inflammation. CRP levels have been found associated with fracture risk including radiographic vertebral fractures[91, 92]. Particular autoimmune diseases in which increased risk of spinal fractures have been confirmed by research data are: rheumatoid arthritis,[93-96] systemic lupus erythematosus,[97, 98] sarcoidosis,[99] inflammatory bowel disease[100, 101] and spondylarthropathies[102-104].

Metabolic and endocrine factors with respect to vertebral fractures

Systemic glucocorticoids have been prescribed world-wide for various diseases since decades, such as the aforementioned autoimmune diseases. Prednisone is the most commonly prescribed synthetic corticosteroid. Although these steroids are a highly effective treatment, they are known to have severe adverse effects on for example the musculoskeletal and gonadal systems,[105, 106] particularly when administered in high doses and for prolonged periods. Natural glucocorticoids are produced by the adrenal glands and are of vital importance. Cushing's disease refers to pituitary disease and Cushing's syndrome includes exogenous administration and for instance, adrenal overproduction. Both endogenous and exogenous hypercortisolism induce osteoporosis and fractures, and as glucocorticoids preferentially affect trabecular bone, the spine is seriously put at risk. Chronic glucocorticoid therapy has unequivocally been linked to BMD loss at the spine and vertebral fracture risk.[107] Although rarer, clinical but also subclinical endogenous hypercortisolism, has repeatedly been connected to lower LS-BMD,[108] worse bone quality as quantified by TBS,[109] incident and prevalent vertebral fractures[110, 111].

Diabetes mellitus affects bone metabolism. It has been demonstrated that patients with type 1 diabetes have low BMD[112] and elevated prevalence of non-spine and asymptomatic vertebral fractures,[113] and these fractures are associated with the presence of type 1 diabetes independently of BMD[114]. Furthermore, both men and women with type 2 diabetes have a higher fracture risk[115] despite a paradoxically systematically higher DXA-BMD at the femoral neck, hip and spine;[116] an effect which seems to be modified by the degree of glucose regulation[117]. The increased fracture risk is most likely

through compromised bone microarchitecture.[118-122] Schwartz et al. established recently that the current World Health Organization's fracture risk assessment tool (FRAX) underestimates osteoporotic fracture risk in individuals with diabetes;[123] reason why diabetes as a risk factor will be considered for inclusion in future iterations of FRAX[124, 125]. It has been observed in different ethnicities that patients with type 2 diabetes may have an increased risk of vertebral fractures independent of BMD or diabetic complication status.[126, 127] A highly complex and heterogeneous pathophysiology seems to underlie vertebral fracture risk in diabetes-related bone disease. Elevated levels of advanced glycation endproducts (AGEs) have been found detrimental,[128] and higher levels of the endogenous secretory receptor for AGEs (esRAGE), acting as a decoy binding AGEs, have protective effects on vertebral fracture risk in diabetes[129]. Other studies showed that lower concentrations of serum insulin-like growth factor-I (IGF-I) levels were associated with a higher number of prevalent vertebral fractures in postmenopausal women with type 2 diabetes.[130, 131]

IGF-I is a hormone similar in molecular structure to insulin and is a primary mediator of the effects of growth hormone. IGF-I is required for the anabolic effects of growth hormone on bone[132, 133] and, specifically in men, free IGF-I levels are positively related with LS-BMD[134]. Furthermore, there is an increased prevalence of radiological spinal deformities in adult patients with growth hormone deficiency, particularly in those patients with untreated disease and longer disease duration.[135] Yet, in acromegaly, a disorder caused by excess growth hormone production by the pituitary gland, an increased prevalence of radiological spinal deformities has also been observed.[136] It has been postulated that the increased frequency of diabetes mellitus in acromegaly may partially explain the higher vertebral fracture risk.[137] However, a very high prevalence of vertebral fractures has also been demonstrated in acromegaly patients with long-term controlled disease, independently of BMD.[138]

Osteoporosis is most prevalent in aging women over the age of 50 yr as the hormonal protective influence of sex steroids such as estrogens on bone health dissipates with the onset of menopause.[20, 21] Therefore, early or premature menopause is a major risk factor for vertebral fractures.[21] In aging premenopausal women, the rate of mineral loss is greater and faster at the trabecular than at the cortical level, and the accelerated phase of trabecular bone loss during menopause also occurs at a greater rate at the trabecular compartment than at the cortical bone.[139] This has a major impact on the spine, as trabecular bone constitutes the greatest part of vertebral bone mass and strength,[140] and it may contribute to the occurrence of vertebral fractures already early on in the disease process of osteoporosis. Furthermore, osteoporosis is being recognized increasingly in men, and undiagnosed clinical hypogonadism is a common cause of osteoporosis in men[141]. Also, an increasing number of patients with cancer acquire hypogonadism as breast and prostate malignancies are treated with antihormonal therapies, such as aromatase inhibitors and anti-androgens; additionally, gonadal damage may occur as an adverse effect of radiation and chemotherapy.[142-144] It has been demonstrated that menopausal transition and treatment with aromatase inhibitors result in decreases in BMD and TBS and higher prevalences of vertebral fractures, particularly in those women with the lowest levels of estradiol and the highest levels of sex hormone-binding globulin (SHBG)[145-147]. Comparably, men with prostate cancer on androgen deprivation therapy suffer from more spine fractures.[148, 149] Intriguingly, vertebral fracture risk in hypogonadism does not seem to be mediated by testosterone but seem dependent on estradiol levels as well[150]. Finally, pregnancy and lactation can cause irreversible bone loss, which may cause vertebral fractures from young up to old age.[151, 152] Levels of the hormone prolactin are normally high during pregnancy and lactation, and prolactin exerts negative feedback on estrogen production in women and testosterone production in men. Prolactin-secreting adenomas account for about half of

all pituitary adenomas and are associated with vertebral fractures in at least men, probably by inducing secondary hypogonadism.[153]

Finally, thyroid hormones are primarily responsible for the regulation of metabolism. Long-standing untreated hyperthyroidism and over suppletion with thyroid hormones have long been known to reduce BMD and elevate risk of fractures of the spine up to nine times compared to euthyroid controls.[154-156] This effect may partially be reversible after treatment of the thyroid disorder.

Miscellaneous systemic conditions associated with risk of vertebral fractures

It seems impossible to be all-comprehensive on this topic; we will briefly mention a few more conditions associated with vertebral fractures in this final subchapter.

Surely, problems in mineral and calcium homeostasis through parathyroid hormone (PTH) and vitamin D metabolism are associated with vertebral fracture risk.[157-159] TBS is compromised in hyperparathyroidism.[160-162] Increased rates of vertebral fractures have been found in chronic kidney disease, where the association with tertiary hyperparathyroidism is weak at most and the causal mechanisms seem complex, but vascular calcification appears to be related to chronic kidney disease-mineral bone disorder (CKD-MBD).[163-165] In a study by Szulc et al. severe abdominal aortic calcification and vertebral fracture, both assessed using DXA, were positively associated independently of bone mineral density.[166] High prevalences of radiological vertebral fractures in HIV-infected patients have been reported, which might be attributable to higher rates of diabetes mellitus, renal insufficiency and steroid use in this group.[167, 168]

Furthermore, decreases in DXA-BMD have been linked to chronic lung disease mortality independent of other risk factors for low BMD in chronic obstructive pulmonary disease (COPD) such as smoking, low BMI or use of corticosteroids.[169] COPD is among the most common chronic lung diseases and mainly is a smoking-related disorder affecting millions of people worldwide.[170] Systemic manifestations of the disease include exercise intolerance, skeletal muscle impairment, osteoporosis and hormonal imbalance.[171] These problems may arise due to inactivity, systemic inflammation, hypoxia and corticosteroid treatment. Many studies have reported on a relationship between COPD and vertebral fractures;[172-176] however, it is uncertain whether the effect of COPD on vertebral fractures is independent from smoking[177].

In chronic liver disease and transplantation patients lower BMD, more spinal and peripheral fractures may be found, although it is still unclear whether these effects are explained through confounders such as immunosuppressive medications, hypogonadism, lower levels of IGF-I or lifestyle factors including vitamin D deficiency, malnutrition, alcohol abuse and decreased outdoor activity.[178-182] Hyponatremia, that is, a reduced serum sodium concentration, is the most common electrolyte disorder in hospitalized patients and has a multifactorial etiology such as diuretic medication use, hyperglycemia in diabetes, liver cirrhosis and cardiac decompensation. Mild hyponatremia in the elderly has been found associated with an increased risk of vertebral fractures and incident non-vertebral fractures but not with DXA-BMD.[183]

Finally, we will list a few rare diseases associated with vertebral fractures. Systemic mastocytosis is a clonal disorder of abnormal mast cells which release the allergy mediator histamine and can accumulate in bone marrow, which has been linked to vertebral fractures in some cases.[184, 185] The autosomal recessive genetic disorder cystic fibrosis is characterized by abnormal transport of chloride and sodium across epithelial cells, leading to thick and viscous secretions. This condition has been linked to higher prevalences of osteopenia, osteoporosis, and vertebral fractures in multiple studies as well.[186-191]

CONCLUSION

Vertebral fractures are associated with high morbidity, mortality, and costs, and radiological diagnosis is not always straightforward. A great variety of systemic diseases are associated with risk of osteoporotic vertebral fractures, illustrating its multi-factorial etiology and underscoring its systemic origin. We have reviewed genetic, immunologic, metabolic, and endocrine contributing factors. Prevalences of these conditions vary from common to extremely rare, and incidence peaks differ according to etiology. Plenty remains to be elucidated in this field, identifying even more associated diseases and further exposing pathophysiological mechanisms underlying osteoporotic vertebral fractures.

REFERENCES

1. De Laet C, Kanis JA, Oden A, Johanson H, Johnell O, Delmas P, et al. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporos Int. 2005 Nov;16(11):1330-8.
2. Krolner B, Toft B. Vertebral bone loss: an unheeded side effect of therapeutic bed rest. Clin Sci (Lond). 1983 May;64(5):537-40.
3. Bergmann P, Body JJ, Boonen S, Boutsen Y, Devogelaer JP, Goemaere S, et al. Loading and skeletal development and maintenance. J Osteoporos. 2010;2011:786752.
4. Hides JA, Lambrecht G, Richardson CA, Stanton WR, Armbrecht G, Pruett C, et al. The effects of rehabilitation on the muscles of the trunk following prolonged bed rest. Eur Spine J. 2011 May;20(5):808-18.
5. Kanis JA, Johnell O, Oden A, Johansson H, De Laet C, Eisman JA, et al. Smoking and fracture risk: a meta-analysis. Osteoporos Int. 2005 Feb;16(2):155-62.
6. Kanis JA, Johansson H, Johnell O, Oden A, De Laet C, Eisman JA, et al. Alcohol intake as a risk factor for fracture. Osteoporos Int. 2005 Jul;16(7):737-42.
7. Kanis JA, Johansson H, Oden A, Johnell O, de Laet C, Melton IL, et al. A meta-analysis of prior corticosteroid use and fracture risk. J Bone Miner Res. 2004 Jun;19(6):893-9.
8. Kanis JA, Johansson H, Oden A, Johnell O, De Laet C, Eisman JA, et al. A family history of fracture and fracture risk: a meta-analysis. Bone. 2004 Nov;35(5):1029-37.
9. Miazgowski T, Kleerekoper M, Felsenberg D, Stepan JJ, Szulc P. Secondary osteoporosis: endocrine and metabolic causes of bone mass deterioration. J Osteoporos. 2012;2012:907214.
10. Brunton S, Carmichael B, Gold D, Hull B, Kauffman T, Papaioannou A, et al. Vertebral compression fractures in primary care: recommendations from a consensus panel. J Fam Pract. 2005 Sep;54(9):781-8.
11. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I:1-65.
12. Gold DT. The clinical impact of vertebral fractures: quality of life in women with osteoporosis. Bone. 1996 Mar;18(3 Suppl):185S-9S.
13. Burger H, Van Daele PL, Grashuis K, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities and functional impairment in men and women. J Bone Miner Res. 1997 Jan;12(1):152-7.
14. Ross PD. Clinical consequences of vertebral fractures. Am J Med. 1997 Aug 18;103(2A):30S-42S; discussion S-3S.
15. Nevitt MC, Ettinger B, Black DM, Stone K, Jamal SA, Ensrud K, et al. The association of radiographically detected vertebral fractures with back pain and function: a prospective study. Ann Intern Med. 1998 May 15;128(10):793-800.
16. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C, et al. Health-related quality of life in postmenopausal women with low BMD with or without prevalent vertebral fractures. J Bone Miner Res. 2000 Jul;15(7):1384-92.
17. Oleksik AM, Ewing S, Shen W, van Schoor NM, Lips P. Impact of incident vertebral fractures on health related quality of life (HRQOL) in postmenopausal women with prevalent vertebral fractures. Osteoporos Int. 2005 Aug;16(8):861-70.

18. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. Journal of the American Geriatrics Society. 2000 Mar;48(3):241-9.
19. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
20. Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2002 Jun;17(6):1051-6.
21. van der Klift M, de Laet CE, McCloskey EV, Johnell O, Kanis JA, Hofman A, et al. Risk factors for incident vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2004 Jul;19(7):1172-80.
22. Schousboe JT, Rosen HR, Vokes TJ, Cauley JA, Cummings SR, Nevitt MC, et al. Prediction Models of Prevalent Radiographic Vertebral Fractures Among Older Men. J Clin Densitom. 2013 Nov 27.
23. Schousboe JT, Rosen HR, Vokes TJ, Cauley JA, Cummings SR, Nevitt M, et al. Prediction models of prevalent radiographic vertebral fractures among older women. J Clin Densitom. 2014 Jul-Sep;17(3):378-85.
24. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, Hofman A, et al. Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. Eur Radiol. 2013 Feb;23(2):476-86.
25. Pothuaud L, Carceller P, Hans D. Correlations between grey-level variations in 2D projection images (TBS) and 3D microarchitecture: applications in the study of human trabecular bone microarchitecture. Bone. 2008 Apr;42(4):775-87.
26. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
27. Ferrar L, Jiang G, Adams J, Eastell R. Identification of vertebral fractures: an update. Osteoporos Int. 2005 Jul;16(7):717-28.
28. Ferrar L, Jiang G, Cawthon PM, San Valentin R, Fullman R, Lambert L, et al. Identification of vertebral fracture and non-osteoporotic short vertebral height in men: the MrOS study. J Bone Miner Res. 2007 Sep;22(9):1434-41.
29. Reynolds J, Pritchard JA, Ludders D, Mason RA. Roentgenographic and clinical appraisal of sickle cell beta-thalassemia disease. Am J Roentgenol Radium Ther Nucl Med. 1973 Jun;118(2):378-400.
30. Schwartz AM, Homer MJ, McCauley RG. "Step-off" vertebral body: Gaucher's disease versus sickle cell hemoglobinopathy. AJR Am J Roentgenol. 1979 Jan;132(1):81-5.
31. Dietz GW, Christensen EE. Normal "Cupid's bow" contour of the lower lumbar vertebrae. Radiology. 1976 Dec;121(3 Pt. 1):577-9.
32. Chan KK, Sartoris DJ, Haghighi P, Sledge P, Barrett-Connor E, Trudell DT, et al. Cupid's bow contour of the vertebral body: evaluation of pathogenesis with bone densitometry and imaging-histopathologic correlation. Radiology. 1997 Jan;202(1):253-6.
33. Tsuji H, Yoshioka T, Sainoh H. Developmental balloon disc of the lumbar spine in healthy subjects. Spine (Phila Pa 1976). 1985 Dec;10(10):907-11.
34. Ali RM, Green DW, Patel TC. Scheuermann's kyphosis. Curr Opin Pediatr. 1999 Feb;11(1):70-5.
35. Hart ES, Merlin G, Harisiades J, Grottkau BE. Scheuermann's thoracic kyphosis in the adolescent patient. Orthop Nurs. 2010 Nov-Dec;29(6):365-71; quiz 72-3.
36. Damborg F, Engell V, Andersen M, Kyvik KO, Thomsen K. Prevalence, concordance, and heritability of Scheuermann kyphosis based on a study of twins. J Bone Joint Surg Am. 2006 Oct;88(10):2133-6.
37. Makurthou AA, Oei L, El Saddy S, Breda SJ, Castano-Betancourt MC, Hofman A, et al. Scheuermann disease: evaluation of radiological criteria and population prevalence. Spine (Phila Pa 1976). 2013 Sep 1;38(19):1690-4.
38. Sachs B, Bradford D, Winter R, Lonstein J, Moe J, Willson S. Scheuermann kyphosis. Follow-up of Milwaukee-brace treatment. J Bone Joint Surg Am. 1987 Jan;69(1):50-7.
39. Wenger DR, Frick SL. Scheuermann kyphosis. Spine (Phila Pa 1976). 1999 Dec 15;24(24):2630-9.
40. Oei L, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJ, et al. Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses. Quant Imaging Med Surg. 2013 Oct;3(5):249-55.
41. Breda SJ, Oei HD, Oei EH, Zillikens MC. [Osteoporotic vertebral fractures or Scheuermann's disease?]. Ned Tijdschr Geneeskd. 2013;157(45):A6479.

42. Popko J, Konstantynowicz J, Kossakowski D, Kaczmarski M, Piotrowska-Jastrzebska J. Assessment of bone density in children with Scheuermann's disease. Rocz Akad Med Bialymst. 1997;42(1):245-50.
43. Lopez RA, Burke SW, Levine DB, Schneider R. Osteoporosis in Scheuermann's disease. Spine (Phila Pa 1976). 1988 Oct;13(10):1099-103.
44. Viola S, Peter F, Gyorgy I, Szecsenyi NL. [Alkaline phosphatase level and bone density in Scheuermann's disease and in adolescent idiopathic scoliosis]. Orv Hetil. 2000 Apr 23;141(17):905-9.
45. Runge H, Fengler F, Franke J, Koall W. [Evaluation of the mineral content of peripheral bones (radius) by photon-absorption technique in normals as well as in patients with various types of bone diseases (author's transl)]. Radiologe. 1980 Oct;20(10):505-14.
46. Castano-Betancourt MC, Oei L, Rivadeneira F, de Schepper EI, Hofman A, Bierma-Zeinstra S, et al. Association of lumbar disc degeneration with osteoporotic fractures; the Rotterdam study and meta-analysis from systematic review. Bone. 2013 Nov;57(1):284-9.
47. Engel CC, von Korff M, Katon WJ. Back pain in primary care: predictors of high health-care costs. Pain. 1996 May-Jun;65(2-3):197-204.
48. Cummings SR, Melton LJ. Epidemiology and outcomes of osteoporotic fractures. Lancet. 2002 May 18;359(9319):1761-7.
49. Jones G, Nguyen T, Sambrook PN, Kelly PJ, Eisman JA. A longitudinal study of the effect of spinal degenerative disease on bone density in the elderly. J Rheumatol. 1995 May;22(5):932-6.
50. Schneider DL, Bettencourt R, Barrett-Connor E. Clinical utility of spine bone density in elderly women. J Clin Densitom. 2006 Jul-Sep;9(3):255-60.
51. Livshits G, Ermakov S, Popham M, Macgregor AJ, Sambrook PN, Spector TD, et al. Evidence that bone mineral density plays a role in degenerative disc disease: the UK Twin Spine study. Ann Rheum Dis. 2010 Dec;69(12):2102-6.
52. Hulme PA, Boyd SK, Ferguson SJ. Regional variation in vertebral bone morphology and its contribution to vertebral fracture strength. Bone. 2007 Dec;41(6):946-57.
53. Fields AJ, Lee GL, Keaveny TM. Mechanisms of initial endplate failure in the human vertebral body. J Biomech. 2010 Dec 1;43(16):3126-31.
54. Grisso JA, Kelsey JL, Strom BL, Chiu GY, Maislin G, O'Brien LA, et al. Risk factors for falls as a cause of hip fracture in women. The Northeast Hip Fracture Study Group. N Engl J Med. 1991 May 9;324(19):1326-31.
55. Scheele J, de Schepper EI, van Meurs JB, Hofman A, Koes BW, Luijsterburg PA, et al. Association between spinal morning stiffness and lumbar disc degeneration: the Rotterdam Study. Osteoarthritis Cartilage. 2012 Sep;20(9):982-7.
56. Schwab F, el-Fegoun AB, Gamez L, Goodman H, Farcy JP. A lumbar classification of scoliosis in the adult patient: preliminary approach. Spine (Phila Pa 1976). 2005 Jul 15;30(14):1670-3.
57. Malfair D, Flemming AK, Dvorak MF, Munk PL, Vertinsky AT, Heran MK, et al. Radiographic evaluation of scoliosis: review. AJR Am J Roentgenol. 2010 Mar;194(3 Suppl):S8-22.
58. Yu WS, Chan KY, Yu FW, Yeung HY, Ng BK, Lee KM, et al. Abnormal bone quality versus low bone mineral density in adolescent idiopathic scoliosis: a case-control study with in vivo high-resolution peripheral quantitative computed tomography. Spine J. 2013 Nov;13(11):1493-9.
59. Hensinger RN. Congenital scoliosis: etiology and associations. Spine (Phila Pa 1976). 2009 Aug 1;34(17):1745-50.
60. Andersen MO, Thomsen K, Kyvik KO. Adolescent idiopathic scoliosis in twins: a population-based survey. Spine (Phila Pa 1976). 2007 Apr 15;32(8):927-30.
61. Gorman KF, Julien C, Moreau A. The genetic epidemiology of idiopathic scoliosis. Eur Spine J. 2012 Oct;21(10):1905-19.
62. Cook SD, Harding AF, Morgan EL, Nicholson RJ, Thomas KA, Whitecloud TS, et al. Trabecular bone mineral density in idiopathic scoliosis. J Pediatr Orthop. 1987 Mar-Apr;7(2):168-74.
63. Zhu F, Qiu Y, Yeung HY, Lee KM, Cheng CY. Trabecular bone micro-architecture and bone mineral density in adolescent idiopathic and congenital scoliosis. Orthop Surg. 2009 Feb;1(1):78-83.

64. Cheng JC, Qin L, Cheung CS, Sher AH, Lee KM, Ng SW, et al. Generalized low areal and volumetric bone mineral density in adolescent idiopathic scoliosis. J Bone Miner Res. 2000 Aug;15(8):1587-95.
65. Yu WS, Chan KY, Yu FW, Ng BK, Lee KM, Qin L, et al. Bone structural and mechanical indices in Adolescent Idiopathic Scoliosis evaluated by high-resolution peripheral quantitative computed tomography (HR-pQCT). Bone. 2014 Jan 8;61.
66. Aebi M. The adult scoliosis. Eur Spine J. 2005 Dec;14(10):925-48.
67. Grubb SA, Lipscomb HJ, Coonrad RW. Degenerative adult onset scoliosis. Spine (Phila Pa 1976). 1988 Mar;13(3):241-5.
68. Kanis JA. Diagnosis of osteoporosis and assessment of fracture risk. Lancet. 2002 Jun 1;359(9321):1929-36.
69. Sigurdsson G, Halldorsson BV, Styrkarsdottir U, Kristjansson K, Stefansson K. Impact of genetics on low bone mass in adults. J Bone Miner Res. 2008 Oct;23(10):1584-90.
70. Michaelsson K, Melhus H, Ferm H, Ahlbom A, Pedersen NL. Genetic liability to fractures in the elderly. Arch Intern Med. 2005 Sep 12;165(16):1825-30.
71. Kemp JP, Medina-Gomez C, Estrada K, St Pourcain B, Heppe DH, Warrington NM, et al. Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment. PLoS Genet. 2014 Jun;10(6):e1004423.
72. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
73. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.
74. Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. J Clin Invest. 2006 May;116(5):1202-9.
75. Boyce BF, Xing L. The RANKL/RANK/OPG pathway. Curr Osteoporos Rep. 2007 Sep;5(3):98-104.
76. Oei L, Hsu YH, Styrkarsdottir U, Eussen BH, de Klein A, Peters MJ, et al. A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus. J Med Genet. 2014 Feb;51(2):122-31.
77. Moayyeri A, Hsu YH, Karasik D, Estrada K, Xiao SM, Nielson C, et al. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Hum Mol Genet. 2014 Jun 1;23(11):3054-68.
78. Liu CT, Karasik D, Zhou Y, Hsu YH, Genant HK, Broe KE, et al. Heritability of prevalent vertebral fracture and volumetric bone mineral density and geometry at the lumbar spine in three generations of the Framingham study. J Bone Miner Res. 2012 Apr;27(4):954-8.
79. Oei L, Estrada K, Duncan EL, Christiansen C, Liu CT, Langdahl BL, et al. Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus. Bone. 2014 Feb;59:20-7.
80. Stankiewicz P, Sen P, Bhatt SS, Storer M, Xia Z, Bejjani BA, et al. Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. Am J Hum Genet. 2009 Jun;84(6):780-91.
81. Winnier GE, Hargett L, Hogan BL. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. Genes Dev. 1997 Apr 1;11(7):926-40.
82. Chu ML, Williams CJ, Pepe G, Hirsch JL, Prockop DJ, Ramirez F. Internal deletion in a collagen gene in a perinatal lethal form of osteogenesis imperfecta. Nature. 1983 Jul 7-13;304(5921):78-80.
83. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell. 2001 Nov 16;107(4):513-23.
84. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. N Engl J Med. 2013 May 9;368(19):1809-16.
85. Styrkarsdottir U, Thorleifsson G, Sulem P, Gudbjartsson DF, Sigurdsson A, Jonasdottir A, et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. Nature. 2013 May 23;497(7450):517-20.

86. van Dijk FS, Zillikens MC, Micha D, Riessland M, Marcelis CL, de Die-Smulders CE, et al. PLS3 mutations in X-linked osteoporosis with fractures. N Engl J Med. 2013 Oct 17;369(16):1529-36.
87. van Dijk FS, Byers PH, Dalgleish R, Malfait F, Maugeri A, Rohrbach M, et al. EMQN best practice guidelines for the laboratory diagnosis of osteogenesis imperfecta. Eur J Hum Genet. 2012 Jan;20(1):11-9.
88. Schett G, Kiechl S, Weger S, Pederiva A, Mayr A, Petrangeli M, et al. High-sensitivity C-reactive protein and risk of nontraumatic fractures in the Bruneck study. Arch Intern Med. 2006 Dec 11-25;166(22):2495-501.
89. Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol. 2009 Jun;201(3):309-20.
90. Breban S, Briot K, Kolta S, Paternotte S, Ghazi M, Fechtenbaum J, et al. Identification of rheumatoid arthritis patients with vertebral fractures using bone mineral density and trabecular bone score. J Clin Densitom. 2012 Jul-Sep;15(3):260-6.
91. Rolland T, Boutroy S, Vilayphiou N, Blaizot S, Chapurlat R, Szulc P. Poor trabecular microarchitecture at the distal radius in older men with increased concentration of high-sensitivity C-reactive protein--the STRAMBO study. Calcif Tissue Int. 2012 Jun;90(6):496-506.
92. Oei L, Campos-Obando N, Dehghan A, Oei EH, Stolk L, van Meurs JB, et al. Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study. Osteoporos Int. 2014 Apr;25(4):1247-54.
93. Dirven L, van den Broek M, van Groenendael JH, de Beus WM, Kerstens PJ, Huizinga TW, et al. Prevalence of vertebral fractures in a disease activity steered cohort of patients with early active rheumatoid arthritis. BMC Musculoskelet Disord. 2012;13:125.
94. Orstavik RE, Haugeberg G, Mowinckel P, Hoiseth A, Uhlig T, Falch JA, et al. Vertebral deformities in rheumatoid arthritis: a comparison with population-based controls. Arch Intern Med. 2004 Feb 23;164(4):420-5.
95. Peel NF, Moore DJ, Barrington NA, Bax DE, Eastell R. Risk of vertebral fracture and relationship to bone mineral density in steroid treated rheumatoid arthritis. Ann Rheum Dis. 1995 Oct;54(10):801-6.
96. Spector TD, Hall GM, McCloskey EV, Kanis JA. Risk of vertebral fracture in women with rheumatoid arthritis. BMJ. 1993 Feb 27;306(6877):558.
97. Bultink IE, Lems WF, Kostense PJ, Dijkmans BA, Voskuyl AE. Prevalence of and risk factors for low bone mineral density and vertebral fractures in patients with systemic lupus erythematosus. Arthritis Rheum. 2005 Jul;52(7):2044-50.
98. Borba VZ, Matos PG, da Silva Viana PR, Fernandes A, Sato EI, Lazaretti-Castro M. High prevalence of vertebral deformity in premenopausal systemic lupus erythematosus patients. Lupus. 2005;14(7):529-33.
99. Heijckmann AC, Drent M, Dumitrescu B, De Vries J, Nieuwenhuijzen Kruseman AC, Wolffenbuttel BH, et al. Progressive vertebral deformities despite unchanged bone mineral density in patients with sarcoidosis: a 4-year follow-up study. Osteoporos Int. 2008 Jun;19(6):839-47.
100. van Staa TP, Cooper C, Brusse LS, Leufkens H, Javaid MK, Arden NK. Inflammatory bowel disease and the risk of fracture. Gastroenterology. 2003 Dec;125(6):1591-7.
101. Vazquez MA, Lopez E, Montoya MJ, Giner M, Perez-Temprano R, Perez-Cano R. Vertebral fractures in patients with inflammatory bowel disease compared with a healthy population: a prospective case-control study. BMC Gastroenterol. 2012;12:47.
102. Mitra D, Elvins DM, Speden DJ, Collins AJ. The prevalence of vertebral fractures in mild ankylosing spondylitis and their relationship to bone mineral density. Rheumatology (Oxford). 2000 Jan;39(1):85-9.
103. Munoz-Ortego J, Vestergaard P, Rubio JB, Wordsworth P, Judge A, Javaid MK, et al. Ankylosing spondylitis is associated with an increased risk of vertebral and nonvertebral clinical fractures: a population-based cohort study. J Bone Miner Res. 2014 Aug;29(8):1770-6.
104. Vosse D, Landewe R, van der Heijde D, van der Linden S, van Staa TP, Geusens P. Ankylosing spondylitis and the risk of fracture: results from a large primary care-based nested case-control study. Ann Rheum Dis. 2009 Dec;68(12):1839-42.
105. Cumming DC, Quigley ME, Yen SS. Acute suppression of circulating testosterone levels by cortisol in men. J Clin Endocrinol Metab. 1983 Sep;57(3):671-3.
106. Kumar P, Kumar N, Thakur DS, Patidar A. Male hypogonadism: Symptoms and treatment. J Adv Pharm Technol Res. 2010 Jul;1(3):297-301.

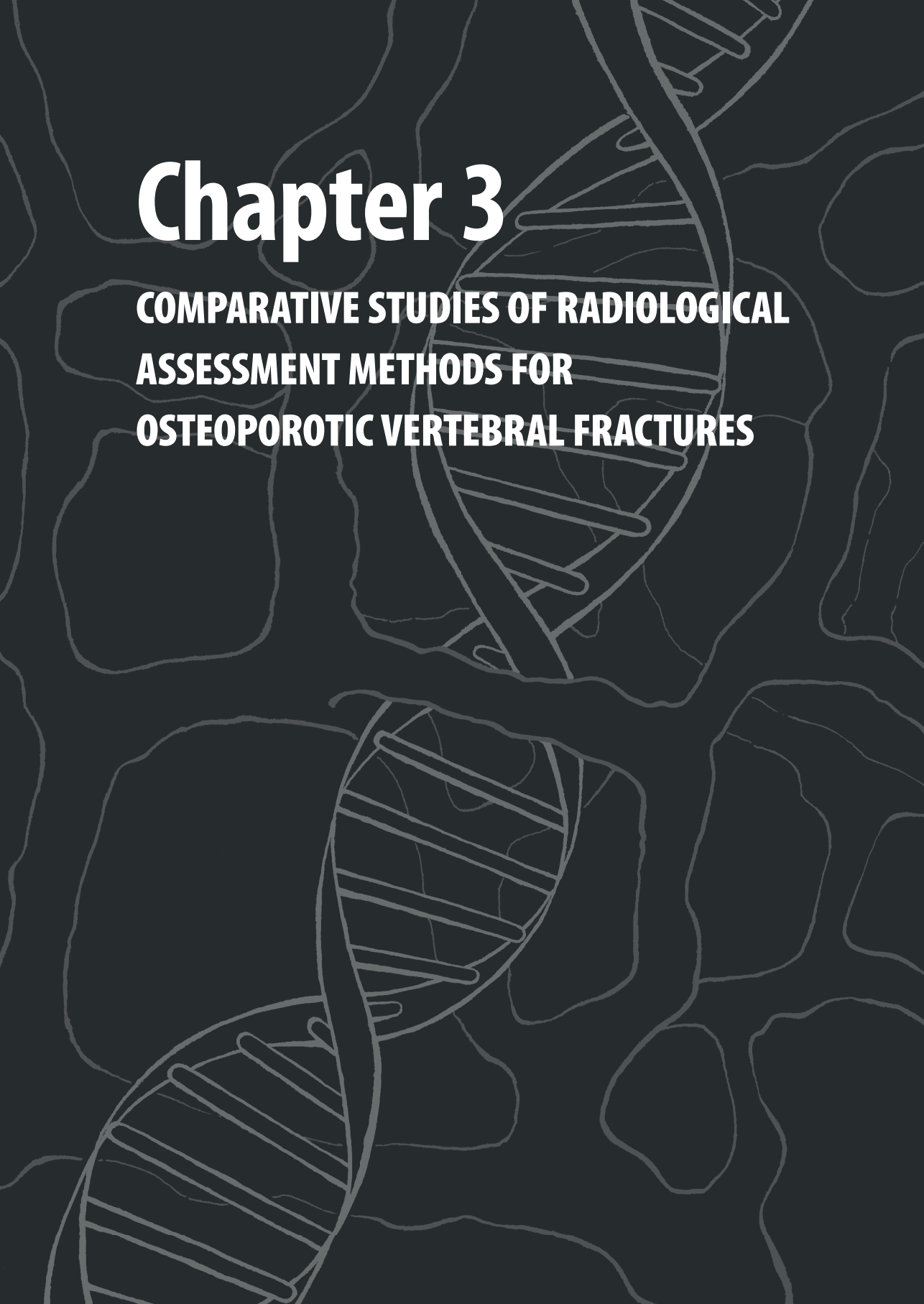
107. Angeli A, Guglielmi G, Dovio A, Capelli G, de Feo D, Giannini S, et al. High prevalence of asymptomatic vertebral fractures in post-menopausal women receiving chronic glucocorticoid therapy: a cross-sectional outpatient study. Bone. 2006 Aug;39(2):253-9.
108. Chiodini I, Tauchmanova L, Torlontano M, Battista C, Guglielmi G, Cammisa M, et al. Bone involvement in eugonadal male patients with adrenal incidentaloma and subclinical hypercortisolism. J Clin Endocrinol Metab. 2002 Dec;87(12):5491-4.
109. Eller-Vainicher C, Morelli V, Ulivieri FM, Palmieri S, Zhukouskaya VV, Cairoli E, et al. Bone quality, as measured by trabecular bone score in patients with adrenal incidentalomas with and without subclinical hypercortisolism. J Bone Miner Res. 2012 Oct;27(10):2223-30.
110. Chiodini I, Morelli V, Masserini B, Salcuni AS, Eller-Vainicher C, Viti R, et al. Bone mineral density, prevalence of vertebral fractures, and bone quality in patients with adrenal incidentalomas with and without subclinical hypercortisolism: an Italian multicenter study. J Clin Endocrinol Metab. 2009 Sep;94(9):3207-14.
111. Morelli V, Eller-Vainicher C, Salcuni AS, Coletti F, Iorio L, Muscogiuri G, et al. Risk of new vertebral fractures in patients with adrenal incidentaloma with and without subclinical hypercortisolism: a multicenter longitudinal study. J Bone Miner Res. 2011 Aug;26(8):1816-21.
112. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int. 2007 Apr;18(4):427-44.
113. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007 Sep 1;166(5):495-505.
114. Zhukouskaya VV, Eller-Vainicher C, Vadzianava VV, Shepelkevich AP, Zhurava IV, Korolenko GG, et al. Prevalence of morphometric vertebral fractures in patients with type 1 diabetes. Diabetes Care. 2013 Jun;36(6):1635-40.
115. de Liefde II, van der Klift M, de Laet CE, van Daele PL, Hofman A, Pols HA. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporos Int. 2005 Dec;16(12):1713-20.
116. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol. 2012 May;27(5):319-32.
117. Oei L, Zillikens MC, Dehghan A, Buitendijk GH, Castano-Betancourt MC, Estrada K, et al. High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study. Diabetes Care. 2013 Jun;36(6):1619-28.
118. Leslie WD, Aubry-Rozier B, Lamy O, Hans D. TBS (trabecular bone score) and diabetes-related fracture risk. J Clin Endocrinol Metab. 2013 Feb;98(2):602-9.
119. Burghardt AJ, Issever AS, Schwartz AV, Davis KA, Masharani U, Majumdar S, et al. High-resolution peripheral quantitative computed tomographic imaging of cortical and trabecular bone microarchitecture in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Nov;95(11):5045-55.
120. Pritchard JM, Giangregorio LM, Atkinson SA, Beattie KA, Inglis D, Ioannidis G, et al. Changes in trabecular bone microarchitecture in postmenopausal women with and without type 2 diabetes: a two year longitudinal study. BMC Musculoskelet Disord. 2013;14:114.
121. Patsch JM, Burghardt AJ, Yap SP, Baum T, Schwartz AV, Joseph GB, et al. Increased cortical porosity in type 2 diabetic postmenopausal women with fragility fractures. J Bone Miner Res. 2013 Feb;28(2):313-24.
122. Farr JN, Drake MT, Amin S, Melton LJ, 3rd, McCready LK, Khosla S. In vivo assessment of bone quality in postmenopausal women with type 2 diabetes. J Bone Miner Res. 2014 Apr;29(4):787-95.
123. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.
124. Giangregorio LM, Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, et al. FRAX underestimates fracture risk in patients with diabetes. J Bone Miner Res. 2012 Feb;27(2):301-8.
125. Leslie WD, Morin SN, Lix LM, Majumdar SR. Does diabetes modify the effect of FRAX risk factors for predicting major osteoporotic and hip fracture? Osteoporos Int. 2014 Aug 5.
126. Yamamoto M, Yamaguchi T, Yamauchi M, Kaji H, Sugimoto T. Diabetic patients have an increased risk of vertebral fractures independent of BMD or diabetic complications. J Bone Miner Res. 2009 Apr;24(4):702-9.
127. Kilpadi KL, Eldabaje R, Schmitz JE, Ehler B, Thames TA, Joshi AP, et al. Type 2 diabetes is associated with vertebral fractures in a sample of clinic- and hospital-based Latinos. J Immigr Minor Health. 2014 Jun;16(3):440-9.

128. Yamamoto M, Yamaguchi T, Yamauchi M, Yano S, Sugimoto T. Serum pentosidine levels are positively associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes. J Clin Endocrinol Metab. 2008 Mar;93(3):1013-9.
129. Yamamoto M, Yamaguchi T, Yamauchi M, Sugimoto T. Low serum level of the endogenous secretory receptor for advanced glycation end products (esRAGE) is a risk factor for prevalent vertebral fractures independent of bone mineral density in patients with type 2 diabetes. Diabetes Care. 2009 Dec;32(12):2263-8.
130. Kanazawa I, Yamaguchi T, Sugimoto T. Serum insulin-like growth factor-I is a marker for assessing the severity of vertebral fractures in postmenopausal women with type 2 diabetes mellitus. Osteoporos Int. 2011 Apr;22(4):1191-8.
131. Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Yano S, Sugimoto T. Serum insulin-like growth factor-I level is associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes mellitus. Osteoporos Int. 2007 Dec;18(12):1675-81.
132. Canalis E, Giustina A, Bilezikian JP. Mechanisms of anabolic therapies for osteoporosis. N Engl J Med. 2007 Aug 30;357(9):905-16.
133. DiGirolamo DJ, Mukherjee A, Fulzele K, Gan Y, Cao X, Frank SJ, et al. Mode of growth hormone action in osteoblasts. J Biol Chem. 2007 Oct 26;282(43):31666-74.
134. Janssen JA, Burger H, Stolk RP, Grobbee DE, de Jong FH, Lamberts SW, et al. Gender-specific relationship between serum free and total IGF-I and bone mineral density in elderly men and women. Eur J Endocrinol. 1998 Jun;138(6):627-32.
135. Mazziotti G, Bianchi A, Bonadonna S, Nuzzo M, Cimino V, Fusco A, et al. Increased prevalence of radiological spinal deformities in adult patients with GH deficiency: influence of GH replacement therapy. J Bone Miner Res. 2006 Apr;21(4):520-8.
136. Bonadonna S, Mazziotti G, Nuzzo M, Bianchi A, Fusco A, De Marinis L, et al. Increased prevalence of radiological spinal deformities in active acromegaly: a cross-sectional study in postmenopausal women. J Bone Miner Res. 2005 Oct;20(10):1837-44.
137. Mazziotti G, Gola M, Bianchi A, Porcelli T, Giampietro A, Cimino V, et al. Influence of diabetes mellitus on vertebral fractures in men with acromegaly. Endocrine. 2011 Aug;40(1):102-8.
138. Wassenaar MJ, Biermasz NR, Hamdy NA, Zillikens MC, van Meurs JB, Rivadeneira F, et al. High prevalence of vertebral fractures despite normal bone mineral density in patients with long-term controlled acromegaly. Eur J Endocrinol. 2011 Apr;164(4):475-83.
139. Isaia GC, Salamano G, Mussetta M, Molinatti GM. Vertebral bone loss in menopause. Exp Gerontol. 1990;25(3-4):303-7.
140. Eswaran SK, Gupta A, Adams MF, Keaveny TM. Cortical and trabecular load sharing in the human vertebral body. J Bone Miner Res. 2006 Feb;21(2):307-14.
141. Ebeling PR. Idiopathic or hypogonadal osteoporosis in men: current and future treatment options. Treat Endocrinol. 2004;3(6):381-91.
142. Shahinian VB, Kuo YF, Freeman JL, Goodwin JS. Risk of fracture after androgen deprivation for prostate cancer. N Engl J Med. 2005 Jan 13;352(2):154-64.
143. Sergi G, Pintore G, Falci C, Veronese N, Berton L, Perissinotto E, et al. Preventive effect of risedronate on bone loss and frailty fractures in elderly women treated with anastrozole for early breast cancer. J Bone Miner Metab. 2012 Jul;30(4):461-7.
144. Dupree K, Dobs A. Osteopenia and male hypogonadism. Rev Urol. 2004;6 Suppl 6:S30-4.
145. Pedrazzoni M, Casola A, Verzicco I, Abbate B, Vescovini R, Sansoni P. Longitudinal changes of trabecular bone score after estrogen deprivation: effect of menopause and aromatase inhibition. J Endocrinol Invest. 2014 Jul 19.
146. Bouvard B, Hoppe E, Soulie P, Georgin-Mege M, Jadaud E, Abadie-Lacourtoisie S, et al. High prevalence of vertebral fractures in women with breast cancer starting aromatase inhibitor therapy. Ann Oncol. 2012 May;23(5):1151-6.

147. Goderie-Plomp HW, van der Klift M, de Ronde W, Hofman A, de Jong FH, Pols HA. Endogenous sex hormones, sex hormone-binding globulin, and the risk of incident vertebral fractures in elderly men and women: the Rotterdam Study. J Clin Endocrinol Metab. 2004 Jul;89(7):3261-9.
148. Greenspan SL, Wagner J, Nelson JB, Perera S, Britton C, Resnick NM. Vertebral fractures and trabecular microstructure in men with prostate cancer on androgen deprivation therapy. J Bone Miner Res. 2013 Feb;28(2):325-32.
149. Saylor PJ, Morton RA, Hancock ML, Barnette KG, Steiner MS, Smith MR. Factors associated with vertebral fractures in men treated with androgen deprivation therapy for prostate cancer. J Urol. 2011 Aug;186(2):482-6.
150. Barrett-Connor E, Mueller JE, von Muhlen DG, Laughlin GA, Schneider DL, Sartoris DJ. Low levels of estradiol are associated with vertebral fractures in older men, but not women: the Rancho Bernardo Study. J Clin Endocrinol Metab. 2000 Jan;85(1):219-23.
151. Bolzetta F, Veronese N, De Rui M, Berton L, Carraro S, Pizzato S, et al. Duration of breastfeeding as a risk factor for vertebral fractures. Bone. 2014 Aug 10;68C:41-5.
152. Campos-Obando N, Oei L, Hoefsloot LH, Kiewiet RM, Klaver CC, Simon ME, et al. Osteoporotic vertebral fractures during pregnancy: be aware of a potential underlying genetic cause. J Clin Endocrinol Metab. 2014 Apr;99(4):1107-11.
153. Mazziotti G, Porcelli T, Mormando M, De Menis E, Bianchi A, Mejia C, et al. Vertebral fractures in males with prolactinoma. Endocrine. 2011 Jun;39(3):288-93.
154. Vestergaard P, Rejnmark L, Weeke J, Mosekilde L. Fracture risk in patients treated for hyperthyroidism. Thyroid. 2000 Apr;10(4):341-8.
155. Vestergaard P, Mosekilde L. Hyperthyroidism, bone mineral, and fracture risk--a meta-analysis. Thyroid. 2003 Jun;13(6):585-93.
156. Mazziotti G, Mormando M, Cristiano A, Bianchi A, Porcelli T, Giampietro A, et al. Association between l-thyroxine treatment, GH deficiency, and radiological vertebral fractures in patients with adult-onset hypopituitarism. Eur J Endocrinol. 2014 Jun;170(6):893-9.
157. Lopes JB, Danilevicius CF, Takayama L, Caparbo VF, Scazufca M, Bonfa E, et al. Vitamin D insufficiency: a risk factor to vertebral fractures in community-dwelling elderly women. Maturitas. 2009 Dec 20;64(4):218-22.
158. Hernandez JL, Olmos JM, Pariente E, Nan D, Martinez J, Llorca J, et al. Influence of vitamin D status on vertebral fractures, bone mineral density, and bone turnover markers in normocalcemic postmenopausal women with high parathyroid hormone levels. J Clin Endocrinol Metab. 2013 Apr;98(4):1711-7.
159. Eller-Vainicher C, Battista C, Guarnieri V, Muscarella S, Palmieri S, Salcuni AS, et al. Factors associated with vertebral fracture risk in patients with primary hyperparathyroidism. Eur J Endocrinol. 2014 Sep;171(3):399-406.
160. Eller-Vainicher C, Filopanti M, Palmieri S, Ulivieri FM, Morelli V, Zhukouskaya VV, et al. Bone quality, as measured by trabecular bone score, in patients with primary hyperparathyroidism. Eur J Endocrinol. 2013 Aug;169(2):155-62.
161. Silva BC, Boutroy S, Zhang C, McMahon DJ, Zhou B, Wang J, et al. Trabecular bone score (TBS)--a novel method to evaluate bone microarchitectural texture in patients with primary hyperparathyroidism. J Clin Endocrinol Metab. 2013 May;98(5):1963-70.
162. Romagnoli E, Cipriani C, Nofroni I, Castro C, Angelozzi M, Scarpiello A, et al. "Trabecular Bone Score" (TBS): an indirect measure of bone micro-architecture in postmenopausal patients with primary hyperparathyroidism. Bone. 2013 Mar;53(1):154-9.
163. Rodriguez-Garcia M, Gomez-Alonso C, Naves-Diaz M, Diaz-Lopez JB, Diaz-Corte C, Cannata-Andia JB. Vascular calcifications, vertebral fractures and mortality in haemodialysis patients. Nephrol Dial Transplant. 2009 Jan;24(1):239-46.
164. Danese MD, Kim J, Doan QV, Dylan M, Griffiths R, Chertow GM. PTH and the risks for hip, vertebral, and pelvic fractures among patients on dialysis. Am J Kidney Dis. 2006 Jan;47(1):149-56.
165. Atsumi K, Kushida K, Yamazaki K, Shimizu S, Ohmura A, Inoue T. Risk factors for vertebral fractures in renal osteodystrophy. Am J Kidney Dis. 1999 Feb;33(2):287-93.

166. Szulc P, Samelson EJ, Sornay-Rendu E, Chapurlat R, Kiel DP. Severity of aortic calcification is positively associated with vertebral fracture in older men--a densitometry study in the STRAMBO cohort. Osteoporos Int. 2013 Apr;24(4):1177-84.
167. Borderi M, Calza L, Colangeli V, Vanino E, Viale P, Gibellini D, et al. Prevalence of sub-clinical vertebral fractures in HIV-infected patients. New Microbiol. 2014 Jan;37(1):25-32.
168. Torti C, Mazziotti G, Soldini PA, Foca E, Maroldi R, Gotti D, et al. High prevalence of radiological vertebral fractures in HIV-infected males. Endocrine. 2012 Jun;41(3):512-7.
169. Campos-Obando N, Castano-Betancourt MC, Oei L, Franco OH, Stricker BH, Brusselle GG, et al. Bone mineral density and chronic lung disease mortality: the rotterdam study. J Clin Endocrinol Metab. 2014 May;99(5):1834-42.
170. Postma DS, Bush A, van den Berge M. Risk factors and early origins of chronic obstructive pulmonary disease. Lancet. 2014 Aug 11.
171. Evans RA, Morgan MD. The systemic nature of chronic lung disease. Clin Chest Med. 2014 Jun;35(2):283-93.
172. Majumdar SR, Villa-Roel C, Lyons KJ, Rowe BH. Prevalence and predictors of vertebral fracture in patients with chronic obstructive pulmonary disease. Respir Med. 2010 Feb;104(2):260-6.
173. Nuti R, Siviero P, Maggi S, Guglielmi G, Caffarelli C, Crepaldi G, et al. Vertebral fractures in patients with chronic obstructive pulmonary disease: the EOLO Study. Osteoporos Int. 2009 Jun;20(6):989-98.
174. Ogura-Tomomatsu H, Asano K, Tomomatsu K, Miyata J, Ohmori N, Kodama M, et al. Predictors of osteoporosis and vertebral fractures in patients presenting with moderate-to-severe chronic obstructive lung disease. COPD. 2012 Aug;9(4):332-7.
175. Kjensli A, Falch JA, Ryg M, Blenk T, Armbrecht G, Diep LM, et al. High prevalence of vertebral deformities in COPD patients: relationship to disease severity. Eur Respir J. 2009 May;33(5):1018-24.
176. Papaioannou A, Parkinson W, Ferko N, Probyn L, Ioannidis G, Jurriaans E, et al. Prevalence of vertebral fractures among patients with chronic obstructive pulmonary disease in Canada. Osteoporos Int. 2003 Nov;14(11):913-7.
177. de Jong WU, de Jong PA, Vliegenthart R, Isgum I, Lammers JW, Oudkerk M, et al. Association of COPD and Smoking Status with Bone Density and Vertebral Fractures in Male Lung Cancer Screening Participants. J Bone Miner Res. 2014 Apr 9.
178. Giouleme OI, Vyzantiadis TA, Nikolaidis NL, Vasiliadis TG, Papageorgiou AA, Eugenidis NP, et al. Pathogenesis of osteoporosis in liver cirrhosis. Hepatogastroenterology. 2006 Nov-Dec;53(72):938-43.
179. Diamond T, Stiel D, Lunzer M, Wilkinson M, Roche J, Posen S. Osteoporosis and skeletal fractures in chronic liver disease. Gut. 1990 Jan;31(1):82-7.
180. Chen CC, Wang SS, Jeng FS, Lee SD. Metabolic bone disease of liver cirrhosis: is it parallel to the clinical severity of cirrhosis? J Gastroenterol Hepatol. 1996 May;11(5):417-21.
181. Collier J. Bone disorders in chronic liver disease. Hepatology. 2007 Oct;46(4):1271-8.
182. Ninkovic M, Skingle SJ, Bearcroft PW, Bishop N, Alexander GJ, Compston JE. Incidence of vertebral fractures in the first three months after orthotopic liver transplantation. Eur J Gastroenterol Hepatol. 2000 Aug;12(8):931-5.
183. Hoorn EJ, Rivadeneira F, van Meurs JB, Ziere G, Stricker BH, Hofman A, et al. Mild hyponatremia as a risk factor for fractures: the Rotterdam Study. J Bone Miner Res. 2011 Aug;26(8):1822-8.
184. Kruger A, Hamann C, Brendel C, Ramaswamy A, Schnabel M, Neubauer A, et al. Multimodal therapy for vertebral involvement of systemic mastocytosis. Spine (Phila Pa 1976). 2009 Aug 1;34(17):E626-8.
185. Manara M, Varenna M, Cantoni S, Parafioriti A, Gallazzi MB, Sinigaglia L. Osteoporosis with vertebral fractures in young males, due to bone marrow mastocytosis: a report of two cases. Clin Exp Rheumatol. 2010 Jan-Feb;28(1):97-100.
186. Paccou J, Zeboulon N, Combescure C, Gossec L, Cortet B. The prevalence of osteoporosis, osteopenia, and fractures among adults with cystic fibrosis: a systematic literature review with meta-analysis. Calcif Tissue Int. 2010 Jan;86(1):1-7.
187. Papaioannou A, Kennedy CC, Freitag A, O'Neill J, Pui M, Ioannidis G, et al. Longitudinal analysis of vertebral fracture and BMD in a Canadian cohort of adult cystic fibrosis patients. BMC Musculoskelet Disord. 2008;9:125.

188. Rossini M, Viapiana O, Del Marco A, de Terlizzi F, Gatti D, Adami S. Quantitative ultrasound in adults with cystic fibrosis: correlation with bone mineral density and risk of vertebral fractures. Calcif Tissue Int. 2007 Jan;80(1):44-9.
189. Stephenson A, Jamal S, Dowdell T, Pearce D, Corey M, Tullis E. Prevalence of vertebral fractures in adults with cystic fibrosis and their relationship to bone mineral density. Chest. 2006 Aug;130(2):539-44.
190. Rossini M, Del Marco A, Dal Santo F, Gatti D, Braggion C, James G, et al. Prevalence and correlates of vertebral fractures in adults with cystic fibrosis. Bone. 2004 Sep;35(3):771-6.
191. Aris RM, Renner JB, Winders AD, Buell HE, Riggs DB, Lester GE, et al. Increased rate of fractures and severe kyphosis: sequelae of living into adulthood with cystic fibrosis. Ann Intern Med. 1998 Feb 1;128(3):186-93.



Chapter 3

COMPARATIVE STUDIES OF RADIOLOGICAL ASSESSMENT METHODS FOR OSTEOPOROTIC VERTEBRAL FRACTURES

Chapter 3.1

Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings

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ABSTRACT

Introduction Osteoporosis is the most common metabolic bone disease; vertebral fractures are the most common osteoporotic fractures.

Methods Several radiological scoring methods using different criteria for osteoporotic vertebral fractures exist. Quantitative morphometry (QM) uses ratios derived from direct vertebral body height measurements to define fractures. Semi-quantitative (SQ) visual grading is performed according to height and area reduction. The algorithm-based qualitative (ABQ) method introduced a scheme to systematically rule out non-fracture deformities and diagnoses osteoporotic vertebral fractures based on endplate depression. The concordance across methods is currently a matter of debate.

Results This article reviews the most commonly applied standardized radiographic scoring methods for osteoporotic vertebral fractures, attaining an impartial perspective of benefits and limitations. It provides image examples and discusses aspects that facilitate large-scale application, such as automated image analysis software and different imaging investigations. It also reviews the implications of different fracture definitions for scientific research and clinical practice.

Conclusion Accurate standardized scoring methods for assessing osteoporotic vertebral fractures are crucial, considering that differences in definition will have implications for patient care and scientific research. Evaluation of the feasibility and concordance among methods will allow establishing their benefits and limitations, and most importantly, optimize their effectiveness for widespread application.

INTRODUCTION

Osteoporosis is the most common metabolic bone disease and vertebral fractures are the most common type of osteoporotic fractures[1]. These fractures are associated with significant morbidity,[2-7] mortality,[8, 9] and high health-care costs. Given the ageing of populations, osteoporotic vertebral fractures are likely to become an increasingly important health issue. The costs of osteoporotic vertebral fractures were estimated to be € 1.5 billion in Europe in 2010[10] and US$ 1.1 billion in the United States in 2005, and they are expected to have increased by more than 50% by 2025.[11]

The etiology of osteoporotic vertebral fractures is believed to be multi-factorial, influenced by genetic and environmental factors.[12, 13] Osteoporosis is a disease characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk.[14] Bone mineral density (BMD) and age are strongly predictive for most osteoporotic fractures, whereas other risk factors may vary according to fracture site.[15] Compared with non-vertebral fractures, which usually occur after a fall, it has been suggested that only ~10-15% of osteoporotic vertebral fractures are preceded by a fall, with many resulting from low-grade trauma apparently derived from insignificant everyday activities.[16]

Importantly, vertebral fractures are strong predictors of future osteoporotic fractures, including both non-vertebral and new vertebral fractures.[17-20] Vertebral fractures can be relatively asymptomatic in some cases, still, asymptomatic vertebral fractures remain strong predictors of subsequent risk of fractures and fracture-associated mortality,[21] reason why radiological detection may be even more valuable. In clinical practice, therefore, prevalent osteoporotic vertebral fractures are considered as a strong indication for anti-osteoporotic treatment.[22] Yet, previous studies have shown that only one third of the patients with vertebral fractures come to clinical attention[23] and that vertebral fractures are commonly underreported in radiological practice.[24-27] The latter implies that applying standardized assessment methods of osteoporotic vertebral fractures might be beneficial to decrease reader subjectivity.

Currently, there is no gold standard for osteoporotic vertebral fracture diagnosis.[28] Several radiological scoring methods for osteoporotic vertebral fractures exist, each using different criteria for diagnosing and grading the fracture. Such grading definitions are currently under debate. This article will review different scoring methods for diagnosing osteoporotic vertebral fractures by discussing the benefits and limitations of the most commonly applied radiographic scoring methods. We will also discuss the role of alternative imaging techniques for assessing these fractures. In addition, this review will illustrate how prevalence of osteoporotic vertebral fractures is influenced by different scoring methods. Finally, application of scoring methods in research and patient care will be discussed.

Vertebral fracture assessment by radiography

Radiography is the standard imaging modality used for initially assessing vertebral fractures. Usually separate anteroposterior and lateral projections of the thoracic and lumbar spine are acquired, sometimes supplemented by additional views focused at the thoracolumbar junction. However, in the scientific research setting, occasionally only lateral radiographs are obtained. Radiographic capture is rapid, image quality is mostly high and radiation dose is relatively low especially compared to CT. It is important that the spine is positioned parallel to the table to enable good assessment of vertebral endplates. Imperfect centering and collimation of the X-ray beam may, however, cause oblique projection and incorrect exposure, resulting in poor image quality.[29] Also, because the X-ray beam is conical, oblique projection is worst at the film areas furthest from the center. This distortion may hinder correct appraisal of vertebral body shape and can in some situations wrongly suggest a biconcave shape.[30]

Because of superimposition of the overlying shoulder girdle, the upper thoracic spinal region cannot be clearly visualized in many cases. The iliac wings of the pelvis can also exert a similar hindrance effect on images of the lower lumbar spine. In addition, superposition of the ribs and pulmonary vasculature on the thoracic vertebrae may occasionally confound the vertebral body margins on the image.

Scoring methods

Measurements of vertebral shape

The first published standardized assessment methods use quantitative morphometry (QM), which entails direct measurement of vertebral body shape. With six-point morphometry, points are placed in the superior and inferior endplates at the anterior, middle and posterior aspects of the vertebral body. For example, two of the more recent and commonly applied QM scoring methods are those described by Eastell-Melton[31] and McCloskey-Kanis[32]. In these methods anterior, central, and posterior vertebral body heights are first measured on a lateral radiograph and ratios between these heights are calculated. These ratios are then used to classify vertebral fractures, using cutoff values based on standard deviation reductions from normal-population means derived from epidemiological studies (Figure 1). Depending on which of the three heights are diminished, the Eastell-Melton[31] method distinguishes three types of fractures (i.e., wedge, biconcavity, or compression) and the McCloskey-Kanis method further classifies the wedge type into anterior and posterior.[32] Using ratios instead of absolute heights is preferable, as anatomical structures farther away from the film may be falsely magnified, depending on the distance of the X-ray tube from the subject. Also, vertebral height is partly associated by a person's body height.[31] It is considered essential to appraise these ratios relative to population reference data, as it has been shown that the derived vertebral height ratios are normally (Gaussian) distributed.[32] In addition, several of these methods relate the values to adjacent vertebra within the same individual, as each vertebra has a different size.[33] Although QM measurements appear more objective and reproducible than visual methods, they are more laborious and time-consuming to acquire. This is an important consideration for large-scale epidemiological research as well as for its implementation in clinical practice.

Semi-quantitative (SQ) method

Currently, the most widely used standardized grading method is the visual SQ method (i.e., according to Genant[34]). It is commonly applied as a surrogate gold standard in research.[35] Vertebral fractures are SQ graded by trained readers, who estimate the percentage of height and/or area reduction subjectively, without direct measurement. Vertebral deformities are graded according to shape and severity (Figure 1). The deformity's shape is classified on the basis of anterior height loss (i.e., wedge), middle height loss (i.e., biconcave), or posterior and anterior height loss (i.e., crush). Severity of vertebral deformities is graded according to the extent of height and area loss, as mildly deformed, moderately deformed, and severely deformed (Table 1). Next, a spinal fracture index (SFI) can be calculated by summing the individual vertebral body grades. Genant et al.[34] have noted that height and area loss determined by morphometry alone fails to capture several other important characteristics of vertebral fracture, including endplate deformity, buckling of cortices, lack of parallelism of endplates and loss of vertical continuity of vertebral morphology.

Algorithm-based qualitative (ABQ) method

The more recent ABQ method by Jiang et al. diagnoses osteoporotic vertebral fractures on the basis of endplate depression, regardless of vertebral height reduction (Figure 2).[36] The key assumption is that the

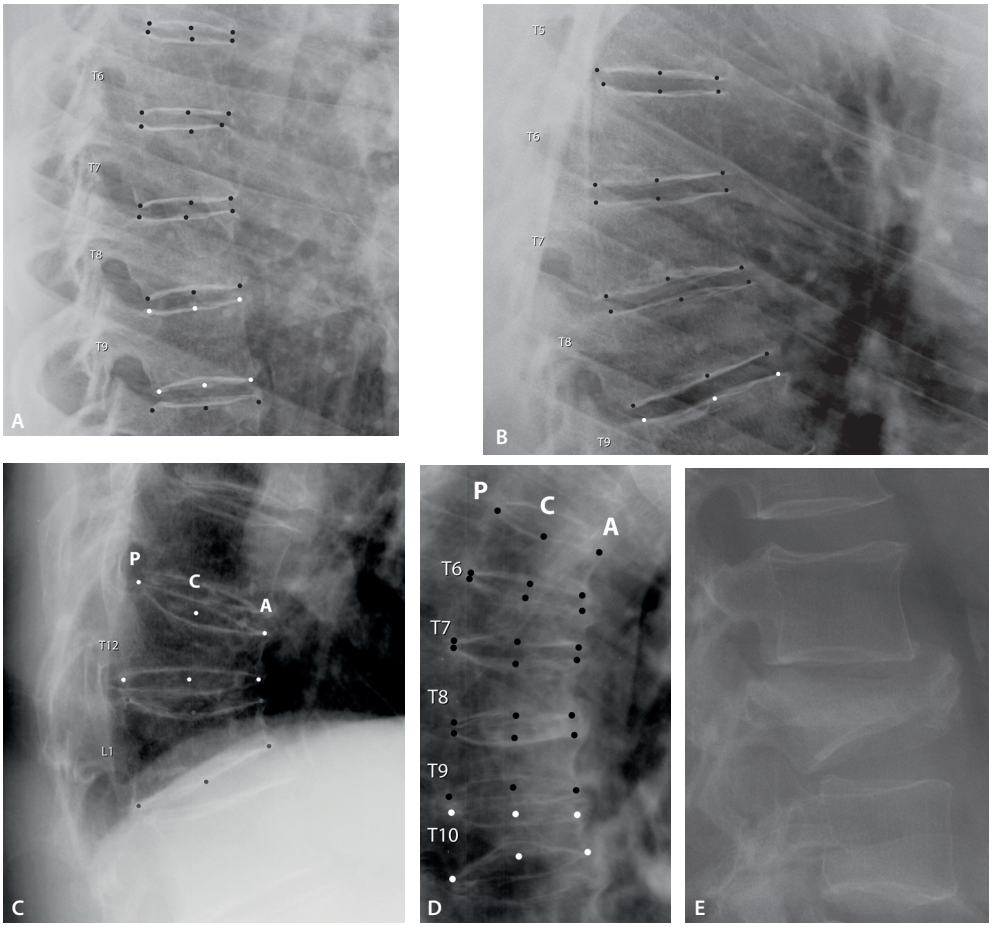


Figure 1. Six-point quantitative morphometry (QM) and semi-quantitative (SQ) method. P, posterior; C, central; A, anterior. **(A)** Normal thoracic vertebrae. **(B)** Mild wedge deformity of T8 and **(C)** severe wedge deformities of T12 and L1. **(D)** Mild wedge deformity of T6, moderate wedge deformity of T7 and moderate biconcave deformities of T8, T9 and T10. **(E)** Crush deformity of L3 in an individual with confirmed history of spinal trauma; Severe vertebral body fracture is seen with slight bulging of the posterior vertebral body margin. This fracture morphology is usually traumatic.

Table 1. Semi-quantitative grading of severity of vertebral fractures according to Genant.[34]

		Reduction of:	
Fracture severity	Grade	Height*	Area
Normal	0		
Uncertain or borderline	0.5		
Mild	1	20-25%	10-20%
Moderate	2	25-40%	20-40%
Severe	3	≥40%	≥40%

*Anterior, middle, and/or posterior height

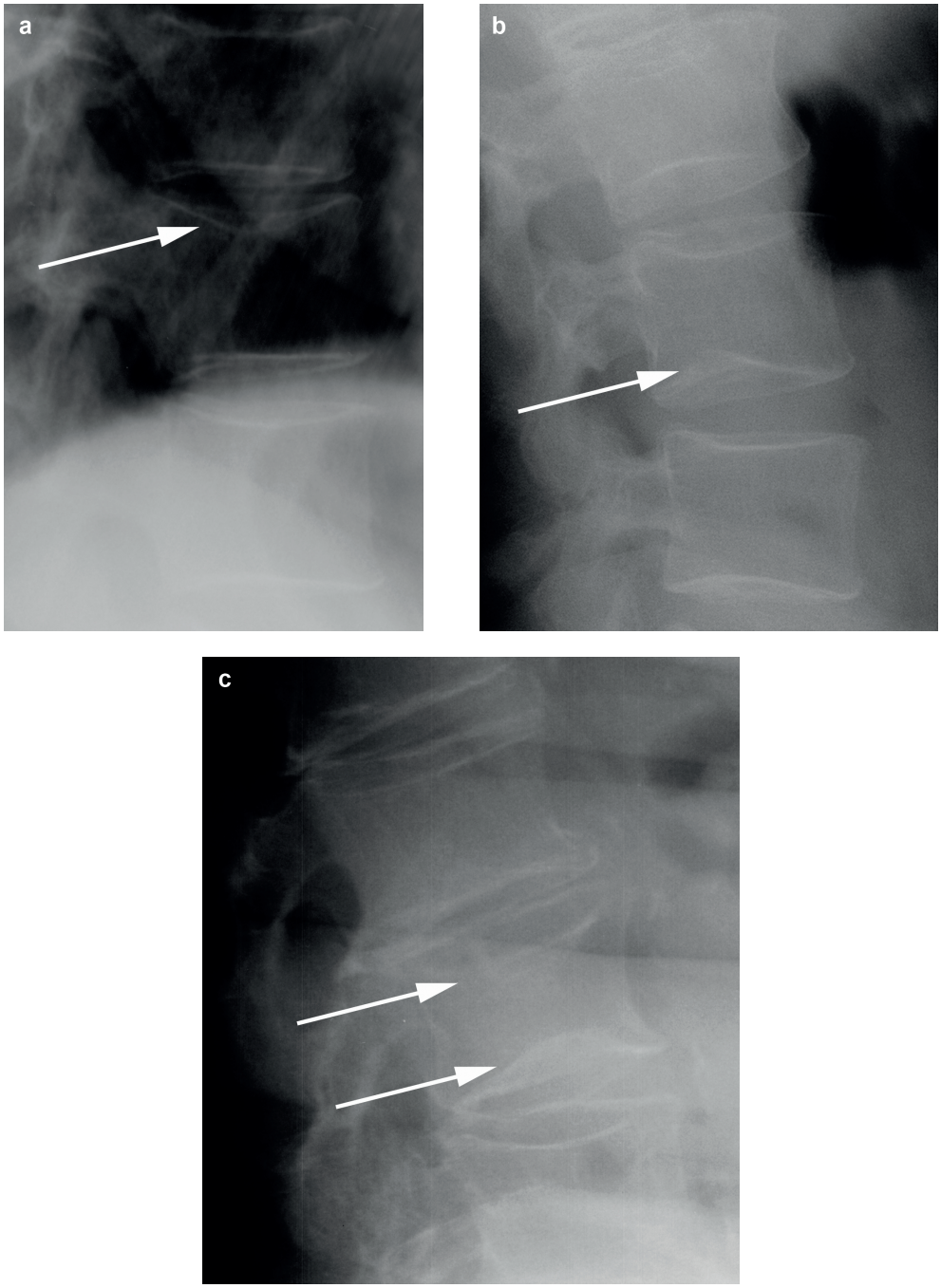


Figure 2. Algorithm-based qualitative (ABQ) method. **(A)** Superior endplate depression of T11. **(B)** Inferior endplate depression of L3. **(C)** Superior and inferior endplate depression of L3.

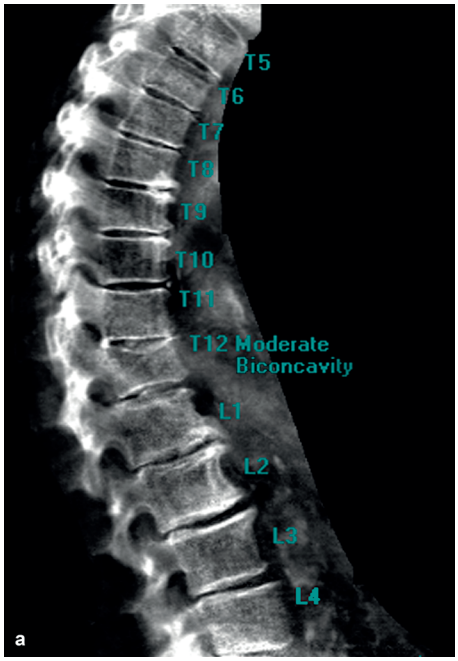
endplate is always deformed in vertebral fractures, and therefore endplate depression has perfect specificity for vertebral fracture. The fracture occurs primarily at the center of the endplate, and thus it follows that the endplate is centrally depressed in all types of vertebral fracture (i.e., concave, wedge, and crush). In addition, vertebral height may appear to be decreased as a result of oblique image projection, certain diseases and anatomical variants that can mimic vertebral fractures. To deal with this misclassification, ABQ uses a flowchart to systematically rule out non-fracture deformities by examining certain radiological features. A skilled ABQ reader is needed to differentiate accurately between vertebral fractures and non-fracture deformities. If images are of poor quality, vertebral fractures with subtle endplate changes can easily be missed.

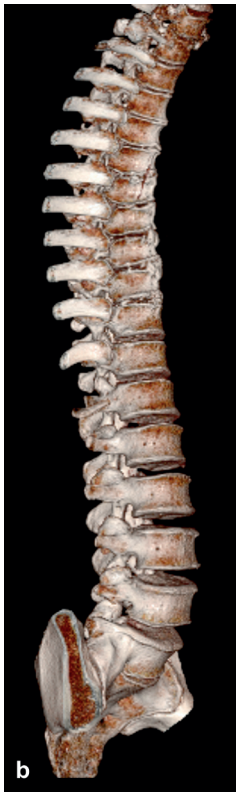
Non-radiographic imaging techniques

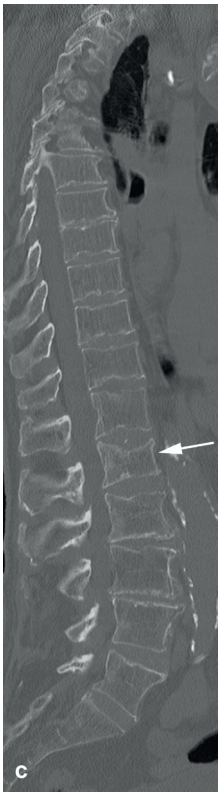
Vertebral fractures can also be detected and graded on radiological imaging investigations other than conventional radiography. Although developed for radiography, which remains the most commonly used technique for vertebral fracture assessment, the scoring methods described above can also be applied to other radiological techniques.

Dual-energy X-ray absorptiometry (DXA) at the lumbar spine and hip to measure BMD is a routine investigation in osteoporosis, because BMD constitutes one of the strongest predictors of future fracture.[37, 38] Several studies have shown that the risk of incident vertebral fractures doubles for each SD reduction of lumbar spine BMD.[37, 39] Note, however, that many fractures occur when BMD is in the osteopenic or normal range of values.[37] In addition to artefacts (i.e., osteophytes, calcifications), BMD measurements of the lumbar spine may be falsely elevated in the presence of vertebral fractures because impacted fracture or fracture healing result in higher areal BMD.[40] The World Health Organization's FRAX® tool can be used to calculate the 10-year fracture risk for individual patients, using validated risk factors (with or without femoral neck DXA BMD).[41] The clinical risk factors used in the calculation include age, gender, height, weight, previous low trauma fracture (including vertebral fractures), parental hip fracture, oral glucocorticoid therapy, rheumatoid arthritis, current smoking, alcohol consumption of more than 3 units per day, secondary causes of osteoporosis. In recent years, the use of densitometers has extended beyond BMD assessment, to identify vertebral fractures from DXA images. The so-called lateral densitometric vertebral fracture assessment (VFA) is gaining popularity due to the considerable improvement in image resolution, and is currently offering complementary and independent information about fracture risk (Figure 3a).[42] The implementation of fan-beam technology in the DXA devices has allowed capturing the whole spine in one image, with virtually no divergent beam issues due to parallax effect. Also, VFA has a low radiation dose, making it very suitable for screening in the clinical setting. For those more recently introduced DXA devices with a rotating C-arm, the lateral examination can even be done without moving the patient from the supine position used for the BMD measurements. In addition, the rotating C-arm may enable three-dimensional DXA scans, allowing the direct measurement of geometric parameters of the vertebrae.[43] It has been demonstrated that image quality can differ greatly between types of densitometers.[44] Still, radiographs have superior spatial resolution, which facilitates identification of more subtle abnormalities.[45]

Unlike two-dimensional radiography, computed tomography (CT) and magnetic resonance imaging (MRI) offer three-dimensional visualization of the vertebra (Figure 3B). In addition, CT and MRI can differentiate between old and recent vertebral fractures, by assessing the integrity and shape of the cortical margins (Figure 3c). MRI does not use ionizing radiation and can demonstrate bone marrow edema which distinguishes recent from old fracture (Figure 3d). The images produced by CT have a







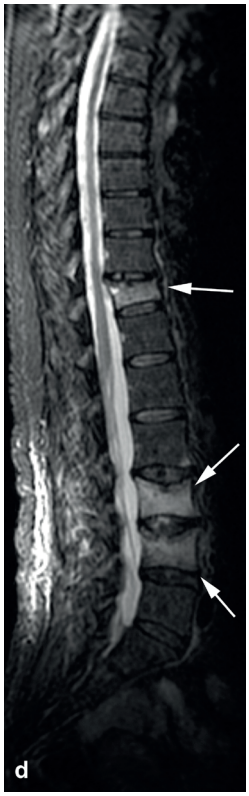


Figure 3. Non-radiographic imaging modalities. **(A)** Lateral VFA shows a biconcave deformity of T12. **(B)** Three-dimensional visualization of the thoracolumbar spine with CT. **(C)** Midline sagittal CT reformation shows an osteoporotic vertebral fracture of L1 (arrow), in addition to degenerative changes and endplate irregularities at multiple levels. **(D)** MRI: Sagittal short tau inversion recovery (STIR) sequence shows endplate deformity, height reduction, and bone marrow edema at the T11, L3, and L4 levels (arrows), indicating recent osteoporotic vertebral fractures.

much higher spatial resolution than those of MRI and DXA. It has been shown that sagittal reformations need to be used to demonstrate vertebral fractures on CT.[21] Despite the introduction of several dose reduction techniques, the ionizing radiation exposure of CT is still substantial, which is a major disadvantage of the imaging technique, especially in the research setting.[46] CT scout images may also be used for assessing vertebral fractures.[47] Novel quantitative and high-resolution CT techniques are being developed to enable separate analysis of trabecular and cortical bone compartments.[48, 49] High-resolution MRI can be used to assess bone trabeculation in the extremities, but this application of MRI at the spine is more challenging.[50] Drawbacks of MRI are, however, the long imaging time and high costs. Hence, MRI is usually used for other conditions that specifically require MRI, such as spinal cord compression and paraspinal soft tissue abnormalities.[51, 52] If a malignant etiology for vertebral fracture is suspected, then MRI or CT have advantages over conventional radiography.[53, 54]

Image analysis software

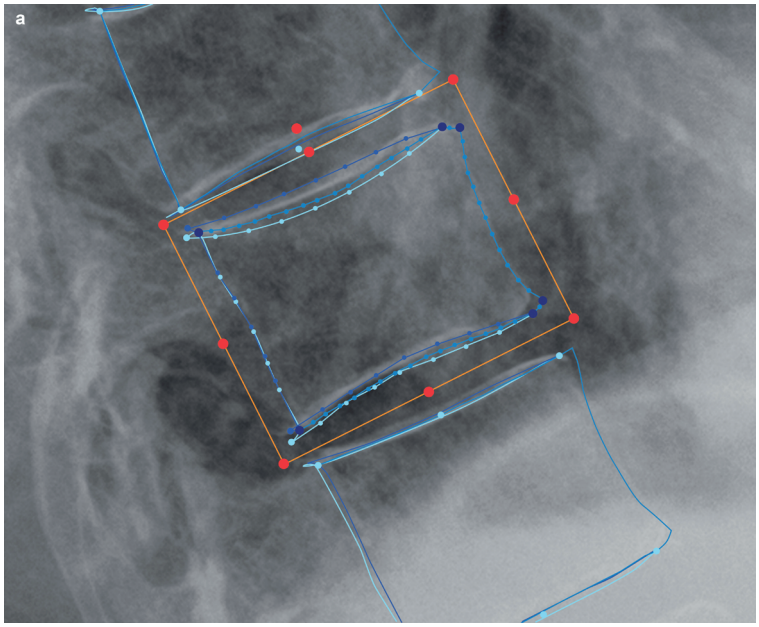
Automated image analysis software packages (e.g., SpineAnalyzer®, Optasia Medical Ltd, Cheadle, UK[55]) have been developed to facilitate efficient and standardized vertebral fracture scoring of large datasets.[56, 57] The software can handle lateral spine radiographs, VFA or CT scout films. So far, software packages have been dedicated to recording QM and SQ.

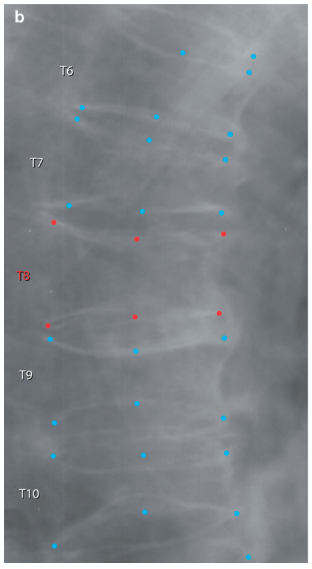
Instead of having to manually define vertebral contours and height, users only need to place one point in the center of each vertebra to define vertebral level. Next, vertebral contours are identified by the software using automated segmentation techniques and vertebral height is measured (Figure 4A). A table with percentage height loss and presence or absence of deformity per vertebral level based on QM and SQ is generated (Figure 4B), after which the data can be exported to a database. The data that can be saved include the exact coordinates of the endplates anteriorly, centrally, and posteriorly. This information can be valuable in the research setting, where analysis of crude vertebral heights could be meaningful to explore optimization of current vertebral fracture definitions. Relatively inexperienced users are deemed to be capable of using the software after a brief training.[58] These software packages can be further improved by incorporating population reference data for QM and by reducing the need for manual adjustment of vertebral contour definition, a procedure that is still required routinely. Automated VFA packages are nowadays integrated in DXA equipment.

Differential diagnosis

There are a number of differential diagnoses that have to be considered in individuals with vertebral deformities.[59] In the 1960s, Hurxthal described several criteria for vertebral measurements.[30] Basically, all artefacts that can interfere with vertebral height measurement should be considered by the reader. Hook-shaped protuberances at the posterosuperior (called uncinate process by some) and posteroinferior borders of the vertebrae, any Schmorl's nodes and osteophytes should be excluded from vertebral height measurement. Six-point morphometry alone is unable to distinguish fractures and vertebral deformities due to other causes. In the description of the SQ method[34] several conditions that can mimic vertebral fracture such as scoliosis and vertebral body remodeling due to degenerative disc disease, are listed. Moreover, the ABQ method introduced a very comprehensive decision-making algorithm, which provides a guideline for systematically assessing various non-fracture deformities.[35]

Normal anatomical variation in the shape of individual vertebrae and of the spinal column as a whole should be taken into account. Viewed laterally, the spine has a natural curvature. Vertebrae in the mid-thoracic region are more wedge-shaped, causing a mild kyphosis. Lumbar vertebrae tend to





Level	Wedge	Biconcave	Crush	Grade
L1	12.2%	15.1%	-18.7%	Normal
L2	-14.8%	6.4%	-4.6%	Normal
L3	4.1%	11.7%	-1.2%	Normal
L4	-7.1%	10.1%	-10.3%	Normal
T4	17.6%	13.8%	-19.3%	Normal
T5	23.4%	18.1%	9.8%	Mild
T6	29.2%	25.4%	-12.8%	Moderate
T7	37.2%	17.5%	16.9%	Moderate
T8	24.3%	25.2%	-25.1%	Moderate
T9	4.2%	35.8%	-1.1%	Moderate
T10	30.9%	36.4%	28.7%	Moderate
T11	18.6%	19.5%	-12.1%	Normal
T12	6.1%	7.4%	-2.7%	Normal

Figure 4. Image analysis software (examinations with SpineAnalyzer®). **(A)** Automated contour detection. **(B)** Automated analysis of shape abnormality.

be biconcave rather than wedge-shaped, and this gives rise to a normal lordotic curve, because of the relatively shorter posterior height. In addition, some adults have vertebrae that have longstanding short anterior height in developmentally small thoracic vertebrae.[35] Therefore, the normal spine shape must be known if SQ and ABQ readers are to avoid false-positive fracture diagnosis. Some QM methods

that compare ratios to population reference data may classify short vertebral height correctly as non-fracture. In addition, the anterior vertebral wedge angle has been shown to increase concurrently with age-related degenerative change. Degenerative signs include degenerative disc disease, osteophytes and endplate irregularities (Figure 5A). The ABQ method incorporates additional differential diagnoses including previous (e.g., during childhood) fractures, metabolic diseases (e.g., osteomalacia), and developmental anomalies, including anterior step deformity (depressions in the anterior portion of the vertebral endplate) in thoracic vertebrae, balloon disc, or cupid's bow with deep inferior endplates in the lumbar vertebrae.[35]

A frequent condition that resembles vertebral fractures is Scheuermann's disease. Radiographic criteria of Scheuermann's disease are a thoracic kyphosis greater than 45 degrees and at least three adjacent wedge-shaped vertebral bodies of 5 degrees or more.[60, 61] Vertebral wedging is frequently associated with endplate irregularity and Schmorl's nodes. Elongated vertebrae and disc space narrowing can also be found in Scheuermann's disease (Figure 5B). This vertebral wedging may be mistaken for mild vertebral fractures by QM or SQ, and Schmorl's nodes may mimic endplate depression. Occasionally, but most importantly, osteoporotic vertebral fractures need to be distinguished from those resulting from malignant etiologies, such as metastases (most commonly of primary breast, kidney, prostate, or lung neoplasms), multiple myeloma, or primary bone tumors.[53] In the majority of osteoporotic vertebral fracture cases, posterior margins of vertebrae maintain a straight or concave shape whereas in malignant etiologies the posterior margin is often convex.

Traumatic fractures should also be distinguished from the typically low-grade trauma osteoporotic fractures. Posterior height loss was regarded as posterior wedge in the McCloskey-Kanis method[32] and as crush deformity in SQ[34]. However, fractures involving the posterior vertebral part are typically attributable to malignancy or high-energy trauma,[62] rather than to low trauma, which is most common in osteoporosis.

Inter- and intra-observer agreement of scoring methods

Inter- and intra-observer agreement seem to vary considerably within and between scoring methods. However, agreement is about precision of a study and may not necessarily relate to its validity. Nevertheless, there are several aspects that need to be considered when comparing methods. Point-placement in SQ and QM is said to be somewhat subjective, and hence inclined to influence fracture discrimination. This is particularly pertinent in the presence of borderline deformities. Also, reproducibility of SQ and ABQ scoring may to some extent depend on the reader's training and experience.[34, 44, 63]

Kim et al. have evaluated intra- and inter-reader agreement of a semi-automated quantitative morphometry software algorithm on lateral CT scout views.[58] They found intraclass correlation coefficients of 0.96 to 0.98 for vertebral heights; while kappa statistics were 0.59 to 0.69 for intra-reader and 0.67 for inter-reader agreement. Agreement for vertebral fracture classification was worse than agreement for height measurements. This was explained by the small variation of height measurement around fracture classification thresholds. Such clinically insignificant variation in height measurement can actually lead to two different fracture classifications in a considerable number of cases. Furthermore, kappa scores did not improve much even when the fracture definition was changed to include only moderate and severe fractures (i.e., deformity ≥25%).

Several publications have evaluated the inter- and intra-observer agreement of SQ alone and compared with QM methods.[34, 36, 63-65] Kappa statistics reported for SQ inter-observer agreement ranged from 0.51 to 0.80 and from 0.76 to 0.93 for intra-observer agreement, respectively. The kappas for agreement

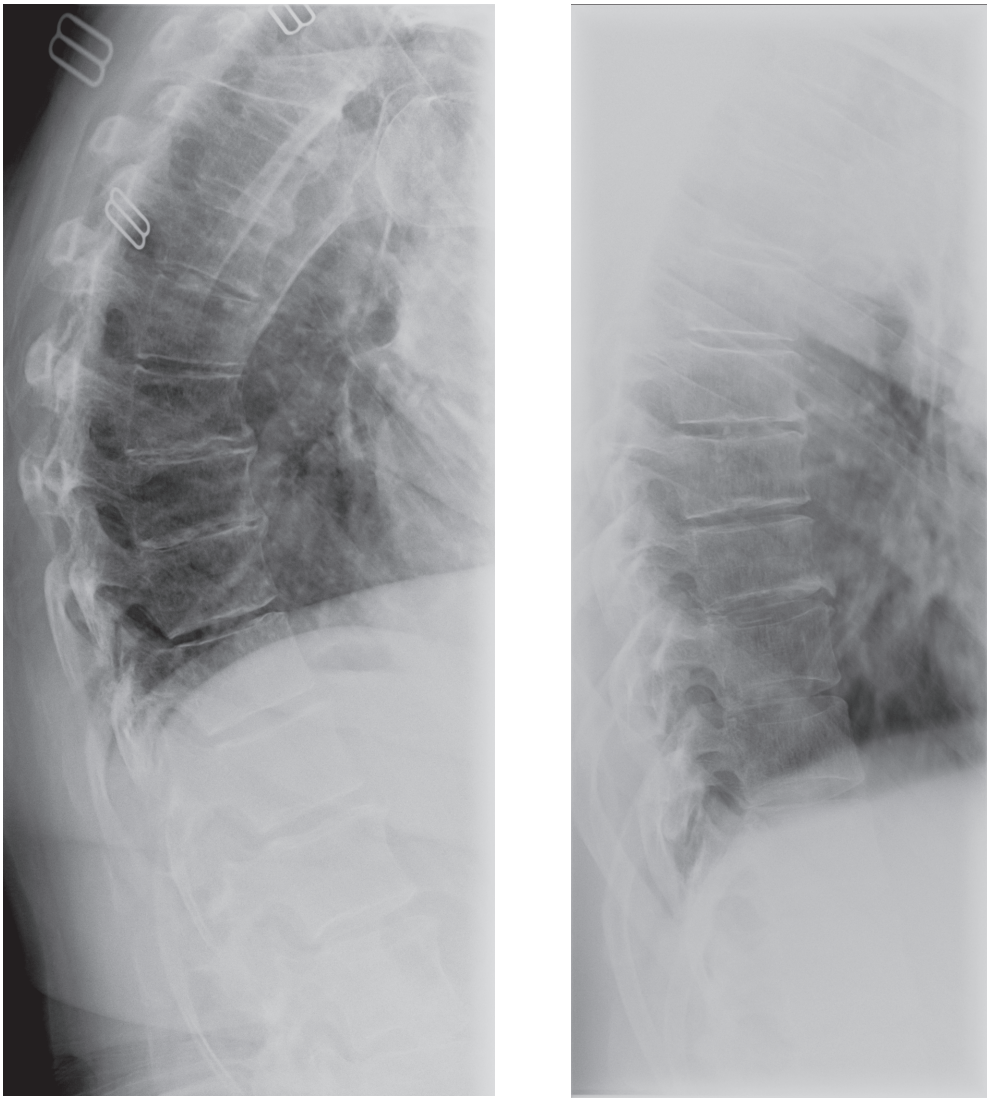


Figure 5. Conditions that mimic vertebral fractures. **(A)** Degenerative changes. Very mild anterior vertebral wedging of two mid-thoracic vertebral bodies is seen along with mild spondylotic changes at the anterior vertebral margins. Note that the endplates are intact and only show mild degenerative irregularities. **(B)** Scheuermann's disease. In addition to marked endplate irregularity, mild anterior wedging of multiple midthoracic vertebrae is seen, resulting in increased thoracic kyphosis.

between SQ and several QM methods have been reported to be lower, ranging from 0.23 to 0.59, with some improvement when fracture definition included only moderate and severe fractures (i.e., deformity ≥25%). Obviously, the agreement between different QM methods will depend on the fracture threshold chosen. Recently, semi-automated QM reading using Genant's criteria by a non-radiologist was compared with conventional SQ grading performed by experienced radiologists, finding a kappa for agreement of 0.78.[66]

Ferrar et al. have examined inter-observer agreement for ABQ diagnosis of prevalent vertebral fracture in approximately 200 elderly women, finding kappa statistics of 0.74 for inter-reader agreement.[63] In general, the ABQ method has displayed low to moderate concordance with other methods. Jiang et al. found kappa statistics between 0.39 and 0.64 comparing ABQ with the QM methods developed by Eastell-Melton and McCloskey.[36] Also, ABQ has been compared with SQ observing kappa statistics of 0.30 to 0.58.[67]

Influence of scoring methods on vertebral fracture prevalence and incidence

All methods assess osteoporotic vertebral fractures with different criteria, which results in different estimates of the prevalence of the disease.[36, 68] For example, QM and SQ would not diagnose vertebral fractures in the case of endplate depression without reduced vertebral height (Figure 6A). Conversely, ABQ would not diagnose a QM-based vertebral fracture with reduced height but intact endplates (Figure 6B). In general, SQ would yield a higher number of fractures than when applying QM, asserting that SQ would be more sensitive particularly for the detection of mild deformities.[68] However, Melton et al. have demonstrated that depending on the morphometric definition used, the prevalence of vertebral fractures ranged from 3 to 90% in their study.[69] Of all the methods, the ABQ reading results in the lowest estimations of vertebral fracture prevalence. The question remains if the higher estimates from other methods are actually due to false-positive classification of non-fracture deformities.[36]

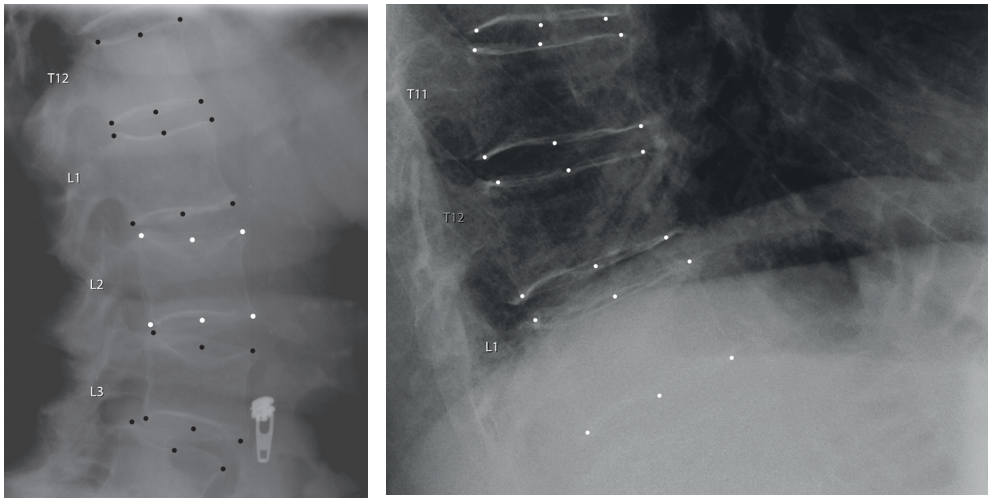


Figure 6. Discrepancy between scoring methods. **(A)** Vertebral fractures diagnosed with ABQ L2 and L3 based on endplate depression, classified as normal with QM because of normal vertebral height. **(B)** Vertebral fracture of T12 diagnosed with QM, based on height reduction, classified as normal with ABQ because of intact endplates.

Research implications

Misclassification of vertebral fractures may result in non-differential information bias, leading to dilution of observed effects. As a consequence, true associations in studies may go undetected. This ascertainment bias can occur both in case-control studies (including clinical trials investigating drug effects) and in observational studies. Therefore, scoring methods should procure the optimal classification of true vertebral fractures.

Large-scale application of standardized scoring can be difficult, with purely morphometric approaches being laborious, while the other methods will require thorough training of observers. In very large studies, especially population-based studies with an expected low prevalence of vertebral fracture cases, a technician triage system may reduce the work burden of scoring thousands of radiographs.[65, 68, 70] Firstly, trained research technicians can triage radiographs as definite vertebral fracture, uncertain fracture, or definite normal. Finally, an expert reader may review the difficult cases and confirm vertebral fractures. Also, a stepwise evaluation process combining morphometry and qualitative assessment represents a possible procedure to achieve a final diagnosis of vertebral osteoporosis.[71]

To date, there have been few large-scale comparisons of vertebral fracture assessment methods. We are currently applying both ABQ and software-assisted QM methods to radiographs from the Rotterdam Study (all image examples included in the present article originate from this study). This study is a prospective population-based cohort, which has been studying disease and disability in more than 15,000 individuals aged 45 and over since 1990.[72] Within the on-going research program, radiographs of approximately 11,000 participants are available, with a follow-up duration of maximally 15 years. An aim of the study is to compare the methods applied for identifying vertebral fractures. In addition, data on numerous outcomes and risk factors are available, including a comprehensive assessment of clinical fractures, BMD, and genetic determinations.

Clinical implications

It is estimated that only about one third of all vertebral fractures come to clinical attention.[23] However, assessment of vertebral fracture status, in addition to BMD, provides practical and relevant clinical information to aid the prediction of subsequent fracture risk.[73] Symptomatic and non-symptomatic vertebral fractures are both associated with decreased quality of life[2-7] and increased mortality risk[8, 9]. In the case of vertebral fracture, pharmacologic therapy is considered necessary to prevent the occurrence of future osteoporotic fractures.[74] However, as all interventions have costs and potential side effects; correct assessment of vertebral fractures is of utmost importance. Over- and underdiagnosis can have major consequences, particularly at the population level. Misdiagnosis of osteoporotic vertebral fractures will result in under- or overtreatment of patients and subsequently unnecessary costs, increased morbidity and higher mortality.

Current definitions used by vertebral fracture scoring methods seem to be based on arbitrary cutoffs. At most, some QM methods have been established by deriving standard deviations from measurements in a sample of healthy individuals, but variation from the mean is not necessarily abnormal. The classifications show association with osteoporosis-related outcomes such as BMD and the risk of future non-vertebral and new vertebral fractures.[17, 36, 65, 70, 75] Yet, from a more clinically oriented perspective the definition of vertebral fractures should be based on cutoffs that were defined based on their ability to predict relevant outcomes, such as future osteoporotic fractures. This will require the optimal combination of true- and false-positive ratios that yield the greatest expected utility for the patient at acceptable costs to society. For optimal appraisal of future osteoporotic fracture risk it might prove necessary to refine currently available vertebral fracture scoring after comprehensive comparative studies and integrate more quantitative information that can be derived from imaging, for example: three-dimensional reconstruction of vertebral shape, BMD, measurements able to appreciate integrity of the endplates and microarchitecture.

In conclusion, standardized and accurate scoring methods for osteoporotic vertebral fractures are desirable. There are several radiological scoring methods for osteoporotic vertebral fractures, which

can be characterized as quantitative, qualitative, or semi-quantitative. Also, these standardized scoring methods can be implemented for different imaging modalities. The scoring methods each use different definitions for the diagnosis of vertebral fracture and the classification of severity. Such differences have implications for patient care and scientific research. Accurate diagnosis of vertebral fractures and differentiation from non-fracture deformities is an important aspect that depends on the expertise of the reader. Future evaluation of the concordance between methods will allow establishing their benefits and limitations, and most importantly, optimize their effectiveness for application in clinical and research scenarios.

KEY POINTS

- Several scoring methods using different criteria for assessing osteoporotic vertebral fractures exist.
- Standardized osteoporotic vertebral fracture assessment should be applicable to different radiological investigations.
- Accurate assessment of osteoporotic vertebral fractures is essential for proper patient management.
- Optimizing feasibility of scoring methods enables wide-spread use in scientific research.
- Assessment of concordance between methods is important for application in patient care.

REFERENCES

1. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I.
2. Burger H, Van Daele PL, Grashuis K, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities and functional impairment in men and women. J Bone Miner Res. 1997 Jan;12(1):152-7.
3. Gold DT. The clinical impact of vertebral fractures: quality of life in women with osteoporosis. Bone. 1996 Mar;18(3 Suppl):185S-9S.
4. Nevitt MC, Ettinger B, Black DM, Stone K, Jamal SA, Ensrud K, et al. The association of radiographically detected vertebral fractures with back pain and function: a prospective study. Ann Intern Med. 1998 May 15;128(10):793-800.
5. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C, et al. Health-related quality of life in postmenopausal women with low BMD with or without prevalent vertebral fractures. J Bone Miner Res. 2000 Jul;15(7):1384-92.
6. Oleksik AM, Ewing S, Shen W, van Schoor NM, Lips P. Impact of incident vertebral fractures on health related quality of life (HRQOL) in postmenopausal women with prevalent vertebral fractures. Osteoporos Int. 2005 Aug;16(8):861-70.
7. Ross PD. Clinical consequences of vertebral fractures. Am J Med. 1997 Aug 18;103(2A):30S-42S; discussion S-3S.
8. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
9. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. J Am Geriatr Soc. 2000 Mar;48(3):241-9.
10. Ström O, Borgström F, Kanis JA, Compston J, Cooper C, McCloskey EV, et al. Osteoporosis: burden, health care provision and opportunities in the EU. Arch Osteoporos. 2011;DOI 10.1007/s11657-011-0060-1.
11. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. 2007 Mar;22(3):465-75.
12. Ralston SH, Uitterlinden AG. Genetics of osteoporosis. Endocr Rev. 2010 Oct;31(5):629-62.

13. van der Klift M, de Laet CE, McCloskey EV, Johnell O, Kanis JA, Hofman A, et al. Risk factors for incident vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2004 Jul;19(7):1172-80.
14. World Health Organization. Assessment of fracture risk and its implication to screening for postmenopausal osteoporosis: Technical report series 843. Geneva: WHO. 1994.
15. Kelsey JL, Samelson EJ. Variation in risk factors for fractures at different sites. Curr Osteoporos Rep. 2009 Dec;7(4):127-33.
16. Cooper C, Atkinson EJ, O'Fallon WM, Melton LJ, 3rd. Incidence of clinically diagnosed vertebral fractures: a population-based study in Rochester, Minnesota, 1985-1989. J Bone Miner Res. 1992 Feb;7(2):221-7.
17. Black DM, Arden NK, Palermo L, Pearson J, Cummings SR. Prevalent vertebral deformities predict hip fractures and new vertebral deformities but not wrist fractures. Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1999 May;14(5):821-8.
18. McCloskey EV, Vasireddy S, Threlkeld J, Eastaugh J, Parry A, Bonnet N, et al. Vertebral fracture assessment (VFA) with a densitometer predicts future fractures in elderly women unselected for osteoporosis. J Bone Miner Res. 2008 Oct;23(10):1561-8.
19. Melton LJ, 3rd, Atkinson EJ, Cooper C, O'Fallon WM, Riggs BL. Vertebral fractures predict subsequent fractures. Osteoporos Int. 1999;10(3):214-21.
20. Lindsay R, Silverman SL, Cooper C, Hanley DA, Barton I, Broy SB, et al. Risk of new vertebral fracture in the year following a fracture. JAMA. 2001 Jan 17;285(3):320-3.
21. Williams AL, Al-Busaidi A, Sparrow PJ, Adams JE, Whitehouse RW. Under-reporting of osteoporotic vertebral fractures on computed tomography. Eur J Radiol. 2009 Jan;69(1):179-83.
22. Lewiecki EM, Laster AJ. Clinical review: Clinical applications of vertebral fracture assessment by dual-energy x-ray absorptiometry. J Clin Endocrinol Metab. 2006 Nov;91(11):4215-22.
23. Cooper C, Shah S, Hand DJ, Adams J, Compston J, Davie M, et al. Screening for vertebral osteoporosis using individual risk factors. The Multicentre Vertebral Fracture Study Group. Osteoporos Int. 1991 Oct;2(1):48-53.
24. Delmas PD, van de Langerijt L, Watts NB, Eastell R, Genant H, Grauer A, et al. Underdiagnosis of vertebral fractures is a worldwide problem: the IMPACT study. J Bone Miner Res. 2005 Apr;20(4):557-63.
25. Gehlbach SH, Bigelow C, Heimisdottir M, May S, Walker M, Kirkwood JR. Recognition of vertebral fracture in a clinical setting. Osteoporos Int. 2000;11(7):577-82.
26. Bartalena T, Giannelli G, Rinaldi MF, Rimondi E, Rinaldi G, Sverzellati N, et al. Prevalence of thoracolumbar vertebral fractures on multidetector CT: underreporting by radiologists. Eur J Radiol. 2009 Mar;69(3):555-9.
27. Kim N, Rowe BH, Raymond G, Jen H, Colman I, Jackson SA, et al. Underreporting of vertebral fractures on routine chest radiography. AJR Am J Roentgenol. 2004 Feb;182(2):297-300.
28. Guglielmi G, Diacinti D, van Kuijk C, Aparisi F, Krestan C, Adams JE, et al. Vertebral morphometry: current methods and recent advances. Eur Radiol. 2008 Jul;18(7):1484-96.
29. Almen A, Tingberg A, Besjakov J, Mattsson S. The use of reference image criteria in X-ray diagnostics: an application for the optimisation of lumbar spine radiographs. Eur Radiol. 2004 Sep;14(9):1561-7.
30. Hurxthal LM. Measurement of anterior vertebral compressions and biconcave vertebrae. Am J Roentgenol Radium Ther Nucl Med. 1968 Jul;103(3):635-44.
31. Eastell R, Cedel SL, Wahner HW, Riggs BL, Melton LJ, 3rd. Classification of vertebral fractures. J Bone Miner Res. 1991 Mar;6(3):207-15.
32. McCloskey EV, Spector TD, Eyres KS, Fern ED, O'Rourke N, Vasikaran S, et al. The assessment of vertebral deformity: a method for use in population studies and clinical trials. Osteoporos Int. 1993 May;3(3):138-47.
33. Davies KM, Recker RR, Heaney RP. Normal vertebral dimensions and normal variation in serial measurements of vertebrae. J Bone Miner Res. 1989 Jun;4(3):341-9.
34. Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res. 1993 Sep;8(9):1137-48.
35. Ferrar L, Jiang G, Adams J, Eastell R. Identification of vertebral fractures: an update. Osteoporos Int. 2005 Jul;16(7):717-28.
36. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.

37. Marshall D, Johnell O, Wedel H. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. BMJ. 1996 May 18;312(7041):1254-9.
38. Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone. 2004 Jan;34(1):195-202.
39. The European Prospective Osteoporosis (EPOS) Study Group. The relationship between bone density and incident vertebral fracture in men and women. J Bone Miner Res. 2002 Dec;17(12):2214-21.
40. Lorente-Ramos R, Azpeitia-Arman J, Munoz-Hernandez A, Garcia-Gomez JM, Diez-Martinez P, Grande-Barez M. Dual-energy x-ray absorptiometry in the diagnosis of osteoporosis: a practical guide. AJR Am J Roentgenol. 2011 Apr;196(4):897-904.
41. Kanis JA, Oden A, Johansson H, Borgstrom F, Strom O, McCloskey E. FRAX and its applications to clinical practice. Bone. 2009 May;44(5):734-43.
42. Cauley JA, Hochberg MC, Lui LY, Palermo L, Ensrud KE, Hillier TA, et al. Long-term risk of incident vertebral fractures. JAMA. 2007 Dec 19;298(23):2761-7.
43. Kolta S, Quiligotti S, Ruyssen-Witrand A, Amido A, Mitton D, Bras AL, et al. In vivo 3D reconstruction of human vertebrae with the three-dimensional X-ray absorptiometry (3D-XA) method. Osteoporos Int. 2008 Feb;19(2):185-92.
44. Buehring B, Krueger D, Checovich M, Gemar D, Vallarta-Ast N, Genant HK, et al. Vertebral fracture assessment: impact of instrument and reader. Osteoporos Int. 2010 Mar;21(3):487-94.
45. Schousboe JT, Debold CR. Reliability and accuracy of vertebral fracture assessment with densitometry compared to radiography in clinical practice. Osteoporos Int. 2006 Feb;17(2):281-9.
46. Damilakis J, Adams JE, Guglielmi G, Link TM. Radiation exposure in X-ray-based imaging techniques used in osteoporosis. Eur Radiol. 2010 Nov;20(11):2707-14.
47. Samelson EJ, Christiansen BA, Demissie S, Broe KE, Zhou Y, Meng CA, et al. Reliability of vertebral fracture assessment using multidetector CT lateral scout views: the Framingham Osteoporosis Study. Osteoporos Int. 2011 Apr;22(4):1123-31.
48. Burghardt AJ, Link TM, Majumdar S. High-resolution computed tomography for clinical imaging of bone microarchitecture. Clin Orthop Relat Res. 2011 Aug;469(8):2179-93.
49. Krug R, Burghardt AJ, Majumdar S, Link TM. High-resolution imaging techniques for the assessment of osteoporosis. Radiol Clin North Am. 2010 May;48(3):601-21.
50. Link TM. Osteoporosis imaging: state of the art and advanced imaging. Radiology. 2012 Apr;263(1):3-17.
51. Van Goethem JW, Maes M, Ozsarlak O, van den Hauwe L, Parizel PM. Imaging in spinal trauma. Eur Radiol. 2005 Mar;15(3):582-90.
52. Wilmink JT. MR imaging of the spine: trauma and degenerative disease. Eur Radiol. 1999;9(7):1259-66.
53. Drevelegas A, Chourmouzi D, Boulogianni G, Sofroniadis I. Imaging of primary bone tumors of the spine. Eur Radiol. 2003 Aug;13(8):1859-71.
54. Erlemann R. Imaging and differential diagnosis of primary bone tumors and tumor-like lesions of the spine. Eur J Radiol. 2006 Apr;58(1):48-67.
55. Brett A, Miller CG, Hayes CW, Krasnow J, Ozanian T, Abrams K, et al. Development of a clinical workflow tool to enhance the detection of vertebral fractures: accuracy and precision evaluation. Spine (Phila Pa 1976). 2009 Oct 15;34(22):2437-43.
56. Guglielmi G, Palmieri F, Placentino MG, D'Errico F, Stoppino LP. Assessment of osteoporotic vertebral fractures using specialized workflow software for 6-point morphometry. Eur J Radiol. 2009 Apr;70(1):142-8.
57. Roberts MG, Oh T, Pacheco EM, Mohankumar R, Cootes TF, Adams JE. Semi-automatic determination of detailed vertebral shape from lumbar radiographs using active appearance models. Osteoporos Int. 2012 Feb;23(2):655-64.
58. Kim YM, Demissie S, Eisenberg R, Samelson EJ, Kiel DP, Bouxsein ML. Intra-and inter-reader reliability of semi-automated quantitative morphometry measurements and vertebral fracture assessment using lateral scout views from computed tomography. Osteoporos Int. 2011 Oct;22(10):2677-88.
59. Link TM, Guglielmi G, van Kuijk C, Adams JE. Radiologic assessment of osteoporotic vertebral fractures: diagnostic and prognostic implications. Eur Radiol. 2005 Aug;15(8):1521-32.

60. Sørensen. Scheuermann's Juvenile Kyphosis: Clinical Appearances, Radiography, Aetiology, and Prognosis. Munksgaard. 1964.
61. Sachs B, Bradford D, Winter R, Lonstein J, Moe J, Willson S. Scheuermann kyphosis. Follow-up of Milwaukee-brace treatment. J Bone Joint Surg Am. 1987 Jan;69(1):50-7.
62. Bensch FV, Kiuru MJ, Koivikko MP, Koskinen SK. Spine fractures in falling accidents: analysis of multidetector CT findings. Eur Radiol. 2004 Apr;14(4):618-24.
63. Ferrar L, Jiang G, Schousboe JT, DeBold CR, Eastell R. Algorithm-based qualitative and semiquantitative identification of prevalent vertebral fracture: agreement between different readers, imaging modalities, and diagnostic approaches. J Bone Miner Res. 2008 Mar;23(3):417-24.
64. Wu CY, Li J, Jergas M, Genant HK. Comparison of semiquantitative and quantitative techniques for the assessment of prevalent and incident vertebral fractures. Osteoporos Int. 1995;5(5):354-70.
65. Black DM, Palermo L, Nevitt MC, Genant HK, Epstein R, San Valentin R, et al. Comparison of methods for defining prevalent vertebral deformities: the Study of Osteoporotic Fractures. J Bone Miner Res. 1995 Jun;10(6):890-902.
66. Kim YM, Demissie S, Genant HK, Cheng X, Yu W, Samelson EJ, et al. Identification of prevalent vertebral fractures using CT lateral scout views: a comparison of semi-automated quantitative vertebral morphometry and radiologist semi-quantitative grading. Osteoporos Int. 2012 Mar;23(3):1007-16.
67. Ferrar L, Jiang G, Cawthon PM, San Valentin R, Fullman R, Lambert L, et al. Identification of vertebral fracture and non-osteoporotic short vertebral height in men: the MrOS study. J Bone Miner Res. 2007 Sep;22(9):1434-41.
68. Genant HK, Jergas M, Palermo L, Nevitt M, Valentin RS, Black D, et al. Comparison of semiquantitative visual and quantitative morphometric assessment of prevalent and incident vertebral fractures in osteoporosis The Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1996 Jul;11(7):984-96.
69. Melton LJ, 3rd, Wenger DE, Atkinson EJ, Achenbach SJ, Berquist TH, Riggs BL, et al. Influence of baseline deformity definition on subsequent vertebral fracture risk in postmenopausal women. Osteoporos Int. 2006;17(7):978-85.
70. Black DM, Palermo L, Nevitt MC, Genant HK, Christensen L, Cummings SR. Defining incident vertebral deformity: a prospective comparison of several approaches. The Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1999 Jan;14(1):90-101.
71. Ziegler R, Scheidt-Nave C, Leidig-Bruckner G. What is a vertebral fracture? Bone. 1996 Mar;18(3 Suppl):169S-77S.
72. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
73. Siris ES, Genant HK, Laster AJ, Chen P, Misurski DA, Krege JH. Enhanced prediction of fracture risk combining vertebral fracture status and BMD. Osteoporos Int. 2007 Jun;18(6):761-70.
74. Chen JS, Sambrook PN. Antiresorptive therapies for osteoporosis: a clinical overview. Nat Rev Endocrinol. 2011;8(2):81-91.
75. Finigan J, Greenfield DM, Blumsohn A, Hannon RA, Peel NF, Jiang G, et al. Risk factors for vertebral and nonvertebral fracture over 10 years: a population-based study in women. J Bone Miner Res. 2008 Jan;23(1):75-85.

Chapter 3.2

Osteoporotic vertebral fracture prevalences vary widely between radiological assessment methods: the Rotterdam Study

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In preparation

ABSTRACT

Vertebral fractures are often a first presentation of osteoporosis, therefore, accurate diagnosis is needed to identify high risk patients to prevent future fractures. Several methods for radiological assessment of vertebral fractures exist, but a gold standard is lacking. The aim of our study was to analyze differences in prevalences when applying two assessment methods for osteoporotic vertebral fractures in the population-based Rotterdam Study, an ongoing prospective cohort study. The algorithm based qualitative (ABQ) method mainly judges endplate integrity, while quantitative morphometry (QM) based methods evaluate vertebral height loss. Trained research assistants assessed lateral spine radiographs (T4-L4), using either ABQ or software-assisted QM (SpineAnalyzer® (SA), applies Genant's classification). With ABQ, radiographs were triaged as normal, uncertain or definite fracture. Definite and uncertain vertebral fractures were re-assessed by a musculoskeletal radiologist. Radiographs were assessed for 2,827 participants aged 45-89 years. With SA, the prevalence was 18.9% (95% CI: 17.4%–20.3%), compared to 3.1% (95% CI: 2.5%–3.8%) with ABQ. Both methods agreed that 80.0% were normal and 1.9% had deformities. 16.9% were assessed as SA deformities but not ABQ fractures; 1.2% were judged fractured according to ABQ but not SA. With ABQ, most fractures were found at the thoraco-lumbar junction, lumbar (T11-L3) and mid-thoracic (T7-T9) regions. With SA, most deformities were at the middle (T7,T8) and lower thoracic regions (T11,T12). The distribution of deformity severity was 70.5% mild, 27.3% moderate, 2.3% severe. Shape of deformity was 95.2% wedge, 2.5% biconcave and 2.3% crush. Most ABQ fractures concerned the superior endplate. The superior endplate was more frequently affected at the thoraco-lumbar junction and lumbar regions (T11-L3), while more inferior endplate fractures were seen at the mid-thoracic spine (T7-T8). In conclusion, osteoporotic vertebral fractures prevalence rates differ significantly between methods. Both QM and ABQ classify a considerable number of deformities that were assessed as normal by the other.

INTRODUCTION

Of all osteoporotic fractures, vertebral fractures are the most common type.[1] Vertebral fractures have been synonymous with the diagnosis of osteoporosis since its earliest description as a metabolic bone disorder.[2] Furthermore, osteoporotic fractures are a major health problem worldwide because of the associated morbidity[3-5] and mortality[6, 7]. Given the ageing of populations, osteoporotic vertebral fractures are likely to become an en even increasingly important health issue. The costs of osteoporotic vertebral fractures were estimated to be € 1.5 billion in Europe in 2010[8] and US$ 1.1 billion in the United States in 2005 and are expected to have increased by more than 50% by 2025[9].

Vertebral fractures may occur in absence of trauma or after only minimal trauma, such as bending, lifting or turning.[1] Clinically detected vertebral fractures only concern one third of radiographically detected vertebral fractures.[10] In other words, two thirds of vertebral fractures do not come to medical attention, nevertheless, these fractures are associated with back pain, functional limitations[4] and mortality[6] as well. These vertebral fractures can only be detected by screening imaging examinations. Vertebral fractures are often a first presentation of osteoporosis, therefore, accurate diagnosis is important to identify high risk patients to prevent future fractures. It has been shown that women with preexisting vertebral fractures have four times greater risk of subsequent vertebral fractures than those without prior fractures, with this risk increasing with the number of prior vertebral fractures.[11-13] Again, it is important to detect these fractures, since anti-osteoporotic therapy has been proven effective in reducing the risk of both non-vertebral and vertebral fractures.[14, 15]

However, the identification of a vertebral fracture on a radiograph is complicated. Several methods for radiological assessment of vertebral fractures exist, but a gold standard is lacking.[16] Quantitative morphometry (QM) based methods evaluate vertebral height loss by measuring the distance between points placed in the superior and inferior endplates at the anterior, middle and posterior aspects of the vertebral bodies. Next, ratios between these heights are calculated to classify vertebral fractures, using cutoff values based on standard deviation reductions from normal-population means derived from epidemiological studies. Alternatively, the Spine Analyzer® software package[17] employs Genant's classification[18] to define vertebral deformities. Finally, the algorithm based qualitative (ABQ) method by Jiang et al.[19] mainly judges endplate integrity, regardless of vertebral height reduction. The key assumption is that the endplate is always deformed in vertebral fractures, and therefore endplate depression has perfect specificity for vertebral fracture. In addition, vertebral height may appear to be decreased as a result of oblique image projection, certain diseases, and anatomical variants that can mimic vertebral fractures.[18-21] To deal with this misclassification, ABQ uses an algorithm to systematically rule out non-fracture deformities by examining certain radiological features.

The aim of our study was to analyze differences in prevalences and fracture location between methods. We applied two methods, i.e., ABQ and SpineAnalyzer® (SA) software-assisted QM, for assessing vertebral fractures in the population-based Rotterdam Study, an ongoing prospective cohort study in elderly persons.

METHODS

The Rotterdam Study

The Rotterdam Study is a prospective population-based cohort studying the determinants of chronic diseases and disability in Dutch men and women. Both the objectives and the study design have been described previously.[22] The study targets investigations on endocrine diseases like osteoporosis amongst

others. It includes 14,926 inhabitants aged 45 years and over of Rotterdam city's Ommoord district in The Netherlands. The present report describes results obtained from the Rotterdam Study-III cohort, which started follow-up in 2006. In short, a baseline home interview on medical history and risk factors for chronic diseases and medication use was taken by trained interviewers. Subsequently, participants were invited to the research center for clinical examination.

Vertebral fracture assessment

X-ray examinations of the spine were obtained by a digitized Fuji FCR system (FUJIFILM Medical Systems).We applied two methods for assessment of osteoporotic vertebral fractures on the radiographs. Two teams of trained research assistants assessed lateral spine radiographs (T4-L4), using either ABQ or software-assisted QM (SpineAnalyzer®, Optasia Medical Ltd, Cheadle, UK).

Each of the assistants applied only one of the two methods. With ABQ, radiographs were triaged as normal, uncertain or definite fracture, according to intactness of the endplates. Definite and uncertain vertebral fractures were re-assessed by a musculoskeletal radiologist. Additionally, endplate fracture localization was recorded (superior, inferior, or both endplates fractured). SpineAnalyzer® software can automatically identify vertebral shape to calculate the exact heights of the vertebrae. After labeling the vertebrae of interest by placing thirteen points at the center of each vertebral body from L4 to T4, SpineAnalyzer® will place six morphometry points around each labeled vertebra, corresponding to the four corners of the vertebral body and the middle. The analyst can make manual adjustments to these six morphometry points to fine-tune their exact locations for accurate measurements. The morphometry points are used to assess reductions in anterior, middle and posterior heights of the vertebrae. The SpineAnalyzer® software output provides a classification for deformities of shape (wedge, biconcave, crush) and severity (mild, moderate, severe) for deformities with a vertebral height reduction of at least 20% according to Genant's classification scheme for osteoporotic vertebral fractures[18].

Additional measurements

During the baseline visit height and weight were measured with indoor clothing and no shoes. Body mass index (BMI) was calculated as weight (in kg)/height (in m^2). Femoral neck and lumbar spine BMD were measured by dual-energy X-ray absorptiometry (DXA), using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA)[23]. BMD was measured directly from mineral mass distributions[24].

Statistical analysis

We compared fracture prevalences and distribution according to vertebral level for QM and ABQ. For QM, SpineAnalyzer® software calculates height reduction and corresponding ratios automatically. Height loss less than 20% is considered normal. Mild fracture (grade 1) is defined as height loss between ≥20% and <25%, moderate fracture (grade 2) between ≥25% and <40% and severe fracture (grade 3) ≥40%[18]. As some believe that most of the grade 1 or mild deformities are not osteoporotic vertebral fractures,[21, 25, 26] we performed secondary analyses by shifting the cut-off of 20% height loss to more conservative thresholds. The wedge ratio is calculated by dividing anterior height by posterior height (h_A/h_P). Biconcavity is calculated by dividing mid height by posterior height (h_M/h_P). The calculation of crush fractures makes use of adjacent vertebral heights, as described before[18]. Agreement between the diagnostic approaches for the identification of prevalent vertebral fractures was analyzed using kappa (κ) statistics. The κ value takes into account the proportion of agreement ascribable to chance alone and

can range from 0 (no agreement) to 1 (complete agreement); values greater than 0.8 are considered satisfactory and values lower than 0.6 poor. SPSS 20 and R software were used for the analyses.

RESULTS

Radiographs were assessed for 2,827 participants (43% men) aged 45-89 years (mean 57, Figure 1). With SA, a prevalence of 18.9% (95% CI: 17.4%–20.3%) was found, compared to 3.1% (95% CI: 2.5%–3.8%) with ABQ. Of all individuals, 80.0% (N=2,261) were identified as having no fractures, while 1.9% (N=55) were labeled as cases according to both methods. 16.9% (N=478) were assessed as having deformities according to SA but not ABQ; 1.2% (N=33) were judged fractured according to ABQ but not SA. The concordance between ABQ and SA was poor (κ=0.13).

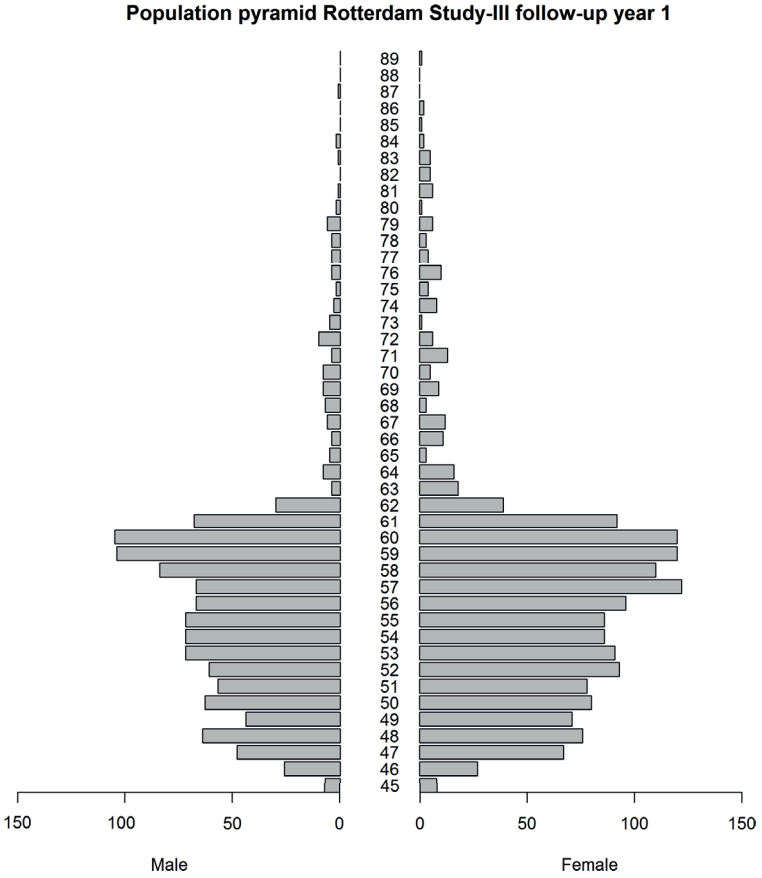

Figure 1. Age at baseline distribution within the Rotterdam Study-III cohort population, stratified by gender.

Fracture distribution according to vertebral levels and shape

In Figure 2, the distribution of osteoporotic vertebral fractures per vertebral level assessed according to ABQ and SA is illustrated. With ABQ, most fractures were found at the thoraco-lumbar junction, lumbar

(T11-L3) and mid-thoracic (T7-T9) regions (illustrated separately in Supplementary Figure 1). With SA, most deformities were at the middle (T7,T8) and lower thoracic regions (T11,T12) (illustrated separately in Supplementary Figure 2). The frequencies for SA deformities' classification of severity was 70.5% mild, 27.3% moderate, 2.3% severe; shape of deformity was 95.2% wedge, 2.5% biconcave and 2.3% crush (Table 1, Supplementary Figures 3 and 4). Most ABQ fractures concerned the superior endplate (58.3% of all ABQ fractures), 29.6% were at the inferior endplates and 12.2% were represented by biconcave fractures. The superior endplate was more frequently affected at the thoraco-lumbar junction and lumbar regions (T11-L3), while more inferior endplate fractures were seen at the mid-thoracic spine (T7-T8) (Figure 3).

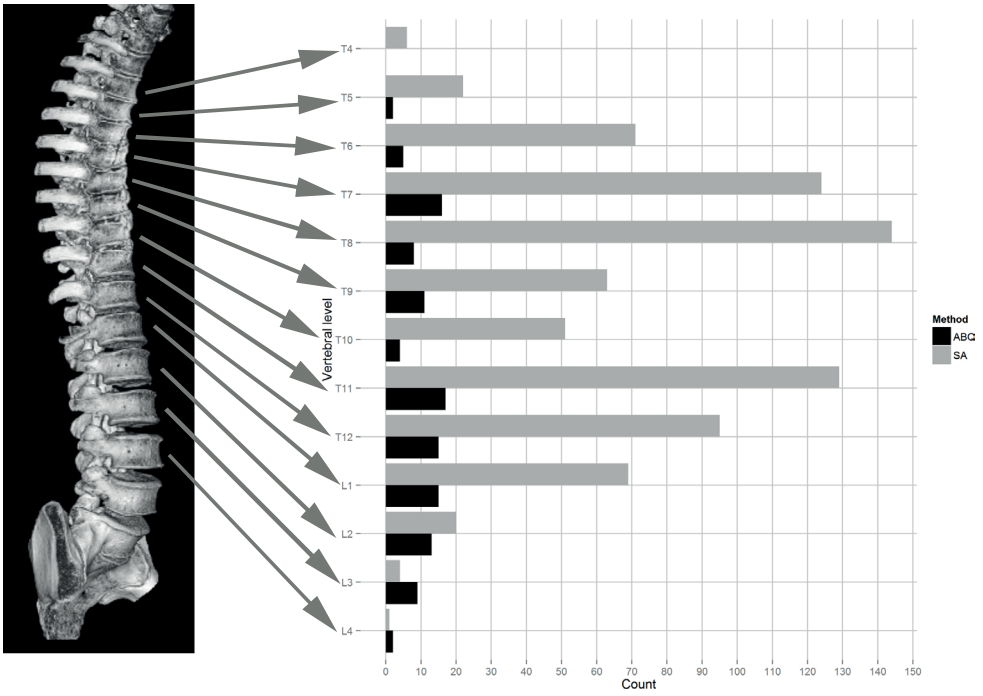

Figure 2. Distribution of osteoporotic vertebral fractures per vertebral level assessed according to the algorithm-based qualitative (ABQ) method and SpineAnalyzer® (SA) software-assisted quantitative morphometry.

Table 1. Frequencies of severity and shape of deformities assessed according to SpineAnalyzer® (SA) software-assisted quantitative morphometry.

		Shape			
		Wedge	Biconcave	Crush	**Total**
Severity	20-25%	541	14	8	**70.5%**
	25-40%	205	5	8	**27.3%**
	>40%	15	1	2	**2.3%**
	Total	**95.2%**	**2.5%**	**2.3%**	799

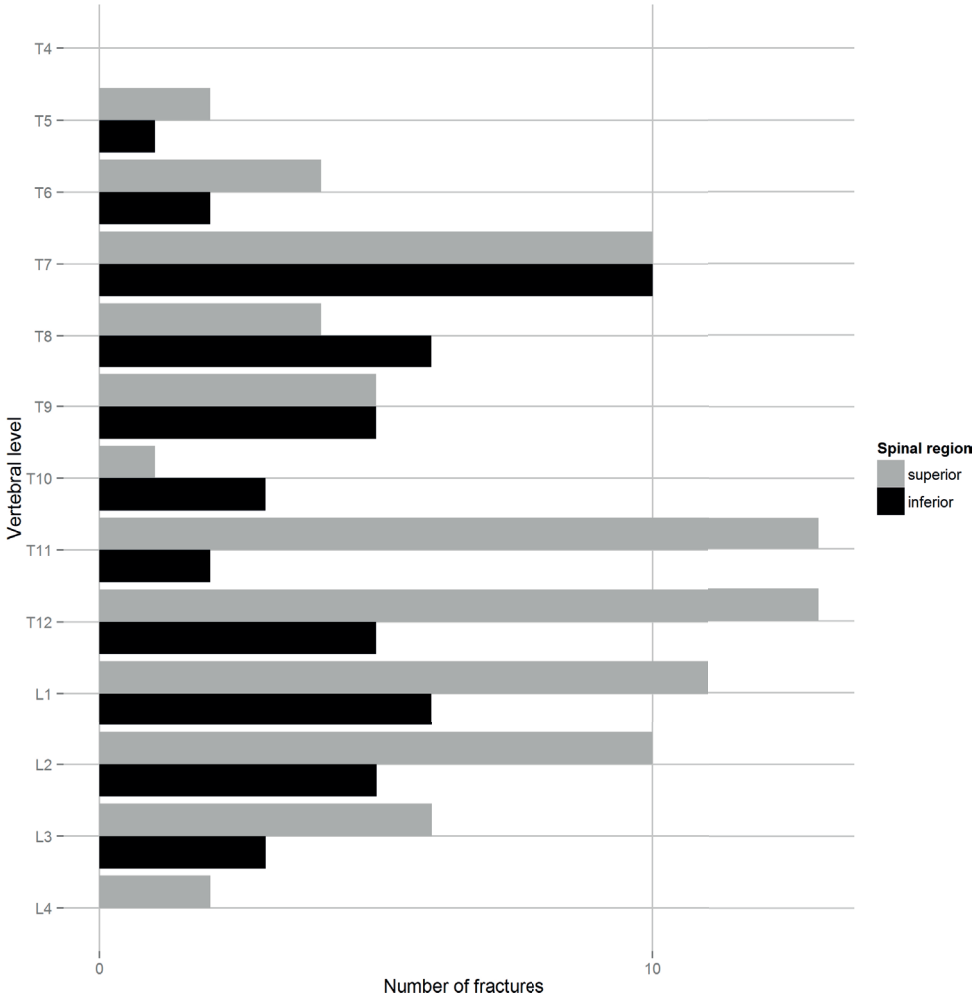

Figure 3. Distribution of osteoporotic vertebral fractures per vertebral level by affected superior and/or inferior endplates, assessed according to the algorithm-based qualitative (ABQ) method.

Varying the cut-off for vertebral height loss

As some believe that most of the grade 1 or mild deformities are not true osteoporotic vertebral fractures[21, 25, 26], we tried to shift the cut-off of 20% height loss to more conservative thresholds (Table 2). Indeed we see an increase in the net agreement between methods, mostly because the deformities with height loss but intact endplates are now regarded as normal; but at the same time there is a decrease in concordance for the fractures positive for the ABQ criteria. Taken altogether, the κ statistic was optimal at a threshold of 30%. The fracture prevalence according to height cut-offs of 25% and 40%, respectively, is displayed by vertebral level distribution in Figure 4.

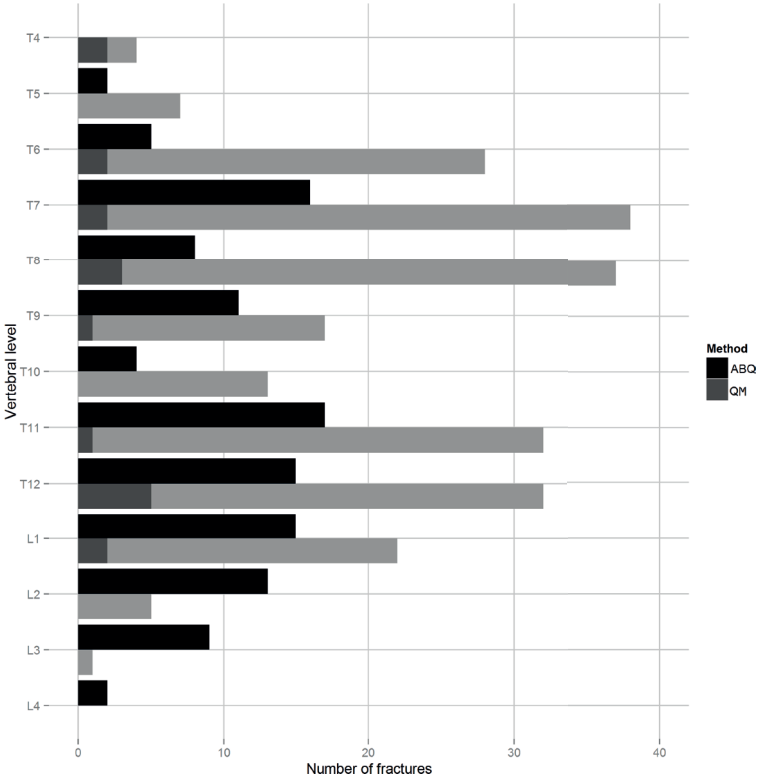

Figure 4. Distribution of osteoporotic vertebral fractures per vertebral level assessed with the algorithm-based qualitative (ABQ) method and SpineAnalyzer® (SA) software-assisted quantitative morphometry applying different cut-offs for vertebral height loss to classify fractures. In SpineAnalyzer® (SA) software-assisted quantitative morphometry readings, moderate fracture (grade 2) is defined as height loss between ≥20% and <25% and severe fracture (grade 3) as height loss ≥40%.

DISCUSSION

Osteoporotic vertebral fractures prevalence rates are significantly different when applying SpineAnalyzer® software-assisted QM or ABQ, because they each classify a considerable number of deformities that were assessed as normal by the other, resulting in poor between-method agreement statistics. In the current Rotterdam Study III cohort, we found that the prevalence of osteoporotic vertebral fractures according to QM was almost seven times higher than for ABQ in the same dataset (18.9% versus 3.1%). Agreement statistics improved when applying more lenient vertebral height cut-offs for QM, but this is most probably at the expense of sensitivity. The optimal agreement statistic was observed with a cut-off of 30%, however, this agreement would still be considered poor. The fracture distribution over the vertebral column was bimodal for both methods in our cohort, with maxima at the mid-thoracic and lower thoracic regions with the thoraco-lumbar junction, where ABQ had a more evenly spread distribution covering the middle lumbar regions in addition. Most ABQ fractures concerned the superior endplate and this type of fracture occurred more frequently in the lower spinal column, while in the higher spinal levels fractures were more equally distributed among the superior and inferior endplates. When assessing QM morphometry, the far majority of deformities were classified as mild wedges.

Table 2. Concordance and discordance between SpineAnalyzer® (SA) software-assisted quantitative morphometry and algorithm-based qualitative (ABQ) methods readings of the same cohort on participant level.

	Concordant		Discordant		
	ABQ + / SA +	ABQ - / SA -	ABQ + / SA -	ABQ - / SA +	Kappa
Cut-off[a]					
20%	55 (1.9%)	2,261 (80.0%)	33 (1.2%)	478 (16.9%)	0.13
25%	41 (1.5%)	2,587 (91.5%)	47 (1.7%)	152 (5.4%)	0.26
30%	30 (1.1%)	2,702 (95.6%)	58 (2.1%)	37 (1.3%)	0.37
35%	20 (0.7%)	2,728 (96.5%)	68 (2.4%)	11 (0.4%)	0.33
40%	12 (0.4%)	2,734 (96.7%)	76 (2.7%)	5 (0.2%)	0.22

[a]Different vertebral height loss cut-offs for SpineAnalyzer® software-assisted quantitative morphometry (QM) were applied and compared to the algorithm-based qualitative (ABQ) method reading.

Our study is the first to compare SpineAnalyzer® software-assisted QM and ABQ. Our vertebral fracture prevalence estimate for the ABQ method seems comparable to previous findings in similar populations, taking into account that we investigated a relatively younger population including both men and women.[19, 27-29] To our knowledge, our study is the first (population-based) prevalence study conducted with SpineAnalyzer® software-assisted QM. We have assessed vertebral levels T4 to L4 as these are the most common sites for osteoporotic fractures. The bimodal fracture distribution over the vertebral column was obvious for both methods in our cohort, with maxima at the mid-thoracic and lower thoracic regions including the thoraco-lumbar junction. This dispersal is conform previous observations with other assessment methods.[30, 31] However, some argue that the more pronounced mid-thoracic peak with QM is to a great extent due to normal anatomical variation (i.e., short vertebral height) and old traumatic fractures,[32] which on the contrary ABQ would be able to discriminate.[20, 21, 33] Ferguson et al. hypothesized an increased fracture risk in the aging spine for the superior endplate because of the observation from biomechanical studies that the superior endplate is generally thinner than the inferior endplate and that sacral and inferior lumbar endplates are stronger than superior lumbar endplates,[34] which we now establish by the discovery that most ABQ fractures concerned the superior endplate in our study.

Several comparative studies putting other combinations of assessment methods side by side have been reported before, but only a few have specifically evaluated SpineAnalyzer® software or ABQ. SpineAnalyzer® software-assisted QM reading by a non-radiologist has been found to agree pretty well with conventional SQ grading performed by experienced radiologists, finding a kappa for agreement of 0.78.[35] ABQ has been compared to QM (Eastell-Melton and McCloskey definitions) finding kappa statistics between 0.39 and 0.64.[19] Most notably, the lowest agreement found to date is between ABQ and Genant's SQ methods, observing kappa statistics of 0.30 to 0.58.[21] The between-method agreement statistics we found are even lower than these evaluative studies. SpineAnalyzer® software-assisted QM and ABQ seem extreme poles apart. ABQ primarily regards endplate depression regardless of vertebral height loss, which is the sole criterion for QM-based fractures. From previous experience we could indeed expect low agreement between ABQ and any method applying Genant's criteria,[21] possibly on top of that, more discordance originates from the lack of non-fracture deformity triage when solely applying SpineAnalyzer® software. This could have been further amplified because we have examined a relatively young and generally healthy population where we might expect more of these (mild) vertebral non-fracture deformities.

Nonetheless, it should be noted that agreement statistics concern precision of a study and may not necessarily relate to its validity. QM would not diagnose vertebral fractures in the case of endplate depression without reduced vertebral height, and conversely, ABQ would not diagnose a QM-based vertebral deformity with reduced height but intact endplates. More research is needed to clarify which of these discordant cases are clinically relevant and which are not at all and should for that reason be regarded as false-positives.

Our aim is to objectively compare radiological assessment methods for osteoporotic vertebral fractures. Strengths of our study are that we systematically applied two very different assessment methods by two independent teams of trained readers in a very large population-based setting. This proved to be very labor-intensive and on top of that, extra consensus meetings, supervision by musculoskeletal radiologists and double readings were performed. Although radiographs were assessed by well-trained reader teams, it was not feasible to have all radiographs assessed by musculoskeletal radiologists. We are aware that more subtle fractures could have been missed. As the Rotterdam Study is deemed representative of the general Dutch middle-aged to elderly population, we believe that our results may be extrapolated to other settings as well.

The semi-automated SpineAnalyzer® software-assisted QM method proved to be an excellent recording tool for research purposes, providing a standardized data output.[36] Surprisingly, ABQ was in our experience even more time-efficient, but this method requires more intensive initial training. Quantitative assessment is based on morphometry alone, which may result in inclusion of deformities into the phenotype definition that are not truly vertebral fractures, reason why one may choose to put the term "deformities" instead of "fractures" for cases defined by QM. Yet, we experienced that further triage for both methods requires a lot of extra effort involving extra double-reading of up to thousands of participants. Further standardization and automation of this triage procedure with clear-cut classification criteria would be very helpful.

Vertebral fractures are often a first presentation of osteoporosis and should be regarded as an opportunity to trace individuals at high risk of more future fractures and other related adverse health outcomes. Accurate vertebral fracture diagnosis is needed to identify these patients with high risk for future fractures to optimize patient management, as many effective treatment options are available.[14, 15]. Contrariwise, at times individuals without true vertebral fractures are unnecessarily treated with medication (e.g., bisphosphonates), associated with high costs and potential adverse effects.[37] Improvement of radiological vertebral fracture definition, clearer criteria for non-fracture deformities differential diagnosis[38] and more wide-spread and consistent application of an optimal method may improve clinical care.

In the future, our work can be extended to projects of more epidemiological nature as thousands of health measurements are available on the Rotterdam Study cohorts which can be correlated to the vertebral fracture assessments reported here.[22] Precise and accurate ascertainment of osteoporotic vertebral fractures seems necessary to optimize the yield of such association studies.[39] We have undertaken meticulous phenotyping, so with some extra efforts we could expand on our ABQ differential diagnoses and SpineAnalyzer® morphometric raw data. For example, different cut-offs and vertebral fracture definitions could be linked to various clinically relevant outcomes. Furthermore, the remaining Rotterdam Study cohorts, which in total will yield ~11,000 subjects aged 45 years and over, will be assessed for the presence of osteoporotic vertebral fractures. In addition, our measurements could serve as population reference data.

In conclusion, we procured an impartial comparison of osteoporotic vertebral fracture assessment methods in the large population-based Rotterdam Study, with extensive recording of distribution ac-

cording to vertebral shape and severity. Osteoporotic vertebral fractures prevalence rates are significantly different when applying either software-assisted QM or ABQ. Both QM and ABQ identify a considerable number of deformities that were assessed as normal by the other. Further work is needed to reveal which of the discordant cases are actually clinically relevant and which are not.

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Supplementary information is available at:
http://www.glimdna.org/publicationdata.html

REFERENCES

1. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I:1-65.
2. Cooper C. Epidemiology and public health impact of osteoporosis. Baillieres Clin Rheumatol. 1993 Oct;7(3):459-77.
3. Burger H, Van Daele PL, Grashuis K, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities and functional impairment in men and women. J Bone Miner Res. 1997 Jan;12(1):152-7.
4. Nevitt MC, Ettinger B, Black DM, Stone K, Jamal SA, Ensrud K, et al. The association of radiographically detected vertebral fractures with back pain and function: a prospective study. Ann Intern Med. 1998 May 15;128(10):793-800.
5. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C, et al. Health-related quality of life in post-menopausal women with low BMD with or without prevalent vertebral fractures. J Bone Miner Res. 2000 Jul;15(7):1384-92.
6. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
7. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. J Am Geriatr Soc. 2000 Mar;48(3):241-9.
8. Ström O, Borgström F, Kanis JA, Compston J, Cooper C, McCloskey EV, et al. Osteoporosis: burden, health care provision and opportunities in the EU. Arch Osteoporos. 2011;DOI 10.1007/s11657-011-0060-1.
9. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. 2007 Mar;22(3):465-75.
10. Cooper C, Shah S, Hand DJ, Adams J, Compston J, Davie M, et al. Screening for vertebral osteoporosis using individual risk factors. The Multicentre Vertebral Fracture Study Group. Osteoporos Int. 1991 Oct;2(1):48-53.

11. Klotzbuecher CM, Ross PD, Landsman PB, Abbott TA, 3rd, Berger M. Patients with prior fractures have an increased risk of future fractures: a summary of the literature and statistical synthesis. J Bone Miner Res. 2000 Apr;15(4):721-39.
12. Burger H, van Daele PL, Algra D, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities as predictors of non-vertebral fractures. BMJ. 1994 Oct 15;309(6960):991-2.
13. Black DM, Arden NK, Palermo L, Pearson J, Cummings SR. Prevalent vertebral deformities predict hip fractures and new vertebral deformities but not wrist fractures. Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1999 May;14(5):821-8.
14. Chen JS, Sambrook PN. Antiresorptive therapies for osteoporosis: a clinical overview. Nat Rev Endocrinol. 2011;8(2):81-91.
15. Lewiecki EM, Laster AJ. Clinical review: Clinical applications of vertebral fracture assessment by dual-energy x-ray absorptiometry. J Clin Endocrinol Metab. 2006 Nov;91(11):4215-22.
16. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, Hofman A, et al. Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. Eur Radiol. 2013 Feb;23(2):476-86.
17. Brett A, Miller CG, Hayes CW, Krasnow J, Ozanian T, Abrams K, et al. Development of a clinical workflow tool to enhance the detection of vertebral fractures: accuracy and precision evaluation. Spine (Phila Pa 1976). 2009 Oct 15;34(22):2437-43.
18. Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res. 1993 Sep;8(9):1137-48.
19. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
20. Ferrar L, Jiang G, Adams J, Eastell R. Identification of vertebral fractures: an update. Osteoporos Int. 2005 Jul;16(7):717-28.
21. Ferrar L, Jiang G, Cawthon PM, San Valentin R, Fullman R, Lambert L, et al. Identification of vertebral fracture and non-osteoporotic short vertebral height in men: the MrOS study. J Bone Miner Res. 2007 Sep;22(9):1434-41.
22. Hofman A, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, et al. The Rotterdam Study: 2014 objectives and design update. Eur J Epidemiol. 2013 Nov;28(11):889-926.
23. Burger H, de Laet CE, van Daele PL, Weel AE, Witteman JC, Hofman A, et al. Risk factors for increased bone loss in an elderly population: the Rotterdam Study. Am J Epidemiol. 1998 May 1;147(9):871-9.
24. Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW. Structural trends in the aging femoral neck and proximal shaft: analysis of the Third National Health and Nutrition Examination Survey dual-energy X-ray absorptiometry data. J Bone Miner Res. 2000 Dec;15(12):2297-304.
25. Vokes TJ, Gillen DL, Pham AT, Lovett JM. Risk factors for prevalent vertebral fractures in black and white female densitometry patients. J Clin Densitom. 2007 Jan-Mar;10(1):1-9.
26. Ziegler R, Scheidt-Nave C, Leidig-Bruckner G. What is a vertebral fracture? Bone. 1996 Mar;18(3 Suppl):169S-77S.
27. Ferrar L, Jiang G, Clowes JA, Peel NF, Eastell R. Comparison of densitometric and radiographic vertebral fracture assessment using the algorithm-based qualitative (ABQ) method in postmenopausal women at low and high risk of fracture. J Bone Miner Res. 2008 Jan;23(1):103-11.
28. Ferrar L, Roux C, Felsenberg D, Gluer CC, Eastell R. Association between incident and baseline vertebral fractures in European women: vertebral fracture assessment in the Osteoporosis and Ultrasound Study (OPUS). Osteoporos Int. 2012 Jan;23(1):59-65.
29. Finigan J, Greenfield DM, Blumsohn A, Hannon RA, Peel NF, Jiang G, et al. Risk factors for vertebral and nonvertebral fracture over 10 years: a population-based study in women. J Bone Miner Res. 2008 Jan;23(1):75-85.
30. Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2002 Jun;17(6):1051-6.
31. Suzuki N, Ogikubo O, Hansson T. The prognosis for pain, disability, activities of daily living and quality of life after an acute osteoporotic vertebral body fracture: its relation to fracture level, type of fracture and grade of fracture deformation. Eur Spine J. 2009 Jan;18(1):77-88.

32. Adams JE, Lenchik L, Roux C, Genant HK. Radiological assessment of vertebral fracture. Vertebral Fracture Initiative Resource Document. 2011;PART II:1-48.
33. Jiang G, Luo J, Pollintine P, Dolan P, Adams MA, Eastell R. Vertebral fractures in the elderly may not always be "osteoporotic". Bone. 2010 Jul;47(1):111-6.
34. Ferguson SJ, Steffen T. Biomechanics of the aging spine. Eur Spine J. 2003 Oct;12 Suppl 2:S97-S103.
35. Kim YM, Demissie S, Genant HK, Cheng X, Yu W, Samelson EJ, et al. Identification of prevalent vertebral fractures using CT lateral scout views: a comparison of semi-automated quantitative vertebral morphometry and radiologist semi-quantitative grading. Osteoporos Int. 2012 Mar;23(3):1007-16.
36. Oei L, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJ, et al. Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses. Quant Imaging Med Surg. 2013 Oct;3(5):249-55.
37. Breda SJ, Oei L, Oei EH, Zillikens MC. [Osteoporotic vertebral fractures or Scheuermann's disease?]. Ned Tijdschr Geneeskd. 2013;157(45):A6479.
38. Makurthou AA, Oei L, Saddy SE, Breda SJ, Castano-Betancourt MC, Hofman A, et al. Scheuermann's Disease: Evaluation of Radiological Criteria and Population Prevalence. Spine (Phila Pa 1976). 2013 Jun 11.
39. Oei L, Estrada K, Duncan EL, Christiansen C, Liu C-T, Langdahl BL, et al. Genome-wide association study for radiographic vertebral fractures: A potential role for the 16q24 BMD locus. Bone. 2014;59(0):20-7.

Chapter 3.3

Prediction of vertebral fracture by trabecular bone score in elderly women of the Rotterdam Study

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In preparation

ABSTRACT

Vertebral fractures are a recognized hallmark of osteoporosis. Trabecular bone score (TBS) is a new technique applied to dual-energy X-ray absorptiometry (DXA) images of the lumbar spine used to assess vertebral microarchitecture. We aimed to examine the association of TBS and risk of prevalent and incident vertebral fractures in a large population based study of Dutch elderly women. We included a selection of 3,128 women from the three Rotterdam Study cohorts (RS-I, RS-II RS-III) to evaluate the association of TBS and vertebral fractures. Cross-sectional radiographic assessments and DXA scans (GE-Lunar Prodigy, Madison) of the lumbar spine were available for RS-I (N=258 cases, 1,152 controls), RS-II (62 cases, 314 controls) and RS-III (N=223 cases, 1,119 controls), while incident clinical vertebral fracture information was available for RS-I (N=12 cases, 950 controls) and RS-II (N=9 cases, 471controls). TBS was derived using the TBS iNsight software (Medimaps, Pessac). Logistic- and Cox- regression models corrected for age, height and weight were used to obtain risk estimates per SD decrease in TBS for prevalent and incident vertebral fractures, respectively. Overall lower TBS scores were significantly associated with 30% increased risk for prevalent vertebral fractures per SD decrease in TBS in the pooled analysis (OR 1.30 95% CI: [1.16–1.45; P<0.001) of the RS-I (OR 1.44 95% CI: [1.24–1.67]; P=$1.17x10^{-6}$), RS-II (OR 1.13 95% CI: [0.83–1.55]; P=0.438) and RS-III (OR 1.25 95% CI: [1.07–1.47]; P=0.006) cohorts. The combined analysis of incident vertebral fractures in RS-I and RS-II did not show a significant association (HR 1.40 95% CI: [0.90–2.20]; P=0.139). Adjustment for covariates including lumbar spine BMD did not modify the risk estimates. In conclusion, trabecular bone scores are strongly associated with prevalent vertebral fractures in women independent of BMD.

INTRODUCTION

Vertebral fractures are known to be the most common osteoporotic fractures .[1] Of all osteoporotic fractures, vertebral fractures are the most common type.[2] Osteoporotic vertebral fractures are a major health problem worldwide because of the associated morbidity,[3-5] disability (such as back pain and functional limitations in mobility) and mortality[6, 7]. Given the ageing of populations, these fractures are likely to become an even increasingly important health issue. The costs of osteoporotic vertebral fractures were estimated to be € 1.5 billion in Europe in 2010[8] and US$ 1.1 billion in the United States in 2005 and are expected to increase by more than 50% by 2025[9].

Early detection and, ideally, prediction of future fractures is important for patients, since anti-osteoporotic therapy has been proven effective in reducing the risk of both non-vertebral and vertebral fractures.[10, 11] In clinical practice dual-energy X-ray absorptiometry (DXA) is used to diagnose osteoporosis, providing accurate estimates of bone mass through the evaluation of bone mineral density (BMD). However, BMD is not always an accurate predictor of fracture.[12] Over 50% of fractures occur in individuals whose BMD is above the osteoporosis threshold of a T score of -2.5.[13] One reason may be that bone mass is not the only contributor to bone strength; BMD provides an assessment of the quantity of bone but does not provide information on bone quality, another important parameter underlying fracture susceptibility. Consequently, evaluating other bone parameters, such as bone microarchitecture, could significantly enhance the assessment of bone strength and fracture risk.[14]

The trabecular bone score (TBS) is a new type of measurement that can be applied to DXA images.[15] It is a measure of bone texture, which correlates with 3D parameters of bone microarchitecture. TBS is based on the measurement of the experimental variogram derived from a gray-level DXA image. TBS can reflect the structural condition of the bone microarchitecture as it is strongly positively correlated with the number and connectivity of trabeculae, and negatively correlated with the space between trabeculae.[14] A high TBS value means that bone microarchitecture is dense, well-connected and with little spaces between trabeculae. Conversely, a low TBS value means that the microarchitecture of bone is incomplete and poorly connected with wide spaces between trabeculae.[16] Bousson et al. showed that this new index emphasizes the failure of the BMD T score to fully capture the fragility fracture risk, but still, there is no sufficient evidence that a TBS measurement alone provides reliable information on the status of the bone microarchitecture for a given patient.[17] Therefore, additional studies will have to be performed to assess the advantages and limitations of the TBS. It has also been demonstrated that TBS is relevant for the assessment of secondary osteoporosis.[15, 18-22] Finally, there is some indication that spine TBS can predict major osteoporotic fractures independent of spine BMD. Combining the TBS microarchitecture index with BMD from conventional DXA has been suggested to improve fracture prediction[16] reason why a large prospective meta-analysis is underway.

The aim of our study was to examine the association of TBS with risk for vertebral fracture in women from the population-based Rotterdam Study, an ongoing prospective cohort study in Dutch elderly individuals. We analyzed prevalent X-ray assessed vertebral fractures and incident clinical vertebral fractures occurring during follow-up.

METHODS

The Rotterdam Study

The Rotterdam Study is a prospective population-based cohort studying the determinants of chronic diseases and disability in Dutch elderly. Both the objectives and the study design have been described

previously.[23] The study targets investigations on endocrine diseases like osteoporosis amongst others. Inhabitants aged 45 years and over of the Rotterdam city's Ommoord district in The Netherlands were invited to participate. Trained interviewers performed a baseline home interview on medical history and risk factors for chronic diseases and medication use. Subsequently, participants were invited to the research center for clinical examination. Our study sample comprised 3,128 women with DXA scans and vertebral fracture assessments. We only studied women, as at the time of measurement the TBS assessments were not optimized for use in men. During the baseline visit height and weight were measured with indoor clothing and no shoes. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m^2).

DXA measurements of BMD and TBS

Lumbar spine BMD was measured by DXA, using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA)[24]. BMD was measured directly from mineral mass distributions[25]. Additionally, TBS was measured in all participants with the DXA scans at the Bone Disease Unit at the University of Lausanne (Lausanne, Switzerland) blinded to clinical parameters and outcomes using the TBS iNsight software (version 1.9; Medimaps, Geneva, Switzerland). TBS was evaluated by determining the variogram of the trabecular bone projected image, calculated as the sum of the squared gray-level differences between pixels at a specific distance and angle. TBS was then calculated as the slope of the log-log transform of this variogram. The mean value of the individual measurements for L1–L4 represents the lumbar spine TBS (unit less).[14] TBS scores were not calibrated and were analyzed as standardized residuals (mean=0, SD=1) adjusted for age, height and weight. Subjects with BMI>35 kg/m^2 were not included in the study because TBS measurements in morbidly obese persons are not accurate.

Vertebral fracture assessment

Radiographic images of the spine were acquired using a digitized radiography system (Fuji FCR, FUJIFILM Medical Systems). We applied two methods for assessment of osteoporotic vertebral fractures on the radiographs. Trained research assistants assessed lateral spine radiographs (T4-L4) by means of software-assisted quantitative morphometry (QM) (SpineAnalyzer®, Optasia Medical Ltd, Cheadle, UK). This software can automatically identify vertebral body contour and shape to calculate the heights of the vertebrae. After labeling the vertebrae of interest by placing points at the center of each vertebral body from T4 to L4, SpineAnalyzer® will place six morphometry points around each labeled vertebra, corresponding to the four corners of the vertebral body and the middle. The analyst can make manual adjustments to these six morphometry points to fine-tune their exact locations. The morphometry points are then used to assess reductions in anterior, middle and posterior heights of the vertebrae. The SpineAnalyzer® software output provides a classification for deformities of shape (wedge, biconcave, crush) and severity (mild, moderate, severe). Deformities with a vertebral height reduction less than 20% are considered normal and vertebral fractures were defined as height loss ≥20% according to Genant's classification scheme for osteoporotic vertebral fractures[26]. A random sample and uncertain vertebral fractures of in total approximately 10% were re-assessed by a musculoskeletal radiologist. Fracture events occurring during follow-up were obtained from computerized records of the general practitioners and hospital registries, which were regularly checked by research physicians who reviewed and coded the fracture information.[13] Subjects were followed from their baseline visit until 1 January 2007 or until a first vertebral fracture or death occurred.

Statistical analysis

Logistic- and Cox- regression models corrected for age, height and weight were used to obtain risk estimates per SD decrease in TBS for prevalent and incident vertebral fractures, respectively. The area under the curve for prevalent and incident vertebral fractures was determined. For this purposes we used SPSS software (IBM SPSS Statistics 20).

RESULTS

The characteristics of participants of the Rotterdam Study cohort included in the analysis of TBS and risk for vertebral fractures are shown in Table 1 by fracture case status. Both TBS and BMD mean levels were significantly lower in fracture cases than non-cases (P<0.001). The Pearson correlation between mean spine TBS and mean spine BMD was between 0.25 and 0.30 across studies (Figure 1).

Table 1. Characteristics of the participants with prevalent radiographic fracture assessments in the Rotterdam Study cohorts.

	Prevalent radiographic vertebral fractures						TOTAL	
	Rotterdam Study I (RSI-4)		Rotterdam Study II (RS II-2)		Rotterdam Study III (RS III-1)		Rotterdam Study I + II + III	
	Controls (N=1,152)	Cases (N=258)	Controls (N=314)	Cases (N=62)	Controls (N=1,119)	Cases (N=223)	Controls (N=2,669)	Cases (N=556)
Age	74.65 (5.86)	77.54 (5.95)	66.64 (5.91)	71.48 (7.81)	56.63 (6.55)	58.78 (7.72)	65.59 (10.49)	68.94 (11.27)
Height	160.89 (6.24)	159.37 (6.47)	161.85 (5.95)	159.81 (6.49)	164.20 (6.27)	164.40 (6.07)	162.49 (6.41)	161.54 (6.75)
Weight	70.22 (10.66)	66.80 (10.52)	70.66 (10.39)	70.44 (10.21)	71.16 (10.86)	72.35 (11.59)	70.69 (10.72)	69.55 (11.24)
TBS_new	1.20 (0.11)	1.17 (0.12)	1.25 (0.11)	1.22 (0.12)	1.29 (0.11)	1.25 (0.13)	1.25 (0.12)	1.21 (0.13)
TBS_old	1.20 (0.11)	1.16 (0.12)	1.25 (0.11)	1.22 (0.12)	1.29 (0.11)	1.25 (0.13)	1.25 (0.12)	1.21 (0.13)
BMD	1.05 (0.19)	0.99 (0.18)	1.07 (0.17)	1.06 (0.18)	1.17 (0.18)	1.14 (0.19)	1.11 (0.19)	1.06 (0.20)

Mean (SD). BMD refers to mean lumbar spine bone mineral density (L1-L4). TBS refers to mean spine trabecular bone score (L1-L4). TBS_old denotes the trabecular bone score without and TBS_new with exclusion of fractured vertebral bodies.

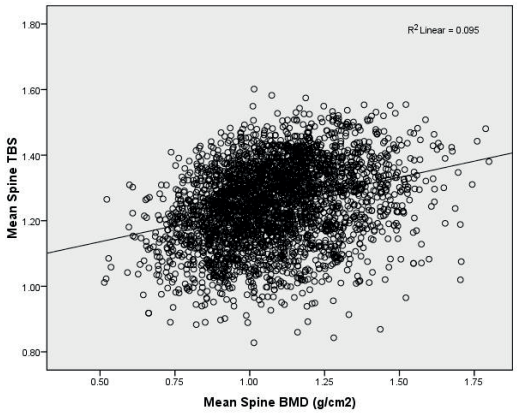

Figure 1. Scatter plot of mean spine TBS and mean spine BMD in RS cohorts.

Radiographs were assessed for 3,128 participants of whom 543 were diagnosed with fracture resulting in a vertebral fracture prevalence of 17.4%. Lower TBS scores were associated with increased odds for prevalent vertebral fractures in RS-I (OR 1.44 95% CI: [1.24–1.67]; P=1.17x10^{-6}), RS-II (OR 1.13 95% CI: [0.83–1.55]; P=0.438) and RS-III (OR 1.25 95% CI: [1.07–1.47]; P=0.006) per SD decrease. Incident clinical vertebral fractures occurring during follow-up (mean 3.03, SD 0.11 years) for RS-I and RS-II participants were available (combined sample size=1,442, 21 events). The combined analysis of incident vertebral fractures in RS-I and RS-II was not significant (HR 1.40 95% CI: [0.90–2.20]; P=0.139). Additional adjustment for lumbar spine BMD did not essentially change these risk estimates. Similarly, the sensitivity analyses where in case of the occurrence of a fracture at L1-L4 the TBS was calculated excluding the level of the fractured vertebral body yielded essentially no different results. The areas under the ROC curves were calculated for prevalent vertebral fractures in models including age, height and weight (area=0.59, 95% CI: 0.56–0.62). Inclusion of LS-BMD and TBS independently in the model resulted in areas under the ROC curve of 0.60 (95% CI: 0.57–0.62) and 0.61 (95% CI: 0.59–0.64), respectively. The area under ROC curve after including both LS-BMD and TBS was 0.61 (95% CI: 0.58–0.64) (Figure 2A). For incident vertebral fractures, areas under the curves of the initial model were 0.65 (95% CI: 0.54–0.77); 0.66 (95% CI: 0.55–0.76) when including LS-BMD; 0.67 (95% CI: 0.56–0.79) including TBS and 0.67 (95% CI: 0.56–0.79) after inclusion of both LS-BMD and TBS (Figure 2B).

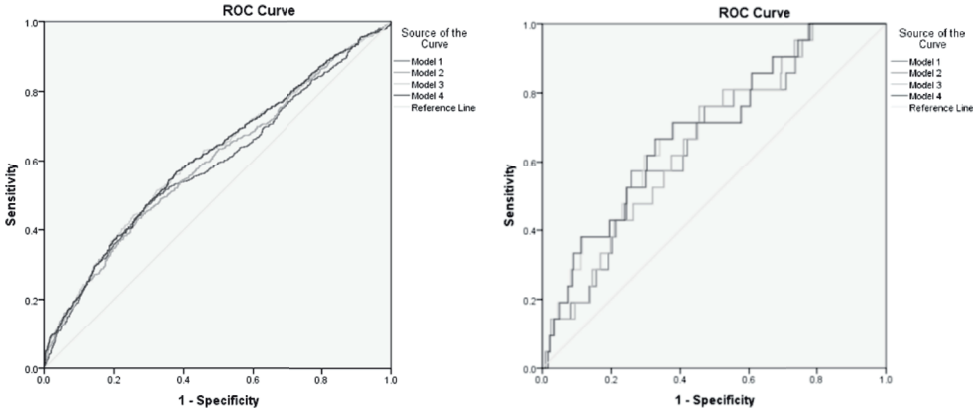

Figure 2. Receiver operating characteristic (ROC) curve for **(A)** prevalent vertebral fractures and **(B)** incident vertebral fractures. Adjustments for: Model 1: age, height, weight; Model 2: bone mineral density, age, height, weight; Model 3: trabecular bone score, age, height, weight; Model 4: trabecular bone score, bone mineral density, age, height, weight.

DISCUSSION

This is to our knowledge the first study examining the trabecular bone score (TBS) in relation to vertebral fracture risk cross-sectionally and prospectively across multiple cohorts. Our research revealed that decreases in TBS are strongly and significantly associated with increased risk of vertebral fractures. Every SD decrease in TBS was significantly associated with an increase of ~30% in risk of prevalent vertebral fractures, but not significantly associated with increased risk for incident vertebral fractures. Furthermore, the TBS associations with vertebral fractures are independent of DXA-based lumbar spine BMD and their combination negligibly improves risk prediction. TBS seems a promising quantitative imaging parameter and might find its way to fracture risk prediction and clinical care, given the fact that it can be easily derived from DXA scans.

TBS was introduced in 2008 as a measure of bone texture that correlates with bone microarchitecture parameters, such as bone volume fraction and mean bone thickness, and together or independent of BMD will better predict fracture risk.[27] It has been shown that the trabecular number (TbN) decreases in the lumbar spine with age (−3.6 % per decade between 30 and 90 years old) and that the trabecular separation (TbSp) increases by 4.9 % per decade for all regions of lumbar spine.[28] Importantly, TBS can differ between two 3D microarchitectures that exhibit the same amount of bone, but different trabecular characteristics.[14, 27] A high correlation has been observed between TBS from DXA and connectivity density (CD) from µCT, with TBS explaining about 67.2% of the variance in connectivity density.[14] In our studies, the correlation between TBS and LS-BMD was relatively low, similar to the results described before.[29, 30] Our results showed that TBS, as evaluated at the spine L1-L4 by DXA, can discriminate subjects with prevalent vertebral fractures from those without, independently of the BMD at L1–L4. We also showed that BMD and TBS measured at the lumbar spine have similar discrimination ability for the prediction of prevalent vertebral fractures, with both TBS and LS-BMD predicting slightly better than LS-BMD alone. For prediction of incident vertebral fractures, TBS had better discrimination ability than LS-BMD, and was similar to TBS and LS-BMD together. These results are in line with few studies showing that lower LS-TBS values were predictive of all types of fragility fractures with an effect of similar magnitude as that reported for LS-BMD. Yet, some of these studies showed that the addition of TBS to age and lumbar spine BMD adds limited information on vertebral fracture risk prediction [30, 31], which is in agreement with our findings. In other studies, matching vertebral fracture cases and controls on BMD still showed significant differences in TBS.[32-34] In such scenario, the diagnostic value of combining BMD and TBS will be higher compared with that of BMD alone. This contention has been shown by some studies reporting incremental improvement in fracture prediction in postmenopausal women after combining the TBS trabecular texture index with BMD.[16, 35] Additionally, TBS has been shown to identify 66-70% of women with all-type of fracture who were not diagnosed with osteoporosis (T score <-2.5) using BMD alone.[29] Despite some controversial findings across studies, our results support the statement that TBS predicts vertebral fractures independently of LS-BMD. A large prospective meta-analysis examining this contention is now underway with the objective of scrutinizing the need of incorporating TBS in individual fracture risk assessment tools.[36] Finally, a major advantage of the TBS and vertebral morphometry measurements used in our study is that their semi-automated operability and even post-hoc use allow large-scale and efficient implementation in research and eventually in clinical practice.[37, 38]

Our study has some limitations. We performed a cross-sectional evaluation of the association of TBS and prevalent vertebral fractures. Hence, we cannot directly imply any causative association between reduced TBS and prevalent vertebral fracture. Nevertheless, incident vertebral fractures were also indicative for the association with TBS. However, larger collections of cases are needed to assess the effect on fracture incidence. Further, for the analysis of prevalent fractures we cannot determine the impact of fracture processes occurring on the vertebral bodies where TBS is measured. Nevertheless, we expect this not to play a significant role as excluding the vertebral bodies with fracture from the analysis did not essentially change the results. Examining differences in TBS values between vertebral bodies where the fracture occurs and those with no fracture will provide additional insight to the observed associations.

More insight into the factors underlying vertebral fracture risk would be helpful to possibly target high-risk sub-groups with preventative interventions before they even fracture. In addition to conventional risk factors,[39] the field of genetics of osteoporotic vertebral fractures should be explored given the fact that heritability estimates up to 69% have been reported[40]. However, directly identifying genetic determinants contributing to the risk of vertebral fractures has proven difficult due to its heterogeneity

in definition, multifactorial nature and occurrence late in life.[41] Most previous genetic epidemiology studies were aimed at the genetics of areal bone mineral density,[42] unable to differentiate genetic determinants from bone microstructural traits even though the genetic variants associated with cortical and trabecular bone parameters may differ.[43] Future genome-wide association studies GWAS studies should be performed for TBS, as analyzing a quantitative trait may prove more powerful than the clinical endpoint of fracture as a dichotomous outcome.

In conclusion, TBS is associated with risk for prevalent vertebral fractures independent of BMD in Dutch women. TBS, as evaluated from standard DXA scans directly, potentially complements BMD in the prediction of osteoporotic vertebral fractures. More studies, preferably of a prospective nature, are necessary to fully evaluate the potential role of TBS as a complementary risk factor for fracture.

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REFERENCES

1. Cooper C, O'Neill T, Silman A. The epidemiology of vertebral fractures. European Vertebral Osteoporosis Study Group. Bone. 1993;14 Suppl 1:S89-97.
2. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I:1-65.
3. Burger H, Van Daele PL, Grashuis K, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities and functional impairment in men and women. J Bone Miner Res. 1997 Jan;12(1):152-7.
4. Nevitt MC, Ettinger B, Black DM, Stone K, Jamal SA, Ensrud K, et al. The association of radiographically detected vertebral fractures with back pain and function: a prospective study. Ann Intern Med. 1998 May 15;128(10):793-800.
5. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C, et al. Health-related quality of life in postmenopausal women with low BMD with or without prevalent vertebral fractures. J Bone Miner Res. 2000 Jul;15(7):1384-92.
6. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
7. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. J Am Geriatr Soc. 2000 Mar;48(3):241-9.
8. Ström O, Borgström F, Kanis JA, Compston J, Cooper C, McCloskey EV, et al. Osteoporosis: burden, health care provision and opportunities in the EU. Arch Osteoporos. 2011;DOI 10.1007/s11657-011-0060-1.

9. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. 2007 Mar;22(3):465-75.
10. Chen JS, Sambrook PN. Antiresorptive therapies for osteoporosis: a clinical overview. Nat Rev Endocrinol. 2011;8(2):81-91.
11. Lewiecki EM, Laster AJ. Clinical review: Clinical applications of vertebral fracture assessment by dual-energy x-ray absorptiometry. J Clin Endocrinol Metab. 2006 Nov;91(11):4215-22.
12. Briot K. DXA parameters: beyond bone mineral density. Joint Bone Spine. 2013 May;80(3):265-9.
13. Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone. 2004 Jan;34(1):195-202.
14. Hans D, Barthe N, Boutroy S, Pothuaud L, Winzenrieth R, Krieg MA. Correlations between trabecular bone score, measured using anteroposterior dual-energy X-ray absorptiometry acquisition, and 3-dimensional parameters of bone microarchitecture: an experimental study on human cadaver vertebrae. J Clin Densitom. 2011 Jul-Sep;14(3):302-12.
15. Silva BC, Leslie WD, Resch H, Lamy O, Lesnyak O, Binkley N, et al. Trabecular bone score: a noninvasive analytical method based upon the DXA image. J Bone Miner Res. 2014 Mar;29(3):518-30.
16. Hans D, Goertzen AL, Krieg MA, Leslie WD. Bone microarchitecture assessed by TBS predicts osteoporotic fractures independent of bone density: the Manitoba study. J Bone Miner Res. 2011 Nov;26(11):2762-9.
17. Bousson V, Bergot C, Sutter B, Levitz P, Cortet B. Trabecular bone score (TBS): available knowledge, clinical relevance, and future prospects. Osteoporos Int. 2012 May;23(5):1489-501.
18. Breban S, Briot K, Kolta S, Paternotte S, Ghazi M, Fechtenbaum J, et al. Identification of rheumatoid arthritis patients with vertebral fractures using bone mineral density and trabecular bone score. J Clin Densitom. 2012 Jul-Sep;15(3):260-6.
19. Eller-Vainicher C, Filopanti M, Palmieri S, Ulivieri FM, Morelli V, Zhukouskaya VV, et al. Bone quality, as measured by trabecular bone score, in patients with primary hyperparathyroidism. Eur J Endocrinol. 2013 Aug;169(2):155-62.
20. Eller-Vainicher C, Morelli V, Ulivieri FM, Palmieri S, Zhukouskaya VV, Cairoli E, et al. Bone quality, as measured by trabecular bone score in patients with adrenal incidentalomas with and without subclinical hypercortisolism. J Bone Miner Res. 2012 Oct;27(10):2223-30.
21. Leslie WD, Aubry-Rozier B, Lamy O, Hans D. TBS (trabecular bone score) and diabetes-related fracture risk. J Clin Endocrinol Metab. 2013 Feb;98(2):602-9.
22. Romagnoli E, Cipriani C, Nofroni I, Castro C, Angelozzi M, Scarpiello A, et al. "Trabecular Bone Score" (TBS): an indirect measure of bone micro-architecture in postmenopausal patients with primary hyperparathyroidism. Bone. 2013 Mar;53(1):154-9.
23. Hofman A, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, et al. The Rotterdam Study: 2014 objectives and design update. Eur J Epidemiol. 2013 Nov;28(11):889-926.
24. Burger H, de Laet CE, van Daele PL, Weel AE, Witteman JC, Hofman A, et al. Risk factors for increased bone loss in an elderly population: the Rotterdam Study. Am J Epidemiol. 1998 May 1;147(9):871-9.
25. Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW. Structural trends in the aging femoral neck and proximal shaft: analysis of the Third National Health and Nutrition Examination Survey dual-energy X-ray absorptiometry data. J Bone Miner Res. 2000 Dec;15(12):2297-304.
26. Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res. 1993 Sep;8(9):1137-48.
27. Pothuaud L, Carceller P, Hans D. Correlations between grey-level variations in 2D projection images (TBS) and 3D microarchitecture: applications in the study of human trabecular bone microarchitecture. Bone. 2008 Apr;42(4):775-87.
28. Stauber M, Muller R. Age-related changes in trabecular bone microstructures: global and local morphometry. Osteoporos Int. 2006;17(4):616-26.
29. Krueger D, Fidler E, Libber J, Aubry-Rozier B, Hans D, Binkley N. Spine trabecular bone score subsequent to bone mineral density improves fracture discrimination in women. J Clin Densitom. 2014 Jan-Mar;17(1):60-5.

30. Del Rio LM, Winzenrieth R, Cormier C, Di Gregorio S. Is bone microarchitecture status of the lumbar spine assessed by TBS related to femoral neck fracture? A Spanish case-control study. Osteoporos Int. 2013 Mar;24(3):991-8.
31. Boutroy S, Hans D, Sornay-Rendu E, Vilayphiou N, Winzenrieth R, Chapurlat R. Trabecular bone score improves fracture risk prediction in non-osteoporotic women: the OFELY study. Osteoporos Int. 2013 Jan;24(1):77-85.
32. Rabier B, Heraud A, Grand-Lenoir C, Winzenrieth R, Hans D. A multicentre, retrospective case-control study assessing the role of trabecular bone score (TBS) in menopausal Caucasian women with low areal bone mineral density (BMDa): Analysing the odds of vertebral fracture. Bone. 2010 Jan;46(1):176-81.
33. Winzenrieth R, Dufour R, Pothuaud L, Hans D. A retrospective case-control study assessing the role of trabecular bone score in postmenopausal Caucasian women with osteopenia: analyzing the odds of vertebral fracture. Calcif Tissue Int. 2010 Feb;86(2):104-9.
34. Pothuaud L, Barthe N, Krieg MA, Mehsen N, Carceller P, Hans D. Evaluation of the potential use of trabecular bone score to complement bone mineral density in the diagnosis of osteoporosis: a preliminary spine BMD-matched, case-control study. J Clin Densitom. 2009 Apr-Jun;12(2):170-6.
35. Lamy O, Metzger M, Krieg MA, Aubry-Rozier B, Stoll D, Hans D. [OsteoLaus: prediction of osteoporotic fractures by clinical risk factors and DXA, IVA and TBS]. Rev Med Suisse. 2011 Nov 2;7(315):2130, 2-4, 6.
36. McCloskey EV, Oden A, Harvey NC, Leslie WD, Hans D, Johansson H, et al. A Meta-Analysis of Trabecular Bone Score in Fracture Risk Prediction and Its Relationship to FRAX. J Bone Miner Res. 2016 May;31(5):940-8.
37. Oei L, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJ, et al. Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses. Quant Imaging Med Surg. 2013 Oct;3(5):249-55.
38. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, Hofman A, et al. Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. Eur Radiol. 2013 Feb;23(2):476-86.
39. Oei L, Zillikens MC, Rivadeneira F, Oei EH. Osteoporotic Vertebral Fractures as Part of Systemic Disease. J Clin Densitom. 2015 Sep 12.
40. Oei L, Zillikens MC, Rivadeneira F, Oei EH. Genetics of Osteoporotic Vertebral Fractures. J Clin Densitom. 2015 Sep 12.
41. Oei L, Estrada K, Duncan EL, Christiansen C, Liu C-T, Langdahl BL, et al. Genome-wide association study for radiographic vertebral fractures: A potential role for the 16q24 BMD locus. Bone. 2014;59(0):20-7.
42. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.
43. Paternoster L, Lorentzon M, Lehtimaki T, Eriksson J, Kahonen M, Raitakari O, et al. Genetic determinants of trabecular and cortical volumetric bone mineral densities and bone microstructure. PLoS Genet. 2013;9(2):e1003247.

Chapter 3.4

Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses

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ABSTRACT

Osteoporotic vertebral fractures are an increasingly active area of research. Oftentimes assessments are performed by software-assisted quantitative morphometry. Here, we will discuss multifunctionality of these data for research purposes. A team of trained research assistants processed lateral spine radiographs from the population-based Rotterdam Study with SpineAnalyzer® software (Optasia Medical Ltd, Cheadle, UK). Next, the raw coordinate data of the two upper corners of T5 and the two lower corners of T12 were extracted to calculate the Cobb's kyphosis angle. In addition, two readers performed independent manual measurements of the Cobb's kyphosis angle between T5 and T12 for a sample (N=99). The mean kyphosis angle and its standard deviation were 53° and 10° for the SpineAnalyzer® software measurements and 54° and 12° by manual measurements, respectively. The Pearson's correlation coefficient was 0.65 [95% confidence interval (CI): 0.53–0.75; $P=2\times10^{-13}$]. There was a substantial intraclass correlation with a coefficient of 0.64 (95% CI: 0.51–0.74). The mean difference between methods was 1° (95% CI: –2°–4°), with 95% limits of agreement of –20°–17° and there were no systematic biases. In conclusion, vertebral fracture morphometry data can be used to derive the Cobb's kyphosis angle. Even more quantitative measures could be derived from the raw data, such as vertebral wedging, intervertebral disc space, spondylolisthesis and the lordosis angle. These measures may be of interest for research into musculoskeletal disorders such as osteoporosis, degenerative disease or Scheuermann's disease. Large-scale studies may benefit from efficient capture of multiple quantitative measures in the spine.

INTRODUCTION

The complaint of back pain is highly prevalent and is among the most common medical conditions.[1] These pain symptoms may impair function and movement of the spinal column, and therefore, back pain is one of the main reasons for healthcare expenditure around the world.[2] Nevertheless, no specific pathology can be identified in up to 85% of patients,[3] as currently employed imaging biomarkers display very limited correlation with symptoms[1, 2, 4] and a better understanding of spine pathology is necessary.

Vertebral fractures are the most common osteoporotic fractures and represent a significant health issue[5] as they are associated with a loss of quality of life,[6] mortality[7] and a considerable financial burden[8, 9].

Hyperkyphosis, i.e., excessive curvature of the spine in the sagittal (anteroposterior) plane, is associated with advanced age[10] and is commonly attributed to osteoporotic vertebral fractures[11]. Additionally, the kyphosis angle is independently associated with decreased mobility, increased propensity to fall and mortality.[12, 13] Other differential diagnoses that display wedging of vertebral bodies, i.e., structural changes involving loss of anterior height, are anatomical variation, degenerative changes or Scheuermann's disease.[14, 15]

Osteoporotic vertebral fractures are an increasingly active area of research, especially in the light of the aging of populations. Oftentimes the presence of spine fractures is assessed by means of software-assisted quantitative morphometry,[16] which can automatically identify vertebral body margins on digital radiography, dual energy X-ray absorptiometry and computed tomography. Some of these software packages allow saving of the quantitative spatial information, for example SpineAnalyzer® software (Optasia Medical Ltd, Cheadle, UK). This data could be used to derive more quantitative measures than osteoporotic vertebral fractures, such as vertebral wedging, dimensions of the intervertebral disc space, spondylolisthesis, kyphosis and lordosis. In this brief report we present an added functionality of these quantitative data for research purposes, illustrated by the example of the Cobb's kyphosis angle.

METHODS

Study sample

The Rotterdam Study is a prospective population-based cohort studying the determinants of chronic diseases, including osteoporosis, and disability in Dutch men and women. Both the objectives and the study design have been described previously.[17] It includes 14,926 inhabitants aged 45 years and over of Rotterdam city's Ommoord district in The Netherlands. The present report describes results obtained from the Rotterdam Study-III cohort baseline visit, which started follow-up in 2006. The Medical Ethics Committee of the Erasmus University Medical Center has approved the Rotterdam Study.

Radiographic assessments

During the periodical research center visits, radiographic examinations of the spine were obtained using a digitized Fuji Computed Radiography system (FUJIFILM Medical Systems). All radiographs were acquired digitally according to a standardized protocol, with a focus to detector distance of 120 cm. A team of trained research assistants processed lateral spine radiographs (vertebral levels T4–L4) with SpineAnalyzer® software (Optasia Medical Ltd, Cheadle, UK).[18] SpineAnalyzer® software can automatically identify vertebral body margins on digital radiography, determine the exact heights of the vertebrae, and calculate the shape and degree of height reduction. After labeling the vertebrae of interest by placing thirteen points at the center of each vertebral body from T4 to L4, the software will automatically outline each labeled vertebra with six morphometry points, corresponding to the four corners of the vertebral

body, as well as the mid-point of the superior and inferior endplate. The analyst can make manual adjustments to these six morphometry points to fine-tune their exact locations for accurate measurements, and thereafter, the pixel coordinates of these points are saved. We extracted the raw coordinate data of the two upper corners of T5 and the two lower corners of T12 to calculate the Cobb's kyphosis angle. For comparison, two readers (A.A.M. and S.S.) performed independent manual measurements of the Cobb's kyphosis angle between T5 and T12 (Figure 1) for a sample of N=99, which had vertebral wedging at a minimum of three levels and presence of vertebral body endplate irregularities, as described previously.[19] The analyses presented in this report concern the sample of N=99 subjects analyzed with both SpineAnalyzer® and the manual measurements of the Cobb's kyphosis angle between T5 and T12.

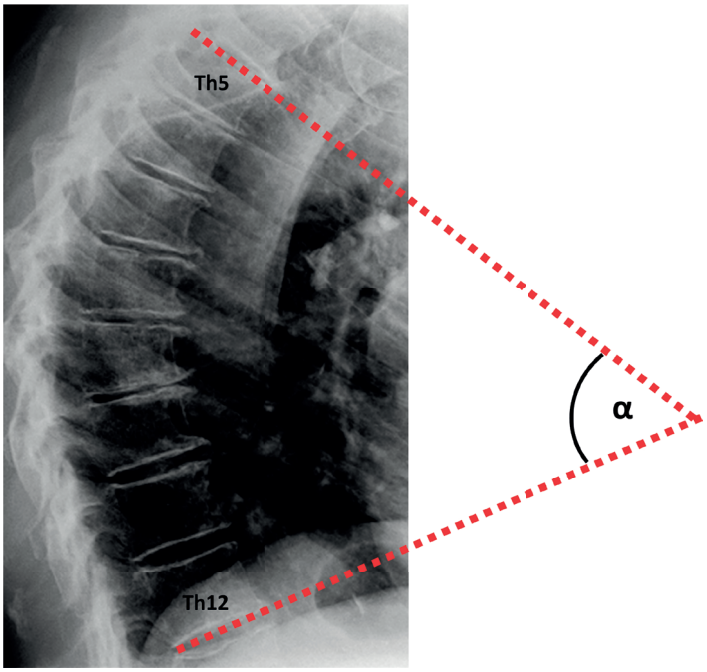

Figure 1. Measurement of the Cobb's kyphosis angle (α) between T5 and T12.

Statistical analysis

The Cobb's kyphosis angle was calculated from the raw coordinates by the formula: $\alpha=\beta_1+\beta_2=\arctan(\Delta y_I/\Delta x_I)+\arctan(\Delta y_{II}/\Delta x_{II})=\arctan[(y_1-y_2)/(x_1-x_2)]+\arctan[(y_3-y_4)/(x_3-x_4)]$ (Figure 2). We computed the Pearson's correlation coefficient r and corresponding t-test statistic between the calculations derived from the SpineAnalyzer® software and the manual measurements for the Cobb's kyphosis angle. In addition, the intraclass correlation coefficient (two way mixed, consistency and agreement) with the matching F-test statistic was determined and classified according to Landis and Koch.[20] Finally, we evaluated if the differences between measurements was different from 0 by a t-test and mapped the results in a Bland-Altman plot including calculation of the interval between the 95% limits of agreement by taking the mean difference plus and minus 2 standard deviations[21] to further evaluate the agreement between the measurements. SPSS statistics software version 20 (IBM, Armonk, NY, USA) and R software version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) were used for the analyses.

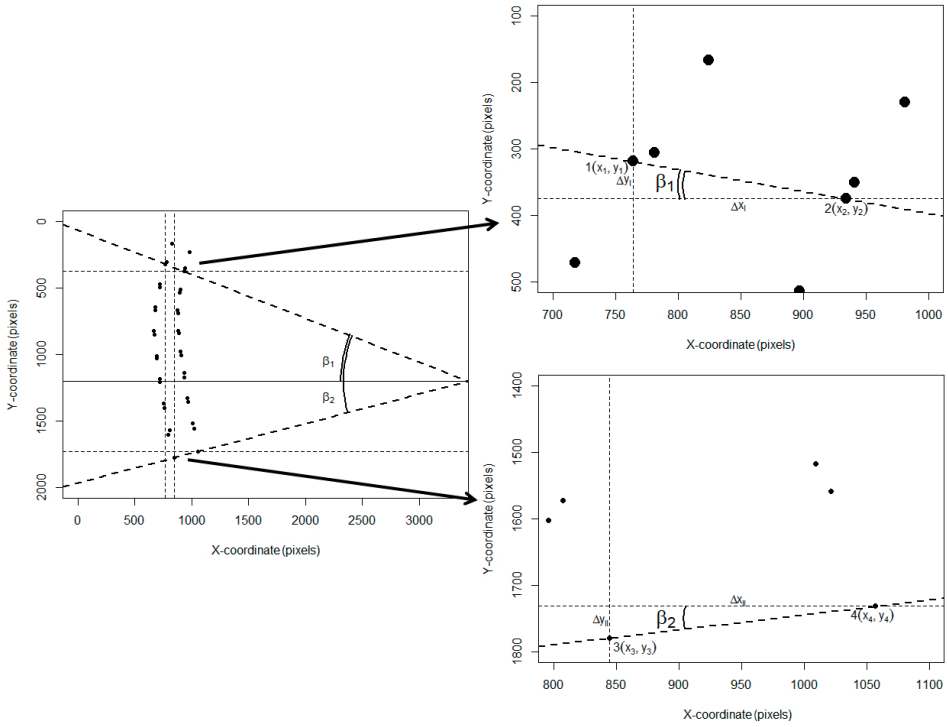

Figure 2. X-Y coordinate plot in pixels of vertebral levels T4–T12 (left panel); zoom-in focused on T5 (upper right panel) and zoom-in focused on T12 (bottom right panel). Calculation of the Cobb's kyphosis angle (α) between T5 and T12 from vertebral fracture morphometry analyses coordinates. The Cobb's kyphosis angle (denoted as α) was calculated from the raw coordinates by the formula: $\alpha=\beta_1+\beta_2$ shown in the left panel; in the upper right panel it is shown that $\beta_1=\arctan(\Delta y_i/\Delta x_i)=\arctan[(y_1-y_2)/(x_1-x_2)]$ and in the bottom right panel it is shown that $\beta_2=\arctan(\Delta y_{ii}/\Delta x_{ii})=\arctan[(y_3-y_4)/(x_3-x_4)]$.

RESULTS

The mean kyphosis angle between the superior endplate of T5 and the inferior endplate of T12 of the sample as determined by the SpineAnalyzer® software measurements was 53° with a standard deviation of 10° and the mean kyphosis angle by manual measurements was 54° with a standard deviation of 12°. The Pearson's correlation coefficient r between the manual measurements and the calculations derived from the SpineAnalyzer® software was 0.65 [95% confidence interval (CI): 0.53–0.75; $P=2\times10^{-13}$] (Figure 3). There was a substantial intraclass correlation with a coefficient of 0.64 for both consistency and absolute agreement (95% CI: 0.51–0.74 $P=5\times10^{-13}$ and $P=4\times10^{-13}$, respectively). The mean difference between methods was 1° (95% CI: –2°–4°) and not different from 0 (P=0.4). The interval between the 95% limits of agreement was –20°–17° where approximately half the individuals (47%) showed a difference of less than 5° and about three quarters less than 10° between the techniques (76%). The Bland-Altman plot did not show systematic biases of proportional error, dependency of variation on the magnitude of measurements, extreme outliers, systematic under- or overestimation (Figure 4).

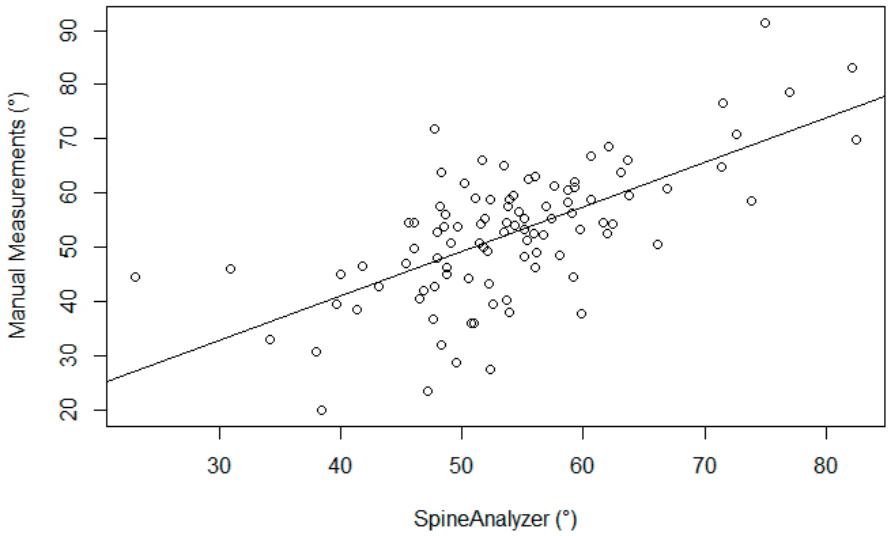

Figure 3. Correlation between the manual measurements (X-axis) and the calculations derived from the SpineAnalyzer® software (Y-axis) of the Cobb's kyphosis angle in degrees between T5 and T12; Pearson's correlation coefficient r=0.65 [95% confidence interval (CI): 0.53–0.75; $P=2\times10^{-13}$].

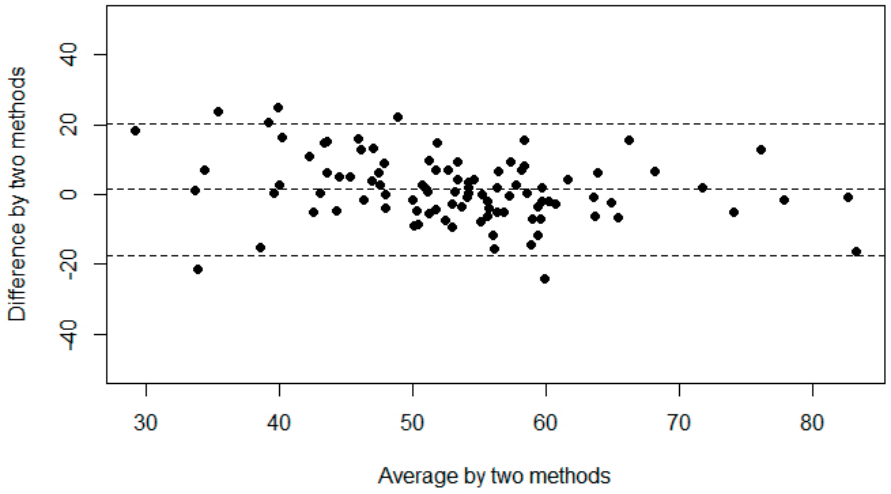

Figure 4. Bland-Altman plot of the average of (X-axis) and the mean difference (Y-axis) of the manual measurements minus the calculations derived from the SpineAnalyzer® software of the Cobb's kyphosis angle in degrees. The dashed lines represent the mean plus two standard deviations and the mean minus two standard deviations, respectively.

DISCUSSION

In the present study we have shown that quantitative vertebral morphometry data derived from lateral spine radiographs from the population-based Rotterdam Study with SpineAnalyzer® software can be used to calculate the Cobb's kyphosis angle. The agreement of this method with independent manual measurements was substantial and there were no systematic biases.

There have been multiple publications comparing different methods for the measurement of Cobb's kyphosis angle and reports of the inter-observer variation. Our results are comparable, but with somewhat lower inter-observer agreement, with the findings of previous comparative studies of other methods for kyphosis angle measurement.[22-24] However, the interval between the 95% limits of agreement we found in our study sample was rather broad, which is an indication of how far apart measurements by the two methods were for most individuals and this limits clinical applicability. It has previously been found that measurement error is primarily due to intra-observer error rather than inter-observer error.[25]

There are several commercially available software packages available for vertebral quantitative morphometry, of which we applied SpineAnalyzer®. A variety of custom or in-house developed software tools are also being used in the research community. These software algorithms do not always exploit automated vertebral detection, but this is not a requirement for our method to calculate Cobb's angle. Even when vertebral body identification is performed manually, the calculation as presented in this paper is possible, provided that the exact pixel coordinates of the superior endplate of T5 and inferior endplate of T12 are available. In addition to kyphosis measurement, more quantitative measures could be derived from the raw morphometry data, such as vertebral wedging,[26, 27] intervertebral disc space[28-30] (Figure 5) and the lordosis angle[31]. Several methods for the assessment of the dimensions of the intervertebral disc space have been proposed and comprise calculations with the measurements of anterior, central and posterior distances between vertebral endplate, disc diameter and disc areas;[30] in addition, inter-vertebral disc angles can be computed. Theoretically, measures of spondylolisthesis could be derived, although obtaining flexion-extension films might be preferable. These measures may be of interest to researchers investigating musculoskeletal disorders such as osteoporosis, degenerative disease or Scheuermann's disease. Extending the work flow to more vertebral levels and to capture more detailed morphological shapes is desirable. For example, the lumbar lordosis angle is usually calculated between T12 and S1 or L5,[31] lumbar disc degeneration is most commonly observed at L4-L5 and L5-S1[32] and spondylolisthesis is most prevalent at levels L4-L5 and L5-S1 as well,[33] which would in fact be the most interesting levels to study from the perspective of degeneration. These levels are, however, usually disregarded by most vertebral morphometry software packages for osteoporotic fractures, because these fractures are rare in the lower lumbar spine. Moreover, information on endplate fractures, Schmorl's nodes and osteophytes cannot be inferred from six point morphometry data, but Spineanalyzer® offers additional 95 point morphometry. However, this registration of 95 points per vertebra has not been studied and validated comprehensively yet and extra manual adjustments of the point placements on the radiographs may be necessary. A limitation of morphometry analyses is that it would not be able to capture qualitative imaging features such as vertebral endplate irregularities and diffuse idiopatic skeletal hyperostosis (DISH). Shortcomings of 2-dimensional radiographical imaging in the current context include failure to represent soft tissues, distortion due to oblique projection of the conical X-ray beams[29] and superimposition of overlying anatomical structures like the shoulder girdle, the iliac wings of the pelvis, the ribs and pulmonary vasculature.

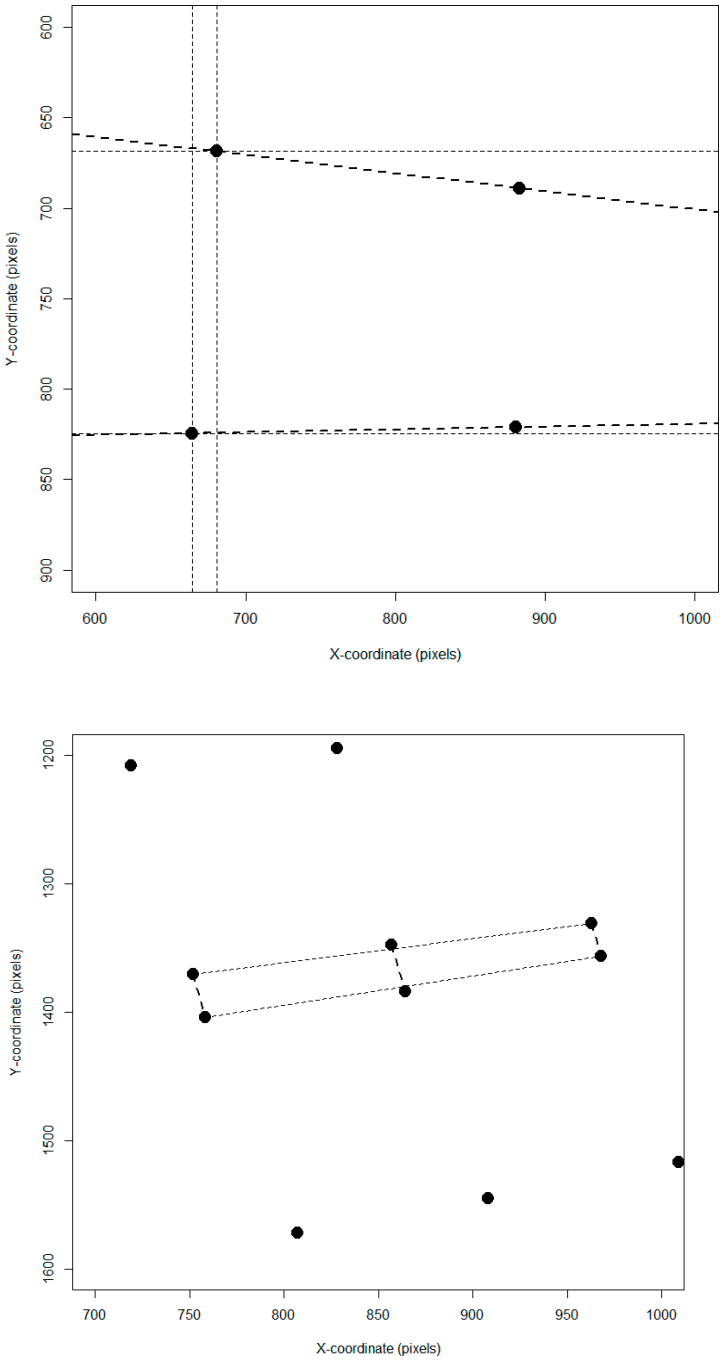

Figure 5. Deriving parameters of vertebral wedging (upper panel, current example concerns wedging of T7) and intervertebral disc space height (bottom panel, current example concerns the intervertebral disc space between T10 and T11).

Large-scale studies may benefit from efficient capture of multiple quantitative measures in the spine. In the future we intend to perform large-scale epidemiological studies with these data in the Rotterdam Study, for example to explore the etiology and associations with health outcomes of degenerative changes. The etiology of these spine diseases is largely unknown. Heritability plays a significant role in various spine diseases with estimates ranging between ~19% and 74%.[34, 35] A better understanding of the genetic susceptibility and epidemiological risk factors for spine diseases has the potential to identify underlying biological mechanisms, improve risk prediction and lead to novel disease interventions.

In conclusion, utilization of vertebral fracture morphometry data to derive the Cobb's kyphosis angle is relatively reliable. Even more quantitative measures could be derived from the raw data, such as vertebral wedging, intervertebral disc space, spondylolisthesis and the lordosis angle, and these parameters may be of interest to research into different musculoskeletal disorders such as osteoporosis or degenerative disease. Efficient capture of multiple quantitative measures in the spine may particularly benefit high-throughput studies and these investigations could contribute to a deeper understanding of spine conditions.

ACKNOWLEDGMENTS

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REFERENCES

1. Koes BW, van Tulder MW, Thomas S. Diagnosis and treatment of low back pain. BMJ. 2006 Jun 17;332(7555):1430-4.
2. Henschke N, Maher CG, Ostelo RW, de Vet HC, Macaskill P, Irwig L. Red flags to screen for malignancy in patients with low-back pain. Cochrane Database Syst Rev. 2013;2:CD008686.
3. Deyo RA, Rainville J, Kent DL. What can the history and physical examination tell us about low back pain? JAMA. 1992 Aug 12;268(6):760-5.
4. Williams CM, Henschke N, Maher CG, van Tulder MW, Koes BW, Macaskill P, et al. Red flags to screen for vertebral fracture in patients presenting with low-back pain. Cochrane Database Syst Rev. 2013;1:CD008643.
5. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I:1-65.
6. Gold DT. The clinical impact of vertebral fractures: quality of life in women with osteoporosis. Bone. 1996 Mar;18(3 Suppl):185S-9S.
7. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. J Am Geriatr Soc. 2000 Mar;48(3):241-9.
8. Burge R. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. [10.1359/jbmr.061113]. 2007;22:465-75.
9. Ström O, Borgström F, Kanis JA, Compston J, Cooper C, McCloskey EV, et al. Osteoporosis: burden, health care provision and opportunities in the EU. Arch Osteoporos. 2011;DOI 10.1007/s11657-011-0060-1.

10. Fon GT, Pitt MJ, Thies AC, Jr. Thoracic kyphosis: range in normal subjects. AJR Am J Roentgenol. 1980 May;134(5):979-83.
11. Katzman WB, Vittinghoff E, Kado DM. Age-related hyperkyphosis, independent of spinal osteoporosis, is associated with impaired mobility in older community-dwelling women. Osteoporos Int. 2011 Jan;22(1):85-90.
12. Kado DM, Lui LY, Ensrud KE, Fink HA, Karlamangla AS, Cummings SR. Hyperkyphosis predicts mortality independent of vertebral osteoporosis in older women. Ann Intern Med. 2009 May 19;150(10):681-7.
13. Katzman WB, Wanek L, Shepherd JA, Sellmeyer DE. Age-related hyperkyphosis: its causes, consequences, and management. J Orthop Sports Phys Ther. 2010 Jun;40(6):352-60.
14. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
15. Scheuermann HW. The classic: kyphosis dorsalis juvenilis. Clin Orthop Relat Res. 1977 Oct(128):5-7.
16. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, A. H, et al. Review of Radiological Scoring Methods of Osteoporotic Vertebral Fractures for Clinical and Research Settings. Eur Rad. 2012;DOI: 10.1007/s00330-012-2622-z.
17. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
18. Brett A, Miller CG, Hayes CW, Krasnow J, Ozanian T, Abrams K, et al. Development of a clinical workflow tool to enhance the detection of vertebral fractures: accuracy and precision evaluation. Spine (Phila Pa 1976). 2009 Oct 15;34(22):2437-43.
19. Makurthou AA, Oei L, Saddy SE, Breda SJ, Castano-Betancourt MC, Hofman A, et al. Scheuermann's Disease: Evaluation of Radiological Criteria and Population Prevalence. Spine (Phila Pa 1976). 2013 Jun 11.
20. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159-74.
21. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986 Feb 8;1(8476):307-10.
22. Chaise FO, Candotti CT, Torre ML, Furlanetto TS, Pelinson PP, Loss JF. Validation, repeatability and reproducibility of a noninvasive instrument for measuring thoracic and lumbar curvature of the spine in the sagittal plane. Rev Bras Fisioter. 2011 Nov-Dec;15(6):511-7.
23. de Oliveira TS, Candotti CT, La Torre M, Pelinson PP, Furlanetto TS, Kutchak FM, et al. Validity and reproducibility of the measurements obtained using the flexicurve instrument to evaluate the angles of thoracic and lumbar curvatures of the spine in the sagittal plane. Rehabil Res Pract. 2012;2012:186156.
24. Goh S, Price RI, Leedman PJ, Singer KP. A comparison of three methods for measuring thoracic kyphosis: implications for clinical studies. Rheumatology (Oxford). 2000 Mar;39(3):310-5.
25. Carman DL, Browne RH, Birch JG. Measurement of scoliosis and kyphosis radiographs. Intraobserver and interobserver variation. J Bone Joint Surg Am. 1990 Mar;72(3):328-33.
26. Crawford MB, Toms AP, Shepstone L. Defining normal vertebral angulation at the thoracolumbar junction. AJR Am J Roentgenol. 2009 Jul;193(1):W33-7.
27. Gaca AM, Barnhart HX, Bisset GS, 3rd. Evaluation of wedging of lower thoracic and upper lumbar vertebral bodies in the pediatric population. AJR Am J Roentgenol. 2010 Feb;194(2):516-20.
28. Farfan HF. Mechanical disorders of the low back. Philadelphia: Lea & Febiger; 1973.
29. Hurxthal LM. Measurement of anterior vertebral compressions and biconcave vertebrae. Am J Roentgenol Radium Ther Nucl Med. 1968 Jul;103(3):635-44.
30. Pope MH, Hanley EN, Matteri RE, Wilder DG, Frymoyer JW. Measurement of intervertebral disc space height. Spine (Phila Pa 1976). 1977;2(4):282–6.
31. Hwang JH, Modi HN, Suh SW, Hong JY, Park YH, Park JH, et al. Reliability of lumbar lordosis measurement in patients with spondylolisthesis: a case-control study comparing the Cobb, centroid, and posterior tangent methods. Spine (Phila Pa 1976). 2010 Aug 15;35(18):1691-700.
32. Toyone T, Takahashi K, Kitahara H, Yamagata M, Murakami M, Moriya H. Vertebral bone-marrow changes in degenerative lumbar disc disease. An MRI study of 74 patients with low back pain. J Bone Joint Surg Br. 1994 Sep;76(5):757-64.

33. Hirano K, Imagama S, Matsuyama Y, Kawakami N, Yukawa Y, Kato F, et al. Surgically Treated Cases of Lumbar Spondylolysis and Isthmic Spondylolisthesis: A Multicenter Study. J Spinal Disord Tech. 2012 Dec 3.
34. Damborg F, Engell V, Andersen M, Kyvik KO, Thomsen K. Prevalence, concordance, and heritability of Scheuermann kyphosis based on a study of twins. J Bone Joint Surg Am. 2006 Oct;88(10):2133-6.
35. Liu CT, Karasik D, Zhou Y, Hsu YH, Genant HK, Broe KE, et al. Heritability of prevalent vertebral fracture and volumetric bone mineral density and geometry at the lumbar spine in three generations of the Framingham study. J Bone Miner Res. 2012 Apr;27(4):954-8.

Chapter 3.5

Scheuermann's disease: evaluation of radiological criteria and population prevalence.

Makurthou AA, Oei L, El Saddy S, Breda SJ, Castaño-Betancourt MC, Hofman A, van Meurs JBJ, Uitterlinden AG, Rivadeneira F, Oei EHG

Spine (Phila Pa 1976). 2013 Sep;38(19):1690-4. PMID: 24509552

ABSTRACT

Study Design Observational population-based study.

Objective To determine the prevalence of radiographical Scheuermann's disease in a Dutch population and evaluate the consistency of diagnostic criteria.

Background Scheuermann's disease is a form of osteochondrosis characterized by increased posterior rounding of the thoracic spine with structural vertebral deformity. Different expert opinion–based radiological criteria exist, yet these have not been validated. The prevalence in the general population reported ranged from 1% to 10%.

Methods Lateral spine radiographs of 2,753 Rotterdam Study participants (aged 45–89 years) were assessed for Scheuermann's disease using Sørensen and Sachs' radiographical criteria in 2 phases. Cohen κ statistics were calculated for interrater agreement. Prevalence estimates were calculated and sex differences were tested with Pearson χ^2 test. We evaluated whether varying the kyphosis angle criterion would change the prevalence estimate.

Results A total of 677 (24.6%) individuals had endplate irregularities and 140 (5.1%) individuals had vertebral wedging. Abnormalities were significantly more prevalent among males ($P<0.05$). The interrater agreement κ statistics were 78.8% for vertebral wedging and 79.4% for endplate irregularity. A total of 127 individuals had both criteria, of which 111 had a kyphosis angle greater than 45°, resulting in a prevalence of 4.0% (95% confidence interval [CI]: 3.3%–4.7%). The disease prevalence was 4.5% in males versus 3.6% in females, yet this difference was not statistically significant ($P=0.23$). Adjustment of the kyphosis angle criterion from 45° to 40° or 35° increased the number of cases marginally, corresponding to prevalence estimates not significantly different from the estimates using original criteria (4.2% [95% CI: 3.3%–4.7%] and 4.4% [95% CI: 3.6%–5.2%]).

Conclusion Our results revealed a prevalence of 4.0% of radiographical Scheuermann's disease in Dutch individuals aged 45 years and older. Although there is no current "gold standard" for the radiographical definition, standardized scoring of independent features resulted in substantial interobserver agreement, and different applications of diagnostic criteria did not significantly alter the classification.

Level of Evidence: 3

INTRODUCTION

Scheuermann's disease is a form of osteochondrosis of the spine and is characterized by increased posterior rounding of the thoracic spine in association with structural deformity of the vertebral elements.[1] Previous prevalence estimates of Scheuermann's disease vary widely, ranging between 0.4% and 10%.[1-6] Scheuermann's disease is diagnosed on the basis of radiographical criteria, yet, there is no diagnostic "gold standard." The criteria by Sørensen and Sachs are the most commonly applied[7, 8] but different radiographical diagnostic criteria exist.[9] These different radiographical criteria include endplate irregularity, thoracic kyphosis greater than 35°[9] or 45°,[10] and at least 1[7, 9] or 3[10] adjacent wedged vertebral bodies each of 5° or more in magnitude[1, 11] (Figure 1). Also, Schmorl's nodes are thought to be a common but not obligate manifestation of Scheuermann's disease.[12] When different criteria are used inconsistently, the estimates of disease prevalence may differ widely and seem unreliable.

Therefore, the purpose of our study was to determine the prevalence of Scheuermann's disease and its radiographical criteria in the Dutch population across sexes and to evaluate the consistency of the diagnostic criteria.

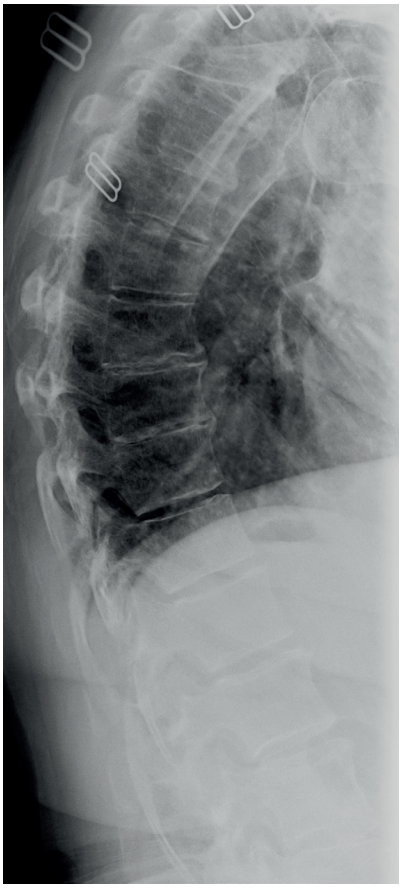

Figure 1. Lateral radiograph showing Scheuermann's disease with marked endplate irregularities and mild anterior wedging of multiple midthoracic vertebrae, resulting in increased thoracic kyphosis.

METHODS

The Rotterdam Study is a prospective population-based cohort study that started in 1990 in Ommoord, a suburb of Rotterdam, the Netherlands. The main objective of the Rotterdam Study is to investigate the prevalence, incidence, and risk factors for chronic disease and disability in elderly individuals aged 55 years and older. After approximately 1 decade, the Rotterdam Study was expanded with a younger cohort (RS-III), including participants aged 45 years and older living in the same suburb. A detailed description of the Rotterdam Study has been reported previously.[13] The Medical Ethics Committee of the Erasmus University Medical Center has approved the Rotterdam Study.

A trained research technician obtained standing lateral radiographs of the thoracolumbar spine of individuals visiting the research center. All radiographs were acquired digitally according to a standardized protocol, with a focus to detector distance of 120 cm (Fujifilm Medical Systems, Stamford, CT). A DICOM viewer was used for radiographical assessment.

To diagnose Scheuermann's disease, the radiographical criteria of Sørensen[7] and Sachs et al.[8] were applied in 2 phases (Table 1). A.A.M. and S.S., research assistants trained by a musculoskeletal radiologist (E.H.G.O.), scored the radiographs. To determine the level of agreement, a randomly selected subset (N=154) was assessed by both readers. The musculoskeletal radiologist (E.H.G.O.) acted as a third reader by assessing inconclusive radiographs to resolve discrepancies. In the first phase, we triaged potential cases from normal radiographs on the basis of 2 criteria: vertebral wedging at a minimum of 3 levels and presence of vertebral body endplate irregularities. Because Schmorl's nodes are actually focal indentations of the vertebral endplate,[12] we scored these as endplate irregularities. We defined potential cases as those with consecutive vertebral wedging in combination with endplate irregularity. In the second phase, we reevaluated all these potential cases by measuring the thoracic kyphosis angle between thoracic vertebral levels T5 and T12. We defined a kyphosis angle of 45° or more to diagnose the Scheuermann's disease cases.[10] In addition, we evaluated the impact of varying the kyphosis angle criterion on prevalence estimation of Scheuermann's disease by adjusting from 45° to 40° or 35°. Finally, we reassessed the levels of vertebral wedging and endplate irregularities in more detail. All radiographs fulfilling these criteria were reassessed by the other reader (A.A.M. or S.S.) to verify the diagnosis of Scheuermann's disease.

Table 1. Radiographical assessment of diagnostic criteria in 2 phases.

Phases	Data Type	Criteria	Specifications
1	Qualitative	Vertebral body endplate irregularities	One or more vertebral levels
	Qualitative	Vertebral wedging	At least 3 adjacent vertebral levels 5° or more per vertebra
2	Quantitative	Kyphosis angle	Between thoracic levels T5 and T12 45° or more in total

Statistical analysis

Frequencies of each of the independent radiological diagnostic criteria were assessed per vertebral and per patient levels, and the prevalence of Scheuermann's disease was determined in the study population. A random subset of radiographs was scored by both readers (A.A.M. and S.S.), and Cohen κ statistics for interrater agreement were calculated for this sample and graded according to Landis and Koch.[14] Sex-specific and sex-combined prevalence estimates were calculated and sex differences were tested with Pearson χ^2 test. Analyses were performed with SPSS statistics software version 20 (IBM, Armonk, NY).

RESULTS

Lateral spine radiographs were available and assessed for 2,753 participants (mean age, 57 years; range, 45–89 years). After triage, we identified 677 (24.6%) cases with endplate irregularities and 140 (5.1%) cases with vertebral wedging (Table 2). The Cohen κ statistics were 78.8% for vertebral wedging and 79.4% for endplate irregularity. We investigated whether the occurrence of endplate irregularities and vertebral wedging differed between sexes. We found a higher prevalence of endplate irregularities among males than in females, but this difference was borderline statistically significant (P=0.06). Also, we observed a significantly higher prevalence of vertebral wedging among males than among females (P=0.02). In addition, most endplate irregularities occurred at thoracic and vertebral level 8 (Figure 2), and vertebral wedging was most common at the midthoracic region (Figure 3). Subsequently, 127 participants were classified as having both endplate irregularities and vertebral wedging (Table 2). The frequency of having both endplate irregularity and vertebral wedging was significantly higher in males than in females (5.6% vs. 3.9%; P=0.04).

Table 2. Frequencies of the radiological diagnostic criteria of Scheuermann's disease.

Variable	Males (N=1,187)	Females (N=1,566)	Total (N=2,753)	P*
Endplate irregularity	313 (26.4%)	364 (23.2%)	677 (24.6%)	0.06
Vertebral wedging	74 (6.2%)	66 (4.2%)	140 (5.1%)	0.02
Endplate irregularity + vertebral wedging	66 (5.6%)	61 (3.9%)	127 (4.6%)	0.04
Scheuermann's disease	54 (4.5%)	57 (3.6%)	111 (4.0%)	0.23

*P value χ^2 males versus females.

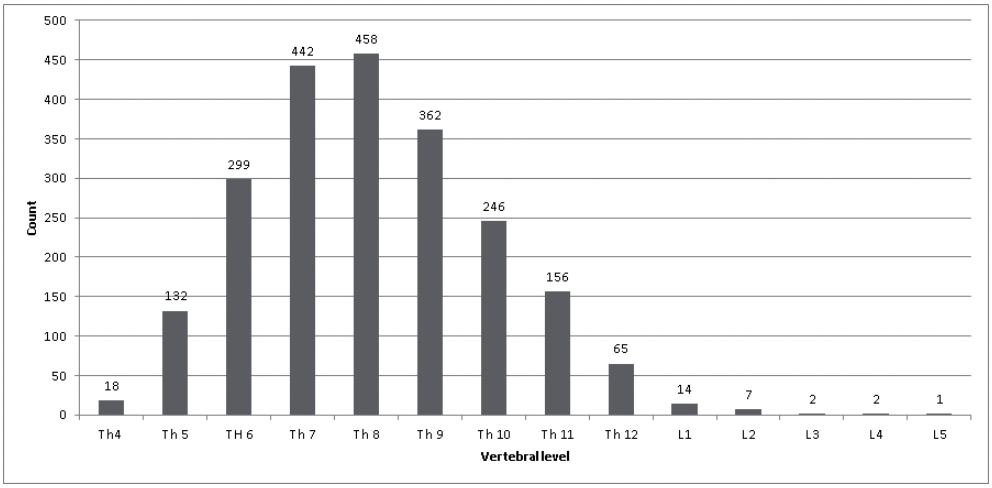

Figure 2. Number of endplate irregularities observed per thoracic (T) and lumbar (L) vertebral level.

Next, we measured the kyphosis angle in the 127 potential cases of Scheuermann's disease (Table 3). A kyphosis angle of 45° or more was found in 111 cases (87% of the participants prioritized by the triage procedure), resulting in a definitive diagnosis of Scheuermann's disease. The prevalence of Scheuermann's disease was estimated to be 4.0% (95% confidence interval [CI]: 3.3%–4.7%), with no significant difference across sexes (P=0.23) (Table 2).

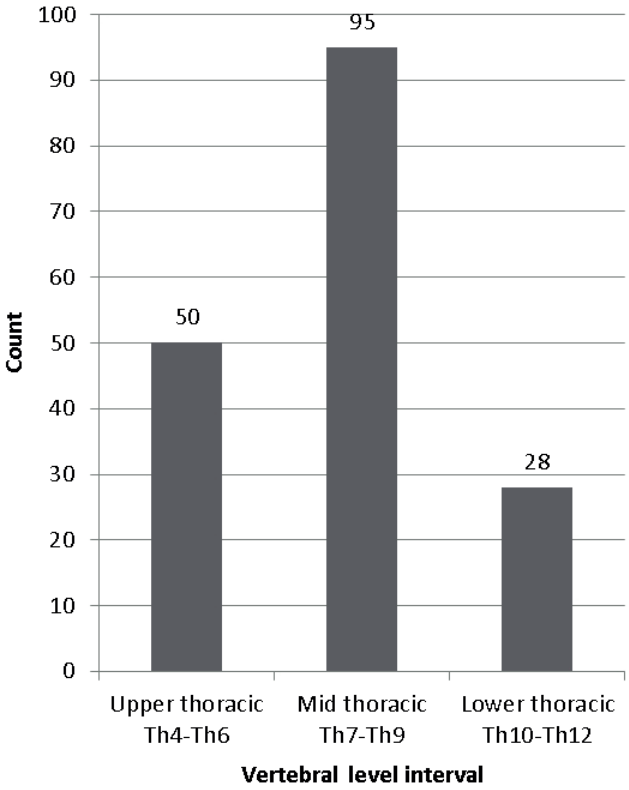

Figure 3. Distribution of vertebral wedging of 3 adjacent vertebrae of 5° or more per thoracic (T) region.

Table 3. Frequencies per kyphotic angle interval for potential cases.

Kyphotic angle interval	Frequency	%
20–25	2	1.6
25–30	1	0.8
30–35	3	2.4
35–40	6	4.7
40–45	4	3.1
45–50	27	21.3
50–55	30	23.6
55–60	31	24.4
60–65	12	9.4
65–70	3	2.4
70–75	4	3.1
75–80	2	1.6
80–85	2	1.6
Total	127	100.0

In addition, we evaluated whether a modification of the diagnostic criteria would influence the prevalence estimate of Scheuermann's disease. By adjusting the kyphosis angle criterion from 45° to 40°, we found that this would increase the total number of Scheuermann's disease cases to 115, resulting in no essential change in prevalence of 4.2% (95% CI: 3.3%– 4.7%). Similarly, no major difference was observed by using a kyphosis angle cutoff of 35°, in which the prevalence was 4.4% (95% CI: 3.6%–5.2%) after just adding 6 additional cases.

DISCUSSION

In this first epidemiological study in the Dutch population, we found a prevalence for radiographical Scheuermann's disease of 4.0%, applying the criteria defined by Sørensen and Sachs. Although no "gold standard" for the radiographical definition exists, standardized scoring of independent features resulted in substantial interobserver agreement, and different definitions of diagnostic criteria did not alter disease classification.

The population-based study design with radiographs of the full thoracolumbar spine and a meticulous scoring system enabled us to identify radiographical Scheuermann's disease cases without clinical complaints, which would have been missed by a clinical-based study. We assessed a large number of radiographs specifically for Scheuermann's disease and recorded each of the diagnostic criteria separately and in detail with high interobserver agreement.

Usually, Scheuermann's kyphosis becomes clinically overt during growth spurt and ceases to progress once axial skeletal maturity is reached.[15, 16] Therefore, all participants with Scheuermann's disease should have displayed radiographical features at the time of our examination. Nevertheless, our study in older individuals could have included some false positive cases because of the coexistence of degenerative disease. However, the most typical features of spine degeneration are disc disease, osteophytosis, and facet joint osteoarthritis,[17] which are not diagnostic criteria for Scheuermann's disease. Furthermore, these degenerative changes occur much less commonly in the thoracic spine than in the cervical and lumbar regions.[18] As mortality rate is unchanged in Scheuermann's disease,[6] our prevalence estimates may also be extrapolated to younger populations.

Different expert opinion–based criteria have been used for diagnosing Scheuermann's disease and these criteria remain controversial.[8] Therefore, we evaluated the effect of modifying the kyphosis angle criterion and found that only few more would be classified as Scheuermann's disease cases without affecting the prevalence. Composite standardized assessment of independent criteria seems to result in sufficient diagnostic consistency.

Our prevalence of 4.0% is within the previously reported range (0.4%–10%)[1-6] and highlights that radiographical Scheuermann's disease is not infrequent in the general population. Each of the diagnostic criteria, that is, endplate irregularities and vertebral wedging, occurred most commonly at the midthoracic region. The frequency of both endplate irregularities and vertebral wedging was higher in males than in females; however, the sex difference in the prevalence was not statistically significant, which could be due to the limited study power with a relatively low number of cases. Some publications have reported prevalences of Scheuermann's disease to be closely similar between the sexes,[19-21] whereas others have observed Scheuermann's disease to be more prevalent among males than in females.[22, 23]

An accurate and precise diagnosis of Scheuermann's disease is important to provide proper treatment and hopefully to avert disability. Although coexistence with osteoporotic vertebral fractures can occur, the disorders should be distinguished as disease treatment is very different.[6, 11, 24, 25] Radiographical find-

ings should be correlated with clinical symptoms that typically started at adolescent age,[26] nonetheless, taking into account recall bias. Some patients with adult Scheuermann's kyphosis are only moderately affected by the disease;[20, 27] future research might elucidate why some individuals have more complaints than others. In addition, the condition is generally not well known among clinical practitioners, which may cause diagnosis of Scheuermann's disease to be missed or delayed. The fairly high disease prevalence underscores that Scheuermann's disease should not be overlooked.

CONCLUSION

In sum, our study revealed a prevalence of 4.0% of Scheuermann's disease in the Dutch population. Standardized scoring of independent features resulted in substantial interobserver agreement, and different applications of diagnostic criteria did not alter disease classification.

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KEY POINTS

- The prevalence of radiographical Scheuermann's disease was 4.0% in a Dutch population sample aged 45 years and older.
- Vertebral wedging and endplate irregularities are significantly more prevalent among males.
- Standardized scoring of independent Scheuermann's disease radiographical features shows substantial interobserver agreement.
- Current diagnostic criteria for Scheuermann's disease seem sufficient, as different applications on a population level did not significantly alter disease classification.

REFERENCES

1. Hart ES, Merlin G, Harisiades J, Grottkau BE. Scheuermann's thoracic kyphosis in the adolescent patient. Orthop Nurs. 2010 Nov-Dec;29(6):365-71; quiz 72-3.
2. Damborg F, Engell V, Andersen M, Kyvik KO, Thomsen K. Prevalence, concordance, and heritability of Scheuermann kyphosis based on a study of twins. J Bone Joint Surg Am. 2006 Oct;88(10):2133-6.
3. Heithoff KB, Gundry CR, Burton CV, Winter RB. Juvenile discogenic disease. Spine (Phila Pa 1976). 1994 Feb 1;19(3):335-40.
4. Nissinen M. Spinal posture during pubertal growth. Acta Paediatr. 1995 Mar;84(3):308-12.

5. Papagelopoulos PJ, Mavrogenis AF, Savvidou OD, Mitsiokapa EA, Themistocleous GS, Soucacos PN. Current concepts in Scheuermann's kyphosis. Orthopedics. 2008 Jan;31(1):52-8; quiz 9-60.
6. Puisto V, Rissanen H, Heliovaara M, Knekt P, Helenius I. Mortality in the presence of a vertebral fracture, scoliosis, or Scheuermann's disease in the thoracic spine. Ann Epidemiol. 2008 Aug;18(8):595-601.
7. Sørensen KH. Scheuermann's Juvenile Kyphosis: Clinical Appearances, Radiography, Aetiology and Prognosis. Copenhagen: Munksgaard. 1964.
8. Sachs B, Bradford D, Winter R, Lonstein J, Moe J, Willson S. Scheuermann kyphosis. Follow-up of Milwaukee-brace treatment. J Bone Joint Surg Am. 1987 Jan;69(1):50-7.
9. Wenger DR, Frick SL. Scheuermann kyphosis. Spine (Phila Pa 1976). 1999 Dec 15;24(24):2630-9.
10. Bradford DS, Moe JH, Montalvo FJ, Winter RB. Scheuermann's kyphosis and roundback deformity. Results of Milwaukee brace treatment. J Bone Joint Surg Am. 1974 Jun;56(4):740-58.
11. Masharawi Y, Rothschild B, Peled N, Hershkovitz I. A simple radiological method for recognizing osteoporotic thoracic vertebral compression fractures and distinguishing them from Scheuermann disease. Spine (Phila Pa 1976). 2009 Aug 15;34(18):1995-9.
12. Pfirrmann CW, Resnick D. Schmorl nodes of the thoracic and lumbar spine: radiographic-pathologic study of prevalence, characterization, and correlation with degenerative changes of 1,650 spinal levels in 100 cadavers. Radiology. 2001 May;219(2):368-74.
13. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
14. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159-74.
15. Fotiadis E, Kenanidis E, Samoladas E, Christodoulou A, Akritopoulos P, Akritopoulou K. Scheuermann's disease: focus on weight and height role. Eur Spine J. 2008 May;17(5):673-8.
16. Lemire JJ, Mierau DR, Crawford CM, Dzus AK. Scheuermann's juvenile kyphosis. J Manipulative Physiol Ther. 1996 Mar-Apr;19(3):195-201.
17. Peterson CK, Bolton JE, Wood AR. A cross-sectional study correlating lumbar spine degeneration with disability and pain. Spine (Phila Pa 1976). 2000 Jan 15;25(2):218-23.
18. Niemelainen R, Battie MC, Gill K, Videman T. The prevalence and characteristics of thoracic magnetic resonance imaging findings in men. Spine (Phila Pa 1976). 2008 Nov 1;33(23):2552-9.
19. Lowe TG, Line BG. Evidence based medicine: analysis of Scheuermann kyphosis. Spine (Phila Pa 1976). 2007 Sep 1;32(19 Suppl):S115-9.
20. Murray PM, Weinstein SL, Spratt KF. The natural history and long-term follow-up of Scheuermann kyphosis. J Bone Joint Surg Am. 1993 Feb;75(2):236-48.
21. Rachbauer F, Sterzinger W, Eibl G. Radiographic abnormalities in the thoracolumbar spine of young elite skiers. Am J Sports Med. 2001 Jul-Aug;29(4):446-9.
22. Fisk JW, Baigent ML, Hill PD. Scheuermann's disease. Clinical and radiological survey of 17 and 18 year olds. Am J Phys Med. 1984 Feb;63(1):18-30.
23. Ristolainen L, Kettunen JA, Heliovaara M, Kujala UM, Heinonen A, Schlenzka D. Untreated Scheuermann's disease: a 37-year follow-up study. Eur Spine J. 2012 May;21(5):819-24.
24. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
25. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, A. H, et al. Review of Radiological Scoring Methods of Osteoporotic Vertebral Fractures for Clinical and Research Settings. Eur Rad. 2012;DOI: 10.1007/s00330-012-2622-z.
26. Summers BN, Singh JP, Manns RA. The radiological reporting of lumbar Scheuermann's disease: an unnecessary source of confusion amongst clinicians and patients. Br J Radiol. 2008 May;81(965):383-5.
27. Wood KB, Melikian R, Villamil F. Adult Scheuermann kyphosis: evaluation, management, and new developments. J Am Acad Orthop Surg. 2012 Feb;20(2):113-21.

Chapter 3.6

Identification of Scheuermann's disease with different radiological scoring methods for osteoporotic vertebral fractures: the Rotterdam Study

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In preparation

ABSTRACT

Purpose Osteoporotic vertebral fractures are a major health issue and should be diagnosed accurately. An important radiological differential diagnosis is Scheuermann's disease. Our aim was to evaluate how different radiological assessment methods perform on independently ascertained cases of radiological Scheuermann's disease.

Methods Two independent groups of trained research assistants applied the algorithm based qualitative (ABQ) or software-assisted quantitative morphometry (QM using SpineAnalyzer® software to measure vertebral height ratios, shape and severity of vertebral deformities classified according to Genant). These methods for osteoporotic vertebral deformities were compared on cases of radiological Scheuermann's disease, which were diagnosed by a third group applying the radiological criteria by Sørensen and Sachs. In addition, thoracic spine indexes according to Masharawi were calculated from the raw QM data. Complete data was available for 2,656 lateral spine radiographs (T4–L4) from the population-based Rotterdam Study (43% men, age 45–89), an ongoing Dutch prospective cohort study.

Results Radiographic Scheuermann's disease was present in 4.1% (95% CI: 3.3%–4.9%; N=109) of the sample. Of these participants identified with radiographic Scheuermann's disease, QM scored 65.1% (95% CI: 56.2%–74.1%; N=71) as having an osteoporotic vertebral deformity, while ABQ classified only 7.3% (95% CI: 2.4%–12.2%; N=8) of the participants with radiographic Scheuermann's disease as having osteoporotic vertebral fractures. The QM-based Masharawi method was unable to discern between osteoporotic vertebral fractures, Scheuermann's disease and controls.

Conclusions The inability of QM to rule out non-fracture deformities explains in part the higher number of osteoporotic vertebral deformities misclassified when applying QM as scoring method compared to ABQ.

INTRODUCTION

Osteoporotic vertebral fractures embody a clinically and publicly relevant health issue[1] as they occur relatively frequently[2] and are associated with various adverse disease outcomes.[3-6] Pharmacological treatment options include bisphosphonates [7-10], selective estrogen receptor modulators[11, 12], denosumab[13] and (in Europe) strontium ranelate[14].

Given the favorable efficacy of anti-osteoporotic therapy, it is important to adequately and accurately detect these spine fractures. Unfortunately, defining and diagnosing these fractures is not that simple as clinical work flows are too infrequently standardized nor is there a gold standard assessment method.[15]

Quantitative morphometry (QM)-based methods evaluate vertebral height loss by measuring the distance between points placed in the superior and inferior endplates at the anterior, middle and posterior aspects of the vertebral bodies.[16] Next, ratios between these heights are calculated to classify vertebral fractures; this process may be (semi-) automated.[17] Alternatively, the algorithm based qualitative (ABQ) method mainly judges endplate integrity visually by an expert reader, regardless of vertebral height reduction.[18]

A number of differential diagnoses complicate the diagnosis of vertebral fractures,[19, 20] among which Scheuermann's disease. Scheuermann's disease, or vertebral osteochondrosis, is a developmental defect of the spine giving rise to morphological changes of the thoracic vertebral column in particular.[21] The typical presentation is a frequently painful thoracic kyphosis and functional restriction of the spine, acquired mostly during adolescence.[22] The pathogenesis is unknown and probably there is a contribution of both genetic and environmental factors.[23] Scheuermann's disease is diagnosed on the basis of radiographical criteria of which the criteria by Sørensen and Sachs[24, 25] are the most commonly applied. There is a thoracic kyphosis greater than 45° and at least three adjacent wedge-shaped vertebral bodies of 5° or more and endplate irregularities with possible vertebral elongation and disc space narrowing. Also, Schmorl's nodes are thought to be a common but not obligate manifestation of Scheuermann's disease. Although coexistence of Scheuermann's disease with osteoporotic vertebral fractures can occur, the disorders should be distinguished as disease treatments differ.[26, 27]

The vertebral wedging or short vertebral height in Scheuermann's disease may be mistaken for mild vertebral fractures particularly by QM-based methods for vertebral fracture assessment, and Schmorl's nodes may mimic endplate depression.[28] The algorithm-based qualitative (ABQ) method for osteoporotic vertebral fractures has introduced a decision-making algorithm, which provides a guideline for systematically assessing various non-fracture deformities. Masharawi et al. introduced a QM-based second derivative index for distinguishing osteoporotic thoracic vertebral fractures from Scheuermann's disease. The aim of our study was to formally and objectively evaluate the discriminative performance for osteoporotic vertebral fractures of QM and ABQ on an independently assembled Scheuermann's disease case collection.

METHODS

The Rotterdam Study

The Rotterdam Study is a population-based cohort that targets the investigation of determinants of numerous chronic diseases and disability in Dutch men and women. The study design has been described in great detail by Hofman et al.[29] The present report describes results obtained from the Rotterdam Study-III cohort. In short, all inhabitants aged 45 years and over of the Ommoord district in the city of Rotterdam in The Netherlands were invited to participate from 2006 onwards. A baseline

home interview on medical history and risk factors for chronic diseases and medication use was taken by trained interviewers. Subsequently, participants were invited to the research center for clinical examination, including X-ray imaging of the spine. A trained research technician obtained standing lateral radiographs of the thoracolumbar spine of individuals visiting the research center. All radiographs were acquired digitally according to a standardized protocol, with a focus to detector distance of 120 cm. The Medical Ethics Committee of the Erasmus University Medical Center has approved the Rotterdam Study.

Vertebral fracture assessment

Radiographic examinations of the spine were obtained by a digitized Fuji FCR system (FUJIFILM Medical Systems). Two separate teams of trained research assistants assessed lateral spine radiographs (Th4–L4), using either ABQ or software-assisted QM (SpineAnalyzer®, Optasia Medical Ltd, Cheadle, UK) for the assessment of osteoporotic vertebral fractures on the complete collection of radiographs. For QM, vertebral bodies with height loss ≥20% were considered fractured. Finally, an additional two research assistants, naive to any osteoporotic vertebral fracture assessment methods, were supervised by a musculoskeletal radiologist in the application of the radiological criteria by Sørensen and Sachs et al.,[25] as described before[30]. To be established as Scheuermann's disease case, the following criteria should be met: 1) thoracic kyphosis greater than 45°; 2) at least three adjacent wedge-shaped vertebral bodies of 5° or more; and 3) endplate irregularities including optionally Schmorl's nodes (Figure 1). All radiographs satisfying these criteria were re-evaluated and confirmed by the second reader. All Scheuermann's disease cases were selected for the analyses presented in this article. A study diagram is presented in Figure 2.

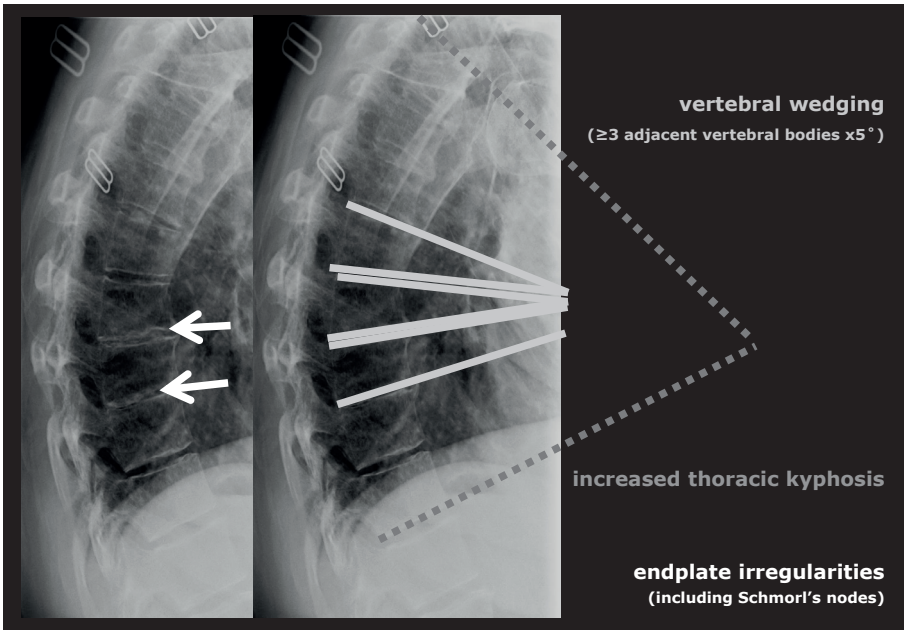

Figure 1. Lateral radiograph showing Scheuermann's disease with marked endplate irregularities (arrows) and mild anterior wedging of multiple midthoracic vertebrae (lines), resulting in increased thoracic kyphosis (dashed arc). The following criteria were applied to define Scheuermann's disease cases: 1) thoracic kyphosis greater than 45°; 2) at least three adjacent wedge-shaped vertebral bodies of 5° or more; and 3) endplate irregularities including optionally Schmorl's nodes.

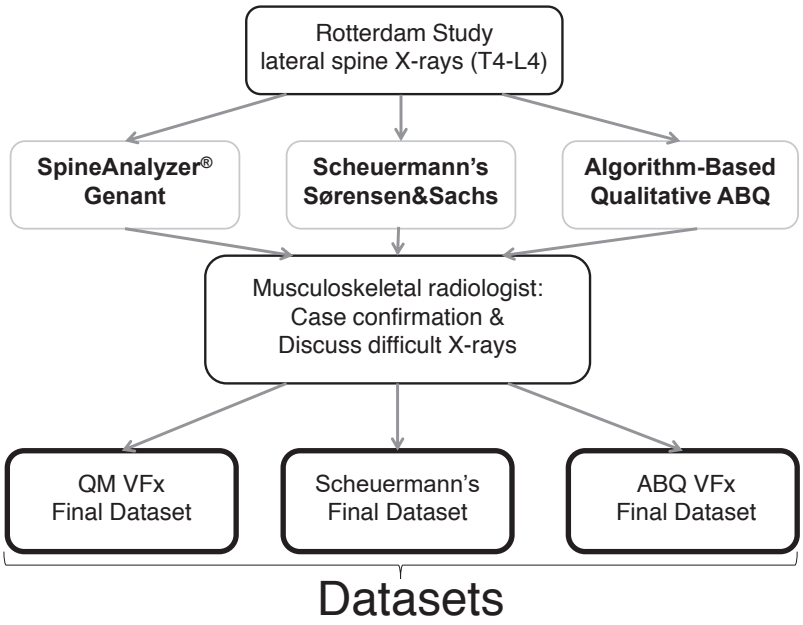

Figure 2. Study diagram: Lateral spine radiographs were assessed from levels T4 to L4 by separate trained assistant teams applying the two osteoporotic vertebral fracture methods, i.e., SpineAnalyzer® software-assisted quantitative morphometry (QM) and the algorithm-based qualitative method (ABQ). Thirdly, independent readers performed the readings for Scheuermann's disease. A musculoskeletal radiologist performed case confirmation and was available to discuss difficult cases. This resulted in two individual datasets for osteoporotic vertebral fractures and a third dataset for Scheuermann's disease describing the same study participants.

Statistical analysis

Prevalences of Scheuermann's disease and of osteoporotic vertebral fractures according to both ABQ and QM were determined in the study population. For QM, vertebral bodies with height loss ≥20% were considered fractured, as defined above. As some believe that most of the grade 1 or mild deformities are not osteoporotic vertebral fractures[31-33] and that Scheuermann's disease may present with only minor height reductions,[27] we performed secondary analyses by shifting the cut-off of 20% height loss to a more conservative threshold of 25% to compare results. Four indexes for the thoracic vertebrae T6-T10 were calculated according to Masharawi et al.: anterior height/posterior height (h_A/h_P, abbreviated as A/P); anterior height/mid height (h_A/h_M, A/M); mid height/posterior height (h_M/h_P, M/P); and a secondary derivate index ((h_A/h_M)/(h_M/h_P), (A/M)/(M/P)) [27]. Categories were created for participants: 1) satisfying the diagnostic characteristics of Scheuermann's disease; 2) meeting the definition stated by either ABQ or QM applying different thresholds; 3) controls which were negative for the aforementioned criteria. After exclusion of cases satisfying criteria for more than one subgroup, t-tests were applied to the subgroup analyses for the QM-based methods. SPSS statistics software version 20 (IBM, Armonk, NY, USA) and R software version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) were used for the analyses. Receiver operating characteristic (ROC) and area under the receiver operating characteristic curve (AUC) statistics were calculated with the pROC R-software package.

RESULTS

In total, 2,656 lateral spine radiographs from the Rotterdam Study were assessed with the three separate workflows (43% men, age 45-89 with a mean of 57 years) for the diagnostic criteria of radiographic Scheuermann's disease, osteoporotic vertebral fracture deformities according to ABQ and QM. Radiographic Scheuermann's disease was present in 4.1% (95% CI: 3.3%–4.9%; N=109) of the study sample. Of these participants identified with radiographic Scheuermann's disease, QM scored 65.1% (95% CI: 56.2%–74.1%; N=71) as having an osteoporotic vertebral deformity, while ABQ classified only 7.3% (95% CI: 2.4%–12.2%; N=8) of the participants with radiographic Scheuermann's disease as having osteoporotic vertebral fractures (Figure 3). For both methods, exclusion of the lumbar vertebral levels resulted in exactly the same outcomes as stated above. For QM, the proportion of radiographic Scheuermann's disease cases with at least one vertebral body with height loss of 25% or greater was 40.4% (95% CI: 31.1–49.6%; N=44).

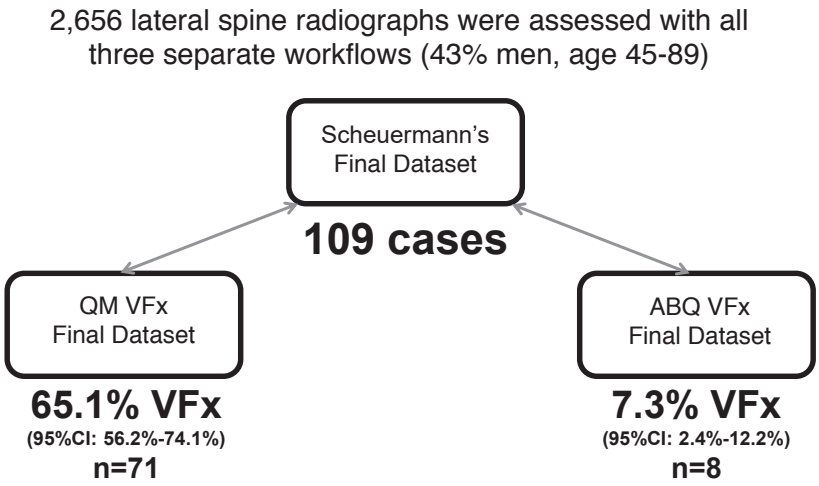

Figure 3. In total, 2,656 lateral spine radiographs were assessed with the three separate workflows for the diagnostic criteria of radiographic Scheuermann's disease, which yielded 109 cases. Of these participants identified with radiographic Scheuermann's disease, QM scored 65.1% (95% CI: 56.2%-74.1%; N=71) as having an osteoporotic vertebral deformity, while ABQ classified 7.3% (95% CI: 2.4%-12.2%; N=8) as having osteoporotic vertebral fractures.

Next, indexes for the thoracic vertebrae T6-T10 were calculated according to the methodology by Masharawi et al., for which results are displayed in Table 1 for the different subgroups.[27] No statistical differences were detected in any indexes of any of the thoracic vertebrae tested between the groups of Scheuermann's disease cases and QM-based osteoporotic vertebral fracture cases regardless of the diagnostic definition applied (Supplementary Tables 1 and 2). The best discriminative ability for distinguishing Scheuermann's disease cases from QM-based osteoporotic vertebral deformities reached was an AUC of 0.60 for a threshold of 0.99 for the (A/M)/(M/P) derivative of vertebra T7 applied to a vertebral height cut-off of 20%, as displayed in Figure 4. Under those conditions, the sensitivity would be 32% (95% CI: 18%–47%) with a specificity of 89% (95% CI: 88%–91%). Full results and ROC curves for the different diagnostic definitions and respective vertebral levels are available in the Supplementary materials.

Table 1. Vertebral body height indices: mean and standard deviations

Studied groups	Thoracic vertebrae	Anterior/ Posterior	Anterior/ Middle	Middle/ Posterior	(Anterior/Middle)/ (Middle/Posterior)
		(SD)	(SD)	(SD)	(SD)
TOTAL	T6	0.91	0.99	0.92	1.08
N=2,656	(N=2,545)	(0.06)	(0.05)	(0.04)	(0.08)
	T7	0.89	0.98	0.92	1.07
	(N=2,632)	(0.06)	(0.05)	(0.04)	(0.08)
	T8	0.89	0.97	0.92	1.06
	(N=2,643)	(0.06)	(0.05)	(0.04)	(0.07)
	T9	0.92	0.99	0.93	1.07
	(N=2,641)	(0.06)	(0.05)	(0.04)	(0.07)
	T10	0.93	1.00	0.93	1.07
	(N=2,643)	(0.06)	(0.05)	(0.04)	(0.06)
	Total N=13,104				
Control	T6	0.91	0.99	0.92	1.08
N=2,547	(N=2,438)	(0.05)	(0.05)	(0.04)	(0.08)
	T7	0.90	0.98	0.92	1.07
	(N=2,523)	(0.05)	(0.05)	(0.04)	(0.07)
	T8	0.90	0.98	0.92	1.07
	(N=2,534)	(0.06)	(0.05)	(0.04)	(0.07)
	T9	0.92	0.99	0.93	1.07
	(N=2,532)	(0.06)	(0.05)	(0.04)	(0.06)
	T10	0.93	1.00	0.93	1.07
	(N=2,534)	(0.06)	(0.05)	(0.04)	(0.06)
	Total N=12,561				
Scheuermann	T6	0.85	0.95	0.89	1.07
N=109	(N=107)	(0.08)	(0.06)	(0.05)	(0.08)
	T7	0.82	0.93	0.88	1.06
	(N=109)	(0.08)	(0.06)	(0.07)	(0.22)
	T8	0.81	0.92	0.88	1.05
	(N=109)	(0.07)	(0.05)	(0.05)	(0.08)
	T9	0.85	0.95	0.90	1.06
	(N=109)	(0.08)	(0.06)	(0.05)	(0.09)
	T10	0.88	0.96	0.91	1.06
	(N=109)	(0.08)	(0.05)	(0.05)	(0.07)
	Total N=543				
Osteoporosis QM	T6	0.88	0.97	0.91	1.07
N=562	(N=534)	(0.08)	(0.06)	(0.05)	(0.08)
	T7	0.86	0.95	0.90	1.07
	(N=558)	(0.08)	(0.06)	(0.05)	(0.11)
	T8	0.86	0.95	0.90	1.06
	(N=560)	(0.08)	(0.06)	(0.05)	(0.07)
	T9	0.89	0.97	0.92	1.06
	(N=559)	(0.07)	(0.06)	(0.04)	(0.07)
	T10	0.90	0.98	0.92	1.06
	(N=559)	(0.07)	(0.05)	(0.04)	(0.07)
	Total N=2,770				

Table 1. Vertebral body height indices: mean and standard deviations (continued)

Studied groups	Thoracic vertebrae	Anterior/ Posterior	Anterior/ Middle	Middle/ Posterior	(Anterior/Middle)/ (Middle/Posterior)
Osteoporosis ABQ	T6	0.88	0.99	0.90	1.10
N=85	(N=80)	(0.08)	(0.06)	(0.06)	(0.09)
	T7	0.85	0.96	0.88	1.12
	(N=83)	(0.11)	(0.06)	(0.09)	(0.23)
	T8	0.87	0.97	0.90	1.08
	(N=83)	(0.10)	(0.06)	(0.07)	(0.08)
	T9	0.91	0.98	0.92	1.07
	(N=83)	(0.06)	(0.05)	(0.04)	(0.07)
	T10	0.91	0.99	0.92	1.07
	(N=83)	(0.08)	(0.06)	(0.05)	(0.07)
	Total N=412				
Osteoporosis QM	T6	0.86	0.96	0.89	1.08
N=184	(N=174)	(0.10)	(0.07)	(0.06)	(0.09)
Moderate: >25%	T7	0.84	0.94	0.89	1.08
	(N=183)	(0.10)	(0.07)	(0.07)	(0.17)
	T8	0.84	0.94	0.89	1.07
	(N=183)	(0.09)	(0.07)	(0.06)	(0.09)
	T9	0.88	0.96	0.91	1.06
	(N=182)	(0.09)	(0.06)	(0.05)	(0.08)
	T10	0.89	0.97	0.92	1.06
	(N=182)	(0.08)	(0.06)	(0.05)	(0.06)
	Total N=904				

Standard deviation (SD); quantitative morphometry (QM); algorithm-based qualitative method (ABQ).

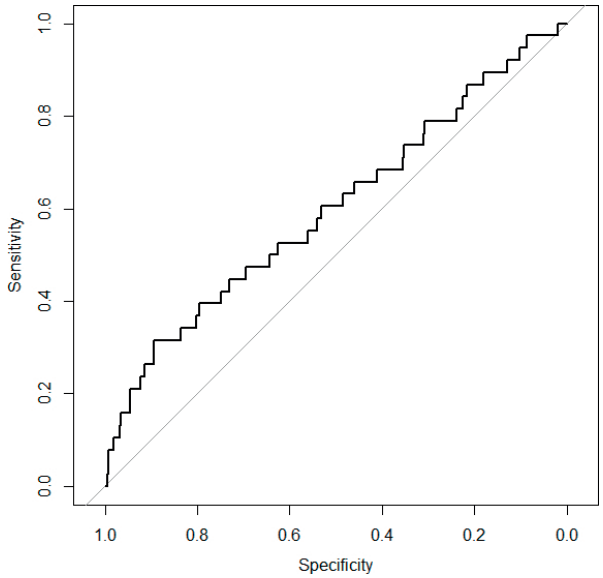

Figure 4. Receiver operating characteristic (ROC) curve for the secondary derivate index of anterior height/mid height relative to mid height/posterior height (A/M)/(M/P) of vertebra T7 applied to a quantitative morphometry (QM) vertebral height cut-off of 20%.

DISCUSSION

Our study evaluated the discriminative performance for osteoporotic vertebral fractures of QM and ABQ on an independently assembled Scheuermann's disease case collection. ABQ assessed a significantly larger proportion of the Scheuermann's diseases cases as non-fractures, whereas QM was less able to differentiate from osteoporotic vertebral fractures, even when applying a specific quantitative method in addition. Sensitivity analyses excluding lumbar levels showed the exact results and adapting the QM cut-off to a moderate level changed the results to a limited extent.

It has been put forward that the vertebral wedging in Scheuermann's disease could easily be differentiated from osteoporotic vertebral fractures by using the derivative of the anterior and mid height divided by the mid and posterior height, because the height reduction in Scheuermann's disease would be less severe and follows a different morphometry.[27] Unfortunately, we were unable to replicate these results yielding an AUC of around 0.60, which may even be an overestimation in a discovery sample, with sensitivities and specificities which would be unsatisfactory in a clinical setting. Regarding the less severe height reduction, although true in a proportion of the cases, almost half of the Scheuermann's disease cases have moderate or even severe vertebral height reduction, thus simply adjusting the vertebral height loss criterion in QM from 20% to for instance 25% will not simply solve the problem.

Limitations of current morphometry analyses include the inability of pattern recognition and the fact that morphometry is not be able to capture qualitative imaging features such as vertebral endplate irregularities, particularly the sclerotic margins[34]. ABQ as a qualitative method mainly assesses central endplate integrity, and unlike QM, is not that much affected by non-fracture deformities with vertebral height loss due to other causes, because there is no minimum requirement for vertebral height loss. ABQ further introduced an algorithm to systematically exclude these non-fracture deformities that may mimic osteoporotic spine fractures. Agreement between diagnostic methods improves by identifying non-fracture deformities such as Scheuermann's disease and it has been demonstrated in the MrOS study that 17% of the discordant cases could be attributed to Scheuermann's disease.[33] Nonetheless, application of qualitative assessment methods on a large-scale is still much more labor-intensive than semi-automated quantitative measures and requires specific training and expertise, thus automation of qualitative means would be desirable.[16, 30]

Strengths of our study are the large-scale and impartial systematic evaluation of several radiological methods in a population-based cohort. The mean age in our study was younger with a more narrow range than in the case-control study sample of Masharawi et al.[27] Nonetheless, our study has some limitations. More variability may have been introduced because of the greater number of readers, which was then again necessary to ensure an unbiased evaluation according to separate work flows. Although radiographs were assessed by well-trained reader teams, it was not feasible to have all radiographs assessed by musculoskeletal radiologists. We are aware that more subtle disease cases could have been misclassified, even in spite of the availability of consensus readings attended by a musculoskeletal radiologist.

Although we cannot exclude that radiographic Scheuermann's disease and osteoporotic vertebral fractures may co-exist, general consensus is that radiographic Scheuermann's disease and osteoporotic vertebral fractures are two different entities that should be diagnosed differentially.[26, 27] Differentiating Scheuermann's disease from osteoporotic vertebral fractures is clinically relevant, thus it is important to be aware of the diagnostic discrepancies high-lighted by our study.[35] Precise vertebral fractures diagnosis is needed to identify patients with high risk for future fractures to optimize patient management, nonetheless, overdiagnosis will result in overtreatment with anti-osteoporotic medication as there is

no evidence that osteoporosis treatment would be effective in Scheuermann's disease. Radiographic Scheuermann's disease and osteoporotic vertebral fractures should be accurately diagnosed differentially by standardized assessments as this supports proper osteoporosis patient management.[33] An increased awareness of the differential diagnoses of osteoporotic vertebral fractures among health professions including radiologists is desirable.

Accurate distinction between different disease entities such as osteoporosis and Scheuermann's disease lays at the basis of etiological investigations. For example, genetics contribute to the occurrence of osteoporotic vertebral fractures.[36, 37] A large-scale global meta-analytical effort found evidence for a vertebral fracture-associated genetic marker, however, the replication phase failed to validate this finding, which may have been due to phenotyping issues.[38] Proper case classification will not only enable genetic studies, but also a myriad of other epidemiological and clinical studies into osteoporotic vertebral fractures. In this line of thinking, the heritability estimate of Scheuermann's disease has been projected highly at 74%, which could encourage further genetic epidemiological studies.[39] Extensive radiological assessments may refine phenotyping and even enable more and better research and unlock new traits worth follow-up.

In conclusion, a significantly higher proportion of radiographic Scheuermann's disease cases were misclassified by SpineAnalyzer® software with Genant criteria (QM) as having vertebral fractures than with the ABQ qualitative method. No QM-based method is yet available to adequately differentiate between radiographic Scheuermann's disease and osteoporotic vertebral fractures. This diagnostic discrepancy is both scientifically and clinically relevant; more developments are needed in this field.

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Supplementary information is available at:
http://www.glimdna.org/publicationdata.html

REFERENCES

1. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I:1-65.
2. Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2002 Jun;17(6):1051-6.

3. Burger H, Van Daele PL, Grashuis K, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities and functional impairment in men and women. J Bone Miner Res. 1997 Jan;12(1):152-7.
4. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. J Am Geriatr Soc. 2000 Mar;48(3):241-9.
5. Nevitt MC, Ettinger B, Black DM, Stone K, Jamal SA, Ensrud K, et al. The association of radiographically detected vertebral fractures with back pain and function: a prospective study. Ann Intern Med. 1998 May 15;128(10):793-800.
6. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C, et al. Health-related quality of life in postmenopausal women with low BMD with or without prevalent vertebral fractures. J Bone Miner Res. 2000 Jul;15(7):1384-92.
7. Black DM, Cummings SR, Karpf DB, Cauley JA, Thompson DE, Nevitt MC, et al. Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group. Lancet. 1996 Dec 7;348(9041):1535-41.
8. Hochberg MC, Ross PD, Black D, Cummings SR, Genant HK, Nevitt MC, et al. Larger increases in bone mineral density during alendronate therapy are associated with a lower risk of new vertebral fractures in women with postmenopausal osteoporosis. Fracture Intervention Trial Research Group. Arthritis Rheum. 1999 Jun;42(6):1246-54.
9. Lyles KW, Colon-Emeric CS, Magaziner JS, Adachi JD, Pieper CF, Mautalen C, et al. Zoledronic Acid in Reducing Clinical Fracture and Mortality after Hip Fracture. N Engl J Med. 2007;357:nihpa40967.
10. Boonen S, Reginster JY, Kaufman JM, Lippuner K, Zanchetta J, Langdahl B, et al. Fracture risk and zoledronic acid therapy in men with osteoporosis. N Engl J Med. 2012 Nov;367(18):1714-23.
11. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA. 1999 Aug 18;282(7):637-45.
12. Siris E, Adachi JD, Lu Y, Fuerst T, Crans GG, Wong M, et al. Effects of raloxifene on fracture severity in postmenopausal women with osteoporosis: results from the MORE study. Multiple Outcomes of Raloxifene Evaluation. Osteoporos Int. 2002 Nov;13(11):907-13.
13. Cummings SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR, et al. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. N Engl J Med. 2009 Aug 20;361(8):756-65.
14. Reginster JY, Felsenberg D, Boonen S, Diez-Perez A, Rizzoli R, Brandi ML, et al. Effects of long-term strontium ranelate treatment on the risk of nonvertebral and vertebral fractures in postmenopausal osteoporosis: Results of a five-year, randomized, placebo-controlled trial. Arthritis Rheum. 2008 Jun;58(6):1687-95.
15. Guglielmi G, Diacinti D, van Kuijk C, Aparisi F, Krestan C, Adams JE, et al. Vertebral morphometry: current methods and recent advances. Eur Radiol. 2008 Jul;18(7):1484-96.
16. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, Hofman A, et al. Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. Eur Radiol. 2013 Feb;23(2):476-86.
17. Brett A, Miller CG, Hayes CW, Krasnow J, Ozanian T, Abrams K, et al. Development of a clinical workflow tool to enhance the detection of vertebral fractures: accuracy and precision evaluation. Spine (Phila Pa 1976). 2009 Oct 15;34(22):2437-43.
18. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
19. Link TM, Guglielmi G, van Kuijk C, Adams JE. Radiologic assessment of osteoporotic vertebral fractures: diagnostic and prognostic implications. Eur Radiol. 2005 Aug;15(8):1521-32.
20. Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res. 1993 Sep;8(9):1137-48.
21. Hart ES, Merlin G, Harisiades J, Grottkau BE. Scheuermann's thoracic kyphosis in the adolescent patient. Orthop Nurs. 2010 Nov-Dec;29(6):365-71; quiz 72-3.

22. Greene TL, Hensinger RN, Hunter LY. Back pain and vertebral changes simulating Scheuermann's disease. J Pediatr Orthop. 1985 Jan-Feb;5(1):1-7.
23. Fotiadis E, Kenanidis E, Samoladas E, Christodoulou A, Akritopoulos P, Akritopoulou K. Scheuermann's disease: focus on weight and height role. Eur Spine J. 2008 May;17(5):673-8.
24. Wenger DR, Frick SL. Scheuermann kyphosis. Spine (Phila Pa 1976). 1999 Dec 15;24(24):2630-9.
25. Sachs B, Bradford D, Winter R, Lonstein J, Moe J, Willson S. Scheuermann kyphosis. Follow-up of Milwaukee-brace treatment. J Bone Joint Surg Am. 1987 Jan;69(1):50-7.
26. Puisto V, Rissanen H, Heliovaara M, Knekt P, Helenius I. Mortality in the presence of a vertebral fracture, scoliosis, or Scheuermann's disease in the thoracic spine. Ann Epidemiol. 2008 Aug;18(8):595-601.
27. Masharawi Y, Rothschild B, Peled N, Hershkovitz I. A simple radiological method for recognizing osteoporotic thoracic vertebral compression fractures and distinguishing them from Scheuermann disease. Spine (Phila Pa 1976). 2009 Aug 15;34(18):1995-9.
28. Ferrar L, Jiang G, Adams J, Eastell R. Identification of vertebral fractures: an update. Osteoporos Int. 2005 Jul;16(7):717-28.
29. Hofman A, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, Ikram MA, et al. The Rotterdam Study: 2014 objectives and design update. Eur J Epidemiol. 2013 Nov;28(11):889-926.
30. Makurthou AA, Oei L, Saddy SE, Breda SJ, Castano-Betancourt MC, Hofman A, et al. Scheuermann's Disease: Evaluation of Radiological Criteria and Population Prevalence. Spine (Phila Pa 1976). 2013 Jun 11.
31. Vokes TJ, Gillen DL, Pham AT, Lovett JM. Risk factors for prevalent vertebral fractures in black and white female densitometry patients. J Clin Densitom. 2007 Jan-Mar;10(1):1-9.
32. Ziegler R, Scheidt-Nave C, Leidig-Bruckner G. What is a vertebral fracture? Bone. 1996 Mar;18(3 Suppl):169S-77S.
33. Ferrar L, Jiang G, Cawthon PM, San Valentin R, Fullman R, Lambert L, et al. Identification of vertebral fracture and non-osteoporotic short vertebral height in men: the MrOS study. J Bone Miner Res. 2007 Sep;22(9):1434-41.
34. Oei L, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJ, et al. Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses. Quant Imaging Med Surg. 2013 Oct;3(5):249-55.
35. Breda SJ, Oei L, Oei EH, Zillikens MC. [Osteoporotic vertebral fractures or Scheuermann's disease?]. Ned Tijdschr Geneeskd. 2013;157(45):A6479.
36. Campos-Obando N, Oei L, Hoefsloot LH, Kiewiet RM, Klaver CC, Simon ME, et al. Osteoporotic vertebral fractures during pregnancy: be aware of a potential underlying genetic cause. J Clin Endocrinol Metab. 2014 Apr;99(4):1107-11.
37. Liu CT, Karasik D, Zhou Y, Hsu YH, Genant HK, Broe KE, et al. Heritability of prevalent vertebral fracture and volumetric bone mineral density and geometry at the lumbar spine in three generations of the Framingham study. J Bone Miner Res. 2012 Apr;27(4):954-8.
38. Oei L, Estrada K, Duncan EL, Christiansen C, Liu C-T, Langdahl BL, et al. Genome-wide association study for radiographic vertebral fractures: A potential role for the 16q24 BMD locus. Bone. 2014;59(0):20-7.
39. Damborg F, Engell V, Andersen M, Kyvik KO, Thomsen K. Prevalence, concordance, and heritability of Scheuermann kyphosis based on a study of twins. J Bone Joint Surg Am. 2006 Oct;88(10):2133-6.

Chapter 3.7

CLINICAL LESSON

Osteoporotic vertebral fractures or Scheuermann's disease

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ABSTRACT

Introduction Recent osteoporotic vertebral fractures are considered as a strong indication for anti-osteoporotic treatment because they are highly predictive of future fracture risk. There are a number of differential diagnoses that have to be considered in individuals with vertebral deformities, including Scheuermann's disease.

Case description A 56-year-old man was incidentally diagnosed with osteoporotic vertebral fractures on a chest radiograph taken for heavy cough and was prescribed bisphosphonate therapy. The patient visited our outpatient clinic for a second opinion because of gastrointestinal complaints. Our re-evaluation with a DXA scan and lateral spine radiographs resulted in the diagnosis of Scheuermann's disease. His gastrointestinal complaints resolved after stopping bisphosphonates. A 42-year-old man with limb-girdle muscular dystrophy type 2B was analyzed because of back pain. Radiographs showed height loss of multiple thoracic vertebrae. Yet, bone mineral density measured by DXA was high-normal and MRI scans were suggestive for Scheuermann's disease. Therefore, there was no indication for osteoporosis medical treatment or vertebroplasty.

Conclusion A number of differential diagnoses have to be considered in individuals with vertebral deformities, including Scheuermann's disease. Recognition is important to avoid unnecessary medical treatment, which should be reserved for patients with osteoporosis. Refined vertebral fracture definitions may help improve diagnostic accuracy.

Ladies and Gentlemen,

The diagnosis of an osteoporotic vertebral fracture may have major consequences for the patient since these fractures in are an important criterion in various clinical guidelines for proceeding with drug therapy for osteoporosis.[1,2] These fractures are associated with increased morbidity and mortality and are important predictors of future fractures.[3] An accurate and timely diagnosis of osteoporotic vertebral fractures is therefore crucial. A disease that can lead to falsely diagnosed osteoporotic vertebral fractures is Scheuermann's disease. We illustrate this with the following two case histories.

Patient A, a 56-year-old man consulted a general practitioner in response to periodic severe cough without sputum. The general practitioner had made a chest radiograph, in which as secondary finding vertebral were reported. The patient was known to have a scoliosis. Since childhood he had no pain and never fractured. The family history mentioned a wrist fracture and substantial loss of height with his mother after age 50, but not hip fractures. There were no other risk factors for osteoporosis.

The patient was referred to the department of internal medicine of a general hospital because of the clinical suspicion of osteoporosis. There therapy with alendronate was started based on the working hypothesis of osteoporotic vertebral fractures with a possible family history of osteoporosis. He was also advised to limit physical exertion to prevent the risk of fractures due to falling. Several months later, the patient was referred on his own request by the general practitioner to the Bone Center of our hospital for a second opinion. He had developed gastrointestinal complaints since the initiation of alendronate treatment and had become insecure by the recommended physical limitations. On physical examination, there was a slight scoliosis and a slightly increased thoracic kyphosis. For the first time, we performed dual-energy X-ray absorptiometry (DXA) scanning and made new radiographs of the thoracolumbar spine. The DXA scan showed normal bone density with a T score of +0.0 SD in the lumbar spine and +0.4 SD in the left femoral neck. An X-ray examination showed a thoracic scoliosis, and also increased kyphosis with a Cobb's angle of 43 degrees with concomitant anterior height loss of the mid-thoracic vertebrae (T7,T8 and T9) of which at least one with more than 25% anterior height loss (T8) (Figure 1). The lumbar lordosis was largely lost and there were Schmorl's nodes present in the endplates of the lumbar spine. We diagnosed the patient with Scheuermann's disease and advised to discontinue the use of alendronate, after which the gastrointestinal symptoms of the patient resolved.

Patient B, a 42-year-old man, who was known with limb-girdle muscular dystrophy type 2B since about twenty years, had been wheelchair bound for three years. A progressive pain in the middle of the back had developed gradually in the last six months, and in recent weeks this had been so severe that he awakes at night. The patient had no prior history of fractures. His paternal grandmother had shrunk to a very small height in later life and she suffered from a hip fracture around her 80th year of life. In addition to the immobilization, the muscular disease and the positive family history, there were no other risk factors for osteoporosis. The rehabilitation physician had ordered radiographs because of the severe back pain, which revealed vertebral deformities of T10, T11 and T12 (Figure 2). The rehabilitation doctor referred the patient to the Bone Center of our hospital for further analysis and treatment. The laboratory examination showed no evidence of secondary osteoporosis. The DXA scan unexpectedly showed a high bone density of the lumbar vertebrae (+ 3.4 SD) and a normal femoral neck BMD (- 0.1 SD). Because of the strong discrepancy between the back pain, the risk factors and vertebral deformities on the one hand, and the (high) normal bone density on the other hand, an MRI of the spine was performed. The findings of this MRI appeared to support the diagnosis of Scheuermann´s disease more than osteoporotic vertebral fractures (Figure 3). There was no indication of drug treatment for osteoporosis or to perform a vertebroplasty. In retrospect, Schmorl´s nodes were also visible on the plain radiographs, but they were not as obvious as in patient A.

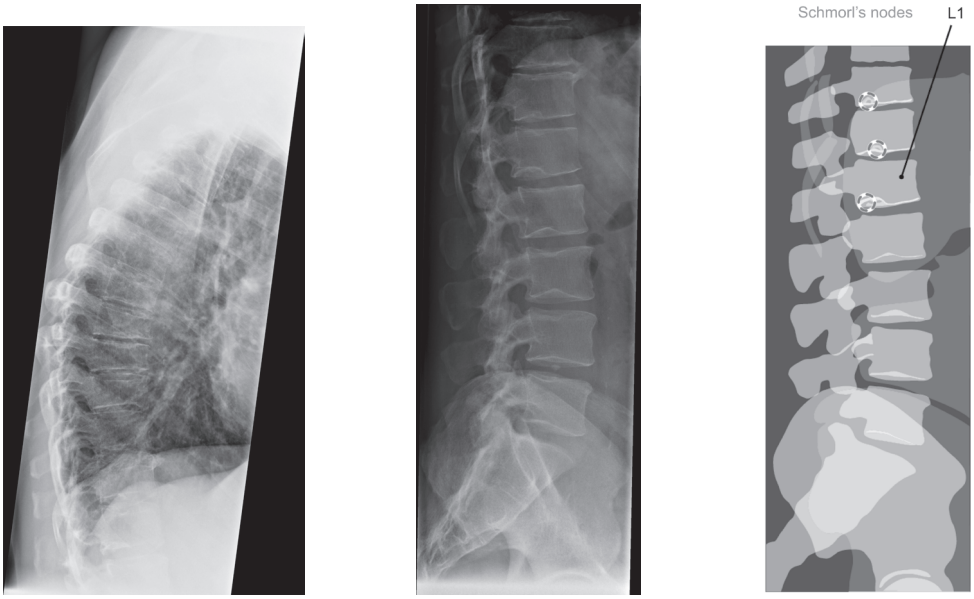


Figure 1. Lateral radiographs of the thoracic and lumbar spine in patient A, a 56-year old man. There is enhanced kyphosis with anterior height loss of mid-thoracic vertebrae T6-9 and Schmorl's nodes on vertebral levels T11 to L1 (arrows). The thoracic vertebral endplates display irregularities and degenerative osteophyte formation.

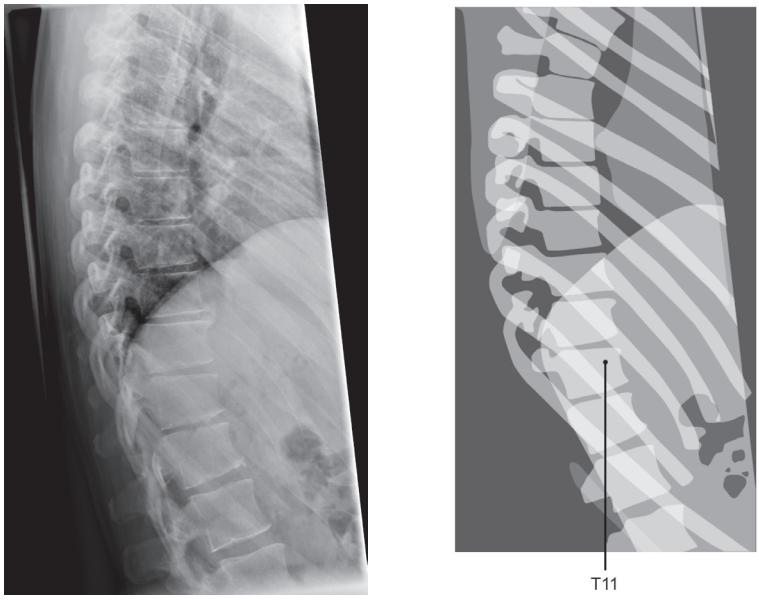


Figure 2. Lateral radiographs of patient B, a 42-year old man, of the mid-thoracic up to the high lumbar spine. There is presence of a wedge deformity with 28% height loss of T11 and to a lesser extent of T10 and T12, with concomitant spondylarthrotic changes of the thoracic spine.

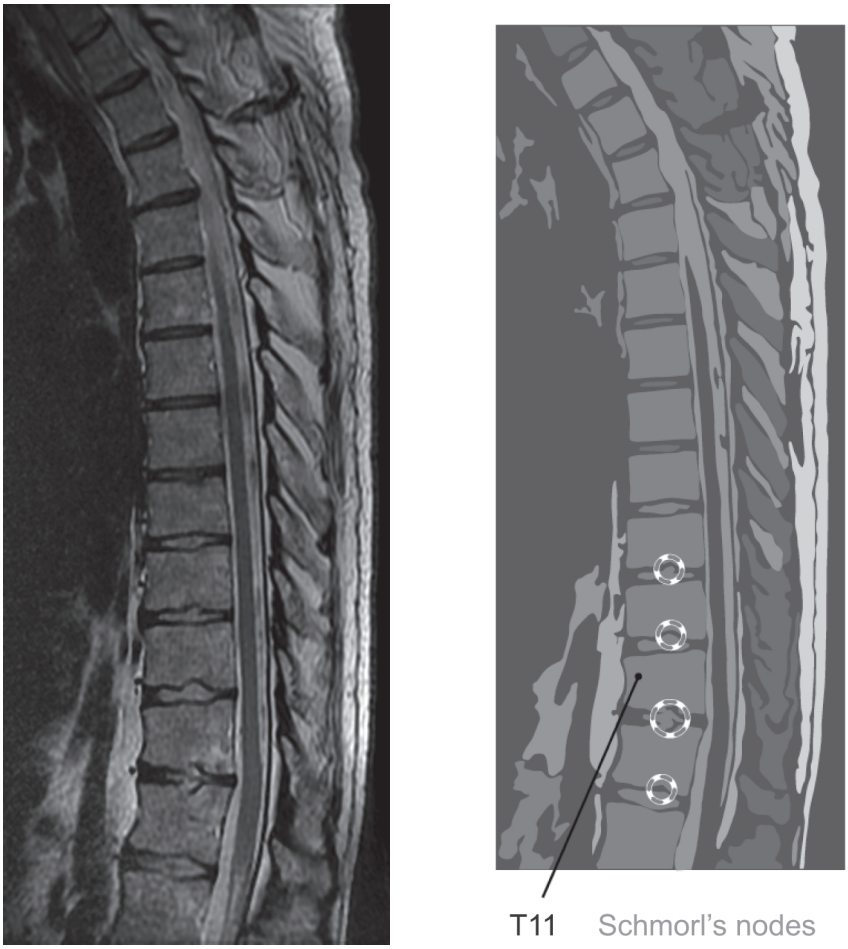


Figure 3. Sagittal T2-weighted MR image of the middle and lower thoracic spine of patient B shows the known anterior wedging of vertebrae T11 and to a lesser extent of T10 and T12. Also, obvious endplate irregularities at the levels of T9 to T12 with multiple Schmorl's nodes (arrows), fitting Scheuermann's disease.

DISCUSSION

A variety of disorders with morphological changes of the vertebrae can complicate the radiologic assessment of the spine.[4] The diagnosis of vertebral fractures on radiographs requires particular expertise in interpreting all possible deformations of the vertebrae. Especially in the mid-thoracic region the differential diagnosis of wedged vertebrae is wide. In addition to osteoporotic spinal fractures, Scheuermann's disease, degenerative disorders and anatomical shape variations are also associated with wedging of the vertebrae. For an online course about osteoporotic vertebral fractures we refer to the website of the International Osteoporosis Foundation (www.iofbonehealth.org/vertebral-fractureteaching-program).

Scheuermann's disease

Scheuermann's disease, or juvenile osteochondrosis of the spine, is a growth disorder of the spinal column that is accompanied by morphological changes, especially of the thoracic spine. Prevalence

estimates vary between 0.4% and 10% and the typical presentation is a (painful) thoracic kyphosis of the spine, evolving in adolescence.[5,6] The pathogenesis is unknown and probably consists of both genetic and environmental factors. Radiological diagnostic criteria for Scheuermann's disease on spinal radiographs include wedging of three or more adjacent vertebrae of each at least five degrees with possible presence of additional criteria such as an enhanced kyphosis angle, narrowing of the intervertebral discs, irregularity of the endplates and Schmorl's nodes. Our unpublished 'Rotterdam Study' data revealed that more than 40% of the patients with Scheuermann's disease has a vertebral body with at least 25% height loss. Usually conservative treatment is sufficient because symptoms often disappear when skeletal development is complete.[5] Other treatment options include posture advice and exercise, wearing a corset, or surgical correction of severe cases.[6]

Osteoporotic vertebral fractures

Osteoporotic vertebral fractures also lead to morphological changes of the spine, however unlike Scheuermann's disease, osteoporosis often is a metabolic bone disease of the elderly population.[3] In the Netherlands it has been estimated that every year more than 80,000 patients aged 50 years and older suffer from a fracture.[1] Vertebral fractures are the most common fractures related to osteoporosis.

There are several radiological assessment methods for the determination of osteoporotic vertebral fractures.[4] The most common method is based on quantification of height loss of the vertebral body according to a modification of the method by Genant, where depressions are defined as wedge (front), biconcave (center) or crush (posterior).[7] The recent CBO guideline 'osteoporosis and fracture prevention' and the NHG guideline 'fracture prevention' recommend a cut-off of at least 25% vertebral height loss for establishing a diagnosis of osteoporotic vertebral fracture.[1,2] However, in addition to the measurement of vertebral height loss it is also important to pay attention to other abnormalities that may fit alternative diagnoses such as in this case Scheuermann's disease. According to a recently propagated algorithm-based qualitative (ABQ) method anterior height loss of more than 20% and even of more than 25% without the collapse of the endplate can also be due to anatomical variants and degenerative conditions.[8]

On the other hand, osteoporotic vertebral fractures are also underreported.[3] It is often difficult to establish the diagnosis of a spinal fracture, as they often occur without prior trauma, unlike the non-vertebral fractures, such as those of the hip or wrist. Also, many of the osteoporotic vertebral fractures are asymptomatic; yet even these fractures are associated with negative health outcomes.[3]

After diagnosis of a vertebral fracture, the risk of a new vertebral fracture is increased 12.6 times and the chance to sustain a hip fracture within 10 years is 22%.[9] Treatment options to reduce risks consist of lifestyle changes, such as increased exercise and sun exposure, quitting smoking and limiting alcohol consumption, adequate intake of calcium and vitamin D and drug therapy. Bisphosphonates are the first choice of pharmacotherapy, selective estrogen receptor modulators (SERMs), strontium ranelate and denosumab.[10] In the Netherlands, PTH-analogs are only reimbursed in postmenopausal women and recently also in men with elevated fracture risk who sustained one or more fractures after already having had two vertebral fractures, or those that cannot tolerate other medication. Again, adequate diagnosis of vertebral fractures is essential.

EPICRISIS

Both guidelines (according to CBO less than one year and according to NHG less than two years ago) consider recent vertebral fractures in people older than 50 years an indication for drug therapy, indepen-

dent of bone mineral density (BMD).[1,2] Concerning fractures of earlier date, a risk assessment according to a points system is advised to decide whether or not to perform DXA scanning.

In patient A vertebral fractures have been reported as a secondary finding. If these were considered old fractures they could have been omitted for lack of a high risk score of >4 points.[1,2] Additionally, according to both the CBO as the NHG guidelines there was no indication for further examinations. Therefore, no osteoporosis drug therapy would have been required. Evidence for the recent nature of the fracture could be found by good history taking including if there was back pain during coughing, comparing with previous radiological examinations, assessment of recent loss of standing height, the absence of trauma in the past, or possibly by evaluation of bone marrow edema on MRI.

Before patient A came to our center, it was assumed that the vertebral deformities had occurred recently during coughing, and therefore, pharmacological treatment was instituted. There were no old radiographs available and the patient could not remember any trauma in the past. A perfectly normal BMD, as was later found by us, would be an argument to doubt the diagnosis of osteoporotic vertebral fractures and to re-examine the radiographs critically.

Despite the fact that the two guidelines advise treatment of vertebral fractures independently of BMD, this case shows that when in doubt about whether or not fractures are recent, a DXA scan can provide additional diagnostic information. This especially concerns young men without an obviously high risk of fractures.

Although the CBO and the NHG guidelines only apply to patients aged 50 years and over, patient B would have most likely been treated with medication in second care because of the suspicion of clinical vertebral fractures with severe back pain. In addition, there was a greatly increased risk of osteoporotic fractures due to the combination of complete immobilization, a muscular disease and a positive family history. The unexpectedly high BMD was reason to doubt the diagnosis of osteoporotic vertebral fractures and to proceed with performing an MRI scan to further examine the vertebrae.

Ladies and Gentlemen, both in the recent CBO guideline 'osteoporosis and fracture prevention' as the NHG guideline 'fracture prevention' great importance is rightfully attached to the presence of vertebral fractures due to the increased risk of associated morbidity, mortality and new fractures. It is therefore important that the diagnosis of vertebral deformities is correct. As is shown in the case histories here, one needs to be aware that also other conditions such as Scheuermann's disease, degenerative changes and anatomical shape variants are associated with wedge formation of the vertebrae. Clinical distinction is important to avoid incorrect treatment. Therefore, there is a need for expertise in evaluating vertebral deformities in both the radiologist and the treating physician who is occupied with fracture prevention and takes into consideration the presence of vertebral fractures. Particularly if there is a clinical presentation atypical for osteoporosis, such as in relatively young men, a normal BMD on DXA scanning can be reason to critically re-examine radiographs and perform additional investigations. In this way, unnecessary medical treatment can be prevented. If the abnormalities on the radiographs at re-evaluation still do not fit a diagnosis well, it could be useful to perform new radiographs or for example do MRI scanning. Finally, future refinements of radiological definitions might improve diagnostic accuracy.

EXPLANATION OF TERMINOLOGY

Dual energy X-ray absorptiometry (DXA)-scan

Method to measure bone density using two bundles of X-rays of different intensities. The results are usually reported as a T score by which bone density measured is compared to values of a reference

population consisting of young, healthy women. A T score of <-1.0 is considered abnormal. Osteoporosis is defined as a T score ≤-2.5.

Cobb's angle

The degree of kyphotic curvature. It is the angle between the top of the upper vertebrae of the curvature and the bottom of lower vertebra of the curvature on a lateral radiograph.

Schmorl's nodes

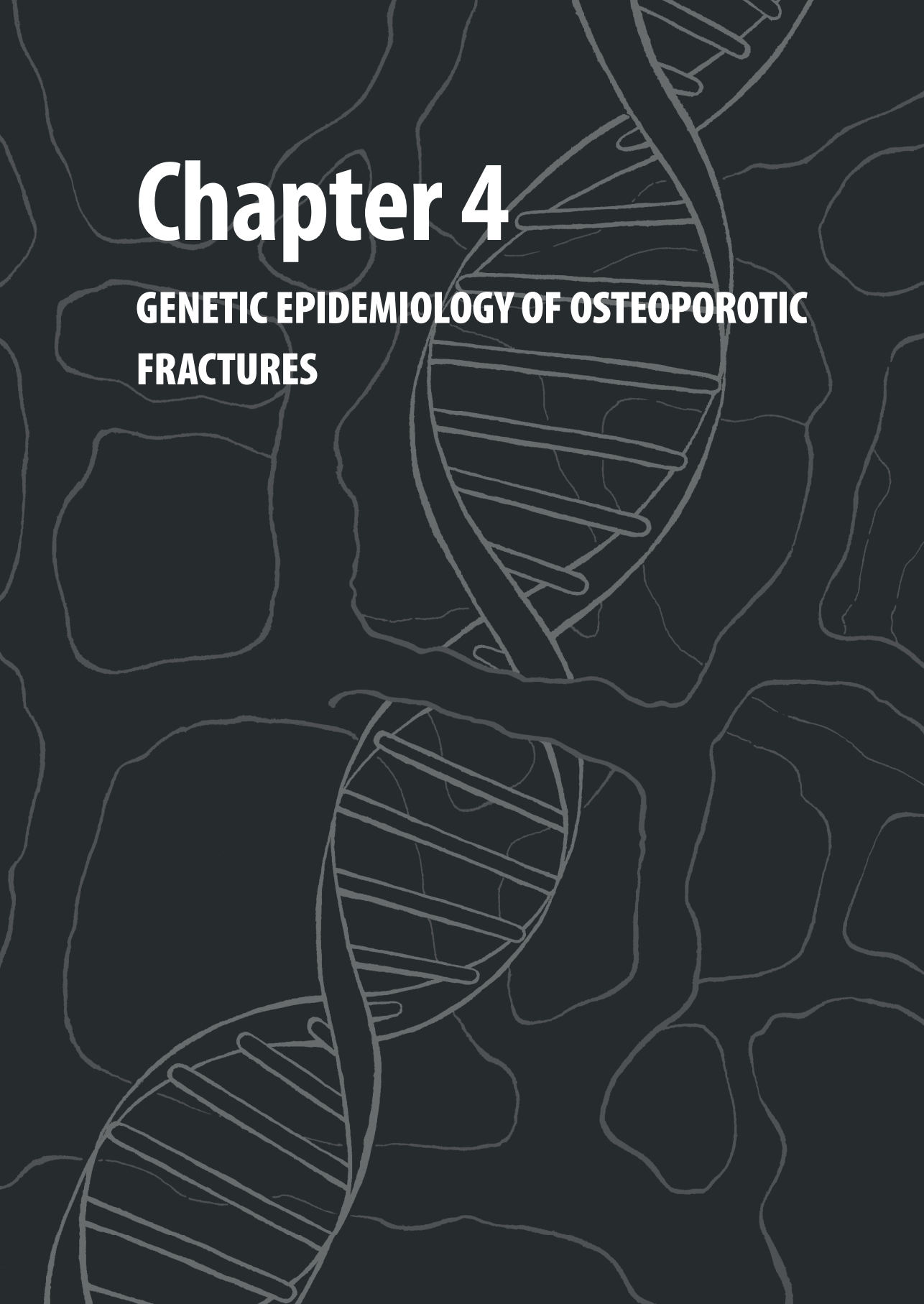
Protrusion of disc cartilage into a vertebral body, which can be noticeable on radiographs.

LESSONS LEARNED

- A disease that can falsely lead to the diagnosis of osteoporotic vertebral fractures is Scheuermann's disease.
- A differential diagnosis for radiological vertebral fractures should be considered especially if the clinical presentation is atypical for osteoporosis and BMD is normal.
- Incorrect diagnosis of osteoporotic vertebral fractures can result in both under- and over-treatment of osteoporosis.
- Particularly in the mid-thoracic region, the differential diagnosis of vertebral deformities is wide.
- In practice it may be difficult to assess whether or not vertebral fractures have occurred recently.

REFERENCES

1. Kwaliteitsinstituut voor de Gezondheidszorg CBO. Richtlijn Osteoporose en Fractuurpreventie. Derde herziene richtlijn. Haarlem: DCHG medische communicatie; 2012.
2. Elders PJM, Dinant GJ, van Geel T, Maartens LWF, Merlijn T, Geijer RMM, et al. NHG-Standaard Fractuurpreventie (tweede herziening). Huisarts Wet. 2012;55(10):452-8.
3. Lems WF, Geusens PP. Klinische relevantie van wervelfracturen. Ned Tijdschr Geneeskd. 2008 Apr 19;152(16):917-9.
4. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, A. H, et al. Review of Radiological Scoring Methods of Osteoporotic Vertebral Fractures for Clinical and Research Settings. Eur Rad. 2012;DOI: 10.1007/s00330-012-2622-z.
5. Haveman LM, van Es HW, ten Berge-Kuipers M. Rugpijnklachten op de kinderleeftijd: behandelbare oorzaken opsporen. Ned Tijdschr Geneeskd. 2008 Feb 16;152(7):353-8.
6. Hart ES, Merlin G, Harisiades J, Grottkau BE. Scheuermann's thoracic kyphosis in the adolescent patient. Orthop Nurs. 2010 Nov-Dec;29(6):365-71; quiz 72-3.
7. Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res. 1993 Sep;8(9):1137-48.
8. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
9. Melton LJ, 3rd, Atkinson EJ, Cooper C, O'Fallon WM, Riggs BL. Vertebral fractures predict subsequent fractures. Osteoporos Int. 1999;10(3):214-21.
10. Chen JS, Sambrook PN. Antiresorptive therapies for osteoporosis: a clinical overview. Nat Rev Endocrinol. 2011;8(2):81-91.



Chapter 4

GENETIC EPIDEMIOLOGY OF OSTEOPOROTIC FRACTURES

Chapter 4.1

Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture

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ABSTRACT

Bone mineral density (BMD) is the most widely used predictor of fracture risk. We performed the largest meta-analysis to date on lumbar spine and femoral neck BMD, including 17 genome-wide association studies and 32,961 individuals of European and East Asian ancestry. We tested the top BMD-associated markers for replication in 50,933 independent subjects and for association with risk of low-trauma fracture in 31,016 individuals with a history of fracture (cases) and 102,444 controls. We identified 56 loci (32 new) associated with BMD at genome-wide significance ($P<5\times10^{-8}$). Several of these factors cluster within the RANK-RANKL-OPG, mesenchymal stem cell differentiation, endochondral ossification and Wnt signaling pathways. However, we also discovered loci that were localized to genes not known to have a role in bone biology. Fourteen BMD-associated loci were also associated with fracture risk ($P<5\times10^{-4}$, Bonferroni corrected), of which six reached $P<5\times10^{-8}$, including at 18p11.21 (*FAM210A*), 7q21.3 (*SLC25A13*), 11q13.2 (*LRP5)*, 4q22.1 (*MEPE*), 2p16.2 (*SPTBN1*) and 10q21.1 (*DKK1*). These findings shed light on the genetic architecture and pathophysiological mechanisms underlying BMD variation and fracture susceptibility.

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased risk of fracture. The disease accounts for approximately 1.5 million new fracture cases each year, representing a huge economic burden on health care systems, with annual costs estimated to be $17 billion in the United States alone and expected to rise 50% by the year 2025.[1] Osteoporosis is defined clinically through the measurement of BMD, which remains the single best predictor of fracture.[2, 3]

Twin and family studies have shown that 50–85% of the variance in BMD is genetically determined.[4] Osteoporotic fractures are also heritable by mechanisms that are partly independent of BMD.[5] Over the past 5 years, genome-wide association studies (GWAS) have revolutionized the understanding of the genetic architecture of common, complex diseases.[6] This approach is providing key insights into the mechanisms of disease, with prospects for the design of effective strategies for risk assessment and the development of new interventions.[7]

Previous GWAS have identified 24 loci that influence BMD variation.[8-14] Whereas several variants in these BMD-associated loci have also been nominally associated with fracture risk,[15, 16] none have shown robust association with genome-wide significance ($P<5\times10^{-8}$). We report here the results of the largest effort to date searching for BMD-associated loci in >80,000 subjects and testing them for association with fracture in >130,000 cases and controls. In addition, we employed bioinformatics tools and gene expression analyses to place the identified variants in the context of pathways relevant to bone biology.

RESULTS

This study was performed across three main stages (Figure 1): (1) the discovery of BMD loci, (2) follow-up replication and (3) association of the BMD-associated loci with fracture.

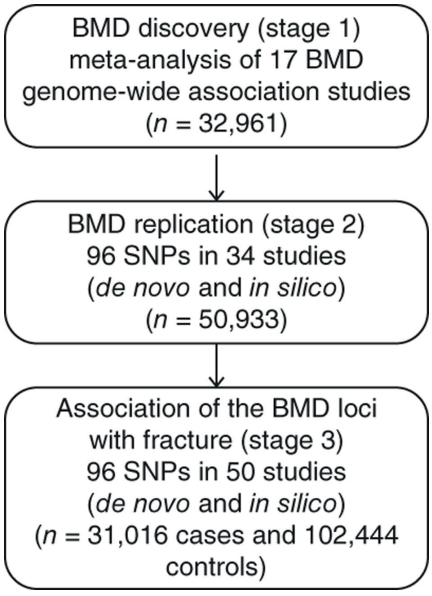

Figure 1. Description of study design. Stage 1: meta-analysis of 17 genome-wide association studies for BMD. Stage 2: 96 top independent SNPs (82 autosomal SNPs with $P<5\times10^{-6}$, 5 SNPs on the X chromosome and 9 SNPs from conditional analysis) were followed up in de novo and in silico replication of the BMD association in 34 studies. Stage 3: the same 96 SNPs were tested for association with fracture in 50 studies with de novo and in silico data.

Discovery of BMD loci (stage 1)

We first performed a meta-analysis of multiple GWAS for BMD of the femoral neck (FN-BMD; N=32,961) and lumbar spine (LS-BMD; N=31,800 cases), including ~2.5 million genotyped or imputed autosomal SNPs from 17 studies of populations across North America, Europe, East Asia and Australia, with a variety of epidemiological designs and subject characteristics (Online Methods). We also performed meta-analysis in men and women separately to identify sex-specific associations. The quantile-quantile plots of the discovery meta-analysis showed strong (and not early) deviation of the observed statistics from the null distribution of no association for both BMD traits (Supplementary Figure 1). After double genomic control correction of the overall ($\lambda_{FN\text{-}BMD\ pooled}$=1.112; $\lambda_{LS\text{-}BMD\ pooled}$=1.127) and sex-stratified ($\lambda_{FN\text{-}BMD\ women}$=1.091; $\lambda_{FN\text{-}BMD\ men}$=1.059; $\lambda_{LS\text{-}BMD\ women}$=1.086; $\lambda_{LS\text{-}BMD\ men}$=1.061) analyses, SNPs in 34 loci surpassed genome-wide significance, whereas a total of 82 loci were associated at $P<5\times10^{-6}$ (Supplementary Figs. 2 and 3). Thirty-eight loci were associated with FN-BMD, 25 with LS-BMD and 19 with both. The overlap reflects correlation between the femoral neck and lumbar spine measurements (Pearson's correlation=0.53). Of these 82 loci, 59, 18 and 5 were prioritized from analyses in the sex-combined, female and male sample sets, respectively (Supplementary Table 1). The meta-analysis was extended to include the evaluation of 76,253 markers on the X chromosome imputed across 14 of the discovery GWAS, for a total of 31,801 participants (Online Methods). Five loci on the X chromosome were associated at $P<5\times10^{-5}$, with four of these derived from the sex-combined analysis and one identified in the analysis of men only (Supplementary Table 1). We further performed genome-wide conditional analyses in all sex-combined stage 1 studies. Each study repeated the GWAS analysis but also adjusted for 82 SNPs representing the autosomal loci associated at $P<5\times10^{-6}$ (Online Methods). We then performed meta-analysis on these studies in the same way as in the primary GWAS meta-analysis. Nine loci showed at least two independent association signals in this conditional analysis (Supplementary Figure 4 and Supplementary Table 2), suggesting that allelic heterogeneity underlies BMD variation. We also assessed all possible pairwise interactions of the 82 SNPs, but none were significant after adjusting for the number of tests (Supplementary Figure 5 and Supplementary Table 3). A total of 96 independent SNPs (82 autosomal SNPs with $P<5\times10^{-6}$, 9 autosomal SNPs from conditional analysis and 5 SNPs on the X chromosome) from 87 genomic loci were selected for further replication (Figure 1).

Follow-up replication (stage 2)

We performed de novo genotyping of these 96 SNPs and tested them for association with BMD in up to 50,933 additional participants from 34 studies (Online Methods). Meta-analysis of the 96 SNPs in the discovery and replication studies (N=83,894) yielded 64 replicating SNPs from 56 associated loci. Of these loci, 32 were newly found to show association (Table 1 and Supplementary Table 4A), and 24 were reported previously[8-14] (Supplementary Table 4b). Thirty-two SNPs did not reach genome-wide significance after replication (Supplementary Table 4C), including 10 markers that remained associated at a suggestive level. Of all the SNPs analyzed, only one (rs9533090 mapping to 13q14.11 near *TNFSF11* (also known as *RANKL*)) showed a high degree of heterogeneity of effects (I^2>50%) across studies, despite being the marker that associated with highest significance ($P=4.82\times10^{-68}$) in the fixed-effect meta-analysis (Supplementary Table 4B). After applying random-effects meta-analysis, this marker was still associated with genome-wide significance ($P=3.98\times10^{-13}$).

Two of the newly identified loci were discovered in the sex-stratified meta-analysis: 8q13.3 in women and Xp22.31 in men; however, only the association at Xp22.31 showed significant evidence for sex specificity, as reflected by significant heterogeneity of effects across sex strata ($P_{het}=1.62\times10^{-8}$). Yet, we

Table 1. Estimated effects of new genome-wide significant SNPs on FN-BMD and LS-BMD across stages

			Functional evidence								FN-BMD				LSBMD				
												Stage 1 (32,961)	Stage 2 (50,933)	Stages 1 and 2 (83,894)		Stage 1 (31,800)	Stage 2 (45,708)	Stages 1 and 2 (77,508)	
SNP	Locus	Closest Gene / Candidate	eQTL	KO mouse	OMIM	function	Tags	GRAIL	Pathway	A	Freq[b]	β[c]	P	P	P	β[a]	P	P	P	P_{het} site[b]
rs479336	1q24.3	*DNM3*								T	0.74	-0.04	1.1×10^{-7}	**1.3×10^{-8}**	**8.5×10^{-15}**	-0.03	0.01	5.0×10^{-4}	2.1×10^{-5}	0.05
rs7584262	2p21	*PKDCC*		·						T	0.23	0.03	1.4×10^{-7}	3.4×10^{-4}	**1.3×10^{-9}**	0.01	0.13	0.28	0.07	0.01
rs17040773	2q13	*ANAPC1*	·							A	0.76	0.03	4.3×10^{-6}	6.1×10^{-5}	**1.5×10^{-9}**	0.01	0.61	0.21	0.19	5.2×10^{-3}
rs1878526	2q14.2	*INSIG2*								A	0.22	0.00	0.70	0.97	0.79	0.04	7.3×10^{-6}	3.4×10^{-6}	**1.2×10^{-10}**	**8.6×10^{-5}**
rs1026364	3q13.2	*KIAA2018*				·				T	0.37	0.03	2.0×10^{-6}	2.5×10^{-5}	**4.1×10^{-10}**	0.02	0.04	7.3×10^{-3}	7.6×10^{-4}	0.11
rs344081	3q25.31	*LEKR1*								T	0.87	0.03	1.1×10^{-4}	2.5×10^{-3}	2.2×10^{-6}	0.06	2.8×10^{-5}	**3.5×10^{-8}**	**4.5×10^{-12}**	0.12
rs3755955	4p16.3	*IDUA*		·		·				A	0.16	-0.05	3.9×10^{-7}	**6.1×10^{-9}**	**1.5×10^{-14}**	-0.05	1.4×10^{-7}	**5.5×10^{-9}**	**5.2×10^{-15}**	0.80
rs11755164	6p21.1	*SUPT3H/RUNX2*		·			·	·		T	0.40	-0.01	0.23	0.12	0.05	-0.03	3.5×10^{-7}	9.2×10^{-6}	**5.6×10^{-11}**	2.1×10^{-3}
rs9466056	6p22.3	*CDKAL1/SOX4*								A	0.38	-0.03	**1.8×10^{-8}**	1.6×10^{-6}	**2.7×10^{-13}**	-0.03	6.5×10^{-5}	1.1×10^{-4}	**3.6×10^{-8}**	0.34
rs3801387	7q31.31	*WNT16*				·	·	·		A	0.74	-0.08	**4.2×10^{-14}**	**2.0×10^{-27}**	**5.0×10^{-40}**	-0.10	**1.4×10^{-16}**	**1.5×10^{-36}**	**3.2×10^{-51}**	0.09
rs13245690[e]	7q31.31	*C7orf58*								A	0.62	0.00	8.6×10^{-5}	0.69	8.2×10^{-4}	0.03	**1.1×10^{-9}**	1.3×10^{-3}	**6.0×10^{-11}**	0.05
rs7812088	7q36.1	*ABCF2*								A	0.13	0.04	1.2×10^{-6}	4.4×10^{-4}	**7.3×10^{-9}**	0.04	2.9×10^{-5}	1.1×10^{-3}	2.2×10^{-7}	0.86
rs7017914[c]	8q13.3	*XKR9/LACTB2*	·			·				A	0.49	0.02	**4.7×10^{-8}**	7.1×10^{-3}	**1.9×10^{-8}**	-0.01	0.35	0.41	0.98	**9.1×10^{-5}**
rs7851693	9q34.11	*FUBP3*								C	0.64	0.05	**3.1×10^{-8}**	**1.4×10^{-15}**	**3.4×10^{-22}**	0.04	0.06	6.7×10^{-8}	6.1×10^{-8}	0.02
rs3905706	10p11.23	*MPP7*								T	0.22	-0.02	0.63	1.7×10^{-3}	0.03	0.05	2.9×10^{-9}	**6.7×10^{-9}**	**2.4×10^{-16}**	**5.8×10^{-11}**
rs1373004	10q21.1	*MBL2/DKK1*		·					·	T	0.13	-0.04	1.4×10^{-5}	1.5×10^{-4}	**1.5×10^{-8}**	-0.05	5.4×10^{-8}	2.2×10^{-6}	1.6×10^{-12}	0.28
rs7071206	10q22.3	*KCNMA1*								T	0.78	0.01	0.29	0.26	0.81	-0.05	**1.5×10^{-12}**	**6.2×10^{-9}**	**5.0×10^{-19}**	**5.9×10^{-9}**
rs7084921	10q24.2	*CPN1*								T	0.39	0.03	1.4×10^{-4}	1.6×10^{-6}	**9.0×10^{-10}**	0.03	0.01	1.9×10^{-5}	9.2×10^{-7}	0.58
rs10835187	11p14.1	*LIN7C*	·							T	0.55	-0.01	0.17	0.08	0.03	-0.02	3.0×10^{-5}	2.4×10^{-4}	**4.9×10^{-8}**	0.03
rs7953528	12p11.22	*KLHDC5/PTHLH*		·	·		·	·		A	0.18	0.04	**5.8×10^{-8}**	2.4×10^{-6}	**1.9×10^{-12}**	-0.02	0.94	0.05	0.13	**2.3×10^{-7}**
rs2887571	12p13.33	*ERC1/WNT5B*						·	·	A	0.76	-0.03	1.1×10^{-4}	1.6×10^{-5}	**6.5×10^{-9}**	-0.04	2.2×10^{-7}	2.9×10^{-6}	**5.6×10^{-12}**	0.37
rs12821008	12q13.12	*DHH*							·	T	0.39	0.03	1.9×10^{-4}	5.2×10^{-4}	3.3×10^{-7}	0.05	1.5×10^{-7}	1.9×10^{-9}	**1.2×10^{-15}**	0.06
rs1053051	12q23.3	*C12orf23*	·							T	0.52	-0.03	1.4×10^{-5}	1.8×10^{-5}	**9.6×10^{-10}**	-0.02	2.5×10^{-6}	2.4×10^{-3}	7.9×10^{-8}	0.76
rs1286083	14q32.12	*RPS6KA5*								T	0.81	-0.05	**2.9×10^{-8}**	**9.3×10^{-9}**	**2.0×10^{-15}**	-0.04	**1.7×10^{-11}**	7.1×10^{-6}	**1.8×10^{-14}**	0.92
rs4985155	16p13.11	*NTAN1*	·			·				A	0.67	-0.03	3.5×10^{-4}	1.4×10^{-7}	**1.7×10^{-10}**	-0.03	8.7×10^{-7}	1.8×10^{-4}	**2.2×10^{-9}**	0.98
rs9921222	16p13.3	*AXIN1*		·				·	·	T	0.48	-0.03	2.5×10^{-7}	2.4×10^{-6}	**5.2×10^{-12}**	-0.04	**2.2×10^{-8}**	**8.3×10^{-10}**	**1.0×10^{-16}**	0.26

Table 1. Estimated effects of new genome-wide significant SNPs on FN-BMD and LS-BMD across stages (continued)

			Functional evidence								FN-BMD				LSBMD				
SNP	Locus	Closest Gene / Candidate	eQTL	KO mouse	OMIM	function Tags	GRAIL	Pathway	A	Freq[b]	β[c]	Stage 1 (32,961) P	Stage 2 (50,933) P	Stages 1 and 2 (83,894) P	β[a]	Stage 1 (31,800) P	Stage 2 (45,708) P	Stages 1 and 2 (77,508) P	P_{het} site[b]
rs13336428	16p13.3	*C16orf38/CLCN7*		·	·				A	0.43	-0.04	2.9×10^{-7}	**1.1×10^{-10}**	**1.5×10^{-16}**	-0.04	5.9×10^{-5}	**5.8×10^{-10}**	**1.7×10^{-13}**	0.80
rs1566045	16q12.1	*SALL1/CYLD*		·					T	0.80	-0.06	**5.0×10^{-12}**	**3.0×10^{-12}**	**1.9×10^{-22}**	-0.01	7.8×10^{-3}	0.55	0.04	**7.5×10^{-6}**
rs1564981[e]	16q12.1	*CYLD*		·					A	0.47	-0.02	1.1×10^{-3}	0.01	4.4×10^{-5}	-0.03	6.2×10^{-8}	5.4×10^{-4}	**2.0×10^{-10}**	0.50
rs4790881	17p13.3	*SMG6*							A	0.69	0.05	**1.7×10^{-8}**	**1.2×10^{-11}**	**9.8×10^{-19}**	0.04	6.0×10^{-4}	1.7×10^{-6}	**3.4×10^{-9}**	0.13
rs7217932	17q24.3	*SOX9*		·	·		·	·	A	0.46	0.03	**3.7×10^{-8}**	2.7×10^{-5}	**1.9×10^{-11}**	0.01	0.31	0.15	0.08	3.8×10^{-3}
rs4796995	18p11.21	*C18orf19*							A	0.63	0.02	3.2×10^{-6}	1.1×10^{-3}	**4.9×10^{-8}**	0.01	5.2×10^{-4}	0.11	6.7×10^{-4}	0.29
rs10416218	19q13.11	*GPATCH1*				·			T	0.73	-0.02	5.7×10^{-6}	7.1×10^{-4}	**5.5×10^{-8}**	-0.03	**9.2×10^{-9}**	1.2×10^{-4}	**6.6×10^{-11}**	0.38
rs5934507[d]	Xp22.31	*FAM9B/KAL1*							A	0.74	-0.08	0.01	8.3×10^{-4}	1.6×10^{-4}	-0.09	5.7×10^{-6}	3.2×10^{-4}	**1.2×10^{-8}**	0.17

Boldface indicates $P<5\times10^{-8}$ or site-specific $P<5\times10^{-4}$. A, allele; β, effect estimates; freq., allele frequency of A. Effect estimates are expressed as standardized values per copy of the SNP allele from fixed-effects meta-analysis. Black dots in the six functional evidence columns indicate, respectively, that the SNP is an eQTL, there is a knockout mouse with skeletal phenotypes (Mouse Genome Informatics (MGI) database 2011), the candidate gene is involved in a monogenic syndrome with skeletal phenotypes (OMIM 2011), the most significant SNP tags a SNP predicted to have impact on function of the cand Boldface indicates P<5×10–8 or site-specific P<5×10–4. A, allele; β, effect estimates; freq., allele frequency of A. Effect estimates are expressed as standardized values per copy of the SNP allele from fixed-effects meta-analysis. Black dots in the six functional evidence columns indicate, respectively, that the SNP is an eQTL, there is a knockout mouse with skeletal phenotypes (Mouse Genome Informatics (MGI) database 2011), the candidate gene is involved in a monogenic syndrome with skeletal phenotypes (OMIM 2011), the most significant SNP tags a SNP predicted to have impact on function of the candidate gene, the gene is the best candidate in GRAIL analysis, and the candidate gene is part of a bone-active pathway. Candidate genes from GRAIL and/or the literature are shown if different from the closest gene. aEffect estimates were calculated in the stage 2 samples. bSite specificity null hypothesis, βLS-BMD=βFN-BMD. crs7017914 was discovered in the meta-analysis of women only. The effects and P value for this marker are for the meta-analysis of women samples. drs5934507 was discovered in the meta-analysis of men only. The effects and P value for this marker are for the meta-analysis of men samples. ers13245690 and rs1564981 were independently associated to their main signals in conditional analysis.

Table 2. Association of identified BMD-associated loci with risk for any type of low-trauma fracture

			Functional evidence							Meta-analysis without studies included in BMD discovery			Combined meta-analysis results			
										25,605 cases, 80,535 controls			31,016 cases, 102,444 controls			
SNP	Locus	Closest Gene/ Candidate	eQTL	KO mouse	OMIM	Tags function	GRAIL	Pathway	Risk Allele	Freq[b]	OR (95% CI)	P	OR (95% CI)	P	Q_{het} P	I^2
Loci significantly associated with fracture risk at P<5x10^{-8}																
rs4233949	2p16.2	*SPTBN1*				·			G	0.63	1.07 (1.04–1.09)	1.4×10^{-7}	1.06 (1.04–1.08)	**2.6×10^{-8}**	0.36	6
rs6532023	4q22.1	*MEPE/SPP1*	·	·			·	·	G	0.67	1.06 (1.04–1.09)	8.8×10^{-7}	1.06 (1.04–1.09)	**1.7×10^{-8}**	1.00	0
rs4727338	7q21.3	*SLC25A13*							G	0.32	1.08 (1.05–1.10)	1.0×10^{-8}	1.08 (1.05–1.10)	**5.9×10^{-11}**	0.03	31
rs1373004	10q21.1	***MBL2/DKK1***		·				·	T	0.13	1.09 (1.06–1.13)	7.2×10^{-7}	1.10 (1.06–1.13)	**9.0×10^{-9}**	0.64	0
rs3736228	11q13.2	*LRP5*		·	·	·	·	·	T	0.15	1.09 (1.05–1.12)	2.1×10^{-6}	1.09 (1.06–1.13)	**1.4×10^{-8}**	0.78	0
rs4796995	18p11.21	***C18orf19***							G	0.39	1.06 (1.04–1.09)	6.4×10^{-7}	1.08 (1.06–1.10)	**8.8×10^{-13}**	0.12	20
Other significant loci associated with fracture risk at P<5x10^{-4} (Bonferroni)																
rs6426749	1p36.12	*ZBTB40*							G	0.83	1.06 (1.03–1.09)	2.4×10^{-4}	1.07 (1.04–1.10)	3.6×10^{-6}	0.07	24
rs7521902	1p36.12[a]	*WNT4*	·				·	·	A	0.27	1.10 (1.06–1.14)	3.5×10^{-6}	1.09 (1.06–1.13)	1.4×10^{-7}	0.87	0
rs430727	3p22.1	*CTNNB1*		·			·	·	T	0.47	1.05 (1.03–1.08)	2.4×10^{-5}	1.06 (1.03–1.08)	2.9×10^{-7}	0.93	0
rs6959212	7p14.1	*STARD3NL*							T	0.33	1.04 (1.02–1.07)	1.0×10^{-3}	1.05 (1.02–1.07)	7.2×10^{-5}	0.43	2
rs3801387	7q31.31	***WNT16***				·	·	·	A	0.74	1.08 (1.05–1.11)	4.9×10^{-9}	1.06 (1.04–1.08)	2.7×10^{-7}	0.69	0
rs7851693	9q34.11	***FUBP3***							G	0.37	1.04 (1.01–1.06)	1.9×10^{-3}	1.05 (1.02–1.07)	3.5×10^{-5}	0.65	0
rs163879	11p14.1	*DCDC5*							T	0.66	1.06 (1.03–1.09)	6.4×10^{-6}	1.05 (1.03–1.07)	3.3×10^{-5}	0.05	28
rs1286083	14q32.12	***RPS6KA5***							T	0.81	1.05 (1.02–1.08)	9.8×10^{-4}	1.05 (1.03–1.08)	7.2×10^{-5}	0.01	34
rs4792909	17q21.31[a]	*SOST*		·	·			·	G	0.62	1.07 (1.04–1.11)	4.0×10^{-5}	1.07 (1.04–1.10)	6.9×10^{-6}	0.31	10
rs227584	17q21.31	*C17orf53*	·						A	0.67	1.05 (1.02–1.08)	2.2×10^{-4}	1.05 (1.03–1.07)	4.1×10^{-5}	0.49	0

Odds ratios (ORs) estimated per risk allele copy for any low-trauma fracture among cases compared with controls. Q_{het} is the Cochran's Q statistic, and I^2 is the measure of heterogeneity. Boldface indicates gene names from new loci and/or those associated with P<5×10^{-8}. Black dots in the six functional evidence columns indicate that, respectively, the SNP is an eQTL, there is a knockout mouse with skeletal phenotypes (MGI database 2011), the candidate gene is involved in a monogenic syndrome with skeletal phenotypes (OMIM 2011), the most significant SNP tags a SNP predicted to have impact on function of the candidate gene, the gene is the best candidate in GRAIL analysis, and the candidate gene is part of a bone-active pathway.

[a]rs7521902 and rs4792909 are secondary independent signals. [b]Freq. is the frequency of the risk allele.

acknowledge that the association at 8q13.3 in women may have been driven by a lower number of men in the discovery and replication data sets (Table 1 and Supplementary Table 5). Furthermore, evidence for BMD site specificity ($P_{het}<5\times10^{-4}$) was observed in a proportion of the loci, including 6 of the 32 new and 4 of the 24 known loci (Table 1 and Supplementary Figure 6). Among the newly identified loci, 2q14 (*INSIG2*), 12p11.22 (*PTHLH*) and 16q12.1 (*CYLD*) showed site specificity with FN-BMD, and 8q13.3 (*LACTB2*), 10p11.23 (*MPP7*) and 10q22.3 (*KCNMA1*) showed site-specificity with LS-BMD.

After replication, the conditional analysis provided significant evidence of association ($P<5\times10^{-8}$) in eight of the nine loci containing secondary signals (Supplementary Figure 4 and Supplementary Table 2). Three loci had variants located less than 40 kb from the initial main signal, suggesting allelic heterogeneity, including at 1p31.3 (represented by rs17482952 near *WLS*), 6q25.1 (rs7751941 near *ESR1*) and 16q12.1 (rs1564981 near *CYLD*). The secondary signal at 16q12.1 (rs1564981) showed a strong association with LS-BMD, whereas the main signal in this locus (rs1566045) was only associated with FN-BMD. The other five secondary signals were represented by variants localized more than 180 kb from the initial main signal and were located in different candidate genes, including at 1p36.12 (rs7521902 near *WNT4*), 7p14.1 (rs10226308 near *SFRP4*), 7q31.31 (rs13245690 near *C7orf58*), 12q13.13 (rs736825 near *HOXC6*) and 17q21.31 (rs4792909 near *SOST*). The secondary signal mapping to the 13q14.11 locus (rs7326472) did not achieve genome-wide significance after replication.

Association of the BMD loci with fracture (stage 3)

We tested the 96 markers for association with fracture in 31,016 cases and 102,444 controls from 50 studies with fracture information. This collection included 5,411 cases and 21,909 controls tested in the BMD GWAS discovery samples, 9,187 cases and 45,057 controls tested by in silico replication and 16,418 cases and 35,478 controls tested by de novo genotyping (Figure 1 and Online Methods). In this fracture meta-analysis, 14 loci were significantly associated with any type of fracture at Bonferroni-corrected significance ($P=5\times10^{-4}$), of which five were new BMD-associated loci. None of the markers showed large estimates of heterogeneity (Table 2, Supplementary Figure 7 and Supplementary Table 6). Markers at six of these loci reached $P<5\times10^{-8}$, including at 18p11.21 (*FAM210A*; also known as *C18orf19*), 7q21.3 (*SLC25A13*), 11q13.2 (*LRP5*), 4q22.1 (*MEPE*), 2p16.2 (*SPTBN1*) and 10q21.1 (*DKK1*). The proportion of the overall fracture risk explained by BMD ranged between 0.09 and 0.40 across markers (Supplementary Table 7) and was estimated in a subset of stage 2 samples (including N=8,594 cases and 23,218 controls) by modeling the effect of BMD-associated SNPs on fracture risk, with and without the inclusion of BMD as a covariate. In general, the effect of these SNPs on BMD was larger than on fracture risk (Figure 2A), except for the most significantly associated locus for fracture at 18p11.21 (Figure 2B). SNPs in genes of the RANK-RANKL-OPG pathway (*TNFRSF11A, TNFSF11* and *TNFRSF11B*, respectively), despite being the strongest loci associated with BMD, were not significantly associated with fracture. All 31 BMD-associated loci that had nominal association with fracture risk ($P<0.05$) showed consistent direction (the allele associated with decreasing BMD was associated with increased risk of fracture). When we performed subgroup analyses using cleaner phenotype definitions generated by limiting subjects to those with clinically validated fractures and stratifying by anatomical site (for example, non-vertebral and vertebral fractures), we did not identify any additional signals (Supplementary Table 8). At a nominally significant level ($P<0.05$), only 3 loci were associated with vertebral fracture, and all 14 BMD-associated loci were associated with non-vertebral fracture, although the difference in effect between fracture sites was not significant. Therefore, the power of our study did not benefit from improving phenotype definitions at the cost of lower sample size.

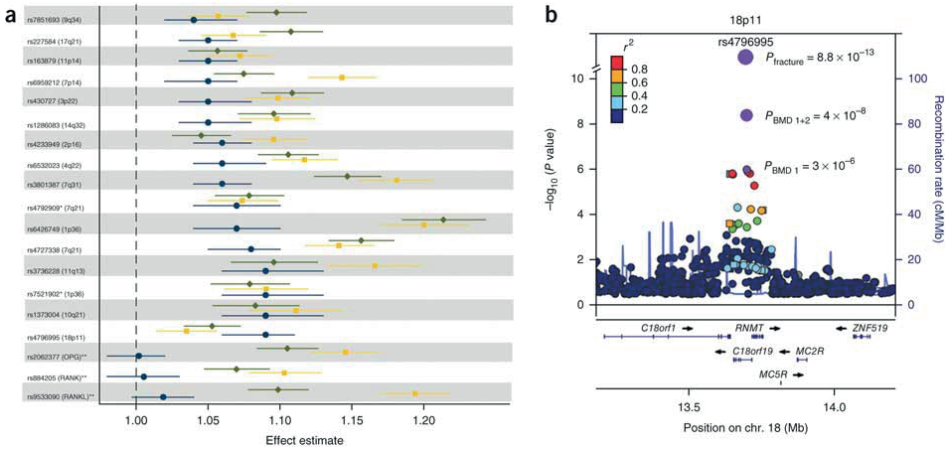

Figure 2. Association of BMD loci with fracture risk. **(A)** Phenotype-wide effects for the BMD loci associated with fracture and those that are part of the RANK-RANKL-OPG pathway. Genetic effect estimates are shown for fracture (blue circles), LS-BMD (yellow squares) and FN-BMD (green diamonds) for the 14 loci associated with fracture risk. Horizontal lines represent 95% confidence limits. Effect estimates are shown after transformation of the standardized mean difference (SMD) in the BMD effect to odds ratio equivalents[34] (for example, a 0.02 SMD in the BMD effect corresponds to an OR of 1.04). Secondary signals for rs227584 and rs6426749 are marked with an asterisk and the signals mapping to the *TNFRSF11B* (also known as *OPG*; rs2062377), *TNFRSF11A* (also known as *RANK*; rs884205) and *TNFSF11* (also known as *RANKL*; rs9533090) genes are marked with a double asterisk. **(B)** Regional association plot for the 18p11.21 locus showing the P value for the top SNP associated with fracture (rs4796995) together with P values from the BMD discovery set (stage 1) and combined with the BMD replication (stage 1+2). SNPs are plotted by position in a 500 kb-window of chromosome 18 against association with FN-BMD ($-\log_{10}$ (P value)). Estimated recombination rates (from HapMap) are plotted in cyan to reflect the local LD structure. SNPs surrounding the most significant SNP are color-coded according to LD between these markers (pairwise r^2). Genes, exons and transcription direction are derived from the UCSC Genome Browser.

Allele risk modeling for osteoporosis and fracture

The combined effect of all significant autosomal SNPs on BMD, osteoporosis and any type of fracture was modeled in the Prospective Epidemiological Risk Factor (PERF) study (N=2,836), a prospective study in postmenopausal Danish women aged 55–86 years.[17] This study represents an independent validation setting, as it was excluded from the overall meta-analysis for this purpose (Supplementary Note). Risk alleles in the score (for example, BMD-decreasing alleles) were weighted by their individual effects on BMD and grouped into five bins (Supplementary Table 9). The difference in mean FN-BMD between individuals in the highest bin of risk score (9% of the population; N=244) and those in the middle bin (34% of the population; N=978) was −0.33 SD (Figure 3A). This analysis was based on data at 63 SNPs and explained 5.8% (95% confidence interval (CI): 4.0%–7.6%) of the total genetic variance in FN-BMD.

The ability of this genetic score to predict the risk for osteoporosis (defined by a T score of≤−2.5) and for fracture was modeled in the PERF study using the middle bin as reference (odds ratio (OR)=1). Women in the highest bin had 1.56 (95% CI: 1.12–2.18) increased odds for osteoporosis (Figure 3B), whereas women in the lowest bin were protected from osteoporosis (OR=0.38, 95% CI: 0.23–0.63). A model based on the 16 BMD-associated SNPs that were also associated with fracture risk showed that women in the highest bin had 1.60 (95% CI: 1.15–2.24) increased odds for fracture, whereas women in the lowest bin had a decreased risk for fracture (OR=0.54, 95% CI: 0.36–0.83) (Figure 3C). Despite

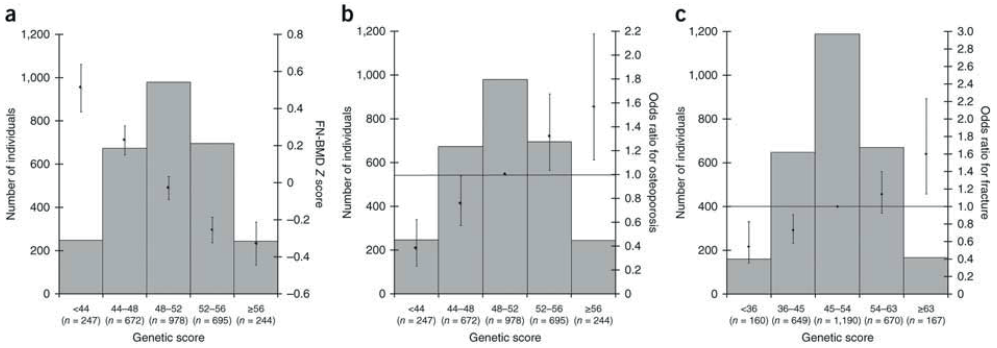

Figure 3. Combined effect of BMD-decreasing alleles and fracture risk–increasing risk alleles modeled in the population-based PERF study (N=2,836 women). **(A–C)** Effects are shown for baseline FN-BMD standardized residuals (Z scores) **(A)**, risk for osteoporosis **(B)** and risk for any type of fracture **(C)**. The genetic score of each individual in **(A)** and **(B)** was based on the 63 SNPs showing genome-wide significant association with BMD (55 main and 8 secondary signals) and in **(C)** was based on the 16 BMD SNPs associated with fracture. Both genetic scores are weighted for relative effect sizes estimated without the PERF study. Weighted allele counts summed for each individual were divided by the mean effect size, making them equivalent to the percent of alleles carried by each individual, and sorted into five bins. Histograms show the numbers of individuals in each genetic score category (left Y-axis). Diamonds (right Y-axis) represent mean FN-BMD standardized levels in **(A)**, risk estimates in the form of odds ratios and osteoporosis (defined as NHANES T score of ≤–2.5) in **(B)** and any type of fracture in **(C)**, using the middle category as reference (OR=1). Vertical lines represent 95% confidence limits.

serving as robust proof of the relationship between BMD-decreasing alleles and the risk of osteoporosis and fracture, prediction ability was modest. Receiver operating characteristics (ROC) analysis showed a significant but relatively small discrimination ability of the genetic score alone, with an area under the curve (AUC) of 0.59 (95% CI: 0.56–0.62) for osteoporosis (Supplementary Figure 8). Adding this score to a model with age and weight alone (AUC=0.75, 95% CI: 0.73–0.77) did not substantially increase discrimination (AUC=0.76, 95% CI: 0.74–0.78). A similar pattern was observed for fracture discrimination, with AUCs of 0.57 (95% CI: 0.55–0.59) in a model with the score alone and 0.62 (95% CI: 0.60–0.64) in a model with age, weight and height. A model considering all 63 SNPs did not change the AUC for fracture risk prediction (0.57, 95% CI: 0.54–0.59).

Functional annotations and pathway analyses

For the purpose of fine mapping and identifying additional SNPs with putative functional implication using linkage disequilibrium (LD), a subset of nine discovery studies (FN-BMD, N=21,699; LS-BMD, N=20,835) used 1000 Genomes Project data (Release June 2010) to re-impute genotypes at the 55 autosomal BMD loci (Supplementary Note). In 13 of the 55 BMD-associated loci (the SNP on the X chromosome was not included), we identified markers in the surrounding 1-Mb region that were imputed from 1000 Genomes Project data and that were more significant than the original HapMap signals (Supplementary Tables 10 and 11), highlighting the benefit of using a denser reference panel of markers. All HapMap markers in LD with variants with functional annotation and showing higher significance in the 1000 Genomes Project meta-analysis are shown (Supplementary Table 12). In 14 of the 56 identified BMD-associated loci, a marker from HapMap imputation was highly correlated (r^2>0.8) with at least one putative functional variant annotated in the 1000 Genomes Project reference. Three of the 14 BMD-associated loci that also associated with fracture contained putative functional variants tagged by

the top SNPs of the BMD meta-analysis. These included the known rs3736228 functional marker in *LRP5* (encoding p.Ala1330Val),[16, 18] the intronic marker rs3779381 within a promoter and/or regulatory region of *WNT16* and one intronic marker (rs4305309) within a promoter and/or regulatory region of *SPTBN1*.

Expression profiles at the BMD loci associated with genome-wide significance were analyzed within four data sets (Supplementary Note). In transiliac bone biopsies, expression of five genes correlated with LS-BMD and/or FN-BMD of the donors with P<0.001, including *PSME4* (2p16.2), *DKK1* (10q21.1), *MIR22HG* (also known as *C17orf91*; 17p13.3), *SOST* (17q21.31_1) and *DUSP3* (17q21.31_1) (Supplementary Table 13). Among these loci, the SNP at *DKK1* (10q21.1) was the most significantly correlated with FN-BMD ($P=1.3\times10^{-5}$) and LS-BMD ($P=3.2\times10^{-4}$). Variants in all these BMD-associated loci (with the exception of *MIR22HG* at 17p13.3) were also associated with fractures.

SNP expression quantitative trait locus (eQTL) analyses were performed across diverse tissues, examining the correlation between marker alleles and transcript levels at the associated BMD loci. Fourteen

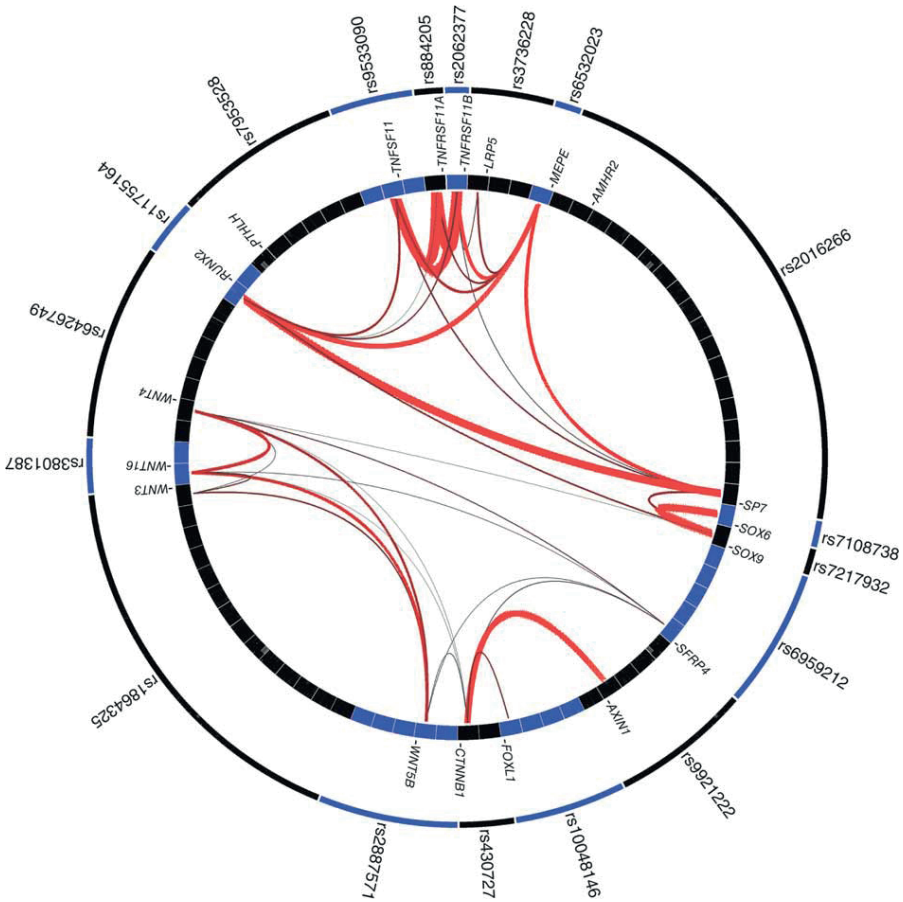

Figure 4. Graphic representation of GRAIL connections between SNPs and corresponding genes for the 18 SNPs, as determined with GRAIL P<0.01. The top ten keywords linking the genes were bone, catenin, signaling, differentiation, rank, osteoblast, diacylglycerol, kappab, development and osteoclast. Thicker redder lines imply stronger literature-based connectivity. Blue and black boxes depict loci boundaries represented for each top-associated marker (outer circle) and for each gene in the region (inner circle).

of the BMD-associated SNPs correlated with the expression of one or more of the nearby genes with $P<5\times10^{-5}$ and were either the strongest *cis* variants or were good surrogates of these for the affected genes (Supplementary Tables 14 and 15). The most significant BMD-associated SNP eQTL was observed for rs10835187[T], resulting in reduced expression of the *LIN7C* gene at the 11p14.1 locus ($P=2.8\times10^{-39}$ in adipose tissue). Of particular interest were BMD-associated SNP *cis* variants at three loci that were also associated with fracture, including 1p36.12, 4q22.1 and 17q21.31. At 1p36.12, rs6426749[G] correlated with reduced *WNT4* expression in fibroblasts, osteoblasts and adipose tissue; at 4q22.1, rs6532023[G] correlated with reduced *SPP1* (encoding osteopontin) expression in adipose tissue; and, at 17q21.31, rs227584[A] correlated with increased *C17orf65* expression in monocytes, adipose tissue, whole blood and lymphoblasts.

We applied the Gene Relationships Across Implicated Loci (GRAIL) text-mining algorithm[19] to investigate connections between genes in the 55 autosomal BMD-associated loci. This analysis revealed significant ($P<0.01$) connections between genes in 18 of the 55 input loci (Figure 4 and Supplementary Table 16). The strongest connections were seen for members of three key biological pathways: the RANK-RANKL-OPG pathway (encoded by *TNFRSF11A, TNFSF11* and *TNFRSF11B*, respectively); mesenchymal stem cell differentiation (*RUNX2, SP7* and *SOX9*); and Wnt signaling (*LRP5, CTNNB1, SFRP4, WNT3, WNT4, WNT5B, WNT16* and *AXIN1*), with the ten most frequently connecting terms being bone, catenin, signaling, differentiation, rank, osteoblast, diacylglycerol, kappab, development and osteoclast. To assess the significance of this biological gene connection enrichment, we applied GRAIL to 2,000 randomly matched sets of 55 SNPs (Supplementary Note) and did not observe any set with 15 or more loci with significantly enriched connectivity (Supplementary Figure 9), providing strong statistical evidence of the significant clustering of our BMD-associated loci ($P<0.0005$).

DISCUSSION

In this report of the largest GWAS for osteoporosis traits to date, we identified 32 new genomic loci, bringing the total number of loci robustly associated with BMD variation to 56. Furthermore, we report that six of these BMD-associated loci are also associated with low-trauma fractures at $P<5\times10^{-8}$, an association that has not previously been detected. In terms of other complex traits, our results indicate that hundreds of variants with small effects may contribute to the genetic architecture of BMD and fracture risk.[20] Our hypothesis-free assessment of common variants of the genome provides new insights into biology, implicating several factors that cluster in bone-active pathways.

Our results highlight the highly polygenic nature of BMD variation and the critical role of several biological pathways influencing osteoporosis and fracture susceptibility (Supplementary Figure 10). In addition to the Wnt factors known to be associated with BMD (*CTNNB1, SOST, LRP4, LRP5, WLS, WNT4* and *MEF2C*), several of the newly discovered loci implicate additional Wnt signaling factors (including *WNT5B, WNT16, DKK1, PTHLH, SFRP4* and *AXIN1*). Another clearly delineated pathway is that involved in mesenchymal stem cell differentiation, including the newly identified *RUNX2, SOX4* and *SOX9* BMD-associated loci along with the previously known *SP7*. Another bone-relevant pathway includes that of endochondral ossification, which involves essential processes during fetal development of the mammalian skeleton and in which several of our identified BMD-associated loci are implicated, including *SPP1, MEF2C, RUNX2, SOX6, PTHLH, SP7* and *SOX9*. In addition, the biological relevance of our associations is accentuated by the identification of genes underlying rare monogenetic forms of osteoporosis and/or high bone mass, such as *SOST, CLCN7* and *LRP5*[21-23] (Supplementary Table 17), which also contain

common variants involved in normal BMD variation at the population level[11,14,16]. This is supportive of a genetic architecture where both common and rare genetic variation may reside in the same locus.[24] Other genes have not been reported to be associated with monogenic forms of osteoporosis but have clear involvement in bone development in animal models. For example, SNPs in the BMD-associated locus at 16q12.1 map near *CYLD*. Human mutations in this gene have been described to cause familial cylindromatosis, a condition without phenotypic skeletal manifestations. However, it has been shown that *Cyld* knockout mice have significant bone loss, leading to a severe osteoporosis phenotype[25] and also that *CYLD* regulates osteoclastogenesis[26]. Moreover, evidence from the GWAS and eQTL analyses also suggests that some loci contain more than one common variant with independent effects on BMD and fracture risk. On the other hand, when no correlation is observed between gene expression and a particular SNP, it is difficult to draw conclusions. A correlation might be missed if the expression of the transcript was not measured in a relevant tissue or if the expression of a particular splice variant was not measured.[27]

BMD and fracture genetic effects correlate to some extent, but some important risk variants for fracture may have minimal impact on BMD and vice versa. This is the case for the signal at 18p11.21 (Figure 2B), which, despite a modest effect on BMD (0.02% variance explained), showed the most significant association with fracture risk (OR=1.08, 95% CI: 1.06–1.10; $P=8.8\times10^{-13}$). This is in contrast to variants that are known to have stronger effects on BMD that were not significantly associated with fracture risk. For example, variants affecting the RANK-RANKL-OPG pathway that has a critical role in osteoclastogenesis had clear associations with BMD but not with fracture risk (Figure 2A). Even though loci discovery was based on the BMD phenotype, these findings reflect the heterogeneous and complex nature of the mechanistic pathways leading to fracture. Therefore, given our study design, we cannot rule out the possibility that unidentified genetic loci influence risk for fracture independently of BMD. Future well-powered GWAS meta-analyses on fracture risk will address this question, while corroborating the associations with fracture that we report for some of the BMD-associated loci (particularly those not associated with fracture at $P<5\times10^{-8}$).

Our study also provides indication that there is sex and site specificity underlying BMD variation. One of the GWAS signals (Xp22.31) was only significant in the sex-stratified analysis in men and showed significant sex heterogeneity ($P_{het}=1.62\times10^{-8}$). This is expected, considering the sexual dimorphism of bone.[28, 29] In fact, in a recent GWAS, the rs5934507 SNP mapping to Xp22.31, which is associated with BMD in the current study, was previously associated with male serum testosterone levels[30]. Thus, it is likely that rs5934507 affects serum testosterone, which in turn regulates BMD. In line with the different types of bone composition at different skeletal sites (predominantly trabecular at the lumbar spine and cortical at the femoral neck), we observed some indication of site specificity in 10 of the 56 BMD loci, suggesting differential genetic influences on BMD determination across skeletal sites. As has been previously shown,[31] we did not find in our results major differences in effect sizes between individuals of European and East Asian ancestry (Supplementary Figure 7). However, this may be due to reduced power, given the smaller number of individuals of East Asian ancestry. We tested a genetic risk score to identify individuals at risk for osteoporosis and fracture and showed that, cumulatively, the identified variants generate a gradient of risk. These gradients reach ORs of 1.56 for osteoporosis and 1.60 for fractures, when comparing participants with the highest risk scores to those having the mean score. Yet, at present, there is limited clinical usefulness for this score, as evidenced by its non-significant contribution to case discrimination when considering clinical risk factors with strong effects on osteoporosis

and fracture risk (like age and weight). This is not unexpected, given the small fraction of genetic risk for either BMD or fracture that has been identified thus far.

Our study has limitations. The identified SNPs are probably not the causal variants; it is more likely that these markers are in LD with the underlying causal variants. Additional analyses on potential functional SNPs identified in this study will be required to determine whether they are causal in these relationships with BMD. Moreover, the causal genes underlying the GWAS signals may be different from the candidate genes we describe, considering that our understanding of the role of these candidate genes in bone biology is limited. Further exploration of these loci with more detailed sequencing, gene expression and translational studies will be required. Such studies can also disentangle the diverse types of complex relationships we currently cannot distinguish in the BMD-associated loci with secondary signals to determine whether these are the result of true allelic heterogeneity or if they are driven by a second gene in the same region.[32] Similarly, despite our large sample size, power limitations still influence the detection of additional associations with smaller effect sizes and/or those arising from rarer variants. Finally, given the different levels of data availability and the difficulty of standardization across studies, we did not evaluate the effect of additional risk factors for osteoporosis, such as menopausal status and smoking, which can influence genetic associations with BMD. Nonetheless, despite these limitations, we have identified many new and previously unsuspected associations with BMD variation and fracture risk.

Finally, the relatively weak effects of the variants discovered by GWAS do not undermine the biological relevance of the genes identified, for proteins currently targeted by new osteoporosis treatments (Supplementary Figure 10). The new genes identified in our study may represent new candidates to target for osteoporosis drug discovery. Most established treatments for osteoporosis focus on curtailing bone resorption (for example, bisphosphonates and RANKL inhibitors), whereas only a few anabolic treatments are currently approved for the treatment of osteoporosis (recombinant truncated or altered PTH). Other anabolic compounds undergoing Phase 2 development include PTHrP fragments and Wnt signaling enhancers, such as antibodies to sclerostin[33]. Several of the variants robustly associated with BMD map in or close to genes that encode proteins involved in these pharmacologic pathways, namely *TNFRSF11B* (encoding osteoprotegerin), *TNFRSF11A* (encoding RANK), *TNFSF11* (encoding RANKL), *PTHLH* (encoding PTHrP), *LRP5* (encoding low-density lipoprotein receptor–related protein 5), *SOST* (encoding sclerostin) and *DKK1* (encoding Dickkopf-1).

In conclusion, these findings highlight the highly polygenic and complex nature of BMD variation, shed light on the pathophysiological mechanisms underlying fracture susceptibility and may contribute to the identification of future drug targets for the treatment of osteoporosis.

URLs. GEFOS Consortium, http://www.gefos.org/; GENOMOS Consortium, http://www.genomos.eu/; HapMap Project, http://hapmap.ncbi.nlm.nih.gov/; 1000 Genomes Project, http://www.1000genomes.org/; LocusZoom, http://csg.sph.umich.edu/locuszoom/; METAL, http://www.sph.umich.edu/csg/abecasis/Metal/.

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ONLINE METHODS

Study design

This study was conducted as part of the GEFOS Consortium, a coalition of teams of investigators dedicated to identifying the genetic determinants of osteoporosis. The discovery samples comprised 17 GWAS (N=32,961) from populations across North America, Europe, East Asia and Australia, with a variety of epidemiological designs (Supplementary Table 18A) and clinical characteristics of individuals

(Supplementary Table 18B); a subset of these had fracture information available (Supplementary Table 18C). Subjects from 34 additional studies with BMD data (N=50,933) were used for replication, and association with fracture was tested across 50 studies with fracture information, most of which were also used for the BMD analysis (N=31,016 cases and 102,444 controls) (Figure 1 and Supplementary Tables 19A-C and 20A-C). All studies were approved by their institutional ethics review committees, and all participants provided written informed consent.

BMD measurements and fracture definition

LS-BMD and FN-BMD were measured in all cohorts using dual-energy X-ray absorptiometry, following standard protocols (Supplementary Tables 18B, 19B and 20B). Three clinically distinct fracture definitions were used: (1) any type, consisting of low-trauma fractures at any skeletal site (except fingers, toes and skull) occurring after age 18 years, assessed by X-ray, radiographic report, clinical record, clinical interview and/or questionnaire, (2) validated non-vertebral, consisting of fractures occurring after age 50 years, with diagnosis confirmed by hospital records and/or radiographs, and (3) radiographic vertebral fractures, from lateral morphometry scored on X-rays. The first definition is most-inclusive, whereas the latter two are more stringent fracture definitions that are commonly used in randomized trials.[35, 36] Controls were defined as individuals without a history of fracture, using for each fracture type the same age limit categories as for the cases.

Stage 1 genome-wide association analysis

Genotyping and imputation

GWAS genotyping was performed by each study following standard protocols, and imputation was then carried out on ~2.5 million SNPs from HapMap[37] Phase 2 release 22 using Genome Build 36. Quality control was performed independently for each study. To facilitate meta-analysis, each group performed genotype imputation with BIM-BAM,[38] IMPUTE[39] or MACH[40] software using genotypes from HapMap Phase 2 release 22 (CEU or Han Chinese in Beijing (CHB) and Japanese in Tokyo (JPT) as appropriate). HapMap release 21 was used as a reference for SNPs residing on the X chromosome, and IMPUTE software was used for imputation. Overall, imputation quality scores for each SNP were obtained from IMPUTE (proper_info) and MACH (rsq_hat) statistics. Details of the genotyping platform, genotype quality control procedures and software for imputation that were used by each study are presented (Supplementary Tables 18D and 19D).

Association analysis with BMD

Each study performed genome-wide association analysis for FN-BMD and LS-BMD, using sex-specific and age-, weight- and principal component–adjusted standardized residuals analyzed under an additive (per allele) genetic model. Analyses of autosomal and X-chromosome markers were performed separately. The analysis of imputed genotype data accounted for uncertainty in each genotype prediction by using either the dosage information from MACH or the genotype probabilities from IMPUTE and BIM-BAM. Studies used MACH2QTL40 directly or via GRIMP[41] (which uses genotype dosage value as a predictor in a linear regression framework), SNPTEST,[39] Merlin,[42] BIM-BAM or the linear mixed-effects model of the Kinship and ProbABEL[43] (Supplementary Tables 18D and 19D). For analysis of the X chromosome, either SNPTEST or R software was used in each participating study. We coded 'effect allele homozygous genotype' as 2 and 'other allele homozygous genotype' as 0 in the genotyped SNPs in men on the X chromosome. The imputed genotypes were coded as continuous variables from 0 to 2 to take

into account imputation uncertainty. The genomic control method[44] was used to correct the standard error (SE) by the square root of the genomic inflation factor (λ): $SE_{corrected}=SEM\times\sqrt{\lambda}$.

Meta-analysis of the GWAS

Before performing meta-analysis on the genome-wide association data, SNPs with poor imputation quality scores (rsq_hat of <0.3 in MACH, proper_info of <0.4 in IMPUTE or a ratio of observed-to-expected dosage variance of <0.3 in BIM-BAM) and markers with a minor allele frequency (MAF) of <1% were excluded from each study. All individual GWAS were genomic control corrected before meta-analysis.[44] Individual study-specific genomic control values ranged from 0.98 to 1.08 (Supplementary Table 18D). A total of 2,483,766 autosomal SNPs were included in meta-analysis across 17, 16 and 13 studies for FN-BMD (pooled, women-only and men-only analyses, respectively) and 16, 13 and 12 studies for LS-BMD (pooled, women-only and men-only analyses, respectively). A total of 76,253 X-linked SNPs were included in meta-analysis across 14, 13 and 10 studies for LS-BMD and FN-BMD (pooled, women-only and men-only analyses, respectively). In our discovery analysis, we chose to implement a fixed-effects model, as it is generally preferable for the purposes of initial discovery, where the aim is to screen and identify as many of the true variants as possible.[45, 46] SNPs present in less than three studies were removed from the meta-analysis, yielding ~2.2 million SNPs in the final results. The genomic inflation factors (λ) were 1.11, 1.09 and 1.06 for FN-BMD (pooled, women-only and men-only analyses, respectively) and 1.13, 1.09 and 1.06 for LS-BMD (pooled, women-only and men-only analyses, respectively). A second genomic control correction was applied to the overall meta-analysis results, although such a second correction is considered overly conservative.[47] Significance for BMD association was set at $P<5\times10^{-8}$, and a Bonferroni correction was used for association with fracture.[48]

Selection of SNPs for replication

We took forward the most significant 96 SNPs for replication. With respect to power estimations, after adding 30,000 samples in stage 2, these variants had a priori power of ≥85% to reach $P=5\times10^{-8}$ in the meta-analysis. Loci were considered independent when separated by at least 1 Mb from a top GWAS signal. The 96 variants included the 82 index SNPs representing each of the 82 loci reaching $P<5\times10^{-6}$ in stage 1, 9 SNPs that were within the same 2-Mb windows as the 82, which were independent from the main signals (secondary signals), and the top 5 most-associated SNPs on the X chromosome (with $P<5\times10^{-5}$).

Association analyses with fracture risk

Effect estimates (odds ratios) for association of allele dosage of the top signals with fracture risk were obtained from logistic regression models adjusted for age, age2, weight, sex, height and four principal components. The proportion of the fracture risk explained by FN-BMD was calculated from the regression coefficients as $(\beta_{unadjusted}-\beta_{BMDadjusted})/\beta_{unadjusted}$ in a subset of replication samples for which both FN-BMD and complete fracture information was available.

STAGE 2 REPLICATION

Samples and genotyping

Fracture association results were also obtained for the 82 most-significant SNPs from 54,244 individuals of European ancestry from 7 GWAS (in silico genotyping) that had not been included in the stage 1

analyses (Supplementary Table 19A–C). Subjects from 34 studies of the GENOMOS Consortium with BMD and/or fracture information were studied in replication analysis (Supplementary Table 3A–C). De novo replication genotyping was performed in the UK (Kbiosciences), Iceland (deCODE Genetics), Australia (University of Queensland Diamantina Institute) and the United States (WHI GeCHIP) using KASPar, Centaurus, OpenArray and iSelect assays, respectively (Supplementary Note). Minimum genotyping quality control criteria were defined as sample call rate of >80%, SNP call rate of >90%, Hardy-Weinberg Equilibrium P value of $>1\times10^{-4}$ and MAF of >1%.

Association analyses and meta-analysis

We tested the association between the 96 SNPs and BMD and fracture risk in each in silico and de novo stage 2 study separately, as described for the stage 1 studies. We subsequently performed meta-analysis of effects and standard errors from the stage 2 studies and then carried out a meta-analysis of the summary statistics of stages 1 and 2 combined using the inverse-variance method in METAL. At the replication stage, where more than 30 studies were synthesized, we chose to first assess the underlying heterogeneity, considering both the Cochran's Q statistic and the I^2 metric. If the heterogeneity was not significant, fixed-effects model were applied. If the Cochran's Q P value was <0.0005 and I^2 was >50%, we used the more conservative random-effects model. Additional analyses. Further analyses were performed for the SNPs carried forward for replication. Each of these analyses is described in detail in the Supplementary Note.

In brief, we performed (1) a conditional genome-wide association analysis to determine whether any of the 82 BMD loci harbored additional independent signals, (2) tested gene-by-gene pairwise interactions between these BMD loci, (3) assessed within the independent setting of the PERF study (for details on study design see Supplementary Table 20A–C) the predictive ability derived from the cumulative effect of the 63 autosomal SNPs associated with BMD with genome-wide significance in relation to BMD levels and osteoporosis risk and that of the 16 BMD SNPs also associated with fracture risk in relation to fracture risk, (4) identified SNPs with r^2 of ≥0.80 with the lead SNP that were potentially functional (for example, nonsense, non-conservative nonsynonymous, synonymous, exonic splicing, transcription factor binding), using regional imputation with 1000 Genomes Project data (June 2010 release), (5) tested the relationship between gene expression profiles from transiliac bone biopsies and BMD in 84 unrelated postmenopausal women[49] and examined *cis* associations between each of the 55 significant BMD SNPs and expression of nearby genes in different tissues, including lymphoblastoid cell lines,[50-52] primary human fibroblasts and osteoblasts,[53] adipose tissue,[54] whole blood[54] and circulating monocytes,[55] and (6) evaluated the connectivity and relationships between identified loci using literature-based annotation with the GRAIL[19] statistical strategy.

Supplementary information is available at:
http://www.nature.com/ng/journal/v44/n5/extref/ng.2249-S1.pdf

REFERENCES

1. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. 2007 Mar;22(3):465-75.
2. Johnell O, Kanis JA, Oden A, Johansson H, De Laet C, Delmas P, et al. Predictive value of BMD for hip and other fractures. J Bone Miner Res. 2005 Jul;20(7):1185-94.

3. Kanis JA, Oden A, Johnell O, Johansson H, De Laet C, Brown J, et al. The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women. Osteoporos Int. 2007 Aug;18(8):1033-46.
4. Peacock M, Turner CH, Econs MJ, Foroud T. Genetics of osteoporosis. Endocr Rev. 2002 Jun;23(3):303-26.
5. Ralston SH, Uitterlinden AG. Genetics of osteoporosis. Endocr Rev. 2010 Oct;31(5):629-62.
6. Hardy J, Singleton A. Genomewide association studies and human disease. N Engl J Med. 2009 Apr 23;360(17):1759-68.
7. Manolio TA. Genomewide association studies and assessment of the risk of disease. N Engl J Med. 2010 Jul 8;363(2):166-76.
8. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008 May 3;371(9623):1505-12.
9. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008 May 29;358(22):2355-65.
10. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
11. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. New sequence variants associated with bone mineral density. Nat Genet. 2009 Jan;41(1):15-7.
12. Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, et al. An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet. 2010 Jun;6(6):e1000977.
13. Kung AW, Xiao SM, Cherny S, Li GH, Gao Y, Tso G, et al. Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies. Am J Hum Genet. 2010 Feb 12;86(2):229-39.
14. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
15. Richards JB, Kavvoura FK, Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, et al. Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture. Ann Intern Med. 2009 Oct 20;151(8):528-37.
16. van Meurs JB, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, et al. Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. JAMA. 2008 Mar 19;299(11):1277-90.
17. Bagger YZ, Rasmussen HB, Alexandersen P, Werge T, Christiansen C, Tanko LB. Links between cardiovascular disease and osteoporosis in postmenopausal women: serum lipids or atherosclerosis per se? Osteoporos Int. 2007 Apr;18(4):505-12.
18. Kiel DP, Ferrari SL, Cupples LA, Karasik D, Manen D, Imamovic A, et al. Genetic variation at the low-density lipoprotein receptor-related protein 5 (LRP5) locus modulates Wnt signaling and the relationship of physical activity with bone mineral density in men. Bone. 2007 Mar;40(3):587-96.
19. Raychaudhuri S, Plenge RM, Rossin EJ, Ng AC, Purcell SM, Sklar P, et al. Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. PLoS Genet. 2009 Jun;5(6):e1000534.
20. Lango Allen H, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F, et al. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature. 2010 Oct 14;467(7317):832-8.
21. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, et al. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet. 2001 Mar;68(3):577-89.
22. Guo YF, Xiong DH, Shen H, Zhao LJ, Xiao P, Guo Y, et al. Polymorphisms of the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with obesity phenotypes in a large family-based association study. J Med Genet. 2006 Oct;43(10):798-803.

23. Kornak U, Kasper D, Bosl MR, Kaiser E, Schweizer M, Schulz A, et al. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. Cell. 2001 Jan 26;104(2):205-15.
24. Panagiotou OA, Evangelou E, Ioannidis JP. Genome-wide significant associations for variants with minor allele frequency of 5% or less--an overview: A HuGE review. Am J Epidemiol. 2010 Oct 15;172(8):869-89.
25. Jin W, Chang M, Paul EM, Babu G, Lee AJ, Reiley W, et al. Deubiquitinating enzyme CYLD negatively regulates RANK signaling and osteoclastogenesis in mice. J Clin Invest. 2008 May;118(5):1858-66.
26. Sundaram K, Shanmugarajan S, Rao DS, Reddy SV. Mutant p62P392L stimulation of osteoclast differentiation in Paget's disease of bone. Endocrinology. 2011 Nov;152(11):4180-9.
27. Grundberg E, Kwan T, Ge B, Lam KC, Koka V, Kindmark A, et al. Population genomics in a disease targeted primary cell model. Genome Res. 2009 Nov;19(11):1942-52.
28. Duan Y, Beck TJ, Wang XF, Seeman E. Structural and biomechanical basis of sexual dimorphism in femoral neck fragility has its origins in growth and aging. J Bone Miner Res. 2003 Oct;18(10):1766-74.
29. Karasik D, Ferrari SL. Contribution of gender-specific genetic factors to osteoporosis risk. Ann Hum Genet. 2008 Sep;72(Pt 5):696-714.
30. Ohlsson C, Wallaschofski H, Lunetta KL, Stolk L, Perry JR, Koster A, et al. Genetic determinants of serum testosterone concentrations in men. PLoS Genet. 2011 Oct;7(10):e1002313.
31. Styrkarsdottir U, Halldorsson BV, Gudbjartsson DF, Tang NL, Koh JM, Xiao SM, et al. European bone mineral density loci are also associated with BMD in East-Asian populations. PLoS One. 2010;5(10):e13217.
32. Wood AR, Hernandez DG, Nalls MA, Yaghootkar H, Gibbs JR, Harries LW, et al. Allelic heterogeneity and more detailed analyses of known loci explain additional phenotypic variation and reveal complex patterns of association. Hum Mol Genet. 2011 Oct 15;20(20):4082-92.
33. Mosekilde L, Torring O, Rejnmark L. Emerging anabolic treatments in osteoporosis. Curr Drug Saf. 2011 Apr;6(2):62-74.
34. Hasselblad V, Hedges LV. Meta-analysis of screening and diagnostic tests. Psychol Bull. 1995 Jan;117(1):167-78.
35. Harris ST, Watts NB, Genant HK, McKeever CD, Hangartner T, Keller M, et al. Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy With Risedronate Therapy (VERT) Study Group. JAMA. 1999 Oct 13;282(14):1344-52.
36. Jackson RD, LaCroix AZ, Gass M, Wallace RB, Robbins J, Lewis CE, et al. Calcium plus vitamin D supplementation and the risk of fractures. N Engl J Med. 2006 Feb 16;354(7):669-83.
37. Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA, et al. A second generation human haplotype map of over 3.1 million SNPs. Nature. 2007 Oct 18;449(7164):851-61.
38. Servin B, Stephens M. Imputation-based analysis of association studies: candidate regions and quantitative traits. PLoS Genet. 2007 Jul;3(7):e114.
39. Marchini J, Howie B, Myers S, McVean G, Donnelly P. A new multipoint method for genome-wide association studies by imputation of genotypes. Nat Genet. 2007 Jul;39(7):906-13.
40. Li Y, Willer C, Sanna S, Abecasis G. Genotype imputation. Annu Rev Genomics Hum Genet. 2009;10:387-406.
41. Estrada K, Abuseiris A, Grosveld FG, Uitterlinden AG, Knoch TA, Rivadeneira F. GRIMP: a web- and grid-based tool for high-speed analysis of large-scale genome-wide association using imputed data. Bioinformatics. 2009 Oct 15;25(20):2750-2.
42. Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. Nat Genet. 2002 Jan;30(1):97-101.
43. Aulchenko YS, Struchalin MV, van Duijn CM. ProbABEL package for genome-wide association analysis of imputed data. BMC Bioinformatics. 2010;11:134.
44. Devlin B, Roeder K, Wasserman L. Genomic control, a new approach to genetic-based association studies. Theor Popul Biol. 2001 Nov;60(3):155-66.
45. Ioannidis JP, Thomas G, Daly MJ. Validating, augmenting and refining genome-wide association signals. Nat Rev Genet. 2009 May;10(5):318-29.
46. Pereira TV, Patsopoulos NA, Salanti G, Ioannidis JP. Discovery properties of genome-wide association signals from cumulatively combined data sets. Am J Epidemiol. 2009 Nov 15;170(10):1197-206.

47. Yang J, Weedon MN, Purcell S, Lettre G, Estrada K, Willer CJ, et al. Genomic inflation factors under polygenic inheritance. Eur J Hum Genet. 2011 Jul;19(7):807-12.
48. Pe'er I, Yelensky R, Altshuler D, Daly MJ. Estimation of the multiple testing burden for genomewide association studies of nearly all common variants. Genet Epidemiol. 2008 May;32(4):381-5.
49. Reppe S, Refvem H, Gautvik VT, Olstad OK, Hovring PI, Reinholt FP, et al. Eight genes are highly associated with BMD variation in postmenopausal Caucasian women. Bone. 2010 Mar;46(3):604-12.
50. Ge B, Pokholok DK, Kwan T, Grundberg E, Morcos L, Verlaan DJ, et al. Global patterns of cis variation in human cells revealed by high-density allelic expression analysis. Nat Genet. 2009 Nov;41(11):1216-22.
51. Montgomery SB, Sammeth M, Gutierrez-Arcelus M, Lach RP, Ingle C, Nisbett J, et al. Transcriptome genetics using second generation sequencing in a Caucasian population. Nature. 2010 Apr 1;464(7289):773-7.
52. Stranger BE, Nica AC, Forrest MS, Dimas A, Bird CP, Beazley C, et al. Population genomics of human gene expression. Nat Genet. 2007 Oct;39(10):1217-24.
53. Grundberg E, Adoue V, Kwan T, Ge B, Duan QL, Lam KC, et al. Global analysis of the impact of environmental perturbation on cis-regulation of gene expression. PLoS Genet. 2011;7(1):e1001279.
54. Emilsson V, Thorleifsson G, Zhang B, Leonardson AS, Zink F, Zhu J, et al. Genetics of gene expression and its effect on disease. Nature. 2008 Mar 27;452(7186):423-8.
55. Zeller T, Wild P, Szymczak S, Rotival M, Schillert A, Castagne R, et al. Genetics and beyond--the transcriptome of human monocytes and disease susceptibility. PLoS One. 2010;5(5):e10693.

Chapter 4.2

Large-scale genome wide meta-analysis identifies genetic determinants for fracture risk

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In preparation

ABSTRACT

Background Bone fractures are considered the most clinically relevant clinical sequelae of osteoporosis. A positive family history is an important risk factor for fractures. The genes that contribute to risk for osteoporotic fractures are largely unknown.

Methods This meta-analysis on any-type of fracture risk included up to 163,292 participants (38,021 cases) across 66 cohorts from Europe, USA, Asia and Australia. Cases were adults with fractures confirmed by medical, radiological or questionnaire reports. The discovery phase comprised 24 GWAS. SNPs surpassing $P<5x10^{-6}$ and previously reported BMD-fracture SNPs were followed-up in a replication phase including both de-novo genotyping and in-silico look ups.

Results Common SNPs explained 0.19 (standard error: 0.09, P=0.02) of the variance in fracture risk. Ten loci replicated at genome-wide significance ($P<5x10^{-8}$), with small to moderate effect sizes (OR 1.06–1.18; 95% CI: 1.04–1.24). The signal on chromosome 21 has not been previously reported and maps upstream of the gene *FLJ45139*, which encodes a protein of yet unknown function. We confirmed the 7q21.3 and 18p11.21 loci and replicated signals at genome-wide significant level at the *SOST*, *CPED1/WNT16*, *SPTBN1*, *MEPE/IBSP/SPP1*, *MBL2/DKK1*, *CTNNB1* and *RSPO3* loci.

Conclusions The top loci in our genome-wide screen were associated with both fracture risk and BMD, reinforcing BMD as a powerful endophenotype and suggesting a considerable proportion of genetic variance in fracture risk is through BMD. Mutations in some of the candidate genes cause skeletal abnormalities and many are involved in Wnt signaling, while the function and therapeutic potential of others remains to be explored.

INTRODUCTION

Osteoporosis is a common skeletal disorder characterized by low bone mineral density (BMD), impaired bone quality, and fragility fractures. Osteoporotic fractures mainly occur at the vertebrae, wrist, pelvis and hip and constitute a major and costly public health problem, affecting hundreds of millions of people worldwide. One of the most important clinical risk factors for osteoporosis is a positive family history.[1] Dozens of genetic loci have been identified by genome-wide association studies (GWAS) studying BMD variation in populations,[2-9] while only a few loci have been shown to underlie the occurrence of rare forms of osteoporosis in families[10-12]. Since only a fraction of the BMD loci have been shown to be associated with fracture we have embarked in performing the largest GWAS meta-analysis to date including 19,414 cases and 83,459 controls targeting the characterization of genetic determinants of osteoporotic fracture risk.

METHODS

Subjects

This study is part of the GEnetic Factors for OSteoporosis consortium (GEFOS), a coalition of teams of investigators dedicated to identify the genetic determinants of osteoporosis (http://www.gefos.org/). The discovery samples comprised 24 GWA studies (N=102,873) from populations across North America, Europe, East Asia and Australia, with a variety of epidemiological designs (Table S1A-C in the Supplementary Appendix) and patient characteristics (Table S2A-C in the Supplementary Appendix). The replication phase including both de-novo genotyping and in-silico look ups in subjects from 42 additional studies with fracture data (maximum N=18,779 cases and 41,845 controls) (Tables S3A-C in the Supplementary Appendix). All studies were approved by their institutional ethics review committees and all participants provided written informed consent.

Fracture definition

Cases were individuals (>18 years) with fractures confirmed by medical, radiological or questionnaire reports. When possible, fractures of the fingers, toes and skull were excluded from analyses. Also, high-trauma fractures were excluded whenever possible, for example high-energy traffic accidents or falls from a height greater than standing height. Controls were defined as individuals without a known history of fracture.

Genotyping and imputation

GWAS genotyping was done by each study following standard manufacturer protocols followed by imputation to ~2.5 million SNPs from HapMap Phase II. De-novo replication genotyping was done in the UK (Kbiosciences). Details of the genotyping procedures are presented in the Supplementary Appendix (Tables S4A-C).

Statistical analysis

Association analyses

Each study performed genome-wide association analysis for fracture risk using logistic regression models adjusted for sex, age (simple and quadratic term), height, weight, testing additive (per allele) genetic effects. Additionally, sex-stratified analyses were performed employing the same statistical models. Before performing meta-analysis, three meta-analytical centers checked the data files independently.

In addition, filtering was applied to each data file as described in detail in the Methods section of the Supplementary Appendix. Genomic control (GC) was applied to correct the SE for the genomic inflation factor (λ) to control for possible inflation of test statistics due to population stratification and cryptic family relations, targeting $\lambda<1.05$.[13] Results were meta-analyzed with inverse variance fixed-effects in METAL.[14]

Additional analyses

GCTA analysis was used to estimate the variance explained by common SNPs in Rotterdam Study I and to detect secondary signals in the meta-analysis using individual-level genotype data of two discovery sets (Rotterdam Study-II and -III).[15] Further analyses were performed including: association with dual-energy X-ray absorptiometry (DXA) femoral neck (FN-) and lumbar spine (LS-BMD),[9] expression quantitative trait loci (eQTL), literature-based annotation with Gene Relationships across Implicated Loci (GRAIL) statistical strategy,[16] functional, clinical and regional annotations. The variance explained by SNPs prioritized from the discovery screenings and correlated phenotypes was determined. These analyses are described in detail in the Methods section of the Supplementary Appendix.

RESULTS

Heritability estimate and association analyses

A moderate proportion of variance in fracture risk could be explained by genome-wide common SNPs (0.19; standard error: 0.09, P value: 0.02). Next, GWAS summary statistics from 24 cohorts (N=102,873, 19,414 fractures) were meta-analyzed for ~2.5 million genotyped and imputed SNPs. In the discovery phase, 35 SNPs from the sex-combined and gender-stratified analyses surpassed $P<5x10^{-6}$ at a satisfactory genomic inflation level (Figures S1 and S2 in the Supplementary Appendix), mapping to 27 distinct loci (Table S5 in the Supplementary Appendix). About half of these loci were also associated with BMD (Table S6 in the Supplementary Appendix) clustering in the Wnt signaling pathway, while others seemed to contain genes involved in hormonal and neurological pathways. No secondary signals were identified by GCTA.

These and previously reported BMD-fracture loci[9] were followed-up in a replication phase, summing to 163,292 participants (38,021 cases) as illustrated in Figure 1. Loci replicating at genome-wide significance are included below in Tables 1 and 2 and full results are available in Table S7 in the Supplementary Appendix). In sum, eight SNPs from the current genome-wide screening mapping to six distinct loci reached genome-wide significance, while six SNPs from the published BMD-fracture loci replicated at this level. Altogether, as two loci coincided, these add up to ten genome-wide significant fracture loci. The proportion of variance explained in fracture risk was 0.02 for the independent genome-wide significantly replicating SNPs in the meta-analysis, where results were validated in a genome-wide association study not part of the discovery screening; full results are shown in Table S8 in the Supplementary Appendix. Including more fracture SNPs by lowering the significance threshold further added to the variance explained. Also, sets of SNPs from GWAS for correlated traits such as BMD and height significantly enlarged the variance explained, which was more than would expect by chance. This effect was not shown for SNPs for other known associated phenotypes such as vitamin D and metabolic traits such as type 2 diabetes mellitus and C-reactive protein.

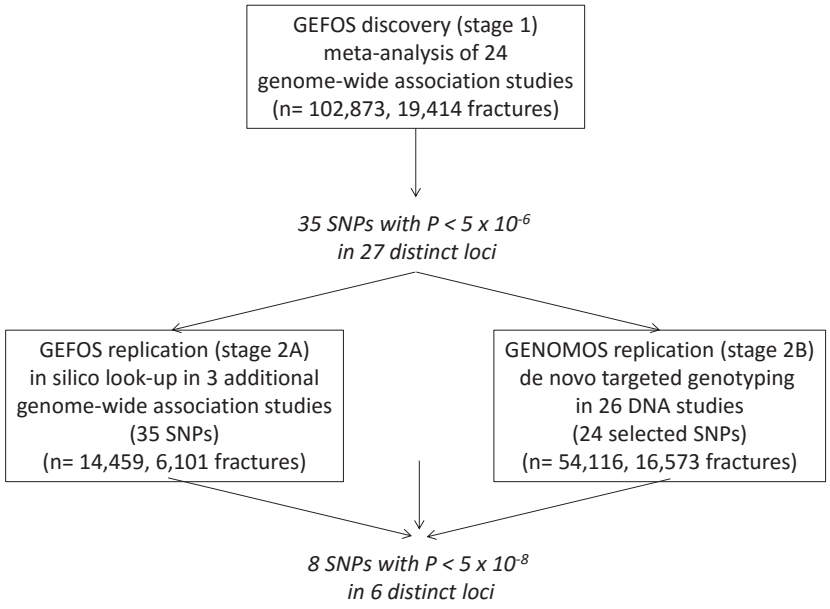

Figure 1. Study design diagram.

Remarkably, the loci replicating consistently were all previously reported as associated with BMD. The signal on chromosome 21q22.2 ($P=3x10^{-10}$; OR=1.07; 95% CI: [1.05–1.10]) has not been previously reported and maps upstream of the gene *FLJ45139* that encodes a protein of yet unknown function. Although not a previously known BMD locus, at least part of the association with fracture risk is probably through BMD as the SNP was associated with DXA FN- and LS-BMD in the GEFOS dataset (rs9982895 $P_{FN\text{-}BMD}=0.01$; $Beta_{FN\text{-}BMD}=-0.02$; $P_{LS\text{-}BMD}=0.0001$; $Beta_{LS\text{-}BMD}=-0.04$).

Functional and clinical annotations

Annotations for the top markers from the fracture GWAS are available in Table S9 in the Supplementary Appendix.

The top signal in the novel locus on chromosome 21 has a strong DNase I-hypersensitive site (DHS) correlation (r=0.58) and is in the same topological domain with *ETS2*. ETS2 is a transcription factor and protooncogene, and is highly expressed in newly formed cartilage in murine development, including skull precursor cells and vertebral primordia. Mice with Ets2 overexpression developed neurocranial, visceral cranial, and cervical skeletal abnormalities. ETS2 has a role in skeletal development and that overexpression is involved in the genesis of some skeletal abnormalities that occur in Down syndrome.[17]

Several of the candidate gene products are part of or closely related to Wnt signaling. The signal replicating on 3p22.1 is in the vicinity of *CTNNB1* ($P=6x10^{-9}$; OR=1.06; 95% CI: [1.04–1.08]), which encodes the factor integral to Wnt signaling beta-catenin. The large linkage disequilibrium (LD) block harboring the 7q21.3 signal includes *SLC25A13* and *SHFM1* ($P=1x10^{-12}$; OR=1.07; 95% CI: [1.05–1.10]). *Slc25a13* is expressed in osteoblasts and osteoclasts in mice (Figure S6 in the Supplementary Appendix). The protein product, Citrin plays a role in transport across the mitochondrial membrane. However, expression of this gene was not correlated with BMD in our data. Genomic re-arrangements take place more frequently in

Table 1. Genome-wide significant signals associated with fracture risk from the discovery and replication phases.

					GEFOS DISCOVERY 19,414 cases 83,459 controls				GENOMOS GENOTYPING 12,678 cases 23,720 controls				IN-SILICO REPLICATION 6,101 cases 8,358 controls				META-ANALYSIS 38,168 cases 115,442 controls				
CHR	POS	SNP	EA	NEA	EAF	BETA	SE	P	EAF	BETA	SE	P	EAF	BETA	SE	P	EAF	BETA	SE	P	I^2
6	127476799	rs6916318	A	T	0.47	0.05	0.01	2.0E-06	0.48	0.09	0.02	1.3E-06	0.46	0.13	0.03	2.9E-05	0.48	0.07	0.01	3.7E-12	71
7	95955854	rs4729260	G	C	0.32	0.09	0.01	3.0E-09					0.3	0.06	0.03	6.2E-02	0.3	0.08	0.01	2.7E-10	0
7	120715028	rs2952556	G	A	0.64	0.07	0.01	6.7E-07	0.6	0.05	0	7.5E-03	0.7	0.10	0.03	1.6E-03	0.6	0.06	0.01	2.7E-10	0
17	39182365	rs2741856	G	C	0.91	0.15	0.03	1.5E-08					0.9	0.28	0.06	7.4E-06	0.9	0.17	0.02	3.7E-12	74
18	13724904	rs3819131	T	C	0.34	0.09	0.01	2.1E-11					0.3	0.06	0.03	5.3E-02	0.34	0.09	0.01	4.0E-12	0
21	39264957	rs9982895	C	T	0.74	0.07	0.01	1.6E-06	0.71	0.07	0.02	6.9E-04	0.73	0.08	0.03	1.9E-02	0.73	0.07	0.01	2.8E-10	0

Abbreviations: GEFOS, Genetic Factors of OSteoporosis consortium; GENOMOS, GENetic Markers of OSteoporosis consortium; CHR, chromosome; POS, position; SNP, single nucleotide polymorphism; EA, effect allele; NEA; non effect allele; EAF, effect allele frequency; BETA, beta-coefficient of effect size; SE, standard error; P, P value; I^2, measure of heterogeneity of effect estimates.

Table 2. Previously published single nucleotide polymorphisms associated with fracture risk reaching genome-wide significance.

					GEFOS DISCOVERY 19,414 cases 83,459 controls				GENOMOS GENOTYPING 16,573 cases 37,543 controls				IN-SILICO REPLICATION 2,045 cases 4,302 controls				META-ANALYSIS 38,021 cases 125,271 controls				
CHR	POS	SNP	EA	NEA	EAF	BETA	SE	P	EAF	BETA	SE	P	EAF	BETA	SE	P	EAF	BETA	SE	P	I^2
2	54513211	rs4233949	G	C	0.62	0.06	0.01	9.4E-07	0.61	0.05	0.02	9.2E-04	0.63	0.02	0.04	7.0E-01	0.62	0.06	0.01	6.2E-09	0
3	41103568	rs430727	T	C	0.46	0.06	0.01	1.0E-05	0.47	0.06	0.02	1.2E-04	0.45	0.02	0.04	6.1E-01	0.46	0.06	0.01	6.3E-09	0
4	88992873	rs6532023	G	C	0.67	0.05	0.01	3.8E-04	0.67	0.08	0.02	2.5E-07	0.69	0.03	0.04	5.4E-01	0.67	0.06	0.01	1.6E-09	39
7	95958611	rs4727338	G	C	0.33	0.08	0.01	1.2E-09	0.32	0.06	0.02	2.4E-04	0.30	0.06	0.04	1.7E-01	0.33	0.07	0.01	1.0E-12	0
10	54097831	rs1373004	T	G	0.13	0.06	0.02	1.2E-03	0.12	0.11	0.02	3.6E-06	0.12	0.11	0.06	6.1E-02	0.13	0.08	0.01	7.7E-09	6
18	13698574	rs4796995	G	T	0.36	0.08	0.01	1.6E-10	0.38	0.06	0.02	1.9E-04	0.36	0.04	0.04	3.3E-01	0.37	0.07	0.01	3.1E-13	17

Abbreviations: GEFOS, Genetic Factors of OSteoporosis consortium; GENOMOS, GENetic Markers of OSteoporosis consortium; CHR, chromosome; POS, position; SNP, single nucleotide polymorphism; EA, effect allele; NEA; non effect allele; EAF, effect allele frequency; BETA, beta-coefficient of effect size; SE, standard error; P, P value; I^2, measure of heterogeneity of effect estimates.

this region, leading to deletion of *DSS1*, *DLX5* and *DLX6*.[18, 19] The latter two code for members of the Wnt signaling pathway, and when both are deleted or mutated, ectrodactyly (also known as split hand/foot malformation) with missing digits, a claw-like appearance of the distal extremities and hypoplasia of the long bones may occur.[20] split hand/foot malformation has been reported with urogenital developmental defects,[21] and double inactivation of *Dlx5* and *Dlx6* in mice leads to decreased testosterone levels and abnormal masculinization.[22]

The closest candidate genes for the association on chromosome 10q21.1 ($P=8x10^{-9}$; OR=1.09; 95% CI: [1.06–1.12]) are *MBL2* and *DKK1*. *DKK1* encodes Dickkopf 1, an inhibitor of Wnt signaling, and anti-DKK1 antibody treatment promotes bone fracture healing in mice.[23] The 17q21.31 signal ($P=1.2x10^{-8}$) is close to *SOST*. *SOST* encodes Sclerostin, which antagonizes bone formation by binding to LRP5/6 receptors and inhibiting Wnt signaling.[24] Also, inactivating mutations of *SOST* have been reported to cause high bone mass syndromes comprising sclerosteosis,[25, 26] van Buchem's disease[27] and craniodiaphyseal dysplasia[28]. Variants on chromosome 6q22.33 upstream from *RSPO3* were first reported as associated at genome-wide significant level with BMD in Australian and Northern European populations,[29] and our GWAS detected a signal for fracture risk ($P=4x10^{-12}$; OR=1.07; 95% CI: [1.05–1.09]). RSpo proteins are activators and regulators of canonical Wnt signaling, with RSpo3 being one of the most potent members.[30]

The signal on chromosome 4q22.1 ($P=2x10^{-9}$; OR=1.06; 95% CI: [1.04–1.08]) points to a cluster of phylogenetically-related genes encoding for matricellular phosphoglycoproteins important for bone formation and mineralization including *MEPE* (matrix extracellular phosphoglycoprotein), *IBSP* (integrin binding sialoprotein) and *SPP1* (osteopontin). All three genes are expressed in bone and exhibit a skeletal phenotype when deleted in mice.[31-33] The signal on 2p16.2 ($P=6x10^{-9}$; OR=1.06; 95% CI: [1.04–1.08]) maps close to *SPTBN1*, which encodes a subform of β-spectrin a major cytoskeletal scaffold protein. In mice it is highly expressed (Figure S7 in the Supplementary Appendix)and targeted inactivation of the mouse homolog results in disruption of TGF-beta signaling.[34] This gene is alternately spliced and expression of this gene yields a mixed correlation with BMD, dependent on the probe used to measure expression.

The *CPED1/WNT16* locus repeatedly appears among the top hits in different GWAS for fracture-related traits,[35, 36] and may thus indicate up to now unknown biological pathways for pharmacological intervention for osteoporosis. Expression of *Cped1* assessed by whole transcriptome RNA sequencing in mouse calvarial osteoblasts increased across differentiation (Figure S8 in the Supplementary Appendix). Expression of *CPED1* in whole bone tissue obtained from iliac crest biopsies from post-menopausal women showed a significantly negative correlation with hip (r=-0.36) and spine (r=-0.32) T score. In addition, osteoblast-derived WNT16 inhibits human and mouse osteoclastogenesis both directly by acting on osteoclast progenitors and indirectly by increasing expression of osteoprotegerin (Opg) in osteoblasts and Wnt16-deficient mice develop spontaneous fractures as a result of low cortical thickness and high cortical porosity.[37] Further, associated SNPs in the 18p11.21 locus map to three genes with unknown function, i.e., *FAM210A, C18orf1* and *RNMT* ($P=3x10^{-13}$; OR=1.07; 95% CI: [1.05–1.09]). *Rnmt* is expressed in mouse osteoblasts (Figure S9 in the Supplementary Appendix). The RNMT protein is involved in mRNA processing and this gene is most highly expressed in the brain. Expression of RNMT in whole bone tissue obtained from iliac crest biopsies from post-menopausal women showed a significantly positive correlation with whole body BMD T score (r=0.25).

To explore any sex-specific associations, additional analyses were performed stratified by gender and by formally testing for interaction effects. The gender-stratified results did not yield any replicating sex-specific associations. The formal testing for sex-interaction effects did not show a significant difference for the genome-wide significant hits, but sex-specific effects may contribute to the high heterogeneity

observed in several suggestive signals, including those at the *ELSPBP1, SHFM1, RASSF4, VLDLR, ALPK3* and *LRP5* loci (Table S9 of the Supplementary Appendix).

DISCUSSION

In this study, the largest GWAS meta-analysis for osteoporosis traits to date, we are among the first to identify genetic loci associated with osteoporotic fracture risk. A significant proportion of the variance of risk of all-type of osteoporotic fracture can be explained by common genetic variants, yet this heritability estimate is of modest size. Additionally, our meta-analytical effort discovered and replicated multiple genetic loci associated with risk of fracture. Discovery analyses suggested the involvement of hormonal and neurological pathways, yet, this could not be replicated. Replicating loci were those found to be associated with BMD levels as well, of which several seem to be involved in Wnt signaling. Our data suggests a high degree of heterogeneity with suggestive evidence for specificity of effects for gender and population. Results were annotated by querying online databases and collaborating with functional biology groups.

In our previous work, we tested 96 BMD markers for association with fracture in 31,016 cases and 102,444 controls from 50 studies with fracture information. In this fracture meta-analysis, 14 loci were significantly associated with any type of fracture at Bonferroni-corrected significance ($P=5\times10^{-4}$), of which six loci surpassed genome-wide significance ($P=5\times10^{-8}$). The genome-wide significant signals mapped to 18p11.21 (*FAM210A*), 7q21.3 (*SLC25A13*), 11q13.2 (*LRP5*), 4q22.1 (*MEPE*), 2p16.2 (*SPTBN1*) and 10q21.1 (*DKK1*). The others at Bonferroni-corrected significance included: two independent signals at 1p36.12 (*ZBTB40* and *WNT4*), 3p22.1 (*CTNNB1*), 7p14.1 (*STARD3NL*), 7q31.31 (*WNT16*), 9q34.11 (*FUBP3*), 11p14.1 (*DCDC5*), 14q32.12 (*RPS6KA5*) and two independent signals at 17q21.31 (*SOST* and *C17orf53*). In our current project we started out directly with GWAS meta-analyses for fracture risk. We confirmed the 7q21.3 and 18p11.21 loci, boosted the signals at the *SOST, CPED1/WNT16, SPTBN1, MEPE/IBSP/SPP1, MBL2/DKK1* and *CTNNB1* loci towards genome-wide significant level, and added the 6q22.33 (*RSPO3*) and 21q22.2 (*FLJ45139*) loci to the list of the now known fracture loci. Altogether, the GWAS design in a well-powered setting has pointed us to 16 loci for fracture risk so far. Compared to other quantitative traits that have been subject of GWAS, investigating BMD seems very promising and prolific (Figure S10 in the Supplementary Appendix). On the other hand, GWAS taking dichotomous disease outcomes as a direct outcome, such as risk of osteoporotic fractures as described in this thesis, have been relatively more challenging (Figure S11 in the Supplementary Appendix). Compared to other GWAS employing a case-control design for different disease outcomes, osteoporotic fracture GWAS have been reasonably successful. From these graphs it becomes clear that generally the number of loci discovered increases along with increases in sample sizes as study power improves. However, the slopes of the graphs and the sample sizes investigated until now for the various phenotypes differ.

As the most prominent genetic loci associated with fracture risk coming out of a hypothesis-free genome-wide screen are also associated with BMD, this serves as proof of BMD being a very powerful endophenotype. From a different perspective, epidemiological studies have shown that a great proportion of persons that fracture have abnormal BMD in either the osteoporotic or osteopenic range, with only 13% of women and 18% of men having a T score >-1.0.[38] These observations bring us to the hypothesis that an abnormally decreased BMD is a prerequisite to fracture in the majority of cases, irrespective of additional mechanisms present. Further, those individuals with BMD in the normal range may have other confounding factors present such as type 2 diabetes mellitus and osteoarthritis.[39, 40]

Nevertheless, screening in view of primary prevention of osteoporotic fractures based only on BMD is not enough, because of the limited specificity as the relative risks are 1.3 for individuals with osteopenic BMD and 2.7 for individuals with osteoporotic BMD, respectively. [38]

Pathway analyses applied to the information derived from our GWAS meta-analyses for BMD and fracture risk highlighted connections between genes in the loci discovered. The discovery results pointed to hormonal and neurological pathways, possibly highlighting the multi-factorial etiology of fracture risk. It should be noted that the GRAIL annotation includes only information what is known so far, therefore, unknown gene functions and pathways will be missed. SOST, RSPO3, WNT16 and DKK1 are known Wnt signaling factors. Wnt signaling has been recognized as a key regulator of bone mass.[41] It is tempting to speculate if defective Wnt signaling is detrimental to fracture risk at older age through a suboptimal peak bone mass established in earlier life. Alternative pathways would be defective maintenance of bone mass and compromised fracture healing at later stages[42, 43].

However, few of the other initial fracture results remained after replication. Particularly the non-BMD loci not replicating could be due to false-positive associations, or the heterogeneity associated with the coarse phenotype definition and variety in study populations. Further, the biological relevance of our associations is accentuated by the identification of genes underlying rare monogenetic forms of musculoskeletal defects or extreme bone mass syndromes which also contain common variants involved in normal BMD variation and fracture risk at the population level.[5, 8, 44] This is supportive of a genetic architecture where both common and rare genetic variation may reside in the same locus.[45]

In contrast to the lack of population-specificity in previous work on BMD,[9, 46] we found suggestive evidence for specificity of genetic variants for fracture risk in Asian versus Caucasian populations. Eurasian populations are efficient for genome scans, and populations of recent African origin (such as African Americans) are efficient for identification of causal polymorphisms within a candidate sequence because of their distinctive linkage disequilibrium (LD) structure.[47] Nevertheless, larger sample sizes will be needed than currently available. Sexual dimorphism in various bone phenotypes is widely observed; however, sex-specific SNP effects, or gene-by-sex interactions, seem infrequent, as only one sex specific BMD variant (Xp22.31 chromosome) has been discovered to date.[9, 48] Our current fracture meta-analysis showed signs of gender-interaction for a few of the loci. Nevertheless, our gender-specific analyses did not seem to be adequately powered to solidly corroborate sex-specific fracture loci in the stratified analyses. Population- and gender-specificity may have contributed to the high heterogeneity of effects present.

There were substantial age differences between studies, and exploring age-specific effects also seems of interest. Alternatively, phenotype measurement differences across studies are a known possible source of heterogeneity complicating replication successes due to non-differential misclassification leading to dilution of effect estimates. The signals we have now identified ought to be very strongly associated, while genetic variants that truly contribute to fracture risk but with small effect sizes may easily be missed, i.e., false negatives. Further, the statistical thresholds for significance in GWAS are very stringent.

For BMD it has been established that several loci display skeletal-site specific effects,[9] and for fracture risk site-specific genetic effects have been proposed as well[49]. Intriguingly, different skeletal site-specificity for BMD has been demonstrated for variants in independent signals within the same locus but in different genes, i.e., *WNT16* and *CPED1*.[50] If genetic risk factors exist that may act only on certain skeletal sites, separate efforts taking specific fracture types as study outcomes may be worthwhile, as pursued in parallel by our consortia. However, so far these approaches have been challenging. For instance, our

GWAS for osteoporotic vertebral fractures identified a genome-wide significant signal in the discovery, however, this could not be convincingly replicated by de-novo genotyping the specific marker in 15 studies world-wide.[51] The current problem is that not enough studies are available with comparable or identical phenotyping procedures and there is a lack of means to harmonize data between cohorts. Analyzing the outcomes of non-vertebral fracture and vertebral fracture in addition to all-type of fracture did not produce any additional signals in our previous effort,[9] demonstrating that the study power did not benefit from improving phenotype definitions at the cost of lower sample size at this stage.

Our study has limitations. The identified SNPs are probably not the causal variants; it is more likely that these markers are in LD with the underlying causal variants. Further, the causal genes underlying the GWAS signals may be different to the candidate genes we describe considering that our understanding of their role in bone biology is limited. Further exploration of these loci with more detailed sequencing, expression, and translational studies will be required. Despite our large sample size, power limitations still play a role for detecting additional associations with smaller effect sizes and/or rarer variants[10, 12]. Nonetheless, we have identified many novel and previously unsuspected associations with osteoporosis and fracture risk.

Future studies could expand on the types of genetic variation under investigation, such as for instance copy number variation.[52] Also, genome-wide analysis of sex-chromosomes has not yet been performed for fracture risk. Analysis of rare variants and fine-mapping may be achieved by 1000 Genomes imputation, regional or whole genome sequencing. The first effort in our GEFOS and GENOMOS consortium encompassing a sequencing-based GWAS meta-analysis has discovered EN1 as a determinant of bone density and fracture.[53] As microarray based GWAS relies on the principle of LD the SNPs found to be associated may not necessarily be the true causal variants or even map to the correct causative gene. Nowadays, advances in bioinformatics and genotyping technology are enabling studies with denser genotype data achieved through imputation, microarrays containing more SNPs or sequencing. New efforts utilizing these means are underway aimed at BMD and fracture risk, yet, the number of studies that have access to these techniques is still somewhat limited. More functional studies (e.g., animal experiments and cell line work) are needed to increase our knowledge about the function of certain genes, for example those in the 7q21.3 and 18p11.21 loci. The genetic markers and loci may serve diagnostic or even therapeutic purposes.

Finally, the relatively weak effects of the variants discovered by GWAS do not undermine the biological relevance of the genes identified as exemplified by the identification of genetic signals at the location of genes coding for proteins currently targeted by novel osteoporosis treatments. From this perspective, one may consider also potential applications of these discoveries towards developing new interventions of osteoporosis. Most established treatments for osteoporosis currently focus on curtailing bone resorption (e.g., bisphosphonates, RANKL inhibitors) while only few anabolic treatments are currently approved for the treatment of osteoporosis (i.e., recombinant truncated or altered PTH). Other anabolic compounds under Phase II development include PTHrP fragments and Wnt-signaling inhibitors such as anti-sclerostin antibodies.[54] Interventional studies exploring the application of sclerostin monoclonal antibodies to osteoporosis treatment are currently ongoing. A significant decrease in bone resorption markers, significant increases in BMD and bone formation markers have been observed in phase 1 and 2 clinical trials, whereas the efficacy to reduce fracture has not yet been studied.[55, 56] Interventional studies exploring the application of sclerostin monoclonal antibodies to osteoporosis treatment are currently ongoing. A significant decrease in bone resorption markers, significant increases in BMD and bone formation markers have been observed in phase 1 and 2 clinical trials, whereas the efficacy to reduce

fracture has not yet been studied. Most clinical trials benefit only those with low BMD; our current results have not evidenced otherwise, as from a genetic perspective the top loci from the first fracture screen are virtually all BMD-associated as well. BMD seems an excellent endophenotype for drug development.

In conclusion, this large-scale GWAS meta-analysis identified multiple genetic loci associated with risk of osteoporotic fracture, of which ten replicated at genome-wide significant level. Many of these loci contain genes involved in the regulation of bone mineral density through Wnt signaling, while the function and therapeutic potential of other candidate genes remains to be explored. These findings highlight the highly polygenic and complex nature underlying osteoporotic fracture risk, shedding light on the pathophysiological mechanisms underlying fracture susceptibility and harboring potential for the future identification of drug targets for the treatment of osteoporosis.

Supplementary information is available at:
http://www.glimdna.org/publicationdata.html

REFERENCES

1. Ralston SH, Uitterlinden AG. Genetics of osteoporosis. Endocr Rev. [10.1210/er.2009-0044]. 2010;31:629-62.
2. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008 May 3;371(9623):1505-12.
3. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008 May 29;358(22):2355-65.
4. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
5. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. New sequence variants associated with bone mineral density. Nat Genet. 2009 Jan;41(1):15-7.
6. Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, et al. An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet. 2010 Jun;6(6):e1000977.
7. Kung AW, Xiao SM, Cherny S, Li GH, Gao Y, Tso G, et al. Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies. Am J Hum Genet. 2010 Feb 12;86(2):229-39.
8. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
9. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012;44(5):491-501.
10. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. N Engl J Med. 2013 May 9;368(19):1809-16.
11. Styrkarsdottir U. New sequence variants associated with bone mineral density. Nat Genet. [10.1038/ng.284]. 2009;41:15-7.
12. van Dijk FS, Zillikens MC, Micha D, Riessland M, Marcelis CL, de Die-Smulders CE, et al. PLS3 mutations in X-linked osteoporosis with fractures. N Engl J Med. 2013 Oct 17;369(16):1529-36.
13. Devlin B, Roeder K, Wasserman L. Genomic control, a new approach to genetic-based association studies. Theor Popul Biol. 2001 Nov;60(3):155-66.
14. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010 Sep 1;26(17):2190-1.

15. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. Am J Hum Genet. 2011 Jan 7;88(1):76-82.
16. Raychaudhuri S, Plenge RM, Rossin EJ, Ng AC, Purcell SM, Sklar P, et al. Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. PLoS Genet. 2009 Jun;5(6):e1000534.
17. Sumarsono SH, Wilson TJ, Tymms MJ, Venter DJ, Corrick CM, Kola R, et al. Down's syndrome-like skeletal abnormalities in Ets2 transgenic mice. Nature. 1996 Feb 8;379(6565):534-7.
18. Crackower MA, Scherer SW, Rommens JM, Hui CC, Poorkaj P, Soder S, et al. Characterization of the split hand/split foot malformation locus SHFM1 at 7q21.3-q22.1 and analysis of a candidate gene for its expression during limb development. Hum Mol Genet. 1996 May;5(5):571-9.
19. van Silfhout AT, van den Akker PC, Dijkhuizen T, Verheij JB, Olderode-Berends MJ, Kok K, et al. Split hand/foot malformation due to chromosome 7q aberrations(SHFM1): additional support for functional haploinsufficiency as the causative mechanism. Eur J Hum Genet. 2009 Nov;17(11):1432-8.
20. Tackels-Horne D, Toburen A, Sangiorgi E, Gurrieri F, de Mollerat X, Fischetto R, et al. Split hand/split foot malformation with hearing loss: first report of families linked to the SHFM1 locus in 7q21. Clin Genet. 2001 Jan;59(1):28-36.
21. Garcia-Ortiz JE, Banda-Espinoza F, Zenteno JC, Galvan-Uriarte LM, Ruiz-Flores P, Garcia-Cruz D. Split hand malformation, hypospadias, microphthalmia, distinctive face and short stature in two brothers suggest a new syndrome. Am J Med Genet A. 2005 May 15;135(1):21-7.
22. Nishida H, Miyagawa S, Vieux-Rochas M, Morini M, Ogino Y, Suzuki K, et al. Positive regulation of steroidogenic acute regulatory protein gene expression through the interaction between Dlx and GATA-4 for testicular steroidogenesis. Endocrinology. 2008 May;149(5):2090-7.
23. Jin H, Wang B, Li J, Xie W, Mao Q, Li S, et al. Anti-DKK1 antibody promotes bone fracture healing through activation of beta-catenin signaling. Bone. 2014 Sep 28.
24. Semenov M, Tamai K, He X. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem. 2005 Jul 22;280(29):26770-5.
25. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, et al. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet. 2001 Mar 1;10(5):537-43.
26. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, et al. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet. 2001 Mar;68(3):577-89.
27. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, et al. Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. J Med Genet. 2002 Feb;39(2):91-7.
28. Kim SJ, Bieganski T, Sohn YB, Kozlowski K, Semenov M, Okamoto N, et al. Identification of signal peptide domain SOST mutations in autosomal dominant craniodiaphyseal dysplasia. Hum Genet. 2011 May;129(5):497-502.
29. Duncan EL. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. [10.1371/journal.pgen.1001372]. 2011;7:e1001372.
30. Kim KA, Wagle M, Tran K, Zhan X, Dixon MA, Liu S, et al. R-Spondin family members regulate the Wnt pathway by a common mechanism. Mol Biol Cell. 2008 Jun;19(6):2588-96.
31. Malaval L, Wade-Gueye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, et al. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. J Exp Med. 2008 May 12;205(5):1145-53.
32. Gowen LC, Petersen DN, Mansolf AL, Qi H, Stock JL, Tkalcevic GT, et al. Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. J Biol Chem. 2003 Jan 17;278(3):1998-2007.
33. Yoshitake H, Rittling SR, Denhardt DT, Noda M. Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. Proc Natl Acad Sci U S A. 1999 Jul 6;96(14):8156-60.
34. Tang Y, Katuri V, Dillner A, Mishra B, Deng CX, Mishra L. Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. Science. 2003 Jan 24;299(5606):574-7.

35. Medina-Gomez C, Kemp JP, Estrada K, Eriksson J, Liu J, Reppe S, et al. Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. PLoS Genet. 2012 Jul;8(7):e1002718.
36. Zheng HF, Tobias JH, Duncan E, Evans DM, Eriksson J, Paternoster L, et al. WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. PLoS Genet. 2012 Jul;8(7):e1002745.
37. Moverare-Skrtic S, Henning P, Liu X, Nagano K, Saito H, Borjesson AE, et al. Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. Nat Med. 2014 Oct 12.
38. Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone. 2004 Jan;34(1):195-202.
39. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol. 2012 May;27(5):319-32.
40. Oei L, Zillikens MC, Dehghan A, Buitendijk GH, Castano-Betancourt MC, Estrada K, et al. High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study. Diabetes Care. 2013 Jun;36(6):1619-28.
41. Baron R, Rawadi G, Roman-Roman S. Wnt signaling: a key regulator of bone mass. Curr Top Dev Biol. 2006;76:103-27.
42. Secreto FJ, Hoeppner LH, Westendorf JJ. Wnt signaling during fracture repair. Curr Osteoporos Rep. 2009 Jul;7(2):64-9.
43. Bethel M, Chitteti BR, Srour EF, Kacena MA. The changing balance between osteoblastogenesis and adipogenesis in aging and its impact on hematopoiesis. Curr Osteoporos Rep. 2013 Jun;11(2):99-106.
44. van Meurs JB, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, et al. Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. Jama. 2008 Mar 19;299(11):1277-90.
45. Panagiotou OA, Evangelou E, Ioannidis JP. Genome-wide significant associations for variants with minor allele frequency of 5% or less--an overview: A HuGE review. Am J Epidemiol. 2010 Oct 15;172(8):869-89.
46. Styrkarsdottir U. European bone mineral density loci are also associated with BMD in East-Asian populations. PLoS ONE. [10.1371/journal.pone.0013217]. 2010;5:e13217.
47. Lonjou C, Zhang W, Collins A, Tapper WJ, Elahi E, Maniatis N, et al. Linkage disequilibrium in human populations. Proc Natl Acad Sci U S A. 2003 May 13;100(10):6069-74.
48. Liu CT, Estrada K, Yerges-Armstrong LM, Amin N, Evangelou E, Li G, et al. Assessment of gene-by-sex interaction effect on bone mineral density. J Bone Miner Res. 2012 Oct;27(10):2051-64.
49. Fox KM, Cummings SR, Powell-Threets K, Stone K. Family history and risk of osteoporotic fracture. Study of Osteoporotic Fractures Research Group. Osteoporos Int. 1998;8(6):557-62.
50. Kemp JP, Medina-Gomez C, Estrada K, St Pourcain B, Heppe DH, Warrington NM, et al. Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment. PLoS Genet. 2014 Jun;10(6):e1004423.
51. Oei L, Estrada K, Duncan EL, Christiansen C, Liu CT, Langdahl BL, et al. Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus. Bone. 2014 Feb;59:20-7.
52. Oei L, Hsu YH, Styrkarsdottir U, Eussen BH, de Klein A, Peters MJ, et al. A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus. J Med Genet. 2014 Feb;51(2):122-31.
53. Zheng HF, Forgetta V, Hsu YH, Estrada K, Rosello-Diez A, Leo PJ, et al. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature. 2015 Oct 1;526(7571):112-7.
54. Mosekilde L, Torring O, Rejnmark L. Emerging anabolic treatments in osteoporosis. Curr Drug Saf. 2011 Apr 1;6(2):62-74.
55. Lewiecki EM. Sclerostin: a novel target for intervention in the treatment of osteoporosis. Discov Med. 2011 Oct;12(65):263-73.
56. Padhi D, Jang G, Stouch B, Fang L, Posvar E. Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. J Bone Miner Res. 2011 Jan;26(1):19-26.

Chapter 4.3

A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus

Oei L, Hsu YH, Styrkarsdottir U, Eussen BH, de Klein A, Peters MJ, Halldorsson B, Liu CT, Alonso N, Kaptoge SK, Thorleifsson G, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Kruk M, Lewis JR, Patel MS, Scollen S, Svensson O, Trompet S, van Schoor NM, Zhu K, Buckley BM, Cooper C, Ford I, Goltzman D, González-Macías J, Langdahl BL, Leslie WD, Lips P, Lorenc RS, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Slagboom PE, Garcia-Ibarbia C, Ingvarsson T, Johannsdottir H, Luben R, Medina-Gómez C, Arp P, Nandakumar K, Palsson ST, Sigurdsson G, van Meurs JBJ, Zhou Y, Hofman A, Jukema JW, Pols HAP, Prince RL, Cupples LA, Marshall CR, Pinto D, Sato D, Scherer SW, Reeve J, Thorsteinsdottir U, Karasik D, Richards JB, Stefansson K, Uitterlinden AG, Ralston SH, Ioannidis JP, Kiel DP, Rivadeneira F, Estrada K

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ABSTRACT

Introduction Osteoporosis is a systemic skeletal disease characterized by reduced bone mineral density and increased susceptibility to fracture; these traits are highly heritable. Both common and rare copy number variants (CNVs) potentially affect the function of genes and may influence disease risk.
Aim To identify CNVs associated with osteoporotic bone fracture risk.

Methods We performed a genome-wide CNV association study in 5,178 individuals from a prospective cohort in the Netherlands, including 809 osteoporotic fracture cases, and performed in silico lookups and de novo genotyping to replicate in several independent studies.

Results A rare (population prevalence 0.14%, 95% confidence interval [CI]: 0.03%–0.24%) 210 kb deletion located on chromosome 6p25.1 was associated with the risk of fracture (OR 32.58, 95% CI: 3.95–1,488.89; $P=8.69\times10^{-5}$). We performed an in silico meta-analysis in four studies with CNV microarray data and the association with fracture risk was replicated (OR 3.11, 95% CI: 1.01–8.22; P=0.02). The prevalence of this deletion showed geographic diversity, being absent in additional samples from Australia, Canada, Poland, Iceland, Denmark, and Sweden, but present in the Netherlands (0.34%), Spain (0.33%), USA (0.23%), England (0.15%), Scotland (0.10%), and Ireland (0.06%), with insufficient evidence for association with fracture risk.

Conclusion These results suggest that deletions in the 6p25.1 locus may predispose to higher risk of fracture in a subset of populations of European origin; larger and geographically restricted studies will be needed to confirm this regional association. This is a first step towards the evaluation of the role of rare CNVs in osteoporosis.

INTRODUCTION

Osteoporosis is a major public health problem in a rapidly aging population. This systemic skeletal disease is characterized by reduced bone mass and microarchitectural deterioration of bone tissue. The disease progresses 'silently' until the increase in bone fragility leads to increased fracture risk.[1, 2] The importance of genetic variation in the regulation of bone mass and bone turnover was first highlighted by linkage analysis in severe Mendelian disorders such as osteoporosis-pseudoglioma syndrome and high bone mass syndrome.[3] In the case of non-Mendelian forms of osteoporosis, common genetic variants have been found to be associated with fracture risk in well powered candidate gene settings.[4] Meta-analysis of single nucleotide polymorphism (SNP) genome-wide association studies (GWAS) for bone mass have identified more than 56 loci independently associated with normal variation of bone mineral density, and some of these studies also found associations with fracture risk.[5-12]

In addition to SNPs, copy number variants (CNVs) have shown associations with complex phenotypes such as schizophrenia, autism, and obesity.[13-16] A study in Chinese individuals suggested an association of a common CNV with osteoporotic fractures;[17] however, the same variant was not replicated in a follow-up study of individuals of European origin,[18] potentially showing population specific effects. Most of the common CNVs are well tagged by common SNPs,[19] and thus are easy to identify with a SNP based GWAS. On the other hand, rare CNVs are difficult to tag and rare and large CNVs have been found to be associated with different diseases.[20] Nevertheless, it is not known whether rare CNVs play a significant role in fracture risk. Thus, we conducted a genome-wide CNV association study on a discovery dataset of 809 fracture cases and 4,369 controls drawn from a prospective cohort study. We further looked for in silico replication of the CNV region showing the most significant association in 1,096 fracture cases and 47,340 controls from four independent studies with CNV microarray data. Finally, using a breakpoint specific genotyping assay we evaluated the association of this deletion in an additional 9,760 fracture cases and 16,542 controls.

METHODS

Subjects

All studies were approved by the institutional ethics review committees of the respective organizations, and all participants provided written informed consent. The Rotterdam Study (RS-I) is a prospective population based cohort study of chronic disabling conditions in Dutch individuals aged 55 years or above (http://www.epib.nl/ergo.htm).[21-23] The Rotterdam Study II (RS-II) is an extension of the Rotterdam Study, which started in 1999 and used the same inclusion criteria and design as the original cohort. Briefly, 3,011 individuals (response rate 67%) who had turned 55 years of age or had moved into the study district of Ommoord, Rotterdam, since the start of the original study in 1990 were included in the extension cohort. The Icelandic deCODE Genetics (dCG) study comprises a population based sample to identify the genetic basis of complex diseases.[10] The Framingham Osteoporosis Study (FOS) is embedded in the Framingham Heart Study, a community based, longitudinal, prospective cohort comprising three generations of individuals in multigenerational pedigrees and additional unrelated individuals (http://www.framinghamheartstudy.org/). The PROSPER study is a randomized controlled clinical trial to test the effect of pravastatin on cardiovascular outcomes in the elderly at risk. In addition, we performed de novo genotyping —that is, targeted locus assessments because no CNV microarray data are available—in 15 studies with a variety of epidemiological designs that are part of the GENOMOS DNA collection (http://www.genomos.eu) across Canada, Europe, and Australia. Given the rarity of this

deletion event, we pursued genotyping only in those largest GENOMOS studies having at least 200 fracture cases and a total sample size of at least 1,000 subjects with phenotype information concerning the fracture status. More information can be found in online Supplementary Tables S1 and S2. All study participants included were of Caucasian ancestry.

Fracture definition

Fracture cases were defined as fractures at any skeletal site (except fingers, toes, and skull) occurring after age 18 years assessed by X-ray screening, clinical radiographic report, clinical record, clinical interview, and/or questionnaire. High trauma fractures were excluded whenever possible, for example, motor vehicle accidents or falls from greater than standing height. Controls were defined as individuals without a history of fracture. Additional information for each study is available in online Supplementary Tables S3 and S4.

GWAS genotyping

The four studies were genotyped using the Illumina Infinium HumanHap550 Beadchip (RS-I, RS-II), Quad660 (PROSPER), the HumanCNV370 Beadchip (dCG) or the Affymetrix Dual NspI/StyI GeneChip 2×250 K with 50 K gene centered MIP set (FOS), all according to manufacturer's protocols and quality control standards. The exclusion/filtering criteria for individuals are described in online Supplementary Table S1A.

CNV analysis of microarray data

Studies used either QuantiSNP[24] or PennCNV[25] to segment CNVs as described below. Quality control (QC) steps for RS-I, RS-II, PROSPER, and FOS are summarized in online Supplementary Figure S1.

RS-I: Log R ratio (LRR) signal intensity and B allele frequency (BAF) were extracted from 5974 samples using BeadStudio 3.1.3. A Hidden-Markov model, implemented in the software QuantiSNP, was used to make CNV calls. A measure of confidence, log Bayes factor, was computed for each CNV call. A correction for local difference in GC content is implemented in the algorithm to adjust for irregularities in signal intensity. We excluded 547 samples with a mean autosomal LRR SD >0.3 or a BAF SD >0.15. We also excluded CNV calls that spanned the centromere (QC1, nCNVs=305,475). We discarded all CNV calls with a log Bayes factor value <10, a CNV size <1 kb or CNVs with less than two consecutive SNPs in the CNV event. This filter effectively reduces the majority of false positive calls, although it has the disadvantage that many putatively real CNV calls might be lost (QC2 nCNVs=58,866). Finally we removed 249 samples with an excess of CNV calls (expressed as upper quartile+1.5×(IQR))=20 CNVs (QC3, nCNVs=49,229 in 5,178 samples).

RS-II: 2,157 samples were used for CNV analyses using QuantiSNP; 154 of these samples with a mean LRR SD >0.35 or a BAF SD >0.15 were excluded. We also excluded CNV calls that spanned the centromere (QC1, nCNVs=129,941). We discarded all CNV calls with a log Bayes factor value <10, a CNV size <1 kb, or only one consecutive SNP in the CNV event (QC2 N=15,266). For samples with a LRR SD between 0.3 and 0.35, we applied a stricter threshold of log Bayes factor=15. Thirty-eight samples with >20 CNVs were excluded (QC3, 13,038 CNVs in 19,65 samples).

dCG: Illumina BeadStudio (V.2.0) was used to call genotypes, normalize the signal intensity data, and establish the LRR and BAF at every SNP according to standard Illumina protocols. All samples passed a standard SNP based QC procedure with an SNP call rate >0.97. PennCNV was used for detection of CNVs. The input data for PennCNV are LRR and BAF. PennCNV employs a hidden Markov model to analyze the

LRR and BAF values across the genome. CNV calls are made on the basis of the probability of a given copy state at the current marker, as well as on the probability of observing a copy state change from the previous marker to the current one.

FOS: A total of 8,734 Framingham participants with genome-wide genotypes using Affymetrix 550 k chips were used for CNV calling on autosomal chromosomes. The raw Affymetrix CEL files were read and normalized with Affymetrix power tools to estimate the LRR and BAF at every SNP probe. All samples passed a standard SNP based QC procedure with average genotype call rate >0.95. We excluded SNP probes with call rate <0.97. We first used PennCNV package (a hidden Markov model) to segment CNV and define the boundaries of a CNV on autosomal chromosomes. The estimated CNVs were then confirmed by another software package, GoldenHelix SVS, with an optimal segmenting algorithm (the Copy Number Analysis Method, CNAM). A principal component analysis was applied to correct for batch effects. We applied several QCs to filter out low quality or questionable quality samples as follows: average LRR SD value >0.35; high total length (10% per chromosome) of CNV; and high total number of CNV (CNV counts >50 per sample). A total of 1,300 samples were excluded. In addition, we excluded CNV with less than three consecutive SNPs; CNV with length <1 kb; CNV in the regions of high GC content (80%); and CNVs in the immunoglobulin regions. Among 7,434 high quality genotyped samples, 112,746 CNVs were assigned. Fracture data were available for 3,529 of these FOS samples.

PROSPER: LRR and BAF measurements were extracted from 5,244 samples using GenomeStudio V2009.1. QuantiSNP v2 was used to make CNV calls. We excluded 446 samples with a mean autosomal LRR SD>0.25 or a BAF SD>0.08. We also excluded CNV calls that spanned the centromere (QC1, nCNVs=2,683,302). We discarded all CNV calls with a log Bayes factor value <10, a CNV size <1 kb or CNVs with less than two consecutive SNPs in the CNV event (QC2 nCNVs=1,228,214). Finally, we removed 89 samples with an excess of CNV calls (N>344 CNVs, QC3, nCNVs=1,195,162 in 4,709 samples).

Association analysis

The genome-wide association analysis on the discovery cohort was carried out using the rare CNV module implemented in Plink V.1.0.5 on binary copy number differences (deletion vs no deletion between cases and controls). Ten million permutations were performed to assess the significance of the genome-wide association results. Each study provided counts for case–control status among carriers and non-carriers. Odds ratio, confidence intervals and P values were calculated using study counts in an exact Cochran-Mantel-Haenszel exact test statistic implemented in the stats package within the R statistical framework.

Quantitative PCR analysis

We validated the deletion that was found to be associated with fractures using quantitative PCR (qPCR) in 12 RS samples where this deletion was found. The primers for the real-time
qPCR experiments were as follows (50–30 direction):
A. forward primer: GGCAGACAGAGAAAAATGTGGC
B. reverse primer: TGTCAGCTTGATGGATTTGTCC
qPCR assays were validated by demonstrating linearity over three orders of magnitude and by observation of a single melt peak by plotting relative fluorescence units (RFU) data with
time (T) (−d(RFU)/dT) on the Y-axis as a function of temperature on the X-axis. Reactions contained 200 nM primer; 1X KAPA SYBR FAST qPCR Master Mix and 5 ng genomic DNA.

All reactions were performed as triplicates on an Applied Biosystems 7300 Real-Time PCR System cycle conditions: 94°C 3 min initial denaturation followed by 30 cycles 5 s 94°C
denaturation and 30 s 60°C primer annealing, extension and RFU data collection. Two reference targets were used normalizing on genomic DNA obtained from healthy individuals.
SEL1L reference
Feb. 2009 (GRCh37/hg19) Assembly chr14:81952705-
81952790 86 bp
5'-GAATGTATGTGAACGAGGCCGttggtctgaaaggcttatgactgcctataacagctataaagatgGCGATTACAATGCTG-CAGTGA-3'
RBM11 reference
Feb. 2009 (GRCh37/hg19) Assembly chr21:15587866
+15587951 86 bp
5'-ACAAAACTGGCTCACTCTCACCagtatatcccttggtatttgctttctcaagttccttttggagtCCACTTAAAACCTCTGC-GACC-3'
Data were analyzed using Applied Biosystems RQ Study Software V1.2.3. A fold-change <0.7 (deletion) or >1.25 (duplication) was considered to constitute a true event. For two subjects the assay was inconclusive (DNA amount was not sufficient for full qPCR cycles).

Determination of the deletion breakpoints

Sanger sequencing was used to map the breakpoints of the 6p25 deletion in a population-control cohort. DNA was obtained from one individual from the SAGE cohort that was genotyped with the Illumina 1M array and was identified as a 6p25 deletion carrier (see online Supplementary Table S5). The cohort and array/CNV analysis have been described previously.[26] The exact breakpoints were found at chromosome 6 in the positions: 4,198,453 and 4,418,843 (NCBI36 hg18).

De novo genotyping

The deletion was genotyped in 15 GENOMOS studies by K-Biosciences (http://www.kbioscience.co.uk) using a competitive allele specific PCR (KASPar) assay designed to identify those individuals with a different sequence at the breakpoint identified by sequencing. Allele specific sequencing to design the probe was set as follows: allele X: 5'-AGGAAAAAAACATGTTAGCAGGCTTCT-3'; allele Y: 5'-GGAAAAAAACATGT-TAGCAGGCTTCC-3'. Given the low frequency of this variant, three positive controls were included in each plate before genotyping. All genotyped plates were evaluated to show signals for the positive controls.

CNV population database query

We queried available CNV population databases to increase the precision for the prevalence estimate of the deletion on a general population level and to check for population specific differences. The resources available were SAGE, OHI, PopGen, WTCCC2, CHOP, the Pharmacogenomics and Risk of Cardiovascular Disease study (PARC), the National Institute for Neurological Disorders and Stroke (NINDS), and the Human Genome Diversity Panel (HGDP), which altogether included 13,441 individuals of European, African American, and Asian descent from various regions throughout the world (see online Supplementary Table S5).[19, 20, 26-30]

RESULTS

We obtained the normalized intensity data on a discovery cohort composed of 5,974 Northwestern European individuals from the RS-I, a population based cohort of individuals aged 55 years and over who had been genotyped with the Illumina 550 K Array (see online Supplementary Table S1). After QC (see Methods), 49,229 CNVs (mapping to 26,162 genomic locations) were identified in 5,178 individuals using a hidden Markov model method to segment CNV regions from microarray DNA intensity data. As expected, we found an inverse correlation between the size and number of CNV events (Figure 1). Nevertheless, 90% of the analyzed subjects presented with at least one large CNV at some position in the genome (length >100 kb).

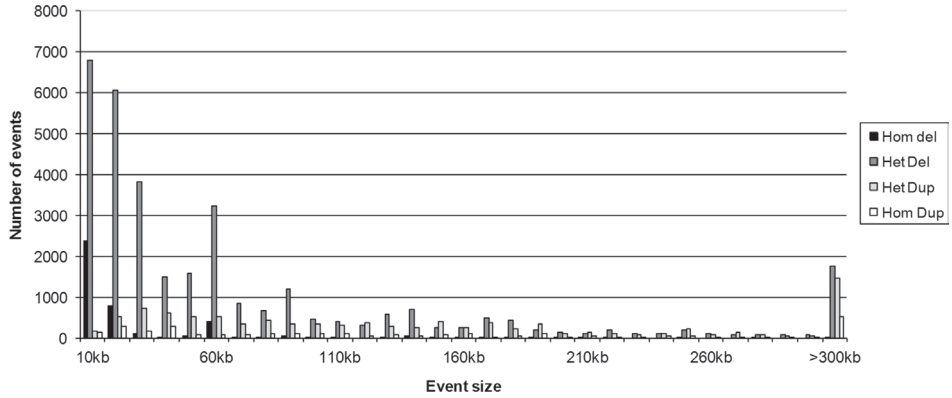
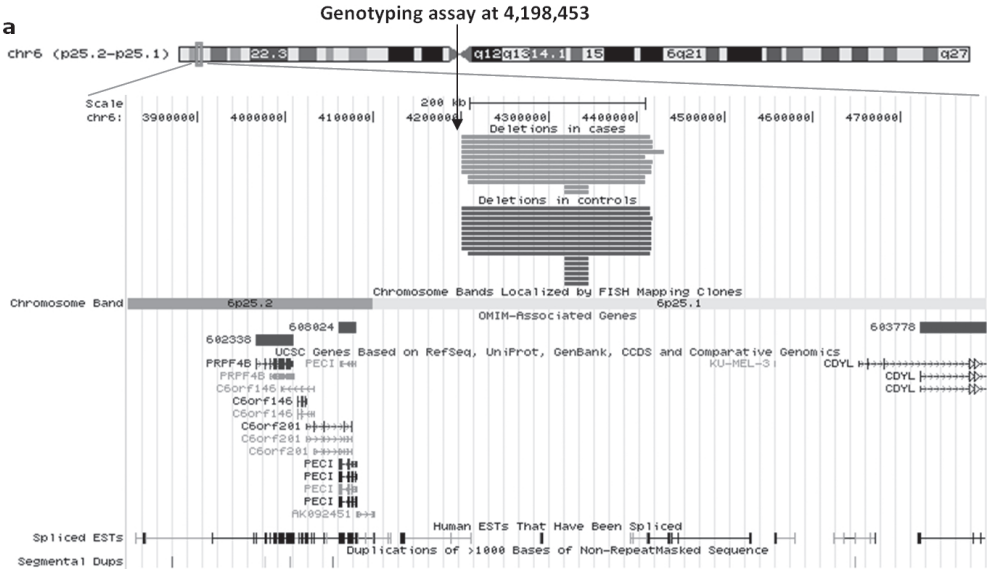


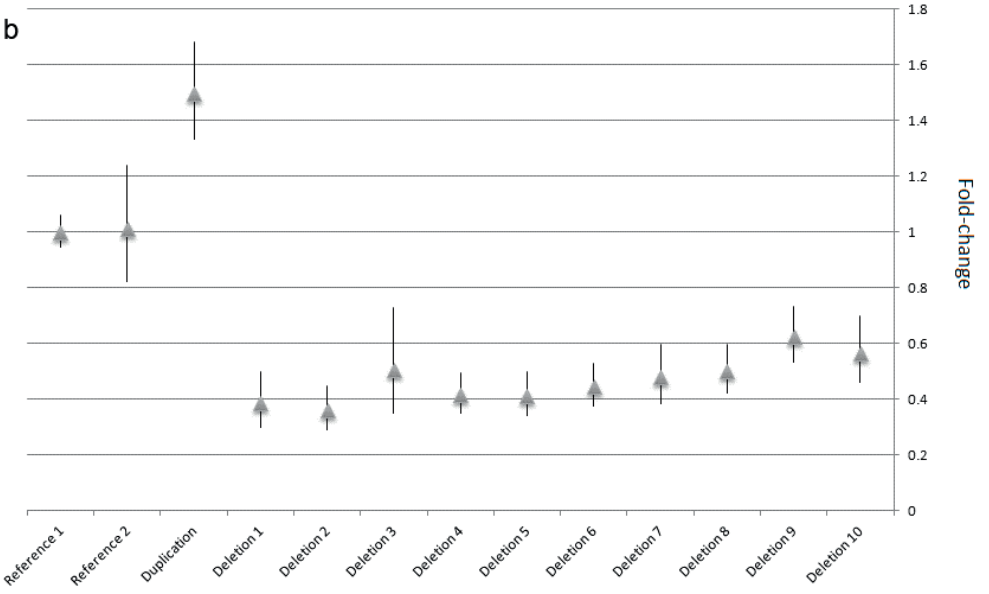
Figure 1. Copy number variant (CNV) type, length, and frequency. CNVs were plotted according to event type (color), length (X-axis), and frequency in the Rotterdam Study (Y-axis, number of samples N=5,178).

First, we investigated if there was a difference in the global burden of CNV events between cases with fractures and controls in the RS-I study. Out of 5,178 individuals, with a mean follow-up of 7.7 years, 809 subjects presented at least one osteoporotic fracture, of which most were fractures of the hip, spine, and wrist. While no difference was found in the overall burden of CNV events between fracture cases and controls, the proportion of fracture cases with at least one or more rare (frequency <1%) deletions was significantly higher compared to controls (OR 1.04, P=0.03).

Next, we tested the association of segmental rare deletions across the genome with fracture risk in the RS-I study. A rare (population prevalence 0.14%, 95% CI: 0.03%–0.24%) 210 kb deletion located on chromosome 6p25.1 (Figure 2A) was the only significantly associated locus with fracture risk after adjusting for multiple testing based on permutations of individual level data (OR 32.58, 95% CI: 3.95–1,488.89; $P=8.69\times10^{-5}$; permuted P=0.027).

We then attempted to replicate this association of the 210 kb deletion on 6p25.1 with fracture in four additional cohort studies with CNV microarray data: the RS II (N=2,157, 161 cases), FOS (N=3,513, 367 cases), deCode Genetics Study (dCG, N=38,250, 178 cases), and a multicenter randomized clinical trial entitled the PROspective Study of Pravastatin in the Elderly at Risk Study (PROSPER_SC, PROSPER_IR, PROSPER_NL, N=4,708, 390 cases). In FOS, we found four cases and 12 non-fracture controls with one copy deletion in the 6p25.1 region.


a
Genotyping assay at 4,198,453
chr6 (p25.2-p25.1)
Deletions in cases
Deletions in controls
Chromosome Bands Localized by FISH Mapping Clones
Chromosome Band
OMIM-Associated Genes
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
Human ESTs That Have Been Spliced
Spliced ESTs
Duplications of >1000 Bases of Non-RepeatMasked Sequence
Segmental Dups


b
Fold-change
Reference 1
Reference 2
Duplication
Deletion 1
Deletion 2
Deletion 3
Deletion 4
Deletion 5
Deletion 6
Deletion 7
Deletion 8
Deletion 9
Deletion 10

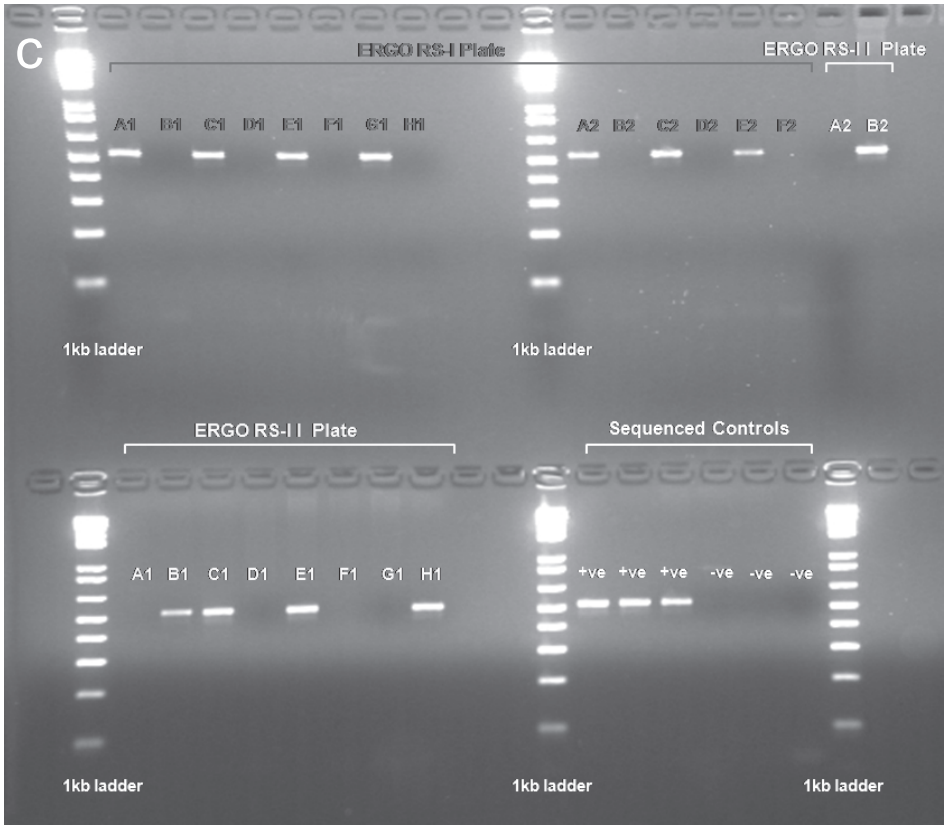


Figure 2. Identification and validation of deletions at 6p25.1. **(A)** 210 kb deletions were first identified in six fracture cases and one control from the Rotterdam Study I (RS-I). Additional carriers from the RS-II and Framingham Osteoporosis Study are also depicted. Refseq genes and OMIM associated genes are depicted. **(B)** Quantitative PCR validation of 12 carriers in the RS (for two samples the assay failed). The third sample, labelled as 'Duplication', is a FISH (fluorescence in situ hybridization) validated complete 6p arm duplication. **(C)** Validation of a sequence based breakpoint detection of the 6p25.1 deletion. Twelve deletion carriers (wells A1, C1, E1, G1, A2, C2, E2, B2, B1, C1, E1, H1) show amplified PCR product exactly with the same length as the sequenced controls.

Table 1. Association results of 6p25.1 deletion with increased fracture risk.

Stage	Study	Country	Deletions/total cases	Frequency in cases (%)	Deletions/total controls	Frequency in controls (%)
Discovery	RS-I	Netherlands	6/809	0.74	1/4,369	0.02
	Frequency (95%CI)		0.74% (0.15–1.33)		0.02 (0–0.20)	
	OR (95%CI)		32.58(3.95–1,488.89) P=8.69x10^{-5}			
In silico	dCG	Iceland	0/178	0	0/38,072	0
Replication	FOS	USA	4/367	1.09	12/3,146	0.38
	PROSPER_IR	Ireland	0/141	0	1/1,650	0.06
	PROSPER_NL	Netherlands	0/57	0	0/756	0
	PROSPER_SC	Scotland	0/192	0	2/1,912	0.10
	RS-II	Netherlands	2/161	1.24	3/1,804	0.17
	Total		6/1,096	0.55	18/47,340	0.04
	Frequency (95%CI)	0.18% (0.11–0.98)		0.04 (0.02–0.05)		
	OR (95%CI)		3.11(1.01–8.22) P=0.02			
Breakpoint	APOSS	Scotland	0/531	0	0/2,129	0
Genotyping	CABRIO-C	Spain	1/327	0.31	3/1,018	0.29
Replication	CABRIO-CC	Spain	4/1,023	0.39	3/1,104	0.27
	CAIFOS	Australia	0/736	0	0/581	0
	CAMOS	Canada	0/235	0	0/1,732	0
	DOPS	Denmark	0/410	0	0/1,242	0
	EDOS	Scotland	0/1,500	0	0/193	0
	EPICNOR	England	0/227	0	1/1,127	0.09
	EPOLOS	Poland	0/231	0	0/446	0
	EPOS	England	1/686	0.15	2/1,289	0.16
	HCS	England	0/339	0	3/2,308	0.13
	LASA	Netherlands	0/313	0	3/562	0.53
	MANMC	Canada	0/750	0	0/0	0
	NOSOS	Scotland	0/342	0	0/740	0
	UFO	Sweden	0/2,110	0	0/2,071	0
	Total		6/9,760	0.06	15/16,542	0.09
	Frequency (95%CI)		0.06% (0.01–0.11)		0.09%(0.04–0.14)	
	OR (95%CI)		0.78 (0.24–2.24) P=0.81			

P, P value computed with an exact Cochran-Mantel-Haenszel test statistic.
dCG, Icelandic deCODE Genetics Study; FOS, Framingham Osteoporosis Study; RS, Rotterdam Study

Among them, two cases and six controls had a smaller deletion (~26 kb) inside the same 6p25.1 region (Figure 2A). The remaining two cases and six controls had exactly the same size of deletion in the 6p25.1 region. Both CNVs were aggregated in families and segregated from parents to offspring in FOS. We included samples with either one of the CNVs in the CNV-fracture association analyses.

The increased prevalence of this 6p25.1 deletion in fracture cases was replicated in RS-II (1.24% in cases, 0.17% in controls) and FOS (1.09% in cases, 0.38% in controls) studies (Table 1). The 6p25.1 deletion was present in three controls from the PROSPER study, but we did not find the 6p25.1 deletion either in

cases or in controls of the dCG study (Table 1). Combining the data from the in silico replication studies (RS-II, FOS, dCG, and PROSPER) using a Cochran-Mantel-Haenszel test yielded a significant threefold increase in the risk of fracture (OR 3.11, 95% CI: 1.01–8.22; P=0.02) (Table 1).

We validated the presence of this variant with qPCR (see Methods) in 12 6p25del carriers of the RS-I and RS-II cohorts. Ten of them showed clear evidence for deletion (Figure 2B). The microarray data in these 12 samples suggested a common breakpoint for all carriers (Figure 2A). To identify the breakpoint at a base pair resolution, we sequenced one sample in which the 6p25del had been identified (see Methods). Validation of the sequence level PCR gel to detect this deletion in the same 12 deletion carriers and 12 controls showed perfect assignment of carrier status as determined from the microarray data (Figure 2B). Thus, we can conclude that all 12 carriers from the Rotterdam Study share exactly the same breakpoint at sequence level.

We then designed a Kaspar genotyping assay using the sequence level breakpoint information to perform de novo genotyping of this deletion in an additional set of 9,760 fracture cases and 16,542 controls from 15 independent studies across Europe, Australia, and Canada (Figure 2C and online Supplementary Table S1). Despite having a large sample size, we could only detect 21 additional 6p25del carriers (frequency <0.1% in both cases/controls) with no significant association with fracture risk (P=0.81) (table 1).

We queried six available CNV population data bases for the prevalence of this deletion (see online Supplementary Table S5; SAGE N=1,287 European, 495 African Americans from USA; OHI N=1,234 European from Canada; PopGen N=1,123 European from Germany; WTCCC2 N=4,783 European from UK; CHOP N=1,320 European, 694 African American, 12 Asian from USA; a combined dataset from PARC, NINDS, and the HGDP N=2,493 individuals from different ethnicities and countries of origin).19 20 26–30 The deletion was identified in five out of six studies: SAGE (1/1,287), OHI (5/1,234), PopGen (0/1,123), WTCCC2=7/4,783, CHOP (4/1,320), PARC (2/936), NINDS (2/671), HGDP (0/886). All carriers were found in samples of European ancestry. The deletion was not found in 886 samples from the Human Genome Diversity Project (51 different world populations) or in two studies of African ancestry (see online Supplementary Table S5).

DISCUSSION

We report here a genome-wide scan for CNVs and risk of fracture assessed in the RS-I cohort. A microdeletion in 6p25.1 was found to be associated with increased risk of fracture and remained significant after permutation testing. The deletion was validated with qPCR and was also replicated in silico in two additional studies: RS-II and FOS; the deletion was only found in three controls of the PROSPER study and it was not found in the deCode study. Combining all four in silico replication studies, the deletion is associated with a threefold higher risk of bone fracture in individuals of mainly Dutch and US American ancestry. Additional replication was pursued using breakpoint genotyping in 15 studies; however, the deletion was only found in six of the 15 studies with no replication of association with fracture risk.

The frequency of this deletion showed regional variation, being present in studies of the Netherlands (0.34%), Spain (0.33%), USA (0.23%), England (0.15%), Scotland (0.10%), and Ireland (0.06%) (Table 1). The deletion seems to be absent or in lower frequency in certain populations such as Iceland (dCG) and Sweden (UFO) where, despite having assessed more than 30,000 and 4,000 subjects for each population, respectively, no additional carriers were found. Founder effects can effectively remove rare variants from the gene pool in a population. These population effects could explain why we did not detect the 6p25.1 microdeletion in 30,000 individuals from a population with relatively similar genetic background

(Iceland) as the one in which we found the microdeletions (Northwestern European from Netherlands and USA).

While replication of the association with fracture risk in two cohorts was achieved using in silico data, the meta-analysis of de novo genotyped studies was not statistically significant. There are several potential explanations for the lack of replication in this subset of studies. First, limitations in study power could make it difficult to identify a significant association (at $P<0.05$) with a variant of such low frequency. Considering an OR=3, and a minor allele frequency (MAF)=0.05% in controls, almost 18,000 cases and an equal number of controls would be needed to reach 80% power. Secondly, it is possible that while 12 of the microarray based carriers in the Rotterdam Study share the same breakpoint, other 6p25del carriers may have different breakpoints (such as the eight carriers with a smaller 26 kb deletion detected in FOS) which were not detected by our specially designed genotyping probe. Thirdly, it may be possible that a two-hit model involving a yet unknown genetic variant is affecting the predisposition of 6p25del carriers to an increased risk for fracture. Finally, some degree of misclassification may have occurred, as carriers currently classified as controls may eventually develop a fracture later in life; this could have potentially affected our results.

The deletion is located in an intergenic region in the subtelomeric region of chromosome 6p (Figure 2A) in the proximity of the *peroxisomal D3,D2-enoylCoA isomerase* (*PECI*) gene which codes for an enzyme relevant for the metabolism of fatty acids. *PECI* was first cloned by using pooled antisera from autoimmune diabetes patients.[31] Hence, it is possible that even though the 6p25.1 microdeletion is 200 kb away from *PECI*, this region may be regulating the expression of *PECI*. Both type 1 and type 2 diabetes are associated with higher risk of fracture, even though bone density is not low.[32-36] Thus, the increased risk we see with individuals with the 6p25del may be mediated by comorbidity with diabetes.

Another candidate hypothesis is that microdeletions in 6p25.1 are disrupting an unidentified gene in the critical region. A similar mechanism was shown in which microdeletions of 1q21.1 disrupted an expressed sequence tag (EST) that was in fact an unknown gene which subsequently increased the risk for neuroblastoma.[37] There are two spliced ESTs that map to the 6p25 region covered by the microdeletions (Figure 2A). One of the ESTs, AL121205.1, shares 33% of its structure with the *KREMEN1* gene. *KREMEN1* encodes a high affinity dickkopf homolog 1 (*DKK1*) transmembrane receptor that cooperates with DKK1 to block Wnt/β-catenin signaling, which is an important pathway in bone biology.[38] The second EST, DB318881.1, shares 38% structural similarity with WDR66, which does not have any clear connection to our findings. Further analyses are required to test whether the deletion of those ESTs are indeed related to the increased risk of fracture.

Other individuals have been reported with the same microdeletion in different populations of Caucasian origin.[20, 29] These reports provide further evidence of the existence of this rare microdeletion in other populations. Similarly, it has been reported that patients with 6p25 microdeletions present a variety of phenotypes such as ocular dysgenesis, hearing impairment, and craniofacial, skeletal, cardiac, and renal malformations.[39-42] However, these deletions are much larger (from 1 to 13 Mb in size), and cover many genes. Therefore, the relation of those larger events with the association we found between the 6p25.1 microdeletion and fractures is not direct.

Our study has three particular strengths: (1) samples from the discovery and in silico replication sets were drawn from cohort studies where cases and controls were genotyped at random in the same laboratory. This is important for avoiding the biases that occur when cases are genotyped at different time points or centers than controls are; (2) DNA was extracted from blood for samples used for the discovery of this CNV—the use of other DNA sources such as cell lines can introduce noise in CNV

analysis; (3) our discovery sample size was large enough to detect rare variants (~1%) with large effects (OR >3) and we also replicated the association in the independent studies in silico; (4) we validated the microdeletion using different technologies (qPCR, Sanger sequencing and Kaspar).

Performing a genome-wide scan for CNVs has limitations. Because the SNP arrays that we used for the discovery phase were not designed to evaluate CNVs, many CNV enriched regions were not covered in this study, and may not have been identified in our scan. Also, to minimize the rate of false positive CNV calls, we used stringent QC thresholds which may have filtered out real CNV calls.

In summary, we have shown that a microdeletion of 6p25.1 is associated with an increased risk of fracture in a group of populations mostly of Dutch origin. Further studies are needed to replicate this variant in populations of similar ancestral background and to identify the specific gene or genes in the region for which this deletion contributes to an increased risk for fracture. Although this event is rare, the effect on fracture risk was substantially greater than the effects usually observed for SNPs. If rare CNVs have similar degrees of effect as the one detected here, it might be possible to identify them with better powered genome scans, not only for fracture risk but also for other human traits and diseases.

ACKNOWLEDGMENTS

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Supplementary information is available at:

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp.pdf

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp_table1.pdf

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp_table2.pdf

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp_table3.pdf

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp_table4.pdf

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp_table5.pdf

http://jmg.bmj.com/content/suppl/2013/12/17/jmedgenet-2013-102064.DC1/jmedgenet-2013-102064supp_table6.pdf

REFERENCES

1. Johnell O, Kanis JA, Oden A, Johansson H, De Laet C, Delmas P, et al. Predictive value of BMD for hip and other fractures. J Bone Miner Res. 2005 Jul;20(7):1185-94.
2. Kanis JA, Oden A, Johnell O, Johansson H, De Laet C, Brown J, et al. The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women. Osteoporos Int. 2007 Aug;18(8):1033-46.

3. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev. 2004 Dec;15(6):457-75.
4. van Meurs JB, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, et al. Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. JAMA. 2008 Mar 19;299(11):1277-90.
5. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
6. Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, et al. An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet. 2010 Jun;6(6):e1000977.
7. Kung AW, Xiao SM, Cherny S, Li GH, Gao Y, Tso G, et al. Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies. Am J Hum Genet. 2010 Feb 12;86(2):229-39.
8. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008 May 3;371(9623):1505-12.
9. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
10. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008 May 29;358(22):2355-65.
11. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. New sequence variants associated with bone mineral density. Nat Genet. 2009 Jan;41(1):15-7.
12. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.
13. Cook EH, Jr., Scherer SW. Copy-number variations associated with neuropsychiatric conditions. Nature. 2008 Oct 16;455(7215):919-23.
14. International Schizophrenia Consortium. Rare chromosomal deletions and duplications increase risk of schizophrenia. Nature. 2008 Sep 11;455(7210):237-41.
15. Marshall CR, Noor A, Vincent JB, Lionel AC, Feuk L, Skaug J, et al. Structural variation of chromosomes in autism spectrum disorder. Am J Hum Genet. 2008 Feb;82(2):477-88.
16. Jacquemont S, Reymond A, Zufferey F, Harewood L, Walters RG, Kutalik Z, et al. Mirror extreme BMI phenotypes associated with gene dosage at the chromosome 16p11.2 locus. Nature. 2011 Oct 6;478(7367):97-102.
17. Yang TL, Chen XD, Guo Y, Lei SF, Wang JT, Zhou Q, et al. Genome-wide copy-number-variation study identified a susceptibility gene, UGT2B17, for osteoporosis. Am J Hum Genet. 2008 Dec;83(6):663-74.
18. Chew S, Mullin BH, Lewis JR, Spector TD, Prince RL, Wilson SG. Homozygous deletion of the UGT2B17 gene is not associated with osteoporosis risk in elderly Caucasian women. Osteoporos Int. 2011 Jun;22(6):1981-6.
19. Craddock N, Hurles ME, Cardin N, Pearson RD, Plagnol V, Robson S, et al. Genome-wide association study of CNVs in 16,000 cases of eight common diseases and 3,000 shared controls. Nature. 2010 Apr 1;464(7289):713-20.
20. Itsara A, Cooper GM, Baker C, Girirajan S, Li J, Absher D, et al. Population analysis of large copy number variants and hotspots of human genetic disease. Am J Hum Genet. 2009 Feb;84(2):148-61.
21. Hofman A, Breteler MM, van Duijn CM, Krestin GP, Pols HA, Stricker BH, et al. The Rotterdam Study: objectives and design update. Eur J Epidemiol. 2007;22(11):819-29.
22. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol. 1991 Jul;7(4):403-22.
23. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.

24. Raychaudhuri S, Iartchouk O, Chin K. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. Nat Genet. 2011.
25. Basu S, Pan W. Comparison of statistical tests for disease association with rare variants. Genet Epidemiol. 2011 Nov;35(7):606-19.
26. Pinto D, Pagnamenta AT, Klei L, Anney R, Merico D, Regan R, et al. Functional impact of global rare copy number variation in autism spectrum disorders. Nature. 2010 Jul 15;466(7304):368-72.
27. Bierut LJ, Agrawal A, Bucholz KK, Doheny KF, Laurie C, Pugh E, et al. A genome-wide association study of alcohol dependence. Proc Natl Acad Sci U S A. 2010 Mar 16;107(11):5082-7.
28. Krawczak M, Nikolaus S, von Eberstein H, Croucher PJ, El Mokhtari NE, Schreiber S. PopGen: population-based recruitment of patients and controls for the analysis of complex genotype-phenotype relationships. Community Genet. 2006;9(1):55-61.
29. Shaikh TH, Gai X, Perin JC, Glessner JT, Xie H, Murphy K, et al. High-resolution mapping and analysis of copy number variations in the human genome: a data resource for clinical and research applications. Genome Res. 2009 Sep;19(9):1682-90.
30. Stewart AF, Dandona S, Chen L, Assogba O, Belanger M, Ewart G, et al. Kinesin family member 6 variant Trp719Arg does not associate with angiographically defined coronary artery disease in the Ottawa Heart Genomics Study. J Am Coll Cardiol. 2009 Apr 21;53(16):1471-2.
31. Mountz JD, Edwards CK, 3rd. Murine models of autoimmunity: T-cell and B-cell defects. Curr Opin Rheumatol. 1992 Oct;4(5):612-20.
32. Rakel A, Sheehy O, Rahme E, LeLorier J. Osteoporosis among patients with type 1 and type 2 diabetes. Diabetes Metab. 2008 Jun;34(3):193-205.
33. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int. 2007 Apr;18(4):427-44.
34. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.
35. Oei L, Zillikens MC, Dehghan A, Buitendijk GH, Castano-Betancourt MC, Estrada K, et al. High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study. Diabetes care. 2013 Jun;36(6):1619-28.
36. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol. 2012 May;27(5):319-32.
37. Diskin SJ, Hou C, Glessner JT, Attiyeh EF, Laudenslager M, Bosse K, et al. Copy number variation at 1q21.1 associated with neuroblastoma. Nature. 2009 Jun 18;459(7249):987-91.
38. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, et al. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature. 2002 Jun 6;417(6889):664-7.
39. DeScipio C. The 6p subtelomere deletion syndrome. Am J Med Genet C Semin Med Genet. 2007 Nov 15;145C(4):377-82.
40. Gould DB, Jaafar MS, Addison MK, Munier F, Ritch R, MacDonald IM, et al. Phenotypic and molecular assessment of seven patients with 6p25 deletion syndrome: relevance to ocular dysgenesis and hearing impairment. BMC Med Genet. 2004 Jun 25;5:17.
41. Kannu P, Oei P, Slater HR, Khammy O, Aftimos S. Epiphyseal dysplasia and other skeletal anomalies in a patient with the 6p25 microdeletion syndrome. Am J Med Genet A. 2006 Sep 15;140(18):1955-9.
42. Mirza G, Williams RR, Mohammed S, Clark R, Newbury-Ecob R, Baldinger S, et al. Refined genotype-phenotype correlations in cases of chromosome 6p deletion syndromes. Eur J Hum Genet. 2004 Sep;12(9):718-28.

Chapter 4.4

Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus

Oei L, Estrada K, Duncan EL, Christiansen C, Liu CT, Langdahl BL, Obermayer-Pietsch B, Riancho JA, Prince RL, van Schoor NM, McCloskey E, Hsu YH, Evangelou E, Ntzani E, Evans DM, Alonso N, Husted LB, Valero C, Hernandez JL, Lewis JR, Kaptoge SK, Zhu K, Cupples LA, Medina-Gómez C, Vandenput L, Kim GS, Hun Lee S, Castaño-Betancourt MC, Oei EHG, Martinez J, Daroszewska A, van der Klift M, Mellström D, Herrera L, Karlsson MK, Hofman A, Ljunggren Ö, Pols HAP, Stolk L, van Meurs JBJ, Ioannidis JP, Zillikens MC, Lips P, Karasik D, Uitterlinden AG, Styrkarsdottir U, Brown MA, Koh JM, Richards JB, Reeve J, Ohlsson C, Ralston SH, Kiel DP, Rivadeneira F

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ABSTRACT

Vertebral fracture risk is a heritable complex trait. The aim of this study was to identify genetic susceptibility factors for osteoporotic vertebral fractures applying a genome-wide association study (GWAS) approach. The GWAS discovery was based on the Rotterdam Study, a population-based study of elderly Dutch individuals aged 55 years; and comprising 329 cases and 2,666 controls with radiographic scoring (McCloskey–Kanis) and genetic data. Replication of one top-associated SNP was pursued by de-novo genotyping of 15 independent studies across Europe, the United States, and Australia and one Asian study. Radiographic vertebral fracture assessment was performed using McCloskey–Kanis or Genant semi-quantitative definitions. SNPs were analyzed in relation to vertebral fracture using logistic regression models corrected for age and sex. Fixed effects inverse variance and Han–Eskin alternative random effects meta-analyses were applied. Genome-wide significance was set at $P<5\times10^{-8}$. In the discovery, a SNP (rs11645938) on chromosome 16q24 was associated with the risk for vertebral fractures at $P=4.6\times10^{-8}$. However, the association was not significant across 5,720 cases and 21,791 controls from 14 studies. Fixed-effects meta-analysis summary estimate was 1.06 (95% CI: 0.98–1.14; P= 0.17), displaying high degree of heterogeneity (I^2=57%; Q_{het} P=0.0006). Under Han–Eskin alternative random effects model the summary effect was significant (P=0.0005). The SNP maps to a region previously found associated with lumbar spine bone mineral density (LS-BMD) in two large meta-analyses from the GEFOS consortium. A false positive association in the GWAS discovery cannot be excluded, yet, the low-powered setting of the discovery and replication settings (appropriate to identify risk effect size >1.25) may still be consistent with an effect size <1.10, more of the type expected in complex traits. Larger effort in studies with standardized phenotype definitions is needed to confirm or reject the involvement of this locus on the risk for vertebral fractures.

INTRODUCTION

Vertebral fractures are the most common osteoporotic fractures and represent a significant health issue.[1, 2] Epidemiological measures derived from population-based studies vary between 1 and 3% per year for incidence and ~10 and 30% for the prevalence in elderly persons, varying by age, gender and geographic region.[3-5] Vertebral fractures are associated with a high morbidity,[6-11] mortality[12, 13] and a considerable financial burden. In the United States the costs of vertebral fractures were estimated to be 1.1 billion dollars in the year 2005, and are expected to rise by more than 50% by the year 2025.[14] A recent report estimated the costs of vertebral fractures in Europe at 1.5billion euros in 2010.[15] Furthermore, vertebral fractures are likely to become an increasingly important health issue with the increasing age of populations[1, 14, 15] and their association with increased risk of future osteoporotic fractures at other skeletal sites[7, 16, 17]. For all of these reasons, a better understanding of the genetic susceptibility to vertebral fracture has the potential to identify underlying biological mechanisms, improve risk prediction and lead to novel disease interventions.

Vertebral fracture risk is a heritable complex trait, also influenced by environmental, and gene–environment interactions.[18, 19] A positive family history for vertebral fracture constitutes an independent risk factor for future fractures,[20] emphasizing the importance of genetics in the pathogenesis of the disease. The hypothesis-free genome-wide association study (GWAS) approach has been particularly successful in identifying loci associated with many diseases and quantitative complex traits,[21] including osteoporosis[18, 22-24].

The aim of our study was to better understand the genetic architecture of radiographic vertebral fractures by conducting the first GWAS for this trait in a large population-based study of elderly Dutch individuals and pursuing replication in a large set of studies across Europe, the United States, Australia and Asia.

METHODS

Datasets assessed

Sample discovery phase

The discovery sample was confined to the original Rotterdam Study cohort, a large population-based study of Dutch men and women aged 55 years and over (mean age at vertebral fracture assessment: 73.5 years). A detailed description of the Rotterdam Study has been reported previously.[25] In short, the study aimed to assess the incidence and determinants of disease and disability in elderly persons. The study has been approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam.

Sample replication phase

The Genetic Factors for Osteoporosis (GEFOS), Genetic Markers for Osteoporosis (GENOMOS) and Anglo-Australasian Osteoporosis Genetics Consortium (AOGC) are three consortia studying the genetic determinants of osteoporosis-related skeletal phenotypes in populations with available DNA and/or GWAS data.[23, 26-29] Within this setting, 15 studies with both DNA samples and lateral morphometry-derived vertebral fracture data participated in the replication phase of this project (Supplementary Table 1). More detailed descriptions are available in the Supplementary material.

Table 1. Vertebral fracture assessment.

					Comparison setting		Cut-off values used		Fractures confirmed by expert[a]
							McCloskey-Kanis	Genant	
Study	Morphometry method used	Number of vertebral fracture cases	Number of vertebral fracture controls	Prevalence or Case:control ratio[b]	Relative to population reference	Absolute height reduction[c]	3 SD relative reduction[d]	Difference in vertebral height ratios of at least:	
RS-I	McCloskey-Kanis	329	2,666	0.11	YES	YES	YES	NA	YES
AOGC	McCloskey-Kanis	686	3,411	0.17	YES	NO	YES	NA	YES
AROS	Genant	335	130	1:0.39[b]	NO	NO	NA	20%	YES
AUSTRIOS-B	Genant	803	1,261	0.39	NO	NO	NA	20%	YES
CABRIO-C	Genant	195	1,185	0.14	NO	NO	NA	20%	YES
CABRIO-CC	Genant	220	354	1:1.61[b]	NO	NO	NA	20%	YES
CAIFOS	McCloskey-Kanis	428	600	0.42	YES	NO	YES	NA	YES
CaMos	McCloskey-Kanis	243	1,785	0.12	YES	NO	NA	NA	YES
DOPS	Genant	108	1,605	0.06	NO	NO	NA	20%	YES
EDOS	Genant	495	523	0.49	NO	NO	NA	20%	YES
EPOS	McCloskey-Kanis	313	1,779	0.15	YES	YES	YES	NA	YES
FOS	Genant	417	2,291	0.15	NO	NO	NA	20%	YES
KorAMC	Genant	101	1,193	0.08	NO	YES	NA	20%	YES
LASA	Genant	237	268	0.47	NO	NO	NA	20%	YES
MrOS Sweden[e]	Genant	309	2,613	0.11	NO	NO	NA	20%	NO
PERF	Genant	830	2,793	0.23	NO	NO	NA	20%	NO

[a]E.g., radiologist/clinician, to rule out artifacts and other etiologies, such as pathological fractures.
[b]Prevalence in population-based studies, case:control ratio in case–control studies.
[c]Any of the three vertebral heights (anterior, central, or posterior) shows a minimum decrease of at least 4mm.
[d]3 SD relative reduction of 2 out of 3 ratios: (ha/hp; hm/hp; hp/hp predicted).
[e]Prevalent X-ray verified vertebral fractures only available for about 1,425 subjects.

The AOGC — Geelong Osteoporosis Study (AOGC-GOS) is a cohort drawn from the Geelong general population. Vertebral fracture imaging was performed in case of a clinical indication.[30, 31] The AOGC — Sheffield (AOGC-SHEFFIELD) study constitutes a large population-based cohort of community-dwelling elderly women aged ≥75 years in Sheffield, UK.[32] AROS (Aarhus Osteoporosis Study) is a case–control study, including 462 osteoporotic patients (vertebral fracture and T score <–2.5) and 336 controls.[33] AUSTRIOS is a prospective cohort study of elderly female patients above 70 recruited in 95 nursing homes in four counties in Austria. The AUSTRIOS-B cohort had vertebral fracture data available and was used for this project.[34] The Cantabria-Camargo (CABRIO-C) and Cantabria Case–Control (CABRIO-CC) studies are based in Northern Spain. CABRIO-C is a community-based study designed to evaluate the prevalence of metabolic bone diseases in postmenopausal women and men older than 50 years attending a primary care center in Santander.[35, 36] CABRIO-CC is a clinic-based study of control individuals and patients with osteoporosis living in Cantabria, a region in Northern Spain.[37, 38] The Calcium Intake Fracture Outcome Study (CAIFOS) is a randomized-controlled trial investigating calcium carbonate supplementation

in ambulatory women older than 70 years recruited in Perth, Australia.[39] The Canadian Multicentre Osteoporosis Study (CaMoS) is a population-based prospective cohort of unrelated men and women followed for osteoporosis and osteoporotic fractures for the past 14 years.[40-42] The Danish Osteoporosis Prevention Study (DOPS) is a population-based study of perimenopausal women. The women were followed for 10 years and approximately 35% were treated with hormone-replacement therapy (HRT).[43] The Edinburgh Osteoporosis Study (EDOS) consists of a clinical referral population of patients assessed for evaluation of osteoporosis in Edinburgh, United Kingdom. The European Prospective Osteoporosis Study (EPOS) is the prospective phase of the European Vertebral Osteoporosis Study (EVOS) in which population-based samples had paired duplicate spinal films. Men and women from 36 centers in 19 European countries were recruited.[5, 44, 45] The Framingham Osteoporosis Study (FOS) is an ancillary study of the Framingham Study, a multigenerational family based cohort study originally initiated to study the risk factors for cardiovascular disease.[46-48] Vertebral fracture assessment was done on multidetector computed tomography (CT) lateral scout views. The Korean osteoporosis study at Asan Medical Center (KorAMC) study is a hospital registered, cross-sectional study of postmenopausal Korean women in Seoul.[49] The Longitudinal Aging Study Amsterdam

(LASA) is an ongoing multidisciplinary cohort study in older persons. A random sample of men and women aged 55 years and over, stratified by age, sex, urbanization grade and expected 5-year mortality rate was drawn from the population register of Amsterdam, The Netherlands.[50] The Osteoporotic Fractures in Men Sweden (MrOS Sweden) study is a multicenter, prospective study including elderly men. Study subjects (men aged 69–80 years) were randomly identified using national population registers, contacted and asked to participate. Eligible subjects had to be able to walk without assistance, provide self-reported data, and sign an informed consent.[51] The Prospective Epidemiological Risk Factor (PERF) Study is based on subjects who were screened for or enrolled into randomized controlled clinical trial to identify genetic and other risk factors of diseases in the elderly in Copenhagen, Denmark.[52]

Phenotyping

Osteoporosis-related skeletal phenotypes in the discovery sample

During the second follow-up visit between 1997 and 1999 all Rotterdam Study participants underwent radiographic screening. A trained research technician obtained lateral radiographs of the thoracolumbar spine following a standard protocol. Radiographs were evaluated morphometrically in Sheffield, UK, by the McCloskey–Kanis method as described previously.[53] Using this method, central collapse, anterior and posterior wedge, and crush deformities were identified based on a cut point of 3 standard deviation height reductions. All vertebral fractures were confirmed by visual interpretation by an expert in the field to rule out artifacts and other etiologies, such as pathological fractures. Cases were defined as those individuals who had at least one vertebral fracture, and controls were defined as those who were free of vertebral fractures. Bone mineral density (BMD) of the femoral neck (FN) and lumbar spine (LS) was measured by dual-energy X-ray absorptiometry (DXA), using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA).

Other measurements (covariates) in the discovery sample

An extensive baseline home interview on medical history, risk factors for chronic diseases, and medication use was performed on all participants by trained interviewers. Smoking habits were coded as "current", "former" and "never". Self-reported age at natural menopause between 40 and 60 years, defined as 12 months after periods ceased, was collected retrospectively. Information on medication use in-

cluded hormone replacement therapy and systemic corticosteroids. Alcohol intake was assessed from a validated semi-quantitative food frequency questionnaire. Height and weight were measured with indoor clothing and no shoes. Body mass index (BMI) was calculated as weight (in kg) / height (in m^2).

Phenotyping replication phase

Vertebral fracture assessments differed by cohorts which applied either the McCloskey–Kanis[53] or the Genant semi-quantitative method[54]. Detailed description of the methods and cut-offs applied by each study is available in Table 1. Four of the replication studies used the McCloskey–Kanis method, which is similar to the discovery (Rotterdam Study), of which one study applied the same additional criterion of absolute height reduction. Phenotyping for covariates was similar to that of the discovery sample.

Genotyping

Genome-wide association data

The Rotterdam Study participants were genotyped using the Illumina Infinium HumanHap550 Beadchip in the Genetic Laboratory of Erasmus MC Department of Internal Medicine, The Netherlands, following manufacturers' protocols and quality control standards.

Single nucleotide polymorphism (SNP) genotyping

The top associated SNP from the discovery phase (rs11645938) was genotyped in 15 studies within three main genotyping centers: deCODE Genetics in Reykjavik, Iceland, Queensland University in Brisbane Australia and KBiosciences, Hertfordshire, U.K. (www.kbioscience.co.uk). Genotyping was carried out by personnel blinded to patient status in all centers. The samples genotyped by KBiosciences were part of the GENOMOS consortium DNA collection, and comprise most of the participating studies. For KBiosciences, a minimum of 1.5µl of DNA at 3.3ng/µl (when quantitated by PicoGreen analysis or 7 ng/µl if quantitated by spectrophotometry) was required for one SNP to be assayed using their proprietary KASPar PCR technique and Taqman (also used by Brisbane University for AOGC samples). Genotype calling was carried out using an automated system, the results of which were checked manually by study personnel using SNPviewer software (KBiosciences). deCODE used the same KASPar assay from KBiosciences to genotype the PERF study samples. To ensure genotyping validity across study centers, a reference plate was shipped from KBiosciences to the AOGC coordinating center. To ensure correct genotyping deCODE Genetics genotyped 92 HapMap samples for comparison with the KASPar assay, and both positive and negative samples were present on all genotyping plates. Additionally, duplicate SNP genotyping was performed in the Rotterdam Study (all samples) and CABRIO-C (random selection of 187 samples) and no discrepancies were found.

Statistical methods

Within the discovery cohort, we tested 2,543,887 genotyped or imputed (HapMap CEU release 22, build 36)[55, 56] SNPs for association with risk of osteoporotic vertebral fractures using a logistic regression model (MACH2DAT)[57, 58] adjusted for age, gender, and admixture principal components (PCs) derived using EIGENSTRAT to adjust for population substructure[59]. Potential effect modifiers for the relationship between genotype and vertebral fracture (i.e., height, weight, BMI, age at menopause, HRT use, corticosteroid use, >3 units alcohol use per day, current and ever smoking) were tested by adding them one at a time to the regression model and evaluating the change in both the effect estimate and significance. The GWAS was performed using a web-based interface (GRIMP) on scalable super-computing grid infrastructures.[60] At a

genome-wide significant α-level of 5×10^{-8}, the design had 0.80 power to detect risk effect sizes (OR) of 1.8 to 2.1 for minor allele frequencies (MAF) of 20% to 10%, respectively.

Replication analyses

Except for the FOS and AOGC studies, all analyses were carried out centrally by the Rotterdam Coordinating Center. Again a logistic regression model adjusting for age and gender was used. Individuals with either missing genotype or phenotype data were excluded from analysis. Initially, fixed effects inverse variance meta-analysis was performed (METAL software[61]). The presence of statistically significant heterogeneity was assessed by Cochran's Q statistic (Q_{het} P) and the extent of the observed heterogeneity was measured by the I^2 metric. Han–Eskin alternative random effects meta-analysis was applied when the I^2metric exceeded 50% as this model is optimized to detect associations under heterogeneity (Metasoft software[62]). SPSS 16.0, PLINK, and R software were used for the rest of the analyses. In addition, the Framingham Study analysis used population-based generalized estimating equation (GEE) approach correcting for correlations owing to family relationships and PCs. The replication setting incorporating 5,720 cases and 21,791 controls from 14 studies was powered to identify a variant with a MAF of 0.10 and risk effect size >1.25, associated at $P<5\times10^{-8}$.

RESULTS

The description of the studies included in the discovery and replication phases is shown in Supplementary Table 1. Description of the vertebral fracture assessment done across studies is presented in Table 1 while baseline characteristics of the study populations are shown in Supplementary Table 2. In the discovery set, 329 of the 2,995 Rotterdam Study participants had at least one vertebral fracture evident on the spinal radiographs. A genotyped SNP (rs11645938) on chromosome 16q24 (MAF=10%) was as-

Table 2. Descriptive information about genotyping of the rs11645938 SNP and association statistics per study.

Study	SNP call rate	P value Hardy-Weinberg Equilibrium	Minor Allele Frequency	Effect estimate (Beta)	Standard error	P value
RS-I	99.9%	0.52	9.65%	0.669	0.122	4.6×10^{-8}
AOGC	93.7%	0.83	9.74%	-0.11	0.11	0.33
AROS	99.3%	0.79	9.76%	0.22	0.31	0.61
AUSTRIOS-B	97.0%	0.96	11.74%	-0.18	0.16	0.26
CABRIO-C	99.1%	0.84	7.71%	-0.51	0.25	0.04
CABRIO-CC	99.1%	0.61	8.98%	-0.16	0.26	0.53
CAIFOS	99.2%	0.02	10.01%	-0.02	0.15	0.91
CaMos	99.0%	0.12	9.57%	0.09	0.16	0.50
DOPS	98.6%	0.17	10.01%	-0.13	0.25	0.59
EDOS	99.4%	0.99	9.45%	0.07	0.16	0.65
EPOS	99.6%	0.80	9.62%	0.15	0.16	0.75
FOS	97.6%	0.86	9.97%	-0.04	0.14	0.78
KorAMC	97.8%	NA	0.00%	NA	NA	NA
LASA	100.0%	0.82	11.16%	-0.06	0.22	0.79
MrOS Sweden	98.6%	0.49	9.77%	0.07	0.16	0.69
PERF	100.0%	0.79	9.45%	0.04	0.10	0.70

sociated at a genome-wide significant level (P=4.6×10^{-8}) with an increased risk of vertebral fractures (Figure 1). Compared to the risk of non-carriers, the odds of the heterozygous carriers of the minor allele (C) was 1.7 times higher (95% confidence interval [CI]: 1.3–2.3) and that of the homozygous carriers was 5.8 times higher (95% CI: 2.7–12.8) (Supplementary Figure 1). Figure 2 shows the regional association plot of the locus, where a cluster of FOX genes maps ~200 kb from the associated SNP, containing *FOXF1, MTHFSD, FOXC2*, and *FOXL1*. Further adjusting for potential confounders did not influence either the effect estimate or the significance of the association between genotype and vertebral fracture risk. Similarly, the association remained significant after adjustment for either LS- or FN-BMD. Sex-stratified association analysis for the SNP, showed similar effect estimates (OR heterozygote men: 1.8 [95% CI: 1.2–2.8] and OR heterozygote women: 1.6 [95% CI: 1.1–2.3]; OR homozygote women: 8.4 [95% CI: 3.0–23.0] and OR homozygote men 3.3 [95% CI: 0.9–12.7]).

The associated SNP rs11645938 was successfully genotyped in 14 of the replication studies (5,722 vertebral fracture cases and 21,793 controls; MAF ~8–12%) while it was found to be monomorphic in the Korean population of the KorAMC study (Table 2). The summary effect estimate for vertebral fracture risk obtained from the meta-analysis was 1.06 (95% CI: 0.98–1.14; P=0.17) and the effect estimate displayed high degree of heterogeneity with I^2=57% and Q_{het} P=0.0006 (Figure 3). When considering a Han–Eskin alternative random effects meta-analysis model the summary effect was significant (p = 0.0005). When applying more stringent genotyping criteria (call rate >95%; Hardy–Weinberg equilibrium P<0.05) the association became significant in both the fixed (P=0.045) and Han–Eskin alternative random effects meta-analysis (P=0.0002).When further restricting analyses only to those studies that used the McCloskey–Kanis assessment a consistent, nonetheless not a statistically significant, effect direction was observed (replication P=0.29).

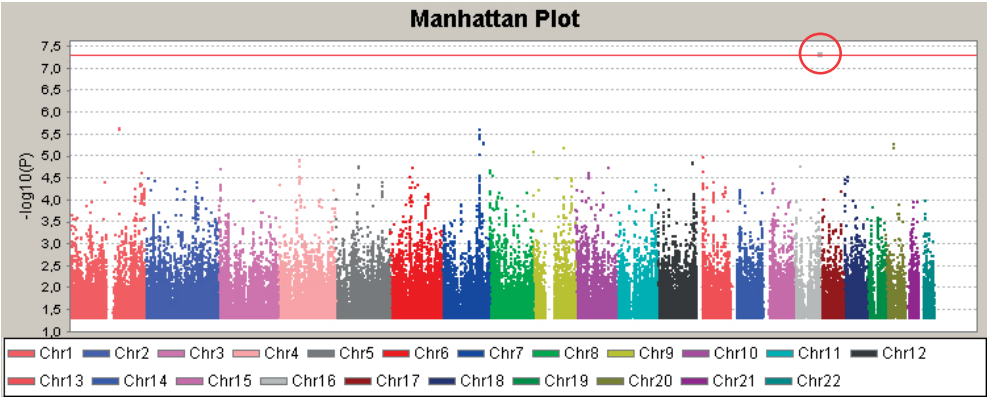

Figure 1. Manhattan plot of negative logarithm P values plotted by chromosome, showing that a SNP on chromosome 16q24 was associated at a genome-wide significant level with osteoporotic vertebral fractures (P=4.6×10^{-8}) in the Rotterdam Study (encircled).

DISCUSSION

To our knowledge, this is the first GWAS for radiographically determined vertebral fracture. A marker on chromosome 16q24 was genome-wide significantly associated with vertebral fracture in the Rotterdam Study discovery set. However, this association was not significant in a replication effort including 15 studies world-wide using conventional statistical analysis techniques.

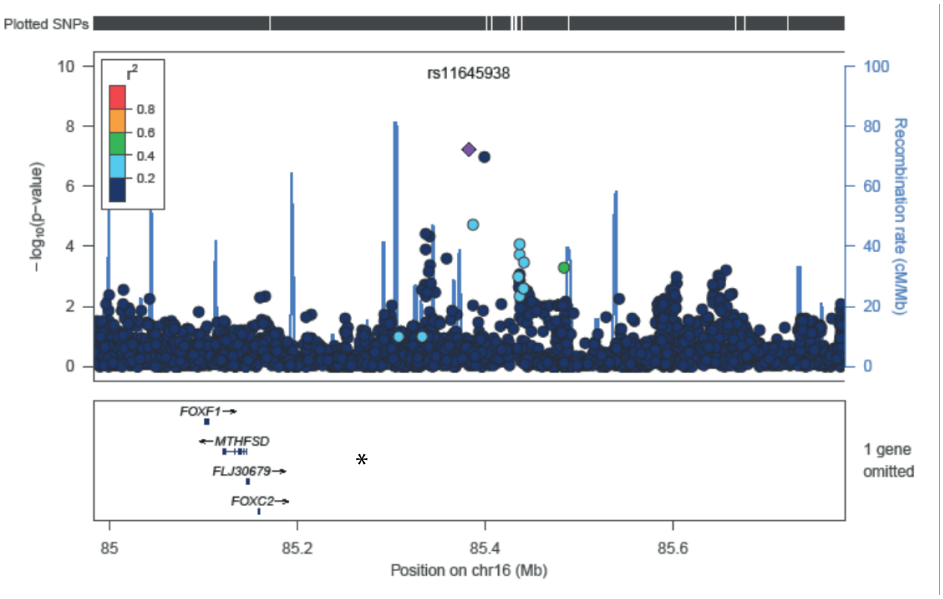

Figure 2. Regional association plot showing position on chromosome 16 and association P values of the analyzed SNPs in the Rotterdam Study with neighboring genes. Included are genotyped, HapMap II and 1000 Genomes imputed SNPs. The rectangle is the SNP of interest, and the circles represent neighboring SNPs with their respective correlation with the topmarker. The spikes depict the recombination rates. The position of the rs10048146 SNP that has previously been found as associated with lumbar-spine bone mineral density is indicated with *.

Work by Stankiewicz et al. implicated deletions/mutations in this 16q24 locus in the VACTERL association (Vertebral anomalies, Anal atresia, Cardiovascular anomalies, TracheoEsophageal fistula, Renal and Radial anomalies, Limb defects), a non-random association of birth defects that includes vertebral defects.[63] *FOXC2*, mapping ~200 kb upstream from the associated SNP, is highly expressed in human bone tissue, and its expression is regulated by bone morphogenetic proteins[64]. The gene is involved in osteoblast differentiation through activation of canonical Wnt/β-catenin signals,[65] and in mice Foxc2 functions as a transcription factor essential for axial skeletogenesis[66]. The vertebral fracture associated SNP maps to a region previously found to be associated with LS-BMD in a meta-analysis of 19,125 individuals[23] and further replicated in 83,894 individuals[22]. However, the vertebral fracture SNP was not associated with either LS- or FN-BMD in our study and this signal was independent of the one previously reported for the BMD SNP rs10048146 (r^2=0.002).

Despite the underlying biological plausibility supporting this association and even with identifying a genome-wide significant signal in the discovery GWAS, replication in independent studies is still needed.[21, 67, 68] Subsequently, de-novo direct genotyping of rs11645938 in 5,720 cases and 21,791 controls, from multiple independent studies around the world, did not provide robust evidence for replication of the association. Therefore, there is a high likelihood of the signal being a false-positive signal. It is expected that discoveries at underpowered settings would have low positive predictive value for true findings and this applies even for signals that pass a stringent genome-wide significance threshold.[69] However, other considerations might have also contributed to an apparent lack of replication of a potentially true association, and these will serve to inform the design of future GWAS of the vertebral fracture phenotype.

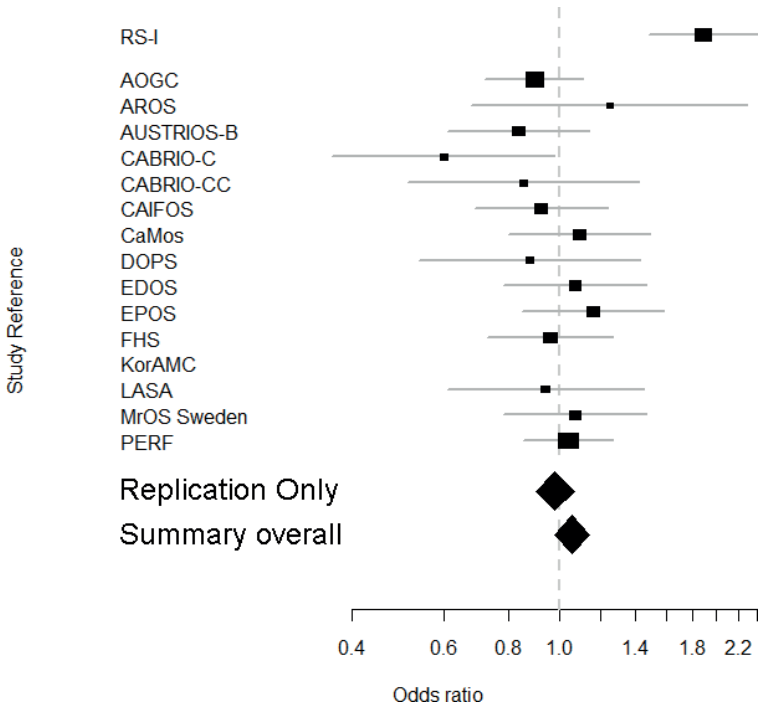

Figure 3. Forest plot showing meta-analysis results of vertebral fracture risk for rs11645938 in discovery and replication studies. Effect estimates represented by squares are displayed on a logarithmic scale, with horizontal lines corresponding to 95% confidence intervals. The center line of the diamond stands for the overall summary measure, and its horizontal line indicates the 95% confidence interval.

Signals in underpowered settings are likely to display inflated effects due to the "winner's curse" phenomenon, where the effect estimate observed in the first study overestimates the actual risk observed at the general population level.[70-72] According to a post-hoc calculation for the replication phase, the current design had merely 0.42 power to detect an OR of 1.2. The study sample should have included more than 8,000 cases to achieve 0.80 power, and we know that typically GWAS of complex traits even requires close to 30,000 cases to identify truly associated SNPs with moderate allele frequencies (e.g., MAF=0.10) in a powered setting. Previous efforts have pointed out that SNPs with MAF<10% tend to be difficult to replicate due to the lack of statistical power.[73] Thus, we cannot yet exclude the possibility that the identified association has a very small, yet genuine effect.[74] Larger-scale GWAS meta-analyses for osteoporotic vertebral fractures are seriously needed.

GWA studies rely on the principle of linkage disequilibrium (LD) where markers are tested under the assumption they tag an underlying causal genetic variant. When the linkage disequilibrium structure in the region differs across populations this may result in decreased power and lack of replication.[75-78] The rs11645938 marker is not in LD with any other marker contained in HapMap and only in moderate LD with one marker (r^2=0.41 with rs11647070) from the 1000 Genomes Project. This observation led us to conclude that existing GWAS without the rs11645938 on their arrays would be poorly imputed, which was the case in the FOS and AOGC studies, and therefore to overcome this, de-novo genotyping of the marker was performed in these studies. However, strictly speaking, genotyping in the Australian

AOGC and CAIFOS studies did not attain conventional criteria for unknown reasons. Further, the SNP is monomorphic in Asian populations.

Despite the fact that all studies used radiological assessments, a critical issue to bear in mind is the phenotype definition, considering that diverse methods and cut-offs exist for the assessment of vertebral fractures.[79] Phenotype measurement differences are a known possible source of heterogeneity, which might be reflected in our study by the great variation in vertebral fracture prevalences among the studies. Noticeably, prevalence estimates varied between 6% and 49% in the cohort studies. Furthermore, quantitative scoring is based on morphometry alone, which may result in inclusion of deformities into the phenotype definition that are not truly vertebral fractures.[80] These non-fracture deformities are frequently labeled as Genant grade 1 or "mild vertebral fractures," when, in fact, they may be normal variations in vertebral shape. Therefore, many studies assign an expert to filter out these non-fracture deformities. Nevertheless, this triage procedure may not have been sufficiently standardized, and this could have introduced the statistically significant heterogeneity between studies. Several methods exist to explore the existence of associations in heterogeneous data and when we applied a Han–Eskin random effects model, more stringent genotyping criteria or sensitivity analyses for phenotype definition, the results became more consistent. Perhaps selecting Genant grade 2 and 3 types including "moderate" and "severe" vertebral fractures[81] could provide a better phenotype definition for future genetic studies. In fact, Liu and colleagues demonstrated that the heritability of a stricter phenotype (when only more severe deformities counted) was higher than considering all vertebral deformities together.[19] Therefore, phenotype standardization among meta-analysis participants can be a key in replication.[71, 82] Unfortunately, data harmonization was not possible because severity grading or qualitative standardized reading to enable data harmonization was not available for most of the studies included in our analysis. This consideration, along with the relatively small sample sizes across replication studies, is a major hurdle to be overcome in future studies focusing on radiographic vertebral fractures. Clinical vertebral fracture is an alternative phenotype definition for future genetic studies, though achieving sufficient sample sizes will be also challenging; considering that only a small fraction of vertebral fractures come to clinical attention (i.e., are symptomatic). In addition, it would be valuable to gain more insight into incident vertebral fractures. Nevertheless, definition of incident vertebral fractures is accompanied by different and possibly greater precision errors than identification of prevalent vertebral fractures. On the other hand, by comparing images at different follow-ups, the radiological reader has the opportunity to correct possible misclassifications, including misattributions of baseline deformities as fracture cases caused by erroneous vertebral height readings due to for example superimposition of other structures or magnification errors.[83-86]

In conclusion, although a GWAS in the population-based Rotterdam Study identified a marker mapping to the 16q24 (*FOXC2*) BMD locus as being genome-wide significantly associated with radiographic vertebral fracture in that population, this could not be conclusively replicated by de-novo genotyping across 15 studies worldwide. A false positive association in the GWAS discovery cannot yet be excluded. However, these results from a low-powered setting may still be consistent with a small true effect size as is common in complex traits. Larger efforts in subsequent GWAS for radiographic vertebral fracture with standardized phenotype definitions may confirm or reject the involvement of this locus on the risk for vertebral fractures.

Supplementary information is available at:

http://www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S8756328213004250/1-s2.0-S8756328213004250-mmc1.doc/271131/html/S8756328213004250/669c20bcd3037605031d66877028a3ba/mmc1.doc

REFERENCES

1. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management2011.
2. Brunton S, Carmichael B, Gold D, Hull B, Kauffman T, Papaioannou A, et al. Vertebral compression fractures in primary care: recommendations from a consensus panel. J Fam Pract. 2005 Sep;54(9):781-8.
3. Samelson EJ, Hannan MT, Zhang Y, Genant HK, Felson DT, Kiel DP. Incidence and risk factors for vertebral fracture in women and men: 25-year follow-up results from the population-based Framingham study. J Bone Miner Res. 2006 Aug;21(8):1207-14.
4. Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2002 Jun;17(6):1051-6.
5. Incidence of vertebral fracture in europe: results from the European Prospective Osteoporosis Study (EPOS). J Bone Miner Res. 2002 Apr;17(4):716-24.
6. Burger H, Van Daele PL, Grashuis K, Hofman A, Grobbee DE, Schutte HE, et al. Vertebral deformities and functional impairment in men and women. J Bone Miner Res. 1997 Jan;12(1):152-7.
7. Ross PD. Clinical consequences of vertebral fractures. Am J Med. 1997 Aug 18;103(2A):30S-42S; discussion S-3S.
8. Nevitt MC, Ettinger B, Black DM, Stone K, Jamal SA, Ensrud K, et al. The association of radiographically detected vertebral fractures with back pain and function: a prospective study. Ann Intern Med. 1998 May 15;128(10):793-800.
9. Gold DT. The clinical impact of vertebral fractures: quality of life in women with osteoporosis. Bone. 1996 Mar;18(3 Suppl):185S-9S.
10. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C, et al. Health-related quality of life in postmenopausal women with low BMD with or without prevalent vertebral fractures. J Bone Miner Res. 2000 Jul;15(7):1384-92.
11. Oleksik AM, Ewing S, Shen W, van Schoor NM, Lips P. Impact of incident vertebral fractures on health related quality of life (HRQOL) in postmenopausal women with prevalent vertebral fractures. Osteoporos Int. 2005 Aug;16(8):861-70.
12. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
13. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. J Am Geriatr Soc. 2000 Mar;48(3):241-9.
14. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res. 2007 Mar;22(3):465-75.
15. Ström O, Borgström F, Kanis JA, Compston J, Cooper C, McCloskey EV, et al. Osteoporosis: burden, health care provision and opportunities in the EU. Arch Osteoporos. 2011;DOI 10.1007/s11657-011-0060-1.
16. Melton LJ, 3rd, Atkinson EJ, Cooper C, O'Fallon WM, Riggs BL. Vertebral fractures predict subsequent fractures. Osteoporos Int. 1999;10(3):214-21.
17. Black DM, Arden NK, Palermo L, Pearson J, Cummings SR. Prevalent vertebral deformities predict hip fractures and new vertebral deformities but not wrist fractures. Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1999 May;14(5):821-8.
18. Ralston SH, Uitterlinden AG. Genetics of osteoporosis. Endocr Rev. 2010 Oct;31(5):629-62.
19. Liu CT, Karasik D, Zhou Y, Hsu YH, Genant HK, Broe KE, et al. Heritability of prevalent vertebral fracture and volumetric bone mineral density and geometry at the lumbar spine in three generations of the framingham study. J Bone Miner Res. 2011 Dec 20.

20. Kanis JA, Johansson H, Oden A, Johnell O, De Laet C, Eisman JA, et al. A family history of fracture and fracture risk: a meta-analysis. Bone. 2004 Nov;35(5):1029-37.
21. Manolio TA. Genomewide association studies and assessment of the risk of disease. N Engl J Med. 2010 Jul 8;363(2):166-76.
22. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.
23. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
24. Richards JB, Zheng HF, Spector TD. Genetics of osteoporosis from genome-wide association studies: advances and challenges. Nat Rev Genet. 2012 Aug;13(8):576-88.
25. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
26. Langdahl BL, Uitterlinden AG, Ralston SH, Trikalinos TA, Balcells S, Brandi ML, et al. Large-scale analysis of association between polymorphisms in the transforming growth factor beta 1 gene (TGFB1) and osteoporosis: the GENOMOS study. Bone. 2008 May;42(5):969-81.
27. Ralston SH, Uitterlinden AG, Brandi ML, Balcells S, Langdahl BL, Lips P, et al. Large-scale evidence for the effect of the COLIA1 Sp1 polymorphism on osteoporosis outcomes: the GENOMOS study. PLoS Med. 2006 Apr;3(4):e90.
28. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
29. Sims AM, Shephard N, Carter K, Doan T, Dowling A, Duncan EL, et al. Genetic analyses in a sample of individuals with high or low BMD shows association with multiple Wnt pathway genes. J Bone Miner Res. 2008 Apr;23(4):499-506.
30. Henry MJ, Pasco JA, Korn S, Gibson JE, Kotowicz MA, Nicholson GC. Bone mineral density reference ranges for Australian men: Geelong Osteoporosis Study. Osteoporos Int. 2010 Jun;21(6):909-17.
31. Henry MJ, Pasco JA, Nicholson GC, Seeman E, Kotowicz MA. Prevalence of osteoporosis in Australian women: Geelong Osteoporosis Study. J Clin Densitom. 2000 Fall;3(3):261-8.
32. McCloskey EV, Beneton M, Charlesworth D, Kayan K, deTakats D, Dey A, et al. Clodronate reduces the incidence of fractures in community-dwelling elderly women unselected for osteoporosis: results of a double-blind, placebo-controlled randomized study. J Bone Miner Res. 2007 Jan;22(1):135-41.
33. Harslof T, Husted LB, Carstens M, Stenkjaer L, Langdahl BL. Genotypes and haplotypes of the estrogen receptor genes, but not the retinoblastoma-interacting zinc finger protein 1 gene, are associated with osteoporosis. Calcif Tissue Int. 2010 Jul;87(1):25-35.
34. Dobnig H, Piswanger-Solkner JC, Roth M, Obermayer-Pietsch B, Tiran A, Strele A, et al. Type 2 diabetes mellitus in nursing home patients: effects on bone turnover, bone mass, and fracture risk. J Clin Endocrinol Metab. 2006 Sep;91(9):3355-63.
35. Martinez J, Olmos JM, Hernandez JL, Pinedo G, Llorca J, Obregon E, et al. Bone turnover markers in Spanish postmenopausal women: the Camargo cohort study. Clin Chim Acta. 2009 Nov;409(1-2):70-4.
36. Olmos JM, Hernandez JL, Martinez J, Pariente E, Llorca J, Gonzalez-Macias J. Bone turnover markers in Spanish adult men The Camargo Cohort Study. Clin Chim Acta. 2010 Oct 9;411(19-20):1511-5.
37. Riancho JA, Valero C, Naranjo A, Morales DJ, Sanudo C, Zarrabeitia MT. Identification of an aromatase haplotype that is associated with gene expression and postmenopausal osteoporosis. J Clin Endocrinol Metab. 2007 Feb;92(2):660-5.
38. Zarrabeitia MT, Hernandez JL, Valero C, Zarrabeitia A, Amado JA, Gonzalez-Macias J, et al. Adiposity, estradiol, and genetic variants of steroid-metabolizing enzymes as determinants of bone mineral density. Eur J Endocrinol. 2007 Jan;156(1):117-22.

39. Prince RL, Devine A, Dhaliwal SS, Dick IM. Effects of calcium supplementation on clinical fracture and bone structure: results of a 5-year, double-blind, placebo-controlled trial in elderly women. Arch Intern Med. 2006 Apr 24;166(8):869-75.
40. Richards JB, Leslie WD, Joseph L, Siminoski K, Hanley DA, Adachi JD, et al. Changes to osteoporosis prevalence according to method of risk assessment. J Bone Miner Res. 2007 Feb;22(2):228-34.
41. Richards JB, Papaioannou A, Adachi JD, Joseph L, Whitson HE, Prior JC, et al. Effect of selective serotonin reuptake inhibitors on the risk of fracture. Arch Intern Med. 2007 Jan 22;167(2):188-94.
42. Tenenhouse A, Joseph L, Kreiger N, Poliquin S, Murray TM, Blondeau L, et al. Estimation of the prevalence of low bone density in Canadian women and men using a population-specific DXA reference standard: the Canadian Multicentre Osteoporosis Study (CaMos). Osteoporos Int. 2000;11(10):897-904.
43. Mosekilde L, Hermann AP, Beck-Nielsen H, Charles P, Nielsen SP, Sorensen OH. The Danish Osteoporosis Prevention Study (DOPS): project design and inclusion of 2000 normal perimenopausal women. Maturitas. 1999 Mar 15;31(3):207-19.
44. Ismail AA, O'Neill TW, Cockerill W, Finn JD, Cannata JB, Hoszowski K, et al. Validity of self-report of fractures: results from a prospective study in men and women across Europe. EPOS Study Group. European Prospective Osteoporosis Study Group. Osteoporos Int. 2000;11(3):248-54.
45. O'Neill TW, Felsenberg D, Varlow J, Cooper C, Kanis JA, Silman AJ. The prevalence of vertebral deformity in european men and women: the European Vertebral Osteoporosis Study. J Bone Miner Res. 1996 Jul;11(7):1010-8.
46. Dawber TR, Meadors GF, Moore FE, Jr. Epidemiological approaches to heart disease: the Framingham Study. Am J Public Health Nations Health. 1951 Mar;41(3):279-81.
47. Kannel WB, Feinleib M, McNamara PM, Garrison RJ, Castelli WP. An investigation of coronary heart disease in families. The Framingham offspring study. Am J Epidemiol. 1979 Sep;110(3):281-90.
48. Splansky GL, Corey D, Yang Q, Atwood LD, Cupples LA, Benjamin EJ, et al. The Third Generation Cohort of the National Heart, Lung, and Blood Institute's Framingham Heart Study: design, recruitment, and initial examination. Am J Epidemiol. 2007 Jun 1;165(11):1328-35.
49. Koh JM, Oh B, Lee JY, Lee JK, Kimm K, Park BL, et al. Association of FLT3 polymorphisms with low BMD and risk of osteoporotic fracture in postmenopausal women. J Bone Miner Res. 2007 Nov;22(11):1752-8.
50. Deeg DJ, van Tilburg T, Smit JH, de Leeuw ED. Attrition in the Longitudinal Aging Study Amsterdam. The effect of differential inclusion in side studies. J Clin Epidemiol. 2002 Apr;55(4):319-28.
51. Mellstrom D, Johnell O, Ljunggren O, Eriksson AL, Lorentzon M, Mallmin H, et al. Free testosterone is an independent predictor of BMD and prevalent fractures in elderly men: MrOS Sweden. J Bone Miner Res. 2006 Apr;21(4):529-35.
52. Bagger YZ, Rasmussen HB, Alexandersen P, Werge T, Christiansen C, Tanko LB. Links between cardiovascular disease and osteoporosis in postmenopausal women: serum lipids or atherosclerosis per se? Osteoporos Int. 2007 Apr;18(4):505-12.
53. McCloskey EV, Spector TD, Eyres KS, Fern ED, O'Rourke N, Vasikaran S, et al. The assessment of vertebral deformity: a method for use in population studies and clinical trials. Osteoporos Int. 1993 May;3(3):138-47.
54. Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res. 1993 Sep;8(9):1137-48.
55. The International HapMap Project. Nature. 2003 Dec 18;426(6968):789-96.
56. Altshuler DM, Gibbs RA, Peltonen L, Dermitzakis E, Schaffner SF, Yu F, et al. Integrating common and rare genetic variation in diverse human populations. Nature. 2010 Sep 2;467(7311):52-8.
57. Li Y, Willer C, Sanna S, Abecasis G. Genotype imputation. Annu Rev Genomics Hum Genet. 2009;10:387-406.
58. Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genet Epidemiol. 2010 Dec;34(8):816-34.
59. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006 Aug;38(8):904-9.
60. Estrada K, Abuseiris A, Grosveld FG, Uitterlinden AG, Knoch TA, Rivadeneira F. GRIMP: a web- and grid-based tool for high-speed analysis of large-scale genome-wide association using imputed data. Bioinformatics. 2009 Oct 15;25(20):2750-2.

61. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010 Sep 1;26(17):2190-1.
62. Han B, Eskin E. Random-effects model aimed at discovering associations in meta-analysis of genome-wide association studies. Am J Hum Genet. 2011 May 13;88(5):586-98.
63. Stankiewicz P, Sen P, Bhatt SS, Storer M, Xia Z, Bejjani BA, et al. Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. Am J Hum Genet. 2009 Jun;84(6):780-91.
64. Nifuji A, Miura N, Kato N, Kellermann O, Noda M. Bone morphogenetic protein regulation of forkhead/winged helix transcription factor Foxc2 (Mfh1) in a murine mesodermal cell line C1 and in skeletal precursor cells. J Bone Miner Res. 2001 Oct;16(10):1765-71.
65. Kim SH, Cho KW, Choi HS, Park SJ, Rhee Y, Jung HS, et al. The forkhead transcription factor Foxc2 stimulates osteoblast differentiation. Biochem Biophys Res Commun. 2009 Aug 28;386(3):532-6.
66. Winnier GE, Hargett L, Hogan BL. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. Genes Dev. 1997 Apr 1;11(7):926-40.
67. Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, Ballinger DG, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature. 2007 Jun 28;447(7148):1087-93.
68. Hoover RN. The evolution of epidemiologic research: from cottage industry to "big" science. Epidemiology. 2007 Jan;18(1):13-7.
69. Panagiotou OA, Ioannidis JP. What should the genome-wide significance threshold be? Empirical replication of borderline genetic associations. Int J Epidemiol. 2012 Feb;41(1):273-86.
70. Ioannidis JP. Why most discovered true associations are inflated. Epidemiology. 2008 Sep;19(5):640-8.
71. Kraft P, Zeggini E, Ioannidis JP. Replication in genome-wide association studies. Stat Sci. 2009 Nov 1;24(4):561-73.
72. Zhong H, Prentice RL. Correcting "winner's curse" in odds ratios from genomewide association findings for major complex human diseases. Genet Epidemiol. 2010 Jan;34(1):78-91.
73. Stromberg U, Bjork J, Vineis P, Broberg K, Zeggini E. Ranking of genome-wide association scan signals by different measures. Int J Epidemiol. 2009 Oct;38(5):1364-73.
74. Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. Nat Genet. 2003 Feb;33(2):177-82.
75. Chen F, Stram DO, Le Marchand L, Monroe KR, Kolonel LN, Henderson BE, et al. Caution in generalizing known genetic risk markers for breast cancer across all ethnic/racial populations. Eur J Hum Genet. 2011 Feb;19(2):243-5.
76. He J, Wilkens LR, Stram DO, Kolonel LN, Henderson BE, Wu AH, et al. Generalizability and epidemiologic characterization of eleven colorectal cancer GWAS hits in multiple populations. Cancer Epidemiol Biomarkers Prev. 2011 Jan;20(1):70-81.
77. Montpetit A, Nelis M, Laflamme P, Magi R, Ke X, Remm M, et al. An evaluation of the performance of tag SNPs derived from HapMap in a Caucasian population. PLoS Genet. 2006 Mar;2(3):e27.
78. Sim X, Ong RT, Suo C, Tay WT, Liu J, Ng DP, et al. Transferability of type 2 diabetes implicated loci in multi-ethnic cohorts from Southeast Asia. PLoS Genet. 2011 Apr;7(4):e1001363.
79. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, Hofman A, et al. Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. Eur Radiol. 2012 Aug 15.
80. Puisto V, Heliovaara M, Impivaara O, Jalanko T, Kroger H, Knekt P, et al. Severity of vertebral fracture and risk of hip fracture: a nested case-control study. Osteoporos Int. 2011 Jan;22(1):63-8.
81. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
82. Kerkhof HJ, Meulenbelt I, Akune T, Arden NK, Aromaa A, Bierma-Zeinstra SM, et al. Recommendations for standardization and phenotype definitions in genetic studies of osteoarthritis: the TREAT-OA consortium. Osteoarthritis Cartilage. 2011 Mar;19(3):254-64.

83. Felsenberg D, Silman AJ, Lunt M, Armbrecht G, Ismail AA, Finn JD, et al. Incidence of vertebral fracture in europe: results from the European Prospective Osteoporosis Study (EPOS). J Bone Miner Res. 2002 Apr;17(4):716-24.
84. Lunt M, Gowin W, Johnell, Armbrecht G, Felsenberg D. A statistical method to minimize magnification errors in serial vertebral radiographs. Osteoporos Int. 2001;12(11):909-13.
85. Lunt M, Ismail AA, Felsenberg D, Cooper C, Kanis JA, Reeve J, et al. Defining incident vertebral deformities in population studies: a comparison of morphometric criteria. Osteoporos Int. 2002 Oct;13(10):809-15.
86. Reeve J, Lunt M, Felsenberg D, Silman AJ, Scheidt-Nave C, Poor G, et al. Determinants of the size of incident vertebral deformities in European men and women in the sixth to ninth decades of age: the European Prospective Osteoporosis Study (EPOS). J Bone Miner Res. 2003 Sep;18(9):1664-73.

Chapter 4.5

TRPV4 deficiency causes sexual dimorphism in bone metabolism and osteoporotic fracture risk

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ABSTRACT

We explored the role of transient receptor potential vanilloid 4 (TRPV4) in murine bone metabolism and association of TRPV4 gene variants with fractures in humans. Urinary and histomorphometrical analyses demonstrated reduced osteoclast activity and numbers in male Trpv4$^{-/-}$ mice, which was confirmed in bone marrow-derived osteoclast cultures. Osteoblasts and bone formation as shown by serum procollagen type 1 amino-terminal propeptide and histomorphometry, including osteoid surface, osteoblast and osteocyte numbers were not affected in vivo. Nevertheless, osteoblast differentiation was enhanced in Trpv4$^{-/-}$ bone marrow cultures. Cortical and trabecular bone mass was 20% increased in male Trpv4$^{-/-}$ mice, compared to sex-matched wild type (Trpv4$^{+/+}$) mice. However, at the same time intracortical porosity was increased and bone matrix mineralization was reduced. Together, these lead to a maximum load, stiffness and work to failure of the femoral bone, which were not different compared to Trpv4$^{+/+}$ mice, while the bone material was less resistant to stress and less elastic. The differential impacts on these determinants of bone strength were likely responsible for the lack of any changes in whole bone strength in the Trpv4$^{-/-}$ mice. None of these skeletal parameters were affected in female Trpv4$^{-/-}$ mice. The T-allele of rs1861809 SNP in the TRPV4 locus was associated with a 30% increased risk (95% CI: 1.1–1.6; P=0.013) for non-vertebral fracture risk in men, but not in women, in the Rotterdam Study. Meta-analyses with the population-based LASA study confirmed the association with non-vertebral fractures in men. This was lost when the non-population-based studies Mr. OS and UFO were included. In conclusion, TRPV4 is a male-specific regulator of bone metabolism, a determinant of bone strength, and a potential risk predictor for fractures through regulation of bone matrix mineralization and intra-cortical porosity. This identifies TRPV4 as a unique sexually dimorphic therapeutic and/or diagnostic candidate for osteoporosis.

INTRODUCTION

The bone is an organ undergoing continuous remodeling, requiring a tight balance between bone resorption (by osteoclasts) and formation (by osteoblasts). The bone stores 99% of total body Ca^{2+}, making it a vital player in Ca^{2+} homeostasis. Members of the transient receptor potential (TRP) superfamily, predominantly the transient receptor potential vanilloid channels (TRPVs), have been implicated in both Ca^{2+} homeostasis and bone metabolism. We have demonstrated earlier in mice that the epithelial Ca^{2+} channel TRPV5 is crucial for renal Ca^{2+} reabsorption and for proper bone resorption.[1, 2] Moreover, mice lacking TRPV6 display disturbed intestinal Ca^{2+} uptake and reduced bone mass.[3-5] In contrast to TRPV5 and TRPV6, TRPV4 is permeable to Ca^{2+} in a non-selective manner.[6] TRPV4 responds to a wide variety of stimuli, including hypotonicity, pH, pain, cell swelling, endocannabinoids and mechanical stretching.[7, 8] Recently, it was shown that activating mutations in the *TRPV4* gene lead to several skeletal phenotypes in humans, including several dysplasias of the brachyolmia, spondylometaphyseal, Kozlowski and metatropic types.[9, 10] Two reports have investigated the role of TRPV4 in the murine skeleton but the data obtained are yet inconclusive.[11, 12] Although both studies used the same mouse model, one study examined male mice while the other study examined female mice. This may implicate that TRPV4 is a potential driver of skeletal sexual dimorphism such as differences in size and strength.[13]

In order to address this, we studied a head-to-head comparison of male and female *Trpv4*$^{-/-}$ mice with respect to the bone phenotype as well as bone cell differentiation patterns. In addition, we tested genetic variants in the *TRPV4* gene locus for association with fracture risk and bone parameters in human cohorts within a meta-analysis.

METHODS

Mice, tissue collection and serum/urine analyses Mice lacking *TRPV4* were generated as described extensively.[14] Briefly, cross-breeding of C57Bl/6 *TRPV4*$^{+/+}$ and *TRPV4*$^{-/-}$ mice resulted in offspring that were heterozygous for TRPV4. This offspring, bred within the Radboud University Nijmegen Medical Centre animal facility, was inter-crossed to obtain *TRPV4*$^{-/-}$ mice. These were subsequently inter-crossed and compared to age-matched *TRPV4*$^{+/+}$ mice. Male and female 20-week-old mice, fed ad libitum, were placed in metabolic cages to collect 24 hour urine. Next, mice were sacrificed and serum was collected. Bones were collected for microcomputed tomography and histomorphometry (left femurs), 3-point bending tests (right femurs) and bone marrow cultures (tibiae). Serum Ca^{2+} was colorimetrically determined with a Ca^{2+} assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's description at 595 nm, using a Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA). Urinary deoxypyridinoline (DPD) as a marker for bone resorption was analyzed using a MetraDPD enzyme immunoassay (Quidel, San Diego, CA, USA). Serum procollagen type 1 amino-terminal propeptide (P1NP) as a marker for bone formation was measured with an EIA (IDS, Boldon, UK). The animal ethics board of the Radboud University Medical Centre Nijmegen approved all experimental procedures.

Microcomputed tomography (µCT)

Femurs from female and male *TRPV4*$^{+/+}$ and *TRPV4*$^{-/-}$mice (N=6) were scanned at a resolution of 9 µm, using a SkyScan 1172 system (Bruker MicroCT, Kontich, Belgium). According to guidelines recently published,[15] the following settings were used: X-ray power and tube current were 40 kV and 0.25 mA, respectively. Beam hardening (20%) was reduced using a 1 mm aluminum filter, ring-artifacts were reduced (set at 5), exposure time was 5.9 s and an average of three pictures was taken at each angle (0.9°) to generate

final images. Using different software packages from Bruker MicroCT (NRecon, CtAn and Dataviewer), bone microarchitectural parameters were assessed in trabecular and cortical bone of all mice (N=14 for both genotypes). The trabecular bone parameters trabecular tissue volume, bone volume, trabecular volume fraction (BV/TV), trabecular thickness, trabecular number and trabecular patterning factor (connectivity of trabeculae) were determined in the distal metaphysis of the femur (scan area 0–4 mm of proximal femur). In the mid-diaphysis (scan area 4–6.2 mm from trochanter), cortical volume, cortical thickness, polar moment of inertia (MOI; proxy for bone strength) and perimeter were analyzed. For image processing, trabecular bone was manually selected and cortical bone was automatically selected. We used a Hamming filter and global thresholding was applied for segmentation, followed by using threshold levels of 150 (lower) and 194 (higher) for trabecular and levels of 0 and 31 for cortical bone measurements. In addition, trabecular and cortical bone mineral density (BMD) was measured on basis of calibration scanning, using two phantoms with known density (0.25 mg/cm^2 and 0.75 mg/cm^2; Bruker MicroCT) under identical conditions as for the femurs (method note from SkyScan provided on website).

Bone mechanical properties (3-point bending)

Femurs were stored in phosphate-buffered saline at –20 °C until further use. Before the 3-point bending test, femurs were scanned according to the settings mentioned above. The procedure was carried out as previously described in detail.[16] Briefly, femurs were placed in a custom made 3-point bending device, with the loading posts 10 mm apart. Mechanical testing was performed, using a Single Column Lloyd LRX System (Lloyd Instruments, Fareham, UK). Displacement (mm) and force (N) were registered. Using the same settings for filtration, segmentation and binarization as mentioned above in the microCT section, the MOI, reflecting the ability of the bone to withstand torsion, was calculated using CtAnalyzer software (Bruker MicroCT). It is the integral of the product of the distance between the area of the cortical bone and the center of gravity on one hand and the cortical bone itself on the other. This was determined in the µCT scan-derived crosssection that corresponded to the fracture site resulting from the bending test. From the resulting displacement to force graphs as well as the MOI values, ultimate force (N), stiffness (N/mm), work to failure (mJ), ultimate stress (N/mm^2) and elastic modulus (GPa) were determined as described before.[17]

Quantitative backscattered electron imaging

The distal half of femoral bone samples were fixed in 70% v/v ethanol, dehydrated in ethanol, and embedded in polymethylmethacrylate. Sample blocks containing grinded and polished surfaces of longitudinal femoral sections were manufactured. Bone mineralization density distribution (BMDD) from the trabecular metaphyseal and epiphyseal as well as from the cortical mid-shaft region was determined using quantitative backscattered electron imaging (qBEI). A digital scanning electron microscope (DSM 962, Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 20 kV, a probe current of 110 pA and equipped with a four-quadrant semiconductor backscattered electron detector, was used. Images with spatial resolution of 1 µm per pixel were acquired for BMDD measurements. This technique is well established and validated and the details of the method have been published elsewhere.[18, 19] The following BMDD parameters were calculated 1) CaMean is the weighted average Ca concentration of the mineralized tissue area, obtained from the integrated area under the BMDD curve. 2) CaPeak is the peak position of the BMDD histogram showing the most frequently occurring wt.% Ca of the measured areas. 3) CaWidth is the width at half-maximum of the BMDD histogram curve indicating the heterogeneity of mineralization and 4) CaLow is the percentage of bone area with a calcium concentration of less than

17.68 wt.% Ca, which reveals the amount of bone area undergoing primary mineralization; and CaHigh, the portion of bone area with a calcium concentration higher than 25.30 wt.% Ca.

Bone histomorphometry

After excision, femurs were routinely embedded in methylmetacrylate as described before.[2] Sections of 6 µm were subjected to tartrate-resistant acid phosphatase (TRAP) staining. Sections were deacrylated, hydrated and rinsed in 0.2 M sodium acetate/50 mM tartaric acid for 5 min. Naphtol AS-MX (0.5 mg/ml) and 1.1 mg/ml Fast red TR salt (both from Sigma, St. Louis, MO, USA) were added and incubated for 120 min at 37 °C. Counterstaining was performed with hematoxylin for 5 s and after air-drying, the sections were embedded in Permount (Thermo Fischer Scientific, Waltham, MA, USA). For osteoid measurements, a von Kossa staining was used. After incubation with 2% w/v silver nitrate (ICN Biomedicals, Irvine, CA, USA) for 5 min in daylight, the sections were counterstained with eosin. The sections were dehydrated and embedded in Entellan (Electron Microscopy Sciences, Hatfield, PA, USA). Eosin-stained osteoid was specifically visualized, using fluorescent imaging with a 365 nm excitation/420 nm emission filter. For osteoblast and osteocyte measurements, sections were stained with a Goldner staining as described before.[20] Images were taken from the TRAP and Goldner stainings with a Nikon Eclipse E400 system (Nikon, Lijnden, the Netherlands) and a Zeiss Axiovert 200 MOT system (Carl Zeiss BV, Jena, Germany) was used for osteoid stainings. Measurements were performed, using the software package Bioquant (Version 7.20; Bioquant image analysis corporation, Nashville, Tennessee, USA).

Quantitative PCR analysis (Q-PCR)

RNA isolation, cDNA syntheses, and Q-PCR were performed as described previously.[21] Primer and probe sequences and concentrations used for Q-PCR are listed in Supplementary Table 1.

Bone marrow cultures

Bone marrow cells derived from *TRPV4*$^{+/+}$ and *TRPV4*$^{-/-}$ mice directed towards osteoclasts and osteoblasts were cultured as described in detail.[2, 22] After 6 days of culture, TRAP and coomassie brilliant blue stainings were used to stain for osteoclasts and resorption pits on bone slices left behind by osteoclasts, respectively.[2] Osteoclast number and resorption surface were measured as well as resorption surface per osteoclast, using the freely available ImageJ software (version 1.41; http://rsbweb.nih.gov/ij/). Alkaline phosphatase and alizarin red staining were performed on osteoblast cultures at days 9 and 21 of culture, respectively, as described earlier.[2] Colony numbers and mineralized area were quantified using Bioquant.

Genetic association studies in humans

To evaluate the effect of genetic variants in *TRPV4* on bone outcomes we first focused on the Rotterdam Study where deep phenotyping on bone parameters is available followed by replication assessment of fracture outcomes in three additional studies.

Rotterdam Study

Individuals were derived from the Rotterdam Study (N=7,983), a single-center prospective population-based cohort study of determinants of disabling chronic diseases in the elderly. The Medical Ethics Committee of Erasmus University Medical School approved the Rotterdam Study, and participants provided written informed consent. Both the rationale and the design of the study have been extensively described previously.[23, 24] In brief, the Rotterdam Study was designed in the mid-1980s as a response to

Table 1. Fracture risks in the Rotterdam Study population for rs1861809.

	Men				Women			
Trait	CC	CT	TT	Trend	CC	CT	TT	Trend
Non-vertebral fractures								
No. fractures / total no. (%)	46/675 (6.8%)	113/1,176 (16.7%)	54/498 (20.0%)	0.013	207/951 (21.7%)	331/1,672 (19.8%)	152/703 (21.7%)	0.785
OR (95% CI)	1	1.5 (1.0–2.1)	1.7 (1.1–2.5)		1	0.9 (0.7–1.1)	1.0 (0.8–1.3)	
Osteoporotic fractures								
No. fractures / total no. (%)	41/675 (6.1%)	89/1,176 (7.6%)	53/498 (10.6%)	0.005	199/951 (20.9%)	319/1,672 (19.1%)	145/703 (20.7%)	0.983
OR (95% CI)	1	1.3 (0.9–1.9)	1.9 (1.2–2.8)		1	0.9 (0.7–1.1)	1.0 (0.8–1.3)	
Fragility fractures								
No. fractures / total no. (%)	15/675 (2.2%)	41/1,176 (3.5%)	26/498 (5.2%)	0.005	84/951 (8.8%)	130/1,672 (7.8%)	63/703 (9.0%)	0.968
OR (95% CI)	1	1.6 (0.9–3.0)	2.5 (1.3–4.8)		1	0.9 (0.6–1.2)	1.0 (0.7–1.5)	
Hip fractures								
No. fractures / total no. (%)	11/675 (1.6%)	27/1,176 (2.2%)	20/498 (4.0%)	0.011	52/951 (5.5%)	83/1,672 (5.0%)	39/703 (5.6%)	0.886
OR (95% CI)	1	1.4 (0.7–2.9)	2.6 (1.2–5.6)		1	0.9 (0.6–1.3)	1.1 (0.7–1.6)	
Wrist fractures								
No. fractures / total no. (%)	5/675 (0.7%)	10/1,176 (0.9%)	12/498 (2.4%)	0.014	62/951 (6.5%)	101/1,672 (6.0%)	47/703 (6.7%)	0.942
OR (95% CI)	1	1.2 (0.4–3.4)	3.3 (1.2–9.5)		1	0.9 (0.7–1.3)	1.0 (0.7–1.5)	

Data are presented as number of fractures as a percentage of total for each genotype, stratified by gender. Odds ratios (OR) are depicted with 95% confidence intervals (CI). All associations were adjusted for gender, age, height and weight. Trend values represent P value for an allele-dose effect.

the demographic changes that were leading to an increase of the proportion of elderly people in most populations. It was clear that this would produce a strong rise in elderly people living with diseases, as most diseases cluster at the end of life, and that to discover the causes of diseases in the elderly one would have to study the risk factors of those diseases. The design of the Rotterdam Study is that of a prospective cohort study among, initially, 7,983 persons living in the well-defined Ommoord district in the city of Rotterdam in The Netherlands (78% of 10,215 invitees). They were all 55 years of age or over and the oldest participant at the start was 106 years. The participants were all examined in some detail at baseline. They were interviewed at home and then had an extensive set of examinations in a specially built research facility in the center of their district. These examinations focused on possible causes of invalidating diseases in the elderly in a clinically state-of the-art manner, as far as the circumstances allowed. The emphasis was put on imaging (of heart, blood vessels, eyes, skeleton and later brain) and on collecting bodily fluids that enabled further in-depth molecular and genetic analyses.

Height and weight were measured in a standing position wearing indoor clothing without shoes. BMI was computed as weight in kilograms divided by height in meters squared (kg/m^2). During the home interview, female participants were asked to recall their age at menopause, and responses were validated as described previously.[25] Assessment of vertebral fracture, incident non-vertebral fractures, bone mineral density (BMD) and bone geometry measurements has been described in detail previously.[26] In short, fractures where derived from general practitioner records and validated by two trained physicians. BMD and hip structural analysis measurements were derived from DXA scans acquired with a GE_Lunar DPX-L scanner.

TRPV4 SNP genotyping

Markers present in the *TRPV4* gene region of interest (chromosome 12q24, positions 108,705,277 to 108,755,595) plus 50 kb up- and downstream of the gene (HapMap release 27, February 2009) were extracted from Illumina HumanHap 550 K beadchip arrays as described earlier[27] and included 32 haplotype tagging SNPs. These cover most of the common genetic variance in the *TRPV4* region spanning chromosome 12 positions 108,623,000 to 108,801,400 and including 78 markers. Markers were excluded if: 1) they deviated significantly from Hardy–Weinberg equilibrium ($P<1\times10^{-4}$; N=0), 2) the low minor allele frequency (MAF) was below 5% (N=2), or 3) they had a call rate <95% (N=3). The exclusion of 5 tagging markers due to low MAF or genotyping call rate did not substantially affect coverage in the region as only 3 additional markers would be missed. This resulted in 27 SNPs from the Illumina array in the *TRPV4* locus area available for gene-wide association analyses, using PLINK v1.05 (Supplementary Figure 1 and Supplementary Table 2). Genomic control was used to correct for potential population stratification using genome-wide data.[28] The genomic inflation factor (based on median chi-squared) ranged between 1.015 (non-vertebral fracture) and 1.049 (femoral neck BMD) across all bone trait analyses providing evidence against the presence of significant population stratification affecting the results.

Association analysis

Association of the rs1861809 SNP with bone mineral density (BMD; femoral neck and lumbar spine BMD) were analyzed. Furthermore, association with osteoporotic, non-vertebral, fragility, hip, wrist and vertebral fracture risk as well as hip structural parameters, including narrow neck (NN) width, NN cortical thickness (Ct.Th), NN cross-sectional moment of inertia (CSMI) and NN buckling ratio (BR) was assessed.

Replication cohorts

For replication, men and women from the prospective population-based cohort study LASA (Longitudinal Aging Study Amsterdam, N=904), the prospective study MrOS Sweden (N=2,829) and the nested case–control study UFO (Umeå Fracture and Osteoporosis, N=2,807) cohorts were genotyped for rs1861809, the most significantly associated marker in the Rotterdam Study using TaqMan Allele discrimination assay (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) and included in the analysis. The Longitudinal Aging Study Amsterdam (LASA) is an ongoing multidisciplinary cohort study in older persons. A random sample of men and women aged 55 years and over, stratified by age, sex, urbanization grade and expected 5-years mortality rate was drawn from the population register of Amsterdam, The Netherlands.[29] Follow-up time of the fractures was 6 years. The Osteoporotic Fractures in Men (MrOS) study is a multicenter, prospective study including elderly men. Study subjects (men aged 69–80 years) were randomly identified using national population registers, contacted and asked to participate. Eligible subjects had to be able to walk without assistance, provide self-reported data, and sign an informed consent. For this study data from the MrOS Sweden cohort was used.[30] Assessments of incident fractures have been described before.[31] The UFO study is a nested case–cohort study investigating associations between genes, lifestyle and osteoporotic fractures (average age 65 years of age). The study is based on the prospective and population-based Northern Sweden Health and Disease Study cohort, initiated to assess risk factors for diabetes and cardiovascular disease.[32, 33]

Statistical analyses

If not stated otherwise, SPSS 15.0 (SPSS, Chicago, IL, USA) was used for the statistical analyses. In all non-genetic experiments values were expressed as mean ± SEM unless stated otherwise. Differences between groups were tested for significance using the Student-t-test. Baseline parameters and bone geometric data from the genetic studies were expressed as mean ± SD. Differences between groups were tested for significance using ANOVA. Values were considered significantly different at $P<0.05$. To estimate the risk of fractures, odds ratios with 95% confidence intervals (95% CI) were calculated using logistic regression models. Trend analysis assuming an underlying additive genetic model was done for the presence of zero, one, or two copies of the associated allele.[34] Since we took only one SNP forward for the replication studies no multiple testing penalty was applied, hence P values <0.05 were considered statistically significant.

RESULTS

Bone phenotype of male and female TRPV4-/- mice

µCT analyses demonstrated a positive effect on bone mass following TRPV4 deficiency in male but not in female mice. Male *Trpv4*$^{-/-}$ mice displayed increased femoral trabecular (Figures 1A and B) and cortical (Figures 1C–E) bone mass compared to female *Trpv4*$^{+/+}$ mice. Femoral bone size was also increased in male *Trpv4*$^{-/-}$ mice as exemplified by larger femoral head volume, diaphyseal volume, perimeter (Figures 1F–H, respectively) and femoral length (Supplementary Table 3). In females, all parameters described above were unaffected. A summary of these and additional µCT parameters are listed in Supplementary Table 3.

We first studied osteoclast function in these *Trpv4*$^{-/-}$ mice. Urinary DPD analysis showed reduced bone resorption in male *Trpv4*$^{-/-}$ mice compared to *Trpv4*$^{+/+}$ (Figure 2A). Histomorphometrical analyses of TRAP staining on bone sections confirmed this (Figure 2B). In femurs from male *Trpv4*$^{-/-}$ mice, osteoclast

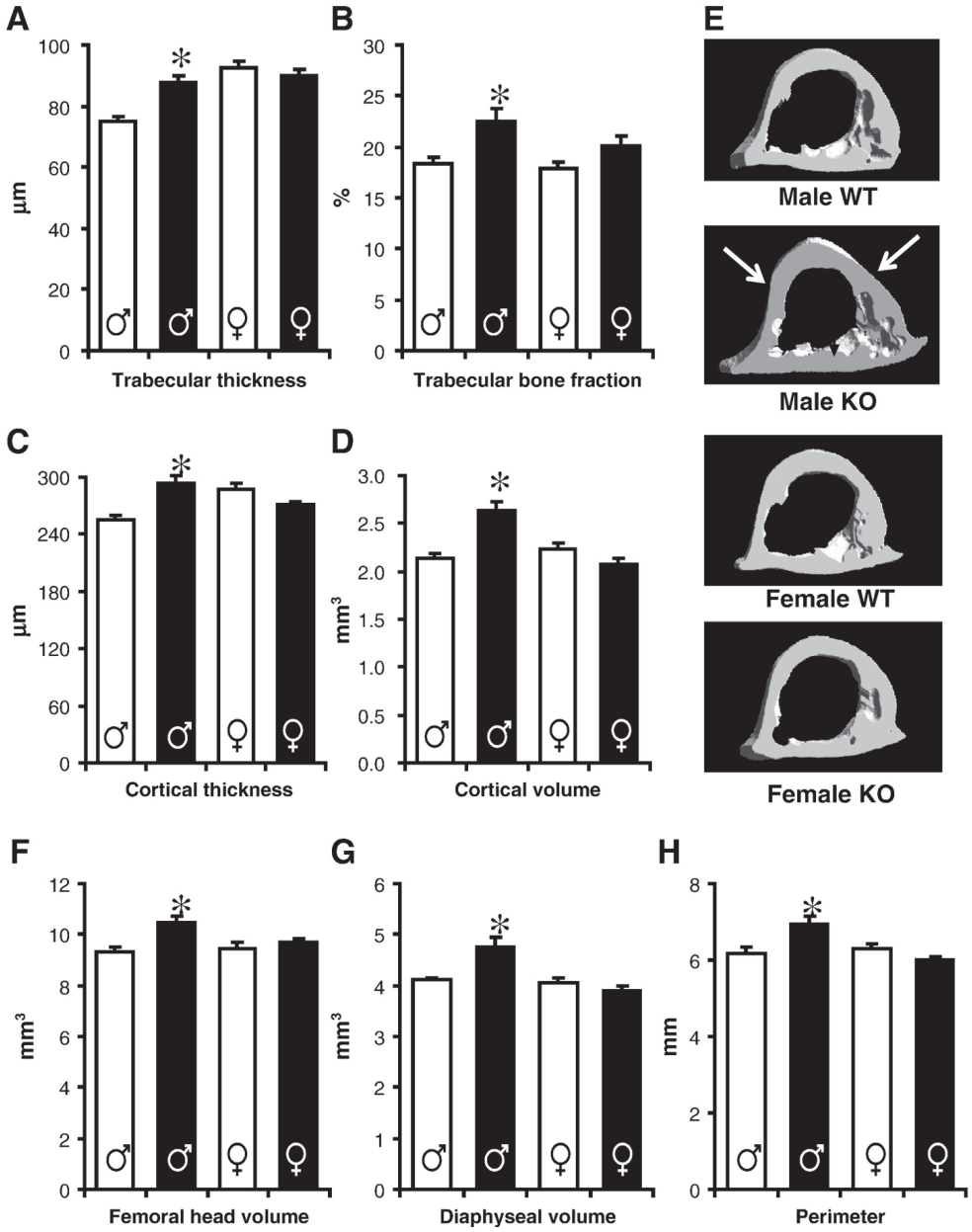

Figure 1. Bone microarchitecture in male and female *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. In the femoral head, (A) trabecular thickness, (B) trabecular bone volume fraction and (F) femoral head volume were determined. Cortical bone parameters included (C) cortical thickness, (D) cortical volume, (G) diaphyseal volume and (H) perimeter. (E) Representative 3D reconstructions for the mid-diaphyseal cortices for each group are shown (arrows indicate thicker cortices in male *Trpv4*$^{-/-}$ mice). White bars: *Trpv4*$^{+/+}$ mice; black bars: *Trpv4*$^{-/-}$ mice. Data are presented as means ± SEM. *P<0.05 versus male *Trpv4*$^{+/+}$ mice (N=6).

number (Figure 2C) and surface area resorbed (data not shown) was significantly reduced. In contrast, no differences in bone resorption and osteoclast number were observed between the female *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (Figure 2A and data not shown).

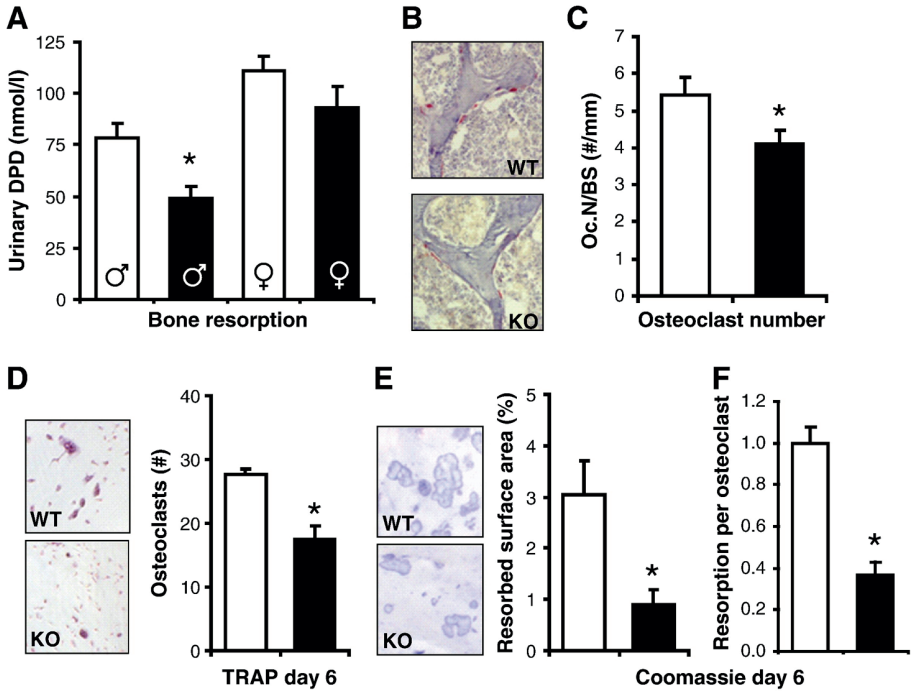

Figure 2. Osteoclast function in *Trpv4*$^{-/-}$ mice. (A) Urinary deoxypyridinoline (DPD) was measured in male and female *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. (B) Representative bone sections were stained for TRAP. (C) Osteoclast numbers corrected for total bone surface (Oc.N/BS) were quantified, using Bioquant software. In osteoclast cultures, (D) number of TRAP positive colonies and (E) coomassie brilliant blue-positive resorption area were quantified as well as (F) resorption surface per osteoclast (*Trpv4*$^{+/+}$ set to 1). White bars: *Trpv4*$^{+/+}$ mice; black bars: *Trpv4*$^{-/-}$ mice. *P<0.05 versus male *Trpv4*$^{+/+}$ mice (N=6).

Bone marrow-derived osteoclast cultures supported the in vivo observations that osteoclast number and resorption is disturbed in male *Trpv4*$^{-/-}$ but not female mice. Fewer osteoclasts developed from male *Trpv4*$^{-/-}$ bone marrow compared to that of *Trpv4*$^{+/+}$ mice (Figure 2D), which is paralleled by a significantly reduced resorption surface area (Figure 2E). Resorption surface per osteoclast analyses demonstrated that osteoclast activity from *Trpv4*$^{-/-}$ cultures is impaired (Figure 2F). None of these differences were found in female *Trpv4*$^{-/-}$ bone marrow-derived osteoclast cultures (e.g., osteoclast numbers: 35.1 ± 3.3 versus 29.6 ± 5.4, P=0.44 for female *Trpv4*$^{+/+}$ versus *Trpv4*$^{-/-}$ mice).

When osteoclast–osteoblast coupling during bone remodeling is intact, reduced osteoclast function should lead to attenuated osteoblast activity. In male *Trpv4*$^{-/-}$ mice, bone formation was unaffected despite reduced bone resorption as shown by serum P1NP analyses (Figure 3A). This is supported by histomorphometrical assessment of bone sections showing no differences in number of osteoblast lining trabecular bone (Figure 3B), percentage osteoid surface (Figure 3C) and osteocytes in cortices (Figure 3D) between male *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice.

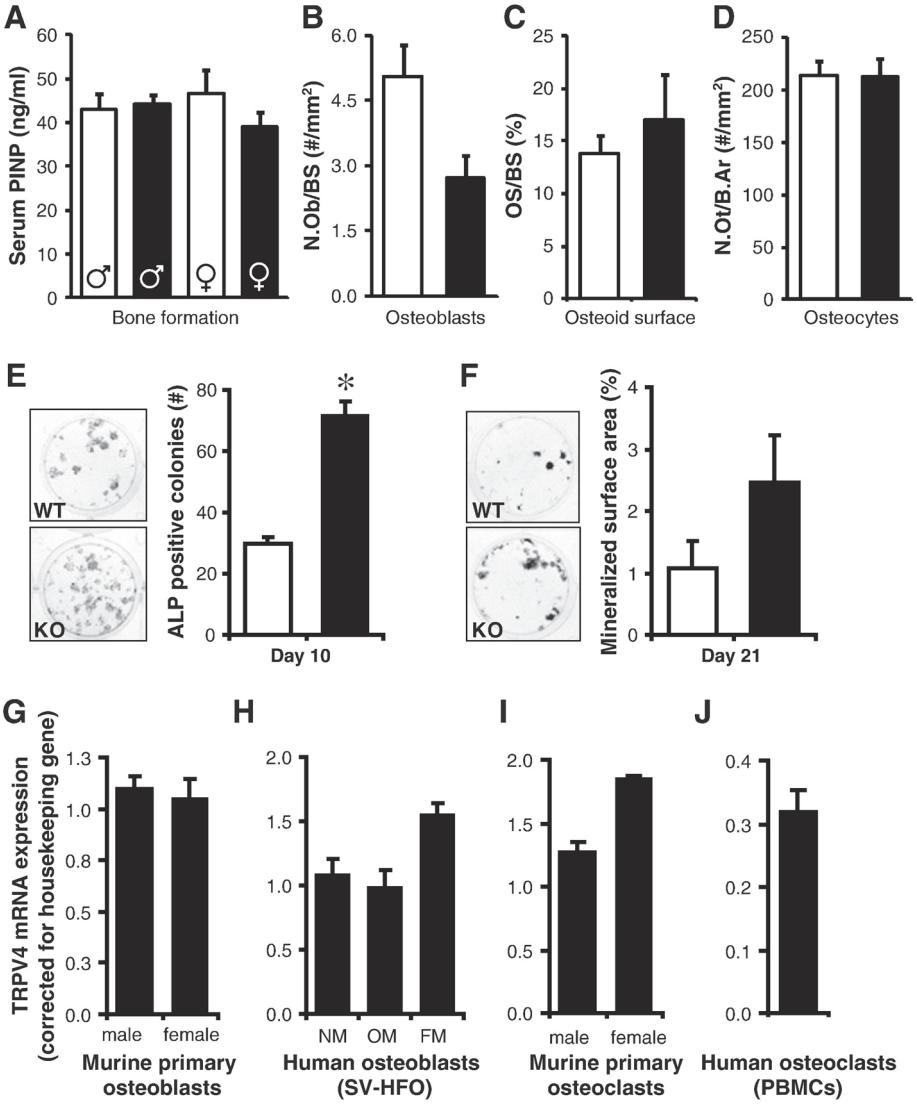

Figure 3. Osteoblast function in *Trpv4*$^{-/-}$ mice. Serum was collected for measurement of P1NP (A). Bone sections were stained for osteoid, using a von Kossa/eosin staining. (B) Osteoblast number along the bone surface (N.Ob/BS), (C) osteoid surface as a percentage of total bone surface (OS/BS)was quantified as well as (D) osteocytes numbers in cortical bone (N.Ot/B.Ar), using Bioquant software. Osteoblast cultures were stained for (E) number of alkaline phosphatase colonies and (F) alizarin red-positive mineralized surface area. White bars: *Trpv4*$^{+/+}$ mice; black bars: *Trpv4*$^{-/-}$ mice. (G) *TRPV4* gene expression in bone marrow-derived osteoblasts from male and female mice, (H) human osteoblast cell-line (SV-HFO), (I) bone marrow-derived osteoclasts from male and female mice (day 6) and (J) human peripheral blood mononuclear cells-derived osteoclasts (day 21). Total RNA was isolated and assessed for *TRPV4* mRNA expression. *P<0.05 versus male *Trpv4*$^{+/+}$ mice (N=5). Abbreviations: NM, no mineralization; OM, onset of mineralization; FM, full mineralization.

While the in vivo findings strongly suggest that osteoblast differentiation and function remain unaffected in *Trpv4*$^{-/-}$ mice, osteoblast differentiation was enhanced in bone marrow cultures. *Trpv4*$^{-/-}$ osteoblast cultures showed a significant increase in the number of alkaline phosphatase positive colonies (Figure 3E) as well as elevated Ca^{2+} deposition, although this did not reach significance (Figure 3F). Colony size was not affected in cultures from male Trpv4$^{-/-}$ mice (0.10 ± 0.02 mm versus 0.15 ± 0.02 mm, P=0.1 for male Trpv4$^{+/+}$ mice). TRPV4 may directly affect osteoblast and osteoclast function as it is abundantly expressed in both cell types (Figures 3G–J). No differences in alkaline phosphatase positive colony numbers and Ca^{2+} deposition were observed between osteoblast cultures from female *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice bone marrow (data not shown).

Resistance to stress and elastic modulus is reduced in male Trpv4–/– mice

To assess whether increased bone mass led to improved bone strength, 3-point bending tests were performed on femurs from male and female *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (Figures 4A–E). Maximum load, stiffness and work to failure were not different between *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (Figures 4A–C). Interestingly, the femurs from *Trpv4*$^{-/-}$ mice were less resistant to stress (Figure 4D) and less elastic (Figure 4E). However, polar moment of inertia was increased in the *Trpv4*$^{-/-}$ mice at the site of fracture (Figure 4F). None of these differences were seen in bones from female mice (e.g., stress: 87.6 ± 2.9 GPa versus 75.0 ± 3.4 GPa, P=0.31 for female *Trpv4*$^{+/+}$ versus *Trpv4*$^{-/-}$ mice).

We assessed cortical porosity by quantifying the holes appearing in the cortical bone (Figure 4G–H). Cortical porosity was more than doubled in the male *Trpv4*$^{-/-}$ mice compared to *Trpv4*$^{+/+}$ mice (Figure 4 and Supplementary Table 3). The diameter of these holes varied between 40 and 160 µm. Increased cortical porosity was not observed in the female mice (Supplementary Table 3). Bone mineral density of the femoral trabecular and cortical compartment was unaltered and slightly but significantly increased, respectively, in the *Trpv4*$^{-/-}$ mice (Figures 4J–K).

Finally, we measured bone mineralization density distribution at three positions in femurs of the male *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (Figure 5A). The bone matrix of *Trpv4*$^{-/-}$ mice was significantly lower mineralized compared to *Trpv4*$^{+/+}$ mice at all skeletal sites analyzed (metaphysis, epiphysis and corticalis) as shown by the significant reduction of CaMean, CaPeak and CaHigh (Figures 5B, C and D). The width of the BMDD curve (CaWidth) is not altered indicating that the heterogeneity in mineralization is not different between the genotypes (Figure 5E). The fraction of lowly mineralized bone areas (CaLow), i.e., areas of ongoing bone formation (primary mineralization), in the *Trpv4*$^{-/-}$ mice is not different from that in *Trpv4*$^{+/+}$ mice (Figure 5F).

Human genetic association studies on TRPV4 and fracture risk

We investigated the contribution of TRPV4 to bone phenotypes in humans by studying the association of genetic variants in the *TRPV4* gene locus with skeletal phenotypes and fracture risk. Baseline characteristics for the Rotterdam Study population are provided in Supplementary Table 4. Using PLINK software, we tested 27 tagging single nucleotide polymorphisms (SNPs) in the *TRPV4* locus for potential association with bone mineral density (BMD), hip geometry and fracture risk in the Rotterdam Study (Supplementary Table 2 and Supplementary Figure 1) as a discovery cohort. Two intronic tagging SNPs located between exons 2 and 3 of the *TRPV4* gene (rs10850783, C to A, MAF=27.4% and rs1861809, C to T, 27.3%; Supplementary Table 2 and Supplementary Figure 1) were found to be associated with osteoporotic fractures (P=0.002). These SNPs were in complete linkage disequilibrium and so rs1861809 was chosen for further analyses. No association was observed for rs1861809 with femoral neck and lumbar

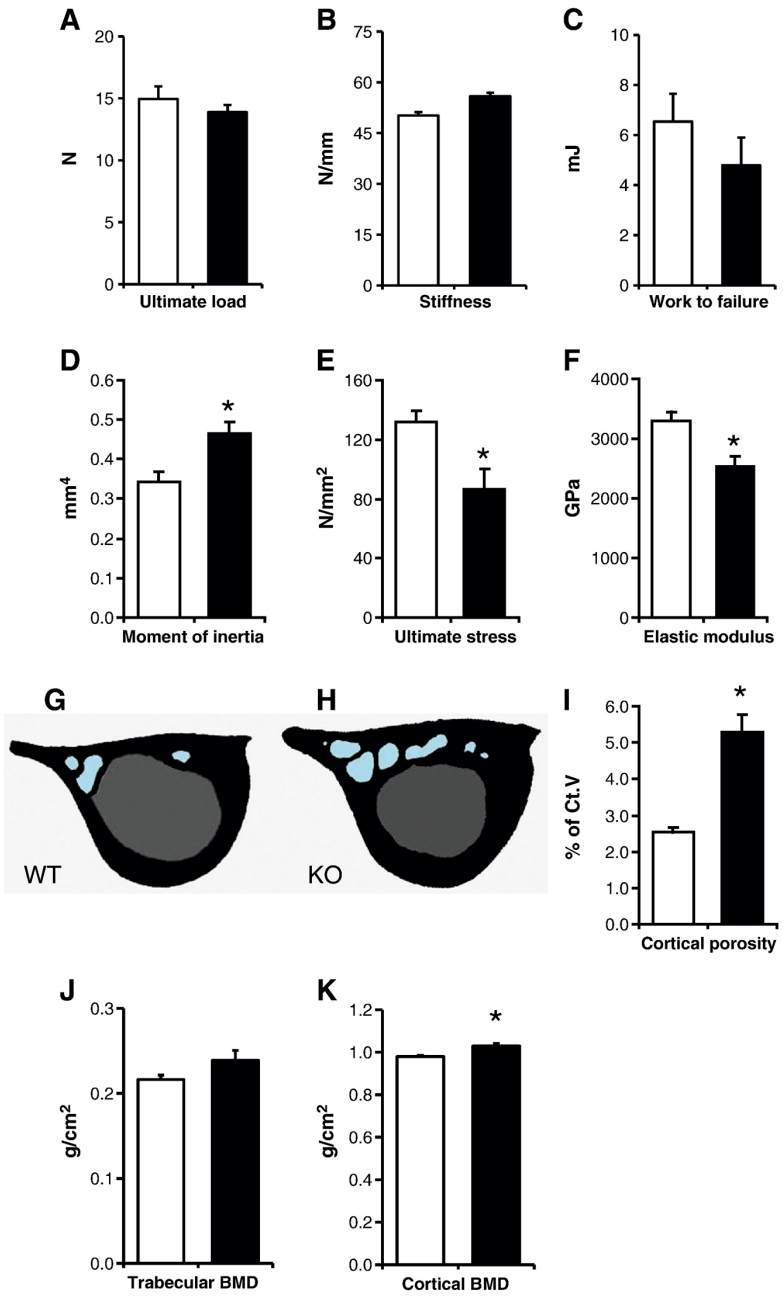

Figure 4. Bone material strength is reduced in mice lacking *Trpv4*. Three-point bending tests were performed in *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice to assess femoral mechanical properties. Besides (A) maximum load, (B) stiffness, (C) work to failure, (D) elastic modulus and (E) ultimate stress were analyzed from the displacement–force curves and (F) moment of inertia was determined from the µCT analyses. Representative images from binarized µCT cross-sections through cortices from male (G) *Trpv4*$^{+/+}$ (WT) and (H) *Trpv4*$^{-/-}$ mice (KO). (I) The percentage volume of the cortices representing holes (white) was quantified as a proxy for cortical porosity. (J) Trabecular and (K) cortical bone mineral density (BMD). White bars: *Trpv4*$^{+/+}$ mice; black bars: *Trpv4*$^{-/-}$ mice. *P<0.05 versus male *Trpv4*$^{+/+}$ mice (N=5).

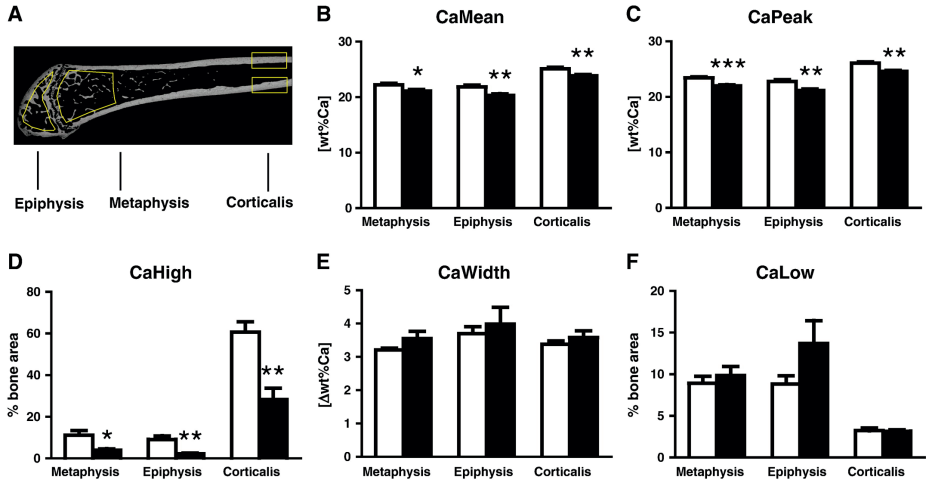

Figure 5. Bone matrix mineralization is reduced in mice lacking Trpv4. Using qBEI, BMDD was measured at 3 locations in femurs from male *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (A). CaPEAK (B), CaMEAN (C), CaHIGH (D), CaWIDTH (E) and CaLOW (F) were measured in the trabecular compartment of the femoral metaphysis and epiphysis as well as in the diaphyseal cortex (corticalis). Data are presented as means ± SEM. *P<0.05 versus *Trpv4*$^{+/+}$ controls (N=6).

spine BMD in either men or women (Supplementary Table 5). The hip bone geometric parameters' narrow neck (NN) width and NN cross-sectional moment of inertia (CSMI) were significantly higher in men with the TT genotype (P=0.006 and P=0.02, respectively), but this effect was not observed in women (P=0.2 and 0.14, respectively; Supplementary Table 5). NN cortical thickness (Ct.Th) and buckling ratio (BR) were not significantly different between the genotypes in either men or women (Supplementary Table 5).

Risk of osteoporotic fractures was 1.9 times higher in men homozygous for the T-allele of rs1861809 (Table 1). Men had a 40% increased risk for osteoporotic fractures per T-risk allele (95% CI: 1.1–1.7, P=0.005). For fragility and hip fractures the risk was 1.6 times (95% CI: 1.1–2.2, P=0.005; and 1.1–2.4, P=0.011, respectively) higher per risk allele, while for wrist fractures the risk was 2 times (95% CI: 1.1–3.5, P=0.014) higher per risk allele. In contrast to men, no association with any type of fracture was observed for women (Table 1).

Next, we sought replication of our genetic associations in other cohorts, including LASA, MrOS and UFO (baseline characteristics in Supplementary Tables 6 and 7). In men from the LASA study the same trend for increased risk for osteoporotic fracture was observed in carriers of the T-allele (OR=1.5, 95% CI: 0.9–2.5, P=0.11) (Supplementary Table 8). Meta-analyses of the Rotterdam and LASA studies together were consistent with a 40% increase in risk for osteoporotic fracture (OR=1.4, 95% CI: 1.1–1.7, P=0.001) per risk allele (Figure 6). However, in the Swedish MrOS and UFO studies, no evidence for association with osteoporotic fracture risk was observed in either study. (Supplementary Table 8 and Figure 6). In the meta-analysis, including all four cohorts, the association of the polymorphism with osteoporotic fracture was lost for men (OR=1.1, 95% CI: 1.0–1.3, P=0.167; Figure 6). As expected, for women the association with osteoporotic fractures remained absent (OR=1.0, 95% CI: 0.9–1.0, P=0.303; Figure 6) nor were any signification associations found for other types of fractures (data not shown).

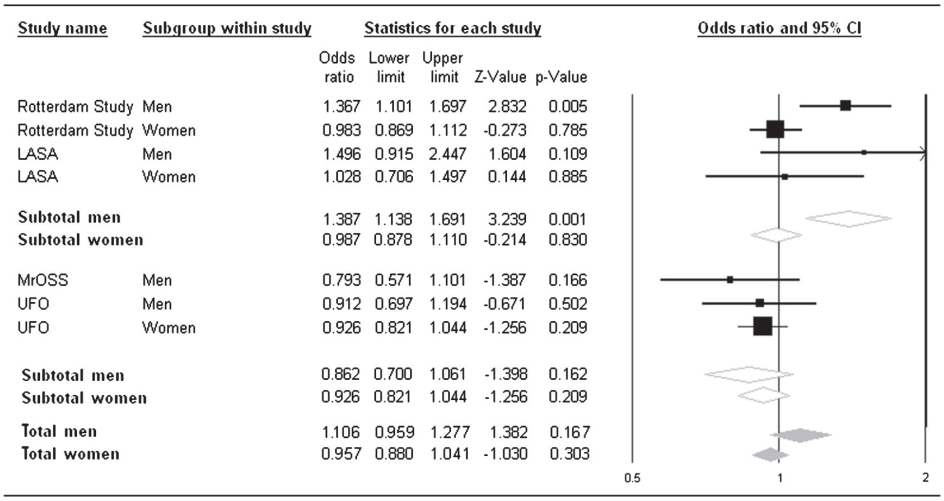

Figure 6. Meta-analysis for osteoporotic fracture risk. Forest plot of the group-wise genotype meta-analysis for osteoporotic fracture risk in men across studies for rs1861809. Provided are the odds ratios for TT versus CC genotype in males and females from the Rotterdam Study, LASA, MrOS Sweden and UFO. All associations were adjusted for age, height and weight.

DISCUSSION

In this multidisciplinary study, using *Trpv4*$^{-/-}$ mice, ex vivo cell biological analyses and genetic association data from human cohorts, we demonstrate that TRPV4 is an important sexually dimorphic factor for determining bone strength, with potentially clinically relevant implications at the population level.

Male *Trpv4*$^{-/-}$ mice display reduced osteoclast function and osteoclast–osteoblast uncoupling

TRPV4 deficiency leads to an increased bone mass phenotype in male, but not in female mice. This predominantly results from decreased osteoclast formation/differentiation and activity, which has.[11,12,35] Considering the importance of osteoblast–osteoclast coupling in bone turnover it was anticipated that impaired osteoclast differentiation would lead to reduced osteoblast differentiation. However, bone formation is not affected in the male *Trpv4*$^{-/-}$ mice in this study, although it is enhanced in ex vivo cultures. This implies that TRPV4 acts indirectly in vivo, through mesenchymal stem cells (MSCs) and limits their differentiation into osteoblasts. *TRPV4* is also abundantly expressed on osteoblasts, which suggests a direct effect; the mechanism through which TRPV4 acts on osteoblasts remains elusive. In contrast to our data, it was demonstrated very recently that bone marrow-derived MSCs from *Trpv4*$^{-/-}$ mice actually were less osteogenic compared to wild type MSCs, whereas the opposite was seen for adipose tissue-derived MSCs.[36] However, these findings are difficult to directly correlate with our data, due to the methodological differences in cell collection (cell sorting), additional passaging of the cells before osteogenic differentiation and culturing under hypoxic conditions, which has been shown to have profound effects on osteogenic differentiation.[37] The reduced bone resorption (DPD as a marker) together with the unchanged bone formation marker (P1NP) demonstrates osteoblast–osteoclast uncoupling following TPRV4 deficiency in male mice only. A summary of the results and a more detailed reasoning

for this conclusion is shown in panels 1–6 in Figure 7. Based on the current data we propose that lack of TPRV4 in male mice, but not female mice, enhances osteoblast development via an osteoblast-intrinsic mechanism (observed: in vitro enhanced osteoblast development; panel 5) that (partially) overrules the in vivo osteoclast–osteoblast coupling signal (observed: in vivo unchanged bone formation; panels 4 and 6) that would have led to reduced osteoblast activity as a consequence of the reduced osteoclast activity and bone resorption (observed: in vivo and in vitro; panels 2 and 6). Overall, these analyses demonstrate a clear sex-specific effect of TRPV4 on bone phenotype, which is due to impaired osteoclast function and disturbed coupling between osteoclasts and osteoblasts.

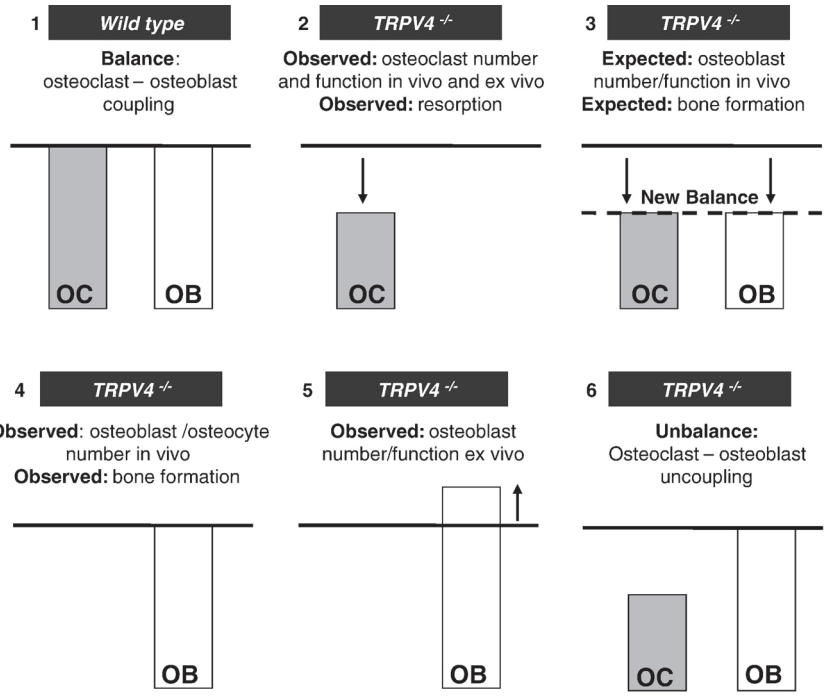

Figure 7. Osteoclast–osteoblast uncoupling in male *Trpv4*$^{-/-}$ mice. Trpv4$^{+/+}$ bone function is characterized by a balance between osteoclastic (OC) bone resorption and osteoblastic (OB) bone formation (panel 1). In male *Trpv4*$^{-/-}$ mice, osteoclast differentiation and function is reduced in vivo and ex vivo (panel 2). In healthy bone metabolism, bone formation is reduced to achieve a new balance (panel 3). However, in the male *Trpv4*$^{-/-}$ mice, bone formation was unaffected (panel 4) and osteoblast differentiation was even enhanced ex vivo (panel 5). Together, these data clearly indicate that uncoupling between bone resorption and formation exists (panel 6), leading to the increased bone mass phenotype observed in male *Trpv4*$^{-/-}$ mice.

Male *Trpv4*$^{-/-}$ mice have increased cortical porosity and reduced matrix mineralization

Despite reduced osteoclast activity, male *Trpv4*$^{-/-}$ mice have increased cortical porosity, an important predictor in diagnosing osteoporosis.[38] In fact, non-vertebral fractures at predominantly cortical sites account for 80% of all fracture age-related osteoporosis.[39] In several mouse models where osteoclast function is increased, such as one where the PTH receptor is constitutively active, or one that overexpresses cathepsin K, intracortical porosity is abundant.[40, 41] In addition, mice lacking the gastrin receptor Cckbr associated with hypochlorhydria also suffer from increased cortical porosity due to low intestinal

Ca^{2+} absorption and secondary hyperparathyroidism.[42] However, cortical porosity may well arise from insufficient bone remodeling during bone development. For bone remodeling, osteoclast activity is required, which is defective in the male *Trpv4*$^{-/-}$ mice. An alternative, intriguing explanation is the process of osteocytic osteolysis, a mechanism by which osteocytes are able to resorb their surrounding perilacunar extracellular matrix, which may lead to intracortical porosity (Reviewed in:[43, 44]). Although osteocyte density was not altered in femoral cortices of our male *Trpv4*$^{-/-}$ mice (Figure 3D) the presence of osteocytic osteolysis remains to be established. However, to achieve this yet robust means to quantitatively assess osteocytic osteolysis need to be developed.

The reduction in matrix mineralization in *Trpv4*$^{-/-}$ mice demonstrates a role for this ion channel in mineralization of bone. The maintenance of cortical bone strength observed in *Trpv4*$^{-/-}$ male mice despite an increased cortical porosity and the observed reduced bone matrix mineralization can be best explained by a compensatory effect of the increase in bone mass, as reflected by an enhanced moment of inertia. In general, reduced bone matrix mineralization causes a lower ultimate stress and elastic modulus and increased intracortical porosity weakens additionally the mechanical competence of whole cortical bone.[45, 46] Interestingly, BMD was only slightly increased in these mice. However, taken into account, that a change in BMD has to be considered as the sum of changes in bone volume and bone matrix mineralization,[47] it seems that the extent of the increase in bone volume fairly compensated for the increase in porosity and the reduction in bone matrix mineralization in these *Trpv4*$^{-/-}$ mice.

The T-allele of rs1861809 is associated with increased fracture risk in men

In the Rotterdam Study we observed that the rs1861809 polymorphism between exons 2 and 3 of *TRPV4* harbors BMD-independent sex-specific effects on osteoporotic fracture risk. Our study and others claim that it is crucial to study men and women independently to determine sex-specific genetic factors that contribute to osteoporosis risk.[48] Similarly, it has been proposed that including bone structural parameters can aid the assessment of fracture risk.[49-51] Nevertheless, performing sex-stratified analysis is a limiting approach resulting in a lower power setting.

Male-specific skeletal findings arising from *Trpv4*$^{-/-}$ mice and the ex vivo cell biology analyses were in line with those observed in men from the Rotterdam Study. In addition, including the LASA study (also of Dutch ancestry) resulted in a consistent, albeit smaller, effect estimate, which is most probably a natural consequence of smaller sample size and less power. The association in two additional studies of Swedish origin and lower number of fracture cases did not follow the same trend observed in the studies of Dutch origin. We do not foresee population-specific effects as allele frequencies of the Rotterdam and LASA studies were similar to those observed in the MrOS Sweden cohort. We did observe different allele frequencies in the UFO cohort as compared to the prospective nature of the other three cohorts, which may be attributable to its case/cohort design. Indeed, having a set of controls enriched with osteoporotic subjects may be an explanation for the observed deviant allele frequencies and also a potential explanation for the lack of association in the UFO cohort. We did not see an effect on BMD but a lower BMD may potentially reflect an enriched set of controls with fracture. Even though the male-specific effect of variants in *TRPV4* in relation to fracture was observed in populations of Dutch origin, further scrutiny of these associations in additional prospective cohorts is warranted to confirm potential translation of the effects seen in mice to men.

TRPV4 has been shown to respond to alterations in osmolarity as well as to hypotonicity (reviewed in:[52]). Of interest, we previously demonstrated in the Rotterdam Study that patients with hyponatremia had a 40% increased risk of getting a non-vertebral fracture.[53] In concordance, others showed a role

for hyponatremia in osteoporosis, most likely through activation of osteoclasts.[54, 55] Future work should point out whether TRPV4 may be a gateway between hyponatremia and fracture risk in the elderly.

Phenotype consistencies and inconsistencies across species

The consistency between the genetic association data in the Rotterdam and LASA Studies and the findings in the *Trpv4*$^{-/-}$ mice – that bone strength is affected in males but not in females – is striking. An explanation for increased fracture risk in men despite an increase in bone mass may reside in reduced bone matrix mineralization and/or increased cortical porosity, as was shown in the male mice. Although there are studies describing the assessment of cortical porosity in the distal radius,[38] we currently have no bone matrix mineralization and cortical porosity data from the Rotterdam Study to corroborate this hypothesis at the population level. Interestingly, increased midpubertal cortical thickness is associated with an increase in forearm fractures but also with elevated cortical porosity,[56] which is more pronounced in boys than in girls.[57] These processes evolve from endocortical bone resorption, most likely due to excess Ca^{2+} requirements in the growing adolescent.[58, 59] It is tempting to speculate that this transient 'weakness' of the long bones, in combination with DNA variations in the *TRPV4* locus may lead to more permanent alterations in bone structure and/or composition resulting in an increased fracture risk for elderly men. There is recent supporting data that reduction in bone matrix mineralization can contribute to the increased fracture risk in men.[60, 61]

Childhood fractures may actually better reflect the porosity phenotype we observe in the male *Trpv4*$^{-/-}$ mice, being bone growth related rather than bone loss related, which occurs in the human aging cohorts that we assessed in this study. Although fracture incidence seems to show a bimodal pattern with an increased fracture incidence during puberty, the incidence is still very low compared to that of the elderly population and studies will lack power. With recently initiated population studies such as the Generation R cohort focusing on children from birth to adulthood,[62] we may be able to study childhood fractures in the forthcoming years.

Dominant mutations in the *TRPV4* gene lead to a comprehensive family of bone dysplasia's, ranging from lethal metatotropic dysplasia to familial arthropathy with brachydactyly.[63] The range and severity of the skeletal conditions together with the knowledge that a single mutation in the *TRPV4* gene leads to different dysplasias, suggests modulation by other parts of the genome. Despite the human mutations, which so far are all dominant and activating of nature, ablation of the whole gene in murine studies (thereby inactivating the gene) displays a surprisingly

mild skeletal phenotype ([11] and our data). Potentially, activating *TRPV4* function by introducing the mutations leading to the various human bone dysplasias in a murine setting will phenocopy what we see in man but this requires extensive mouse genetic approaches.

TRPV4 deficiency is sexually dimorphic

The current study demonstrates a role for TRPV4 in explaining sexual dimorphism in bone metabolism and maintenance of bone strength. The underlying mechanism is unclear and currently purely speculative but other examples of a gender-specific bone phenotype have been described, for example in myeloid-specifically ablated leptin receptor knockout mice.[64] A role for sex steroid hormones such as androgens or estrogens seems logical but there is no data to support an interaction with TRPV4. It has been reported that *TRPV4* is expressed in the testes of male rats[6, 65] but a relation with sex steroid production has not been shown. Although we cannot fully explain the current sex-specific findings in the *Trpv4*$^{-/-}$ animals we did find an induction of *TRPV4* mRNA expression by 17β-estradiol in cultured osteoblasts

from male, but not female mice, suggesting a difference in sensitivity to sex steroids between males and females. It is worth mentioning that although sex was not mentioned in the majority of the reports describing a phenotype in *Trpv4*$^{-/-}$ mice, the ones that did, actually used male mice in their studies.[11, 66, 67] Of interest, in a recent review it was stated that sex differences also occur in the absence of hormonal changes through sex chromosome-mediated epigenetic regulation of autosomal chromosomes, such as DNA methylation and histone modifications[68].

CONCLUSION

In conclusion, TRPV4 is a male-specific determinant of bone strength. TRPV4 influences bone by uncoupling of osteoclast and osteoblast activity and increase in bone mass in a sexually dimorphic manner. In addition, TRPV4 plays a role in bone matrix mineralization, which is reduced, and together with enhanced cortical porosity, may lead to reduced elasticity of bone. The increased bone mass and moment of inertia observed in the male *Trpv4*$^{-/-}$ mice seem to preserve bone strength, but this compensation mechanism may be lost during aging, potentially leading to reduced bone strength and fracture risk. Finally, the human genetic association analyses, which support a role of TRPV4 in male but not female osteoporosis, need to be replicated and verified. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2013.09.017.

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Rotterdam Study

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LASA

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MrOS

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UFO

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Supplementary information is available at:

http://www.sciencedirect.com/science/article/pii/S8756328213003712#gr8

http://www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S8756328213003712/1-s2.0-S8756328213003712-mmc1.docx/271131/html/S8756328213003712/f5ac9af6f648e6e-484eee847f4109283/mmc1.docx

REFERENCES

1. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, et al. Renal Ca2+ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. J Clin Invest. 2003 Dec;112(12):1906-14.
2. van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, et al. The epithelial Ca2+ channel TRPV5 is essential for proper osteoclastic bone resorption. Proc Natl Acad Sci U S A. 2005 Nov 29;102(48):17507-12.
3. Bianco SD, Peng JB, Takanaga H, Suzuki Y, Crescenzi A, Kos CH, et al. Marked disturbance of calcium homeostasis in mice with targeted disruption of the trpv6 calcium channel gene. JBone MinerRes. 2007;22(2):274-85.
4. van der Eerden BC, Weissgerber P, Fratzl-Zelman N, Olausson J, Hoenderop JG, Schreuders-Koedam M, et al. The transient receptor potential channel TRPV6 is dynamically expressed in bone cells but is not crucial for bone mineralization in mice. J Cell Physiol. 2011 Jul 5;227(5):1951-9.
5. Lieben L, Benn BS, Ajibade D, Stockmans I, Moermans K, Hediger MA, et al. Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis. Bone. 2010 Aug;47(2):301-8.
6. Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. NatCell Biol. 2000;2(10):695-702.
7. Nilius B. TRP channels in disease. BiochimBiophysActa. 2007;1772(8):805-12.
8. Liedtke W. Transient receptor potential vanilloid channels functioning in transduction of osmotic stimuli. JEndocrinol. 2006;191(3):515-23.
9. Rock MJ, Prenen J, Funari VA, Funari TL, Merriman B, Nelson SF, et al. Gain-of-function mutations in TRPV4 cause autosomal dominant brachyolmia. NatGenet. 2008;40(8):999-1003.
10. Bellido T, Huening M, Raval-Pandya M, Manolagas SC, Christakos S. Calbindin-D28k is expressed in osteoblastic cells and suppresses their apoptosis by inhibiting caspase-3 activity. J Biol Chem. 2000 Aug 25;275(34):26328-32.

11. Masuyama R, Vriens J, Voets T, Karashima Y, Owsianik G, Vennekens R, et al. TRPV4-mediated calcium influx regulates terminal differentiation of osteoclasts. Cell Metab. 2008;8(3):257-65.
12. Mizoguchi F, Mizuno A, Hayata T, Nakashima K, Heller S, Ushida T, et al. Transient receptor potential vanilloid 4 deficiency suppresses unloading-induced bone loss. JCell Physiol. 2008;216(1):47-53.
13. Callewaert F, Venken K, Kopchick JJ, Torcasio A, van Lenthe GH, Boonen S, et al. Sexual dimorphism in cortical bone size and strength but not density is determined by independent and time-specific actions of sex steroids and IGF-1: evidence from pubertal mouse models. J Bone Miner Res. 2010 Mar;25(3):617-26.
14. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. J Biol Chem. 2003 Jun 20;278(25):22664-8.
15. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J Bone Miner Res. 2010 Jul;25(7):1468-86.
16. Westbroek I, Waarsing JH, van Leeuwen JP, Waldum H, Reseland JE, Weinans H, et al. Long-term fluoxetine administration does not result in major changes in bone architecture and strength in growing rats. J Cell Biochem. 2007 May 15;101(2):360-8.
17. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone. 1993 Jul-Aug;14(4):595-608.
18. Roschger P, Paschalis EP, Fratzl P, Klaushofer K. Bone mineralization density distribution in health and disease. Bone. 2008 Mar;42(3):456-66.
19. Roschger P, Fratzl P, Eschberger J, Klaushofer K. Validation of quantitative backscattered electron imaging for the measurement of mineral density distribution in human bone biopsies. Bone. 1998 Oct;23(4):319-26.
20. Gruber HE. Adaptations of Goldner's Masson trichrome stain for the study of undecalcified plastic embedded bone. Biotech Histochem. 1992 Jan;67(1):30-4.
21. Eijken M, Hewison M, Cooper MS, de Jong FH, Chiba H, Stewart PM, et al. 11beta-Hydroxysteroid dehydrogenase expression and glucocorticoid synthesis are directed by a molecular switch during osteoblast differentiation. Mol Endocrinol. 2005 Mar;19(3):621-31.
22. de Vries TJ, Schoenmaker T, Beertsen W, van der Neut R, Everts V. Effect of CD44 deficiency on in vitro and in vivo osteoclast formation. J Cell Biochem. 2005 Apr 1;94(5):954-66.
23. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. EurJEpidemiol. 1991;7(4):403-22.
24. Hofman A, van Duijn CM, Franco OH, Ikram MA, Janssen HL, Klaver CC, et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol. 2011 Aug;26(8):657-86.
25. Weel AE, Uitterlinden AG, Westendorp IC, Burger H, Schuit SC, Hofman A, et al. Estrogen receptor polymorphism predicts the onset of natural and surgical menopause. J Clin Endocrinol Metab. 1999 Sep;84(9):3146-50.
26. Rivadeneira F, van Meurs JB, Kant J, Zillikens MC, Stolk L, Beck TJ, et al. Estrogen receptor beta (ESR2) polymorphisms in interaction with estrogen receptor alpha (ESR1) and insulin-like growth factor I (IGF1) variants influence the risk of fracture in postmenopausal women. J Bone Miner Res. 2006 Sep;21(9):1443-56.
27. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
28. Devlin B, Roeder K. Genomic control for association studies. Biometrics. 1999 Dec;55(4):997-1004.
29. Huisman M, Poppelaars J, van der Horst M, Beekman AT, Brug J, van Tilburg TG, et al. Cohort profile: the Longitudinal Aging Study Amsterdam. Int J Epidemiol. 2011 Aug;40(4):868-76.
30. Mellstrom D, Johnell O, Ljunggren O, Eriksson AL, Lorentzon M, Mallmin H, et al. Free testosterone is an independent predictor of BMD and prevalent fractures in elderly men: MrOS Sweden. J Bone Miner Res. 2006 Apr;21(4):529-35.
31. Mellstrom D, Vandenput L, Mallmin H, Holmberg AH, Lorentzon M, Oden A, et al. Older men with low serum estradiol and high serum SHBG have an increased risk of fractures. J Bone Miner Res. 2008 Oct;23(10):1552-60.
32. Hallmans G, Agren A, Johansson G, Johansson A, Stegmayr B, Jansson JH, et al. Cardiovascular disease and diabetes in the Northern Sweden Health and Disease Study Cohort - evaluation of risk factors and their interactions. Scand J Public Health Suppl. 2003;61:18-24.

33. Englund U, Nordstrom P, Nilsson J, Bucht G, Bjornstig U, Hallmans G, et al. Physical activity in middle-aged women and hip fracture risk: the UFO study. Osteoporos Int. 2011 Feb;22(2):499-505.
34. Rivadeneira F, Houwing-Duistermaat JJ, Beck TJ, Janssen JA, Hofman A, Pols HA, et al. The influence of an insulin-like growth factor I gene promoter polymorphism on hip bone geometry and the risk of nonvertebral fracture in the elderly: the Rotterdam Study. J Bone Miner Res. 2004 Aug;19(8):1280-90.
35. Masuyama R, Mizuno A, Komori H, Kajiya H, Uekawa A, Kitaura H, et al. Calcium/calmodulin-signaling supports TRPV4 activation in osteoclasts and regulates bone mass. J Bone Miner Res. 2012 Aug;27(8):1708-21.
36. O'Conor CJ, Griffin TM, Liedtke W, Guilak F. Increased susceptibility of Trpv4-deficient mice to obesity and obesity-induced osteoarthritis with very high-fat diet. Ann Rheum Dis. 2013 Feb;72(2):300-4.
37. Nicolaije C, Koedam M, van Leeuwen JP. Decreased oxygen tension lowers reactive oxygen species and apoptosis and inhibits osteoblast matrix mineralization through changes in early osteoblast differentiation. J Cell Physiol. 2012 Apr;227(4):1309-18.
38. Zebaze RM, Ghasem-Zadeh A, Bohte A, Iuliano-Burns S, Mirams M, Price RI, et al. Intracortical remodelling and porosity in the distal radius and post-mortem femurs of women: a cross-sectional study. Lancet. 2010 May 15;375(9727):1729-36.
39. Kanis JA, Johnell O, Oden A, Dawson A, De Laet C, Jonsson B. Ten year probabilities of osteoporotic fractures according to BMD and diagnostic thresholds. Osteoporos Int. 2001 Dec;12(12):989-95.
40. Ohishi M, Chiusaroli R, Ominsky M, Asuncion F, Thomas C, Khatri R, et al. Osteoprotegerin abrogated cortical porosity and bone marrow fibrosis in a mouse model of constitutive activation of the PTH/PTHrP receptor. AmJPathol. 2009;174(6):2160-71.
41. Morko J, Kiviranta R, Hurme S, Rantakokko J, Vuorio E. Differential turnover of cortical and trabecular bone in transgenic mice overexpressing cathepsin K. Bone. 2005;36(5):854-65.
42. Schinke T, Schilling AF, Baranowsky A, Seitz S, Marshall RP, Linn T, et al. Impaired gastric acidification negatively affects calcium homeostasis and bone mass. Nat Med. 2009 Jun;15(6):674-81.
43. Teti A, Zallone A. Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited. Bone. 2009 Jan;44(1):11-6.
44. Bonewald LF. The amazing osteocyte. J Bone Miner Res. 2011 Feb;26(2):229-38.
45. Seeman E. Bone quality: the material and structural basis of bone strength. J Bone Miner Metab. 2008;26(1):1-8.
46. Fratzl P, Gupta HS, Paschalis EP, Roschger P. Structure and mechanical quality of the collagen-mineral nano-composite in bone. Journal of Materials Chemistry. [10.1039/B402005G]. 2004;14(14):2115-23.
47. Fratzl P, Roschger P, Fratzl-Zelman N, Paschalis EP, Phipps R, Klaushofer K. Evidence that treatment with rise-dronate in women with postmenopausal osteoporosis affects bone mineralization and bone volume. Calcif Tissue Int. 2007 Aug;81(2):73-80.
48. Karasik D, Ferrari SL. Contribution of gender-specific genetic factors to osteoporosis risk. AnnHumGenet. 2008;72(Pt 5):696-714.
49. Deng HW, Mahaney MC, Williams JT, Li J, Conway T, Davies KM, et al. Relevance of the genes for bone mass variation to susceptibility to osteoporotic fractures and its implications to gene search for complex human diseases. GenetEpidemiol. 2002;22(1):12-25.
50. Kanis JA, Oden A, Johnell O, De Laet C, Jonsson B. Excess mortality after hospitalisation for vertebral fracture. OsteoporosInt. 2004;15(2):108-12.
51. van Meurs JB, Schuit SC, Weel AE, van der KM, Bergink AP, Arp PP, et al. Association of 5' estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. HumMolGenet. 2003;12(14):1745-54.
52. Cohen DM. The transient receptor potential vanilloid-responsive 1 and 4 cation channels: role in neuronal osmosensing and renal physiology. Curr Opin Nephrol Hypertens. 2007 Sep;16(5):451-8.
53. Hoorn EJ, Rivadeneira F, van Meurs JB, Ziere G, Stricker BH, Hofman A, et al. Mild hyponatremia as a risk factor for fractures: the Rotterdam Study. J Bone Miner Res. 2011 Aug;26(8):1822-8.
54. Verbalis JG, Barsony J, Sugimura Y, Tian Y, Adams DJ, Carter EA, et al. Hyponatremia-induced osteoporosis. J Bone Miner Res. 2010 Mar;25(3):554-63.

55. Barsony J, Sugimura Y, Verbalis JG. Osteoclast response to low extracellular sodium and the mechanism of hyponatremia-induced bone loss. J Biol Chem. 2011 Mar 25;286(12):10864-75.
56. Kirmani S, Christen D, van Lenthe GH, Fischer PR, Bouxsein ML, McCready LK, et al. Bone Structure at the Distal Radius During Adolescent Growth. JBone MinerRes. 2008.
57. Nishiyama KK, Macdonald HM, Moore SA, Fung T, Boyd SK, McKay HA. Cortical porosity is higher in boys compared with girls at the distal radius and distal tibia during pubertal growth: an HR-pQCT study. J Bone Miner Res. 2012 Feb;27(2):273-82.
58. Seeman E. Structural basis of growth-related gain and age-related loss of bone strength. Rheumatology(Oxford). 2008;47 Suppl 4:iv2-iv8.
59. Parfitt AM. The two faces of growth: benefits and risks to bone integrity. OsteoporosInt. 1994;4(6):382-98.
60. Fratzl-Zelman N, Roschger P, Misof BM, Nawrot-Wawrzyniak K, Potter-Lang S, Muschitz C, et al. Fragility fractures in men with idiopathic osteoporosis are associated with undermineralization of the bone matrix without evidence of increased bone turnover. Calcif Tissue Int. 2011 May;88(5):378-87.
61. Misof BM, Patsch JM, Roschger P, Muschitz C, Gamsjaeger S, Paschalis EP, et al. Intravenous treatment with ibandronate normalizes bone matrix mineralization and reduces cortical porosity after two years in male osteoporosis: a paired biopsy study. J Bone Miner Res. 2014 Feb;29(2):440-9.
62. Diderich KE, Nicolaije C, Priemel M, Waarsing JH, Day JS, Brandt RM, et al. Bone fragility and decline in stem cells in prematurely aging DNA repair deficient trichothiodystrophy mice. Age (Dordr). 2012 Aug;34(4):845-61.
63. Nishimura G, Lausch E, Savarirayan R, Shiba M, Spranger J, Zabel B, et al. TRPV4-associated skeletal dysplasias. Am J Med Genet C Semin Med Genet. 2012 Aug 15;160C(3):190-204.
64. Scheller EL, Song J, Dishowitz MI, Hankenson KD, Krebsbach PH. A potential role for the myeloid lineage in leptin-regulated bone metabolism. Horm Metab Res. 2012 Jan;44(1):1-5.
65. Xu ZP, Gao WC, Wang HP, Wang XH. [Expression of transient receptor potential subfamily mRNAs in rat testes]. NanFang YiKeDaXueXueBao. 2009;29(3):519-20.
66. Chen X, Alessandri-Haber N, Levine JD. Marked attenuation of inflammatory mediator-induced C-fiber sensitization for mechanical and hypotonic stimuli in TRPV4-/- mice. MolPain. 2007;3:31.
67. Lee H, Caterina MJ. TRPV channels as thermosensory receptors in epithelial cells. Pflugers Arch. 2005;451(1):160-7.
68. Wijchers PJ, Festenstein RJ. Epigenetic regulation of autosomal gene expression by sex chromosomes. Trends Genet. 2011 Apr;27(4):132-40.

Chapter 4.6

Osteoporotic vertebral fractures during pregnancy: be aware of a potential underlying genetic cause

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ABSTRACT

Introduction Although the baby growing in its mother's womb needs calcium for skeletal development, osteoporosis and fractures very rarely occur during pregnancy.

Case Presentation A 27-year-old woman in the seventh month of her first pregnancy contracted midthoracic back pain after lifting an object. The pain was attributed to her pregnancy, but it remained postpartum. Her past medical history was uneventful, except for severely reduced vision of her left eye since birth. Family history revealed that her maternal grandmother had postmenopausal osteoporosis and her half-brother had three fractures during childhood after minor trauma. Her height was 1.58 m; she had no blue sclerae or joint hyperlaxity. Laboratory examination including serum calcium, phosphate, alkaline phosphatase, creatinine, β-carboxyterminal cross-linking telopeptide of type I collagen, 25-hydroxyvitamin D, and TSH was normal. Multiple thoracic vertebral fractures were diagnosed on X-ray examination, and dual-energy X-ray absorptiometry scanning showed severe osteoporosis (Z scores: L2–L4, -5.6 SD; femur neck, -3.9 SD). DNA analyses revealed two compound heterozygous missense mutations in *LRP5*.The patient's mother carried one of the *LRP5* mutations and was diagnosed with osteoporosis. Her half-brother, treated with cabergoline for a microprolactinoma, also had osteoporosis of the lumbar spine on dual-energy X-ray absorptiometry and carried the same *LRP5* mutation. The patient was treated with risedronate for 2.5 years. Bone mineral density and back pain improved. She stopped bisphosphonate use 6 months before planning a second pregnancy.

Conclusion Our patient was diagnosed with osteoporosis pseudoglioma syndrome/familial exudative vitreoretinopathy. Potential underlying genetic causes should be considered in pregnancy associated osteoporosis with implications for patients and relatives. More studies regarding osteoporosis treatment preceding conception are desirable.

INTRODUCTION

Pregnancy- and lactation-associated osteoporosis (PLO) with the occurrence of fragility fractures mainly of the vertebral bodies was first described as a syndrome by Nordin and Roper in 1955.[1] It is most commonly observed in the third trimester or early postpartum in women presenting with severe and prolonged back pain and sometimes height loss. The prevalence is unknown, and so far about 120 case reports have been reported.[2] The etiology is also not known, although a role of calciotropic hormones such as PTHrP has been suggested.[3, 4] Most of the cases have been reported in primigravid women.[3] There are no guidelines for treatment due to the lack of controlled trials. Another form of rare pregnancy-associated osteoporosis is called transient osteoporosis of pregnancy. Transient osteoporosis of pregnancy usually presents in the third trimester of pregnancy, sometimes with very severe pain while walking or standing, usually localized in the hip, and sometimes leading to hip fracture.[5] Radiographs can show severe localized loss of bone mass, whereas only edema may be visible in magnetic resonance imaging in early stages. This condition usually fades within a few months after delivery.

Additionally, pregnancy and lactation might lead to bone loss in patients with pre-existent osteoporosis attributable to genetic causes of low bone mineral density (BMD). As a consequence, these patients may become clinically manifest and develop fractures during this period. In this case report, we describe the clinical picture of a 27-year-old woman diagnosed with vertebral fractures and osteoporosis shortly after pregnancy. We will discuss potential causes of pregnancy-associated osteoporosis, its clinical consequences, and issues to take into account concerning patient management.

CASE PRESENTATION

A 27-year-old Caucasian woman in the seventh month of her first pregnancy complained of midthoracic back pain after bending over to lift a nonheavy object. The pain remained with differing intensity and was attributed to her pregnancy. After the delivery of a healthy child, the back pain prevented her from lifting her baby. She breastfed her baby for about 4 weeks. Because physical therapy had no effect on the pain, she was referred to an internist about 3 months after delivery. Her past medical history was uneventful without fractures, but she reported a severely reduced vision of her left eye since birth of unknown etiology, treated unsuccessfully with patches on the right eye. She consumed two to three dairy products daily. There was no history of abnormal menstrual cycle, smoking, alcohol, or medication use (such as corticosteroids) except for over-the-counter calcium and vitamin D supplements. Family history revealed that her maternal grandmother had postmenopausal osteoporosis, her grandfather had ankylosing spondylitis, and her only sibling (a half-brother) had experienced three fractures during childhood after minor trauma. On physical examination, her height was 1.58m (5 ft 2 in), her weight was 53 kg (117 lb), and she had no blue sclerae and no joint or skin hyperlaxity. Her maximally corrected visual acuity was 0.16 + + left (S-6.50=C-2.75×22) and 1.0- right (S-5.75=C-1.25×170). Further ophthalmological examination revealed amblyopia in the left eye and changes compatible with a mild form of familial exudative vitreoretinopathy (FEVR) in both eyes. There was normal form and function of the spinal column, which was slightly painful during flexion and extension. Except for an increase in bone-specific alkaline phosphatase and low urinary calcium excretion, there were no abnormalities on laboratory examination (Table 1). Spinal X-ray showed endplate compressions of thoracic vertebrae (T7, -9, -10, and -12; Figure 1). Dual-energy X-ray absorptiometry (DXA) scanning performed approximately 3 months after delivery showed severe osteoporosis (Z scores: L2–L4, -5.6 SD; femur neck, -3.9 SD) (Table 1). A biopsy of the iliac crest revealed coarse trabeculae with loss of connectivity and a strongly increased bone turnover,

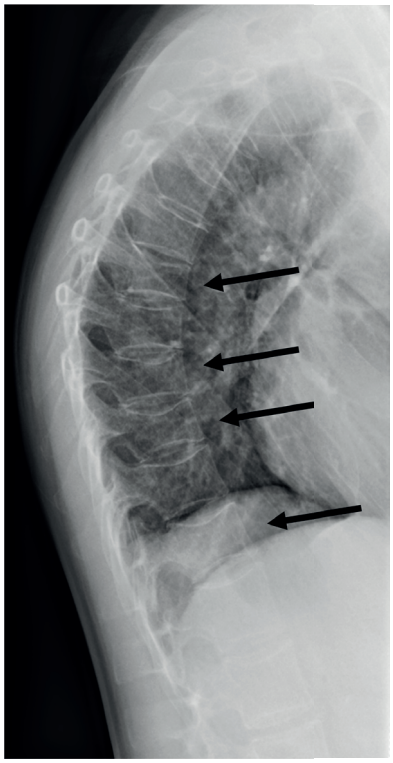

Figure 1. Lateral spinal X-ray image of the index patient. The black arrows show endplate compressions of thoracic vertebrae at T7, T9, T10, and T12.

but no evidence for mastocytosis or osteomalacia. After obtaining informed consent, DNA analysis was performed and showed no mutations in the *COL1A1* or *COL1A2* genes, only a polymorphism in the *COL1A1* gene that has been reported in juvenile osteoporosis but also in nonaffected family members. This makes a mild form of osteogenesis imperfecta unlikely. It is important, however, to notice that 10% of patients with clinical osteogenesis imperfecta have no detectable mutations in the exons for *COL1A1* and *COL1A2*.[6] DNA analyses of the *LRP5* gene revealed two compound heterozygous mutations, c.1519G>A (p.Gly507Ser) and c.3758G>T (p.Cys1253Phe). Subsequently, family screening with DXA and DNA analyses were performed (Figure 2). The mother of the patient, recently postmenopausal, is a carrier of the *LRP5* c.3758G>T mutation and was diagnosed with osteoporosis on a DXA scan (Z scores: lumbar spine, -2.8 SD; femoral neck, 0.0 SD). Spine radiography showed mild anterior wedging (less than 25%) of three thoracic vertebrae. The patient's half-brother, treated with cabergoline for a microprolactinoma, carried the same *LRP5* c.3758G>T mutation. He also had osteoporosis on the DXA scan (Z scores: lumbar spine, -2.1 SD; femoral neck, 0.0 SD) and had sustained three fractures after minimal trauma at a young age, as described before. He had no vertebral fractures on spine radiography. The mother and half-brother had no visual impairments. The father of the patient was deceased and could therefore not be tested. Surprisingly, the c.3758G>T mutation was not detected in DNA from the maternal grandmother with osteoporosis. This indicates that the mutation was inherited from the maternal grandfather or a de novo mutation and that the grandmother may have had common osteoporosis. The patient was treated with risedronate for 2.5 years. BMD and back pain improved. She stopped the use of bisphosphonate 6 months before planning a second pregnancy.

Table 1. Laboratory and imaging studies.

Lab Values	Reference values	Index patient, III:2	Patient's mother, II:2	Patient's half-brother, III:4
Serum				
Calcium, mmol/L	2.25-2.65	2.35	2.26	2.31
Phosphate, mmol/L	0.8-1.4	1.39	0.96	1.19
Creatinine, µmol/L	55-90	67	66	82
TSH, mU/L	0.4–4.3	1.26	4.10	0.55
ALP, U/L	<97	83	76	71
Bone-specific ALP, µg/L	<20.1	**22.6**	NA	**21.8**
25-Hydroxyvitamin D, nmol/L	>50	59	101	46
bCTX, µg/L	<0.56	0.11	NA	0.88
Urine				
24-h calcium, mmol/24 h	2.5-7.5	**1.9**	7.2	NA
Urine spot sample, mmol/L		NA	NA	3.55
DXA scan	**Cut-off for osteoporosis**			
Lumbar spine L2-L4 (T score)	≤-2.5 SD	**-5.7**	**-3.2**	-1.4
Lumbar spine L2-L4 (Z score)	≤-2.0 SD	**-5.6**	**-2.8**	**-2.1**
Femoral neck (T score)	≤-2.5 SD	**-3.9**	-0.5	0.0
Femoral neck (Z score)	≤-2.0 SD	**-3.9**	0.0	-0.8

Abbreviations: ALP, alkaline phosphatase; bCTX, β-carboxyterminal cross-linking telopeptide of type I collagen; NA, measurement not available. Values outside of reference range are marked bold.

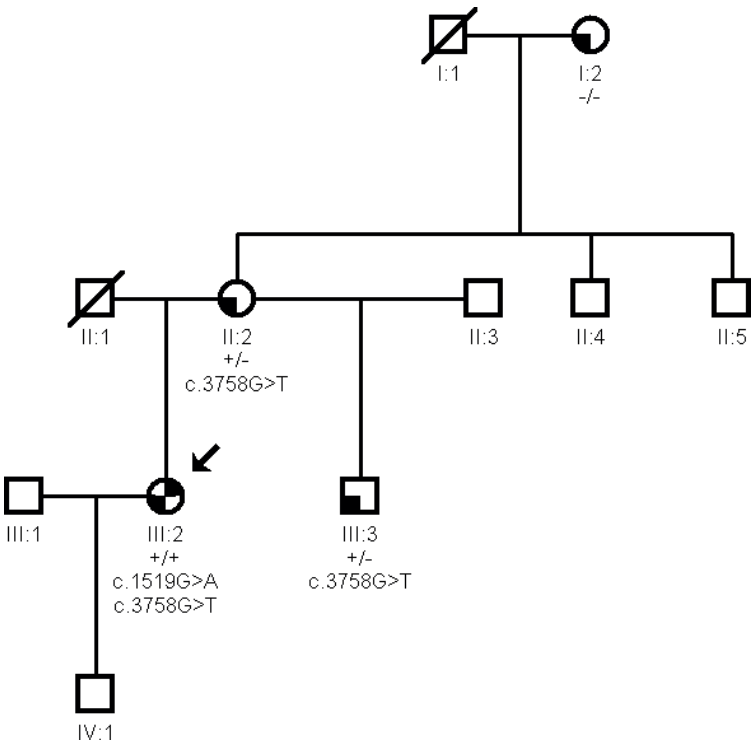

Figure 2. Pedigree and genotypes of the family. Index patient (III:2) is indicated with an arrow. She is compound heterozygous for the c.1519G>A and the c.3758G>T mutations. Subjects II:2 and III:3 are carriers of the c. 3758G>T mutation. Black squares within circles and square represent low BMD (below left) and mild exudative vitreoretinopathy (above right).

DISCUSSION

In this case report we describe the clinical picture of a young woman without a history of fractures. She presented in the third trimester of her first pregnancy with disabling back pain that persisted after delivery and was caused by fractures of multiple thoracic vertebrae. She had a severely reduced BMD on DXA scanning. We considered the diagnosis of PLO, but we identified a genetic cause underlying her condition. PLO is a rare heterogeneous disorder of unknown etiology. It is characterized by the occurrence of fragility fractures mostly in the spine and severe back pain presenting typically in the third trimester of gestation or early postpartum period.[3] In PLO, whereas some patients improve spontaneously after giving birth or stopping lactation, others need medical treatment and continue to have decreased BMD.[7] Pre-existing secondary causes of osteoporosis, such as vitamin D deficiency, celiac disease, anorexia nervosa, mastocytosis, and hyper(para)thyroidism, should always be ruled out. Pregnancy and lactation may lead to up to 5–10% loss of mainly trabecular bone, especially during breast-feeding. However, almost complete recovery occurs in most cases within 6 to 12 months[8] and thus cannot explain the very low BMD in our patient unless BMD was already compromised before pregnancy due to other reasons. In patients with PLO, a high prevalence of fractures has been reported in their mothers[9] and of osteopenia in their offspring,[10] leading to the suggestion of an underlying (genetically determined) low peak bone mass[8, 9].

In our patient, we suspected an underlying monogenetic bone disease due to the severity of her osteoporosis. Analysis of her half-brother and mother confirmed a familial component. The history of severely reduced vision in one eye since birth led to suspicion of osteoporosis pseudoglioma (OPPG) syndrome, an autosomal recessive disorder characterized by early onset osteoporosis and blindness (OMIM no. 259770). OPPG is a rare disease with an estimated incidence of 1:2,000,000 and a carrier frequency of 1:700,[11] caused by biallelic loss of function mutations in *LRP5*[12]. LRP5 (low-density lipoprotein receptor-related protein 5) is a cell-surface protein receptor that plays a key role in several intracellular signaling pathways, mainly Wnt and Norrin signaling.[12] Mutations in *LRP5* are also involved in FEVR[13] (FEVR/exudative vitreoretinopathy 4, OMIM no. 133780), a hereditary blinding disorder with a highly variable phenotype even within the same family[14]. Both autosomal recessive and autosomal dominant inheritance can occur. FEVR caused by *LRP5* mutations is associated with low bone mass, in contrast to FEVR caused by mutations in other genes (e.g., *FZD4* or *NDP*).[14] OPPG and FEVR caused by *LRP5* mutations are therefore disorders with an overlapping phenotype. It has been suggested by Qin et al.[14] that OPPG and FEVR caused by mutations in *LRP5* are part of a single phenotypic spectrum with both ocular and bone manifestations. DNA analysis in our patient showed compound heterozygosity for two missense mutations in the *LRP5* gene. The c.1519G>A (p.Gly507Ser) mutation is predicted to induce a minor chemical change of an evolutionary strongly conserved amino acid with introduction of an alternate splice acceptor site, and when present in homozygous state induces OPPG with very low BMD levels.[15] On the other hand, c.3758G>T (p.Cys1253Phe) is predicted to induce a major chemical change of an evolutionary strongly conserved amino acid and has been previously described in recessive FEVR.[13] Because most patients with OPPG are congenitally blind or become blind by the age of 25 years,[11, 15-17] it is remarkable that our patient had relatively mild signs of exudative vitreoretinopathy, and a diagnosis of recessive FEVR might be considered as well,[18] although osteoporosis is usually less severe than in OPPG[11, 14, 15]. The mother and half-brother carrying the *LRP5* c.3758G>T mutation that has been previously described in recessive FEVR also had decreased BMD. Although OPPG follows an autosomal recessive pattern of inheritance, heterozygous carriers can exhibit mildly reduced BMD[19].

Heterozygous mutations in *LRP5* are associated with primary osteoporosis in children.[20] Moreover, in genome-wide meta-analyses the *LRP5* locus was significantly associated with BMD and fracture risk,[21] broadening the spectrum of bone abnormalities related to genetic variation in *LRP5*.

We treated our patient with risedronate after she told us she did not want to get pregnant for at least 2 years, and she continued the use of oral contraceptives. Bisphosphonates are contraindicated in pregnancy. Animal studies with high doses have shown maternal and fetal toxicity, and there is concern of treating premenopausal women with these drugs because they are retained in bone for several years.[22] A recent study of the literature that identified 78 cases of pregnancies involving exposure to bisphosphonates before conception or during pregnancy did not demonstrate serious adverse effects. Despite this, cases of increased spontaneous abortions, shortened gestational age, low neonatal birth weight, and transient hypocalcemia of the newborn were reported.[23] Although bisphosphonates share the same core structure, their binding affinity to hydroxyapatite crystals varies among them; those with higher affinity display longer skeletal retention. It has been found that the ranking order for hydroxyapatite affinity from highest to lowest is zoledronate > alendronate > ibandronate > risedronate > etidronate.[24] We chose a bisphosphonate with relatively low skeletal retention. We advised the patient to stop treatment at least 6 months before stopping birth control because risedronate levels have not been detected in urine 5 months after cessation of therapy.[25] We would nevertheless advise close monitoring of pregnancy and intrauterine growth, check for neonatal hypocalcemia, and report on outcome. Also, we advised our patient to limit or avoid lactation after a subsequent pregnancy to prevent further maternal bone loss associated with breast-feeding.[8] Alternatively, newer medications without long-term bone retention could be considered as off-label treatment in premenopausal women at very high risk for fractures who wish to become pregnant. However, in theory, stopping these drugs before becoming pregnant could lead to increased bone loss during pregnancy.

CONCLUSION

We report the clinical picture of a 27-year-old woman who suffered from disabling back pain during pregnancy and was diagnosed with multiple vertebral fractures and severe osteoporosis after delivery. We made the diagnosis of severe osteoporosis due to compound heterozygous mutations in the *LRP5* gene with mild exudative vitreoretinopathy as part of a spectrum of diseases named "osteoporosis pseudoglioma syndrome" and "familial exudative vitreoretinopathy." Thus, our patient was genetically predisposed, and pregnancy further exacerbated her osteoporosis, resulting in vertebral fractures. We propose screening for an underlying monogenetic bone disorder in patients with PLO and one of the following features: a severely reduced BMD (Z scores < -2.0 SD); a family history of osteoporosis or fragility fractures, joint hypermobility, blue sclerae, congenital blindness, or severely reduced vision; or a history of fractures before pregnancy (e.g., testing for mutations in *collagen 1A1* and *1A2* genes, *LRP5, WNT1*,[26] and *LGR4*,[27] and for the recently reported *PLS3* gene[28]). A genetic diagnosis has implications for the patient and relatives. More studies regarding bisphosphonate treatment and newer osteoporosis drugs preceding conception are desirable.

REFERENCES

1. Nordin BE, Roper A. Post-pregnancy osteoporosis; a syndrome? Lancet. 1955 Feb 26;268(6861):431-4.

2. Choe EY, Song JE, Park KH, Seok H, Lee EJ, Lim SK, et al. Effect of teriparatide on pregnancy and lactation-associated osteoporosis with multiple vertebral fractures. J Bone Miner Metab. 2012 Sep;30(5):596-601.
3. O'Sullivan SM, Grey AB, Singh R, Reid IR. Bisphosphonates in pregnancy and lactation-associated osteoporosis. Osteoporos Int. 2006;17(7):1008-12.
4. Sowers MF, Hollis BW, Shapiro B, Randolph J, Janney CA, Zhang D, et al. Elevated parathyroid hormone-related peptide associated with lactation and bone density loss. JAMA. 1996 Aug 21;276(7):549-54.
5. Maliha G, Morgan J, Vrahas M. Transient osteoporosis of pregnancy. Injury. 2012 Aug;43(8):1237-41.
6. van Dijk FS, Dalgleish R, Malfait F, Maugeri A, Rusinska A, Semler O, et al. Clinical utility gene card for: osteogenesis imperfecta. Eur J Hum Genet. 2013 Jun;21(6).
7. Lampropoulou-Adamidou K, Trovas G, Stathopoulos IP, Papaioannou NA. Case report: Teriparatide treatment in a case of severe pregnancy -and lactation- associated osteoporosis. Hormones (Athens). 2012 Oct-Dec;11(4):495-500.
8. Kovacs CS. Calcium and bone metabolism disorders during pregnancy and lactation. Endocrinol Metab Clin North Am. 2011 Dec;40(4):795-826.
9. Dunne F, Walters B, Marshall T, Heath DA. Pregnancy associated osteoporosis. Clin Endocrinol (Oxf). 1993 Oct;39(4):487-90.
10. Carbone LD, Palmieri GM, Graves SC, Smull K. Osteoporosis of pregnancy: long-term follow-up of patients and their offspring. Obstet Gynecol. 1995 Oct;86(4 Pt 2):664-6.
11. Ai M, Heeger S, Bartels CF, Schelling DK. Clinical and molecular findings in osteoporosis-pseudoglioma syndrome. Am J Hum Genet. 2005 Nov;77(5):741-53.
12. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell. 2001 Nov 16;107(4):513-23.
13. Nikopoulos K, Venselaar H, Collin RW, Riveiro-Alvarez R, Boonstra FN, Hooymans JM, et al. Overview of the mutation spectrum in familial exudative vitreoretinopathy and Norrie disease with identification of 21 novel variants in FZD4, LRP5, and NDP. Hum Mutat. 2010 Jun;31(6):656-66.
14. Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H. Complexity of the genotype-phenotype correlation in familial exudative vitreoretinopathy with mutations in the LRP5 and/or FZD4 genes. Hum Mutat. 2005 Aug;26(2):104-12.
15. Tuysuz B, Bursali A, Alp Z, Suyugul N, Laine CM, Makitie O. Osteoporosis-pseudoglioma syndrome: three novel mutations in the LRP5 gene and response to bisphosphonate treatment. Horm Res Paediatr. 2012;77(2):115-20.
16. Gong Y, Vikkula M, Boon L, Liu J, Beighton P, Ramesar R, et al. Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. Am J Hum Genet. 1996 Jul;59(1):146-51.
17. Somer H, Palotie A, Somer M, Hoikka V, Peltonen L. Osteoporosis-pseudoglioma syndrome: clinical, morphological, and biochemical studies. J Med Genet. 1988 Aug;25(8):543-9.
18. Downey LM, Bottomley HM, Sheridan E, Ahmed M, Gilmour DF, Inglehearn CF, et al. Reduced bone mineral density and hyaloid vasculature remnants in a consanguineous recessive FEVR family with a mutation in LRP5. Br J Ophthalmol. 2006 Sep;90(9):1163-7.
19. Laine CM, Chung BD, Susic M, Prescott T, Semler O, Fiskerstrand T, et al. Novel mutations affecting LRP5 splicing in patients with osteoporosis-pseudoglioma syndrome (OPPG). Eur J Hum Genet. 2011 Aug;19(8):875-81.
20. Hartikka H, Makitie O, Mannikko M, Doria AS, Daneman A, Cole WG, et al. Heterozygous mutations in the LDL receptor-related protein 5 (LRP5) gene are associated with primary osteoporosis in children. J Bone Miner Res. 2005 May;20(5):783-9.
21. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.
22. Chan B, Zacharin M. Maternal and infant outcome after pamidronate treatment of polyostotic fibrous dysplasia and osteogenesis imperfecta before conception: a report of four cases. J Clin Endocrinol Metab. 2006 Jun;91(6):2017-20.

23. Stathopoulos IP, Liakou CG, Katsalira A, Trovas G, Lyritis GG, Papaioannou NA, et al. The use of bisphosphonates in women prior to or during pregnancy and lactation. Hormones (Athens). 2011 Oct-Dec;10(4):280-91.
24. Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, et al. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone. 2006 May;38(5):617-27.
25. Peris P, Torra M, Olivares V, Reyes R, Monegal A, Martinez-Ferrer A, et al. Prolonged bisphosphonate release after treatment in women with osteoporosis. Relationship with bone turnover. Bone. 2011 Oct;49(4):706-9.
26. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. N Engl J Med. 2013 May 9;368(19):1809-16.
27. Styrkarsdottir U, Thorleifsson G, Sulem P, Gudbjartsson DF, Sigurdsson A, Jonasdottir A, et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. Nature. 2013 May 23;497(7450):517-20.
28. van Dijk FS, Zillikens MC, Micha D, Riessland M, Marcelis CL, de Die-Smulders CE, et al. PLS3 mutations in X-linked osteoporosis with fractures. N Engl J Med. 2013 Oct 17;369(16):1529-36.

Chapter 4.7

Genetics of osteoporotic vertebral fractures

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ABSTRACT

Our understanding of the genetic control of skeletogenesis and bone remodeling is increasing, and in addition to various non-genetic risk factors, a positive family history confers an increased risk of fracture. Vertebral fractures are the most common osteoporotic fractures and they are often a first manifestation of osteoporosis. This review presents the current state of knowledge on the genetic basis of osteoporotic vertebral fractures and, additionally, of structural vertebral deformities resembling osteoporotic vertebral fractures but which may have their own genetic basis. We conclude that, apart from tentative screening for rare monogenic forms of osteoporosis in very unusual case presentations, not enough is currently known to encourage routine genetic screening in regular osteoporotic vertebral fracture cases.

INTRODUCTION

Our understanding of the genetic control of skeletogenesis and bone remodeling is increasing. Normally, bone resorption and bone formation are balanced and regulated by hormones, growth factors and cytokines. In addition to various non-genetic risk factors, a positive family history confers an increased risk of fracture.[1]

Vertebral fractures are the most common osteoporotic fractures and often a first manifestation of osteoporosis. They represent a significant health issue[2] being associated with a high morbidity[3, 4]. There may be skeletal-site specific effects of fracture determinants, meriting the study of vertebral fractures independently of non-vertebral fractures, as discussed below.

In this review, we discuss the genetics of osteoporotic vertebral fractures and touch on other structural vertebral deformities. This is important because these conditions may have their own genetic basis, and an accurate diagnosis is important to prevent case misclassification that may in turn hamper genetic discoveries.

Structural vertebral deformities and fractures

Several methods for radiological assessment of vertebral fractures exist, but a gold standard is lacking.[5] Traditionally, conventional radiography has been the imaging modality of choice. Two advantages of DXA-based vertebral fracture assessment over conventional radiography are the lower radiation dose and capture of the whole spine in one image with virtually no parallax distortion particularly because DXA imaging resolution has improved immensely. However, a number of diseases complicate the diagnosis of vertebral fractures, including degenerative disease, anatomical variation, and anomalies.[6] More is becoming known about these conditions and their possible inter-relationships with osteoporosis and fractures, as discussed subsequently. Therefore, we begin by discussing the definition of osteoporotic vertebral fractures and mimics that should not be confused with vertebral fractures.

Non-fractural deformities represented by anatomical variation and developmental abnormalities have been reviewed by Ferrar et al.[7] On a lateral view, the spine has a natural curvature such that vertebrae in the mid-thoracic region tend to be wedge-shaped, in keeping with the normal mild kyphosis. Lumbar vertebrae have a relatively shorter posterior height and tend to be biconcave. Some individuals have developmentally smaller or shorter vertebrae (Figure 1), particularly anteriorly and most commonly in the mid-thoracic region. This is thought to be because of either congenital variation or inhibited growth of the vertebral body during childhood or adolescence and it is believed that this variant is not because of fracturing[8]. In so-called "step-like" or "step-off" endplates (Figure 2) the central endplate is deeper with an abrupt transition to the more normal periphery. This is in contrast to the appearance of the fractured endplate in osteoporosis, in which a concave depression usually extends from corner to corner of the vertebral body.[7] These "step-off" endplates seem to be the consequence of growth retardation in the central portion of the endplate. In contrast, the periphery of the growth plate has a different blood supply through short arteries, in which vaso-occlusion and microinfarction may lead to avascular necrosis and further developmental disruption of the vertebral body.[9] Implicated in such a process are Gaucher's disease, hemolytic anemias including hereditary spherocytosis, and sickle cell and thalassemia among the hemoglobinopathies.[10] The cortical margins of the inferior endplates of lumbar vertebral bodies also frequently have paired parasagittal concavities, when viewed in the frontal projection, resembling the curvature of an aimed bow[11] (Figure 3). When viewed in the lateral projection, the concavities are superimposed and lie in the posterior portion of the inferior endplate and might be confused with fractures.[7] This anomaly called "cupid's bow" is a normal anatomic variant. Histologic examination in cadavers

reveals thickened bone in the cupid's bow endplate with annular fibers inserting into this region, which was detected at multiple lumbar and thoracic levels, with the highest frequency in the lower lumbar spine in 34 out of 64 thoracolumbar spines.[12] Furthermore, the endplate indentations tend to become progressively deeper distally. Other commonly seen normal variants are a deep inferior endplate (Figure 4) and a balloon disc, where there is an occurrence of an unusually concave disc-vertebral border at multiple levels. A Japanese study has reported a prevalence of balloon disc up to 14% in the healthy population, with an association with male gender and height but a lack of a relation with back pain or age, yet, to our knowledge no replication and validation studies have been published.

A specific example of a vertebral abnormality that might be confused with fracturing is Scheuermann's disease (SD) (Figure 5). With reported prevalence rates of up to 10%, the disease is frequently mentioned in the differential diagnosis of osteoporotic vertebral fractures.[7] It is a form of osteochondrosis of the spine characterized by increased thoracic kyphosis in association with structural deformity.[13, 14] SD usually first appears during adolescence at the time of puberty, resulting in permanent vertebral distortion and back pain in many cases. The etiology is unknown, but heredity may well be important[15]: genetic surveys are underway. SD is diagnosed on the basis of radiographic criteria of which those defined by Sørensen and Sachs are the most commonly applied: a thoracic kyphosis >45°; at least three adjacent wedge-shaped vertebral bodies of ≥5°; endplate irregularities with possible vertebral elongation; and disc space narrowing. Schmorl's nodes are thought to be a common but not obligate manifestation of SD (Figure 6).[16-19] In a population survey by Makurthou et al. the frequency of the separate radiological criteria of endplate irregularities and vertebral wedging was higher in males than in females.[16] Some studies suggest SD to be more prevalent in men,[20, 21] whereas others have reported no significant difference between the sexes[16, 22-24]. Although coexistence

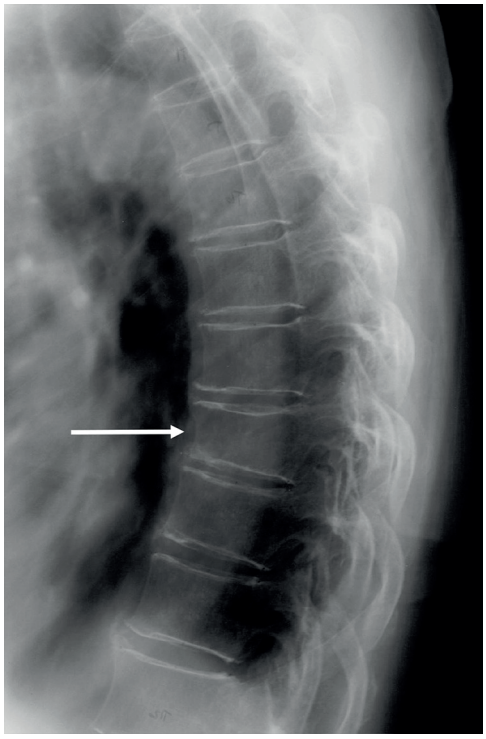

Figure 1. Lateral thoracic spine radiograph demonstrating an example of a developmentally short vertebra (arrow). This vertebra was unchanged for 15 years and the patient had no other evidence of disease. Therefore it was presumed to be a developmentally short vertebra.

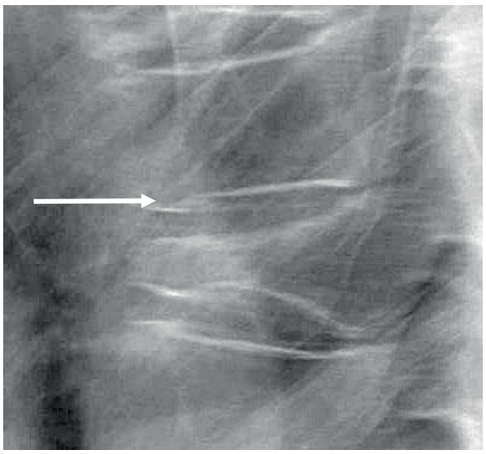

Figure 2. Lateral radiography showing a "step-like" or "step-off" endplate (arrow). An osteoporotic vertebral fracture is also visible one level below.

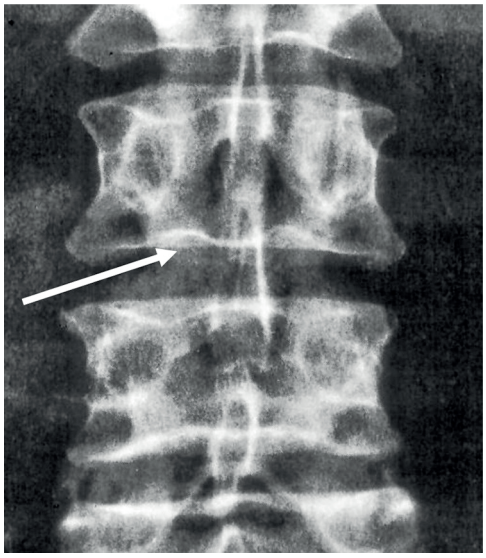

Figure 3. "Cupid's bow" anomaly shown on a vertebral fracture assessment image (arrow).

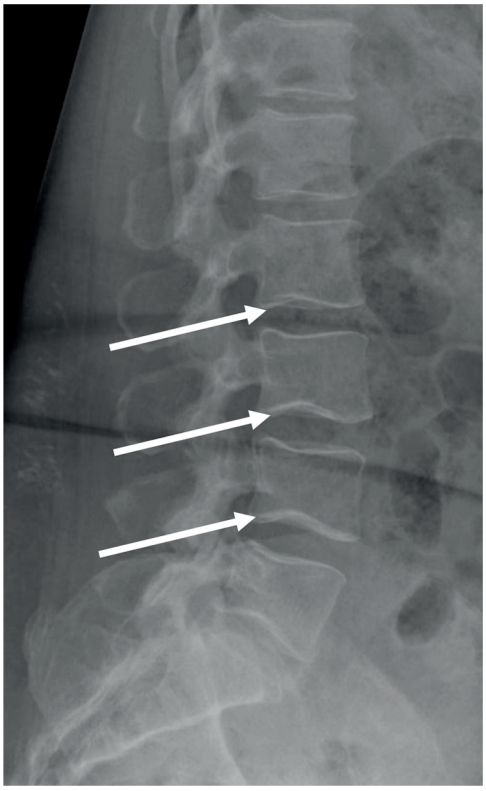

Figure 4. Lateral radiograph of the lumbar spine demonstrates deep inferior endplates at multiple levels (arrows).

of SD with osteoporotic vertebral fractures may occur, it is thought that the disorders and their treatment are different.[25] Nonetheless, few data exist on a possible connection. Some studies with small patient numbers have been conducted a long time ago, some of which suggest that patients with SD have generalized lower bone mineral density (BMD)[26, 27]. This may be a transient effect that resolves in adulthood,[28] whereas other analyses have found no difference in BMD between those with SD and controls[29, 30]. However, no investigations have looked into osteoporotic vertebral fracture risk in SD.

A few percent of the general population has a scoliosis as diagnosed by a Cobb angle of $>10^0$ in the frontal plane.[31, 32] A scoliosis may impair DXA BMD measurement and assessment for vertebral fractures.[7, 33] Scoliosis can be categorized according to the etiology:

- It may occasionally be congenital, arising during embryonic development and oftentimes part of other abnormalities.[34]
- Adolescent idiopathic scoliosis represents most cases and is the most common pediatric skeletal disease. The etiology remains largely unknown, but population and twin studies strongly suggest a genetic contribution.[35] So far, findings from linkage and candidate gene association studies implicate genes related to connective tissue structure, bone formation and metabolism, melatonin signaling pathways, puberty and growth, and axon guidance pathways, but these results remain to be replicated.[36] Two genome-wide association studies (GWAS) containing a replication phase have been published and describe associations with loci containing the candidate genes ladybird homeobox 1 (*LBX1*) and G protein–coupled receptor 126 (*GPR126*), of which the functions remain to be elucidated further. Bone quality deterioration and lower bone mass have been reported at

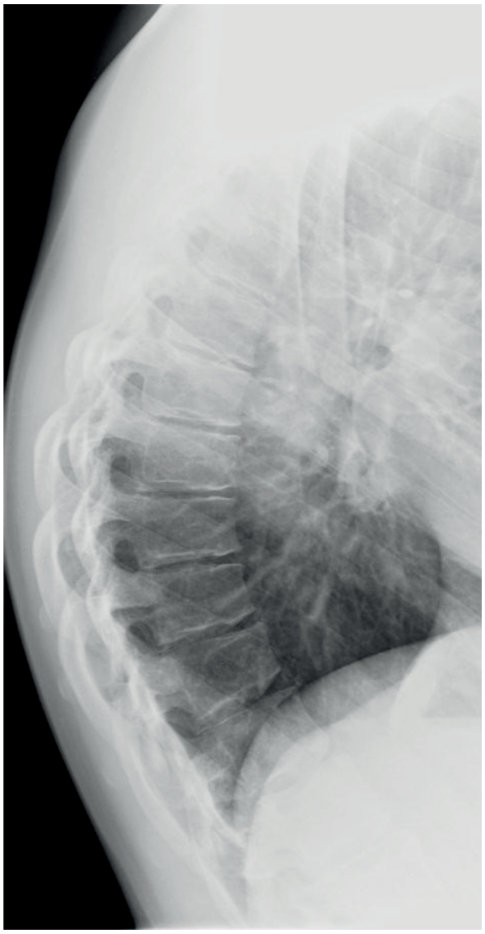

Figure 5. Scheuermann's disease demonstrated on a lateral thoracic spine radiograph. There is increased kyphosis, mild wedging of multiple adjacent thoracic vertebral bodies that demonstrate irregular endplates.

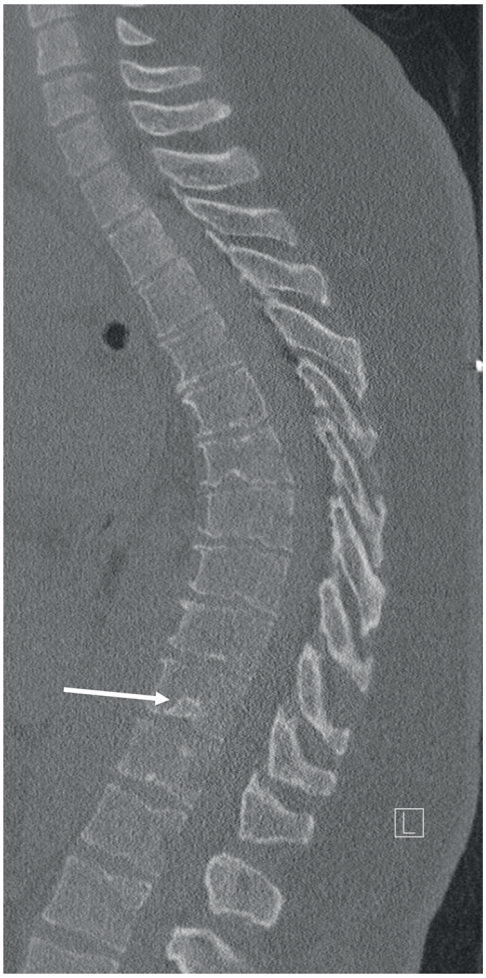

Figure 6. Scheuermann's disease and Schmorl's node (arrow) visualized on a sagittally reconstructed computed tomography image.

the hip, spine and other peripheral sites in adult idiopathic scoliosis patients.[33, 37-40] Whether this decrease in bone mass is associated with an increased risk of osteoporotic spine fractures has not yet been investigated.

- Third, the clinically most important factors in adult scoliosis are primary and secondary degenerative scoliosis.[41] In the so-called primary degenerative scoliosis (de novo form) there is asymmetric disc and facet joint degeneration, mostly located at the thoracolumbar junction or lumbar spine, and associated with aging.[42] Secondary degenerative scoliosis may appear as a consequence of pelvic obliquity because of a leg length discrepancy, hip disease or a neuromuscular disorder.
- Finally, osteoporotic vertebral fractures may bring about an asymmetric configuration with the appearance of kyphosis, scoliosis or both.[41]

Genetics of vertebral fractures

As mentioned, an important risk factor for osteoporosis and fractures is a positive family history.[43] Studies have reported estimates of heritability of BMD and fractures of up to 66% and 46%, respectively.[44, 45] Furthermore, a recent report has found a mixture of shared and specific genetic influences for distinct BMD traits, where the strength of genetic variants may differ in their association and magnitude of effect across different skeletal sites and, moreover, some loci seem to act in certain skeletal locations while they are irrelevant at others.[46] Such a difference in genetic basis had previously been proposed for lumbar BMD compared with femoral neck BMD in GWAS.[47] Although a fracture is the most important clinical outcome in osteoporosis, identifying genetic determinants contributing to the risk of fracture has been difficult because of its multifactorial nature and occurrence late in life. For this reason, correlated intermediate phenotypes such as BMD have attracted the interest of researchers. The most recent GWAS meta-analysis concluded that genetic factors related to BMD measured in the spine and proximal femur by dual-energy X-ray absorptiometry cluster in 3 key biologic pathways: mesenchymal stem cell differentiation; Wnt signaling; and RANK-RANKL-OPG (receptor activator of nuclear factor kappa-B–receptor activator of nuclear factor kappa-B ligand–osteoprotegerin).[48] Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes. Wnt signaling consists of a group of signal transduction pathways in which proteins that pass signals from the extracellular space through cell surface receptors to the inside of the cell. It plays an essential role in development and maintenance of numerous organs and tissues including bone, especially in osteoblastogenesis.[49] The RANK-RANKL-OPG system is crucial for well-balanced bone remodeling through activation of osteoclasts by osteoblasts.[50] Another critical process highlighted in this study was endochondral ossification and the accumulation of intracellular calcium by chondrocytes to form calcified bone tissue in the developing skeleton.

A growing number of GWAS have revealed genetic loci for fracture risk of any type.[48, 51, 52] A study by Liu et al. has found heritability estimates of prevalent moderate and severe vertebral fractures (semi-quantitative (SQ) grade ≥2) as assessed on computed tomography (CT) ranging from 43% to 53% and increasing to approximately 69% when adjusted for volumetric BMD and cross-sectional area.[53] The very first GWAS for radiographic vertebral fractures discovered a single nucleotide polymorphism SNP on chromosome 16q24 at genome-wide significant level.[54] The single nucleotide polymorphism maps to a region previously found associated with DXA LS-BMD in two large meta-analyses.[47, 48] From a biomedical perspective, deletions/mutations in this 16q24 locus are implicated in a combination of birth defects that includes vertebral defects in both humans and mice.[55] Furthermore, the forkhead box C2 (*FOXC2*) candidate gene located nearby in the locus encodes a transcription factor essential for axial skeletogenesis in mice.[56] However, this GWAS association could not be convincingly replicated by de novo genotyping the specific marker in a large-scale global replication effort, displaying a high degree of heterogeneity of effects[54]. It was speculated that, apart from the possibility of the signal being a false positive association, phenotype definition as discussed elsewhere in this issue may have undermined establishment of a firm correlation. For instance, it is thought that many mild vertebral deformities are not caused by fractures but by degenerative changes, etc. Indeed in the study by Liu et al., after adding mild grade 1 vertebral deformities into the analyses, the heritability was much lower (aforementioned 43% to 69% dropping to 19% to 27%) with a lower intrareader agreement (kappa =0.56–0.59 versus kappa=0.68–0.72 for grade ≥2).[53]

There are a multitude of additional candidate genes that might be screened for in very unusual clinical presentations of vertebral fractures, for instance genetic mutations that are known to cause monogenic

forms of osteoporosis or osteogenesis imperfecta, for example: *COL1A1*,[57] *COL1A2, LRP5*,[58] *WNT1*,[59] *LGR4*,[60], *PLS3*[61], *CRTAP, FKBP10, LEPRE1, PLOD2, PPIB, SERPINF1, SERPINH1* and *SP7*[62].

In conclusion, vertebral fractures are associated with high morbidity, mortality and costs. Radiological diagnosis is not always straightforward. We have reviewed what is known so far about the genetic basis of osteoporotic vertebral fractures, and, additionally, other spinal conditions that have phenotypes resembling osteoporotic vertebral fractures while the underlying genetics are probably different. Recent discoveries in this field are likely to be the tip of the iceberg. Apart from tentatively screening for rare monogenic forms of osteoporosis in very unusual case presentations, not enough is currently known to encourage routine genetic screening in typical patients with osteoporotic vertebral fractures.

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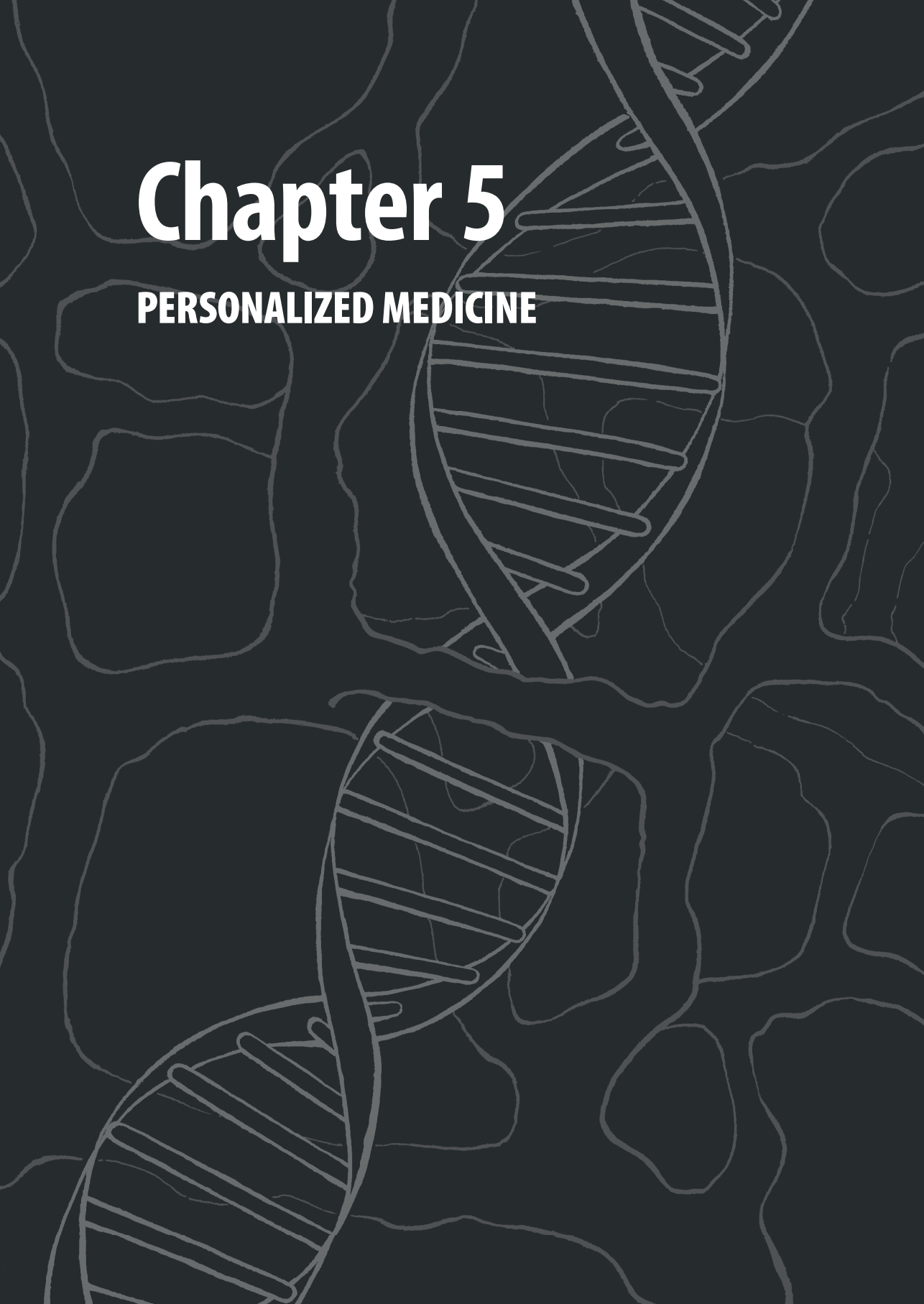
REFERENCES

1. Kanis JA, Johansson H, Oden A, Johnell O, De Laet C, Eisman JA, et al. A family history of fracture and fracture risk: a meta-analysis. Bone. 2004 Nov;35(5):1029-37.
2. Brunton S, Carmichael B, Gold D, Hull B, Kauffman T, Papaioannou A, et al. Vertebral compression fractures in primary care: recommendations from a consensus panel. J Fam Pract. 2005 Sep;54(9):781-8.
3. Ensrud KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. Journal of the American Geriatrics Society. 2000 Mar;48(3):241-9.
4. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA. 2009 Feb 4;301(5):513-21.
5. Oei L, Rivadeneira F, Ly F, Breda SJ, Zillikens MC, Hofman A, et al. Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. Eur Radiol. 2013 Feb;23(2):476-86.
6. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
7. Ferrar L, Jiang G, Adams J, Eastell R. Identification of vertebral fractures: an update. Osteoporos Int. 2005 Jul;16(7):717-28.
8. Ferrar L, Jiang G, Cawthon PM, San Valentin R, Fullman R, Lambert L, et al. Identification of vertebral fracture and non-osteoporotic short vertebral height in men: the MrOS study. J Bone Miner Res. 2007 Sep;22(9):1434-41.
9. Reynolds J, Pritchard JA, Ludders D, Mason RA. Roentgenographic and clinical appraisal of sickle cell beta-thalassemia disease. Am J Roentgenol Radium Ther Nucl Med. 1973 Jun;118(2):378-400.
10. Schwartz AM, Homer MJ, McCauley RG. "Step-off" vertebral body: Gaucher's disease versus sickle cell hemoglobinopathy. AJR Am J Roentgenol. 1979 Jan;132(1):81-5.
11. Dietz GW, Christensen EE. Normal "Cupid's bow" contour of the lower lumbar vertebrae. Radiology. 1976 Dec;121(3 Pt. 1):577-9.
12. Chan KK, Sartoris DJ, Haghighi P, Sledge P, Barrett-Connor E, Trudell DT, et al. Cupid's bow contour of the vertebral body: evaluation of pathogenesis with bone densitometry and imaging-histopathologic correlation. Radiology. 1997 Jan;202(1):253-6.
13. Ali RM, Green DW, Patel TC. Scheuermann's kyphosis. Curr Opin Pediatr. 1999 Feb;11(1):70-5.
14. Hart ES, Merlin G, Harisiades J, Grottkau BE. Scheuermann's thoracic kyphosis in the adolescent patient. Orthop Nurs. 2010 Nov-Dec;29(6):365-71; quiz 72-3.
15. Damborg F, Engell V, Andersen M, Kyvik KO, Thomsen K. Prevalence, concordance, and heritability of Scheuermann kyphosis based on a study of twins. J Bone Joint Surg Am. 2006 Oct;88(10):2133-6.

16. Makurthou AA, Oei L, El Saddy S, Breda SJ, Castano-Betancourt MC, Hofman A, et al. Scheuermann disease: evaluation of radiological criteria and population prevalence. Spine (Phila Pa 1976). 2013 Sep 1;38(19):1690-4.
17. Sachs B, Bradford D, Winter R, Lonstein J, Moe J, Willson S. Scheuermann kyphosis. Follow-up of Milwaukee-brace treatment. J Bone Joint Surg Am. 1987 Jan;69(1):50-7.
18. Wenger DR, Frick SL. Scheuermann kyphosis. Spine (Phila Pa 1976). 1999 Dec 15;24(24):2630-9.
19. Oei L, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJ, et al. Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses. Quant Imaging Med Surg. 2013 Oct;3(5):249-55.
20. Fisk JW, Baigent ML, Hill PD. Scheuermann's disease. Clinical and radiological survey of 17 and 18 year olds. Am J Phys Med. 1984 Feb;63(1):18-30.
21. Ristolainen L, Kettunen JA, Heliovaara M, Kujala UM, Heinonen A, Schlenzka D. Untreated Scheuermann's disease: a 37-year follow-up study. Eur Spine J. 2012 May;21(5):819-24.
22. Lowe TG, Line BG. Evidence based medicine: analysis of Scheuermann kyphosis. Spine (Phila Pa 1976). 2007 Sep 1;32(19 Suppl):S115-9.
23. Murray PM, Weinstein SL, Spratt KF. The natural history and long-term follow-up of Scheuermann kyphosis. J Bone Joint Surg Am. 1993 Feb;75(2):236-48.
24. Rachbauer F, Sterzinger W, Eibl G. Radiographic abnormalities in the thoracolumbar spine of young elite skiers. Am J Sports Med. 2001 Jul-Aug;29(4):446-9.
25. Breda SJ, Oei HD, Oei EH, Zillikens MC. [Osteoporotic vertebral fractures or Scheuermann's disease?]. Ned Tijdschr Geneeskd. 2013;157(45):A6479.
26. Popko J, Konstantynowicz J, Kossakowski D, Kaczmarski M, Piotrowska-Jastrzebska J. Assessment of bone density in children with Scheuermann's disease. Rocz Akad Med Bialymst. 1997;42(1):245-50.
27. Lopez RA, Burke SW, Levine DB, Schneider R. Osteoporosis in Scheuermann's disease. Spine (Phila Pa 1976). 1988 Oct;13(10):1099-103.
28. Viola S, Peter F, Gyorgy I, Szecsenyi NL. [Alkaline phosphatase level and bone density in Scheuermann's disease and in adolescent idiopathic scoliosis]. Orv Hetil. 2000 Apr 23;141(17):905-9.
29. Runge H, Fengler F, Franke J, Koall W. [Evaluation of the mineral content of peripheral bones (radius) by photon-absorption technique in normals as well as in patients with various types of bone diseases (author's transl)]. Radiologe. 1980 Oct;20(10):505-14.
30. Gilsanz V, Gibbens DT, Carlson M, King J. Vertebral bone density in Scheuermann disease. J Bone Joint Surg Am. 1989 Jul;71(6):894-7.
31. Schwab F, el-Fegoun AB, Gamez L, Goodman H, Farcy JP. A lumbar classification of scoliosis in the adult patient: preliminary approach. Spine (Phila Pa 1976). 2005 Jul 15;30(14):1670-3.
32. Malfair D, Flemming AK, Dvorak MF, Munk PL, Vertinsky AT, Heran MK, et al. Radiographic evaluation of scoliosis: review. AJR Am J Roentgenol. 2010 Mar;194(3 Suppl):S8-22.
33. Yu WS, Chan KY, Yu FW, Yeung HY, Ng BK, Lee KM, et al. Abnormal bone quality versus low bone mineral density in adolescent idiopathic scoliosis: a case-control study with in vivo high-resolution peripheral quantitative computed tomography. Spine J. 2013 Nov;13(11):1493-9.
34. Hensinger RN. Congenital scoliosis: etiology and associations. Spine (Phila Pa 1976). 2009 Aug 1;34(17):1745-50.
35. Andersen MO, Thomsen K, Kyvik KO. Adolescent idiopathic scoliosis in twins: a population-based survey. Spine (Phila Pa 1976). 2007 Apr 15;32(8):927-30.
36. Gorman KF, Julien C, Moreau A. The genetic epidemiology of idiopathic scoliosis. Eur Spine J. 2012 Oct;21(10):1905-19.
37. Cook SD, Harding AF, Morgan EL, Nicholson RJ, Thomas KA, Whitecloud TS, et al. Trabecular bone mineral density in idiopathic scoliosis. J Pediatr Orthop. 1987 Mar-Apr;7(2):168-74.
38. Zhu F, Qiu Y, Yeung HY, Lee KM, Cheng CY. Trabecular bone micro-architecture and bone mineral density in adolescent idiopathic and congenital scoliosis. Orthop Surg. 2009 Feb;1(1):78-83.
39. Cheng JC, Qin L, Cheung CS, Sher AH, Lee KM, Ng SW, et al. Generalized low areal and volumetric bone mineral density in adolescent idiopathic scoliosis. J Bone Miner Res. 2000 Aug;15(8):1587-95.
40. Yu WS, Chan KY, Yu FW, Ng BK, Lee KM, Qin L, et al. Bone structural and mechanical indices in Adolescent Idiopathic Scoliosis evaluated by high-resolution peripheral quantitative computed tomography (HR-pQCT). Bone. 2014 Jan 8;61.

41. Aebi M. The adult scoliosis. Eur Spine J. 2005 Dec;14(10):925-48.
42. Grubb SA, Lipscomb HJ, Coonrad RW. Degenerative adult onset scoliosis. Spine (Phila Pa 1976). 1988 Mar;13(3):241-5.
43. Kanis JA. Diagnosis of osteoporosis and assessment of fracture risk. Lancet. 2002 Jun 1;359(9321):1929-36.
44. Sigurdsson G, Halldorsson BV, Styrkarsdottir U, Kristjansson K, Stefansson K. Impact of genetics on low bone mass in adults. J Bone Miner Res. 2008 Oct;23(10):1584-90.
45. Michaelsson K, Melhus H, Ferm H, Ahlbom A, Pedersen NL. Genetic liability to fractures in the elderly. Arch Intern Med. 2005 Sep 12;165(16):1825-30.
46. Kemp JP, Medina-Gomez C, Estrada K, St Pourcain B, Heppe DH, Warrington NM, et al. Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment. PLoS Genet. 2014 Jun;10(6):e1004423.
47. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
48. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012 May;44(5):491-501.
49. Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. J Clin Invest. 2006 May;116(5):1202-9.
50. Boyce BF, Xing L. The RANKL/RANK/OPG pathway. Curr Osteoporos Rep. 2007 Sep;5(3):98-104.
51. Oei L, Hsu YH, Styrkarsdottir U, Eussen BH, de Klein A, Peters MJ, et al. A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus. J Med Genet. 2014 Feb;51(2):122-31.
52. Moayyeri A, Hsu YH, Karasik D, Estrada K, Xiao SM, Nielson C, et al. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Hum Mol Genet. 2014 Jun 1;23(11):3054-68.
53. Liu CT, Karasik D, Zhou Y, Hsu YH, Genant HK, Broe KE, et al. Heritability of prevalent vertebral fracture and volumetric bone mineral density and geometry at the lumbar spine in three generations of the Framingham study. J Bone Miner Res. 2012 Apr;27(4):954-8.
54. Oei L, Estrada K, Duncan EL, Christiansen C, Liu CT, Langdahl BL, et al. Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus. Bone. 2014 Feb;59:20-7.
55. Stankiewicz P, Sen P, Bhatt SS, Storer M, Xia Z, Bejjani BA, et al. Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. Am J Hum Genet. 2009 Jun;84(6):780-91.
56. Winnier GE, Hargett L, Hogan BL. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. Genes Dev. 1997 Apr 1;11(7):926-40.
57. Chu ML, Williams CJ, Pepe G, Hirsch JL, Prockop DJ, Ramirez F. Internal deletion in a collagen gene in a perinatal lethal form of osteogenesis imperfecta. Nature. 1983 Jul 7-13;304(5921):78-80.
58. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell. 2001 Nov 16;107(4):513-23.
59. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. N Engl J Med. 2013 May 9;368(19):1809-16.
60. Styrkarsdottir U, Thorleifsson G, Sulem P, Gudbjartsson DF, Sigurdsson A, Jonasdottir A, et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. Nature. 2013 May 23;497(7450):517-20.
61. van Dijk FS, Zillikens MC, Micha D, Riessland M, Marcelis CL, de Die-Smulders CE, et al. PLS3 mutations in X-linked osteoporosis with fractures. N Engl J Med. 2013 Oct 17;369(16):1529-36.
62. van Dijk FS, Byers PH, Dalgleish R, Malfait F, Maugeri A, Rohrbach M, et al. EMQN best practice guidelines for the laboratory diagnosis of osteogenesis imperfecta. Eur J Hum Genet. 2012 Jan;20(1):11-9.



Chapter 5

PERSONALIZED MEDICINE

Chapter 5.1

Personalized sequencing and the future of medicine: discovery, diagnosis and defeat of disease

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ABSTRACT

The potential for personalized sequencing to individually optimize medical treatment in diseases such as cancer and for pharmacogenomic application is just beginning to be realized, and the utility of sequencing healthy individuals for managing health is also being explored. The data produced requires additional advancements in interpretation of variants of unknown significance to maximize clinical benefit. Nevertheless, personalized sequencing, only recently applied to clinical medicine, has already been broadly applied to the discovery and study of disease. It is poised to enable the earlier and more accurate diagnosis of disease risk and occurrence, guide prevention and individualized intervention as well as facilitate monitoring of healthy and treated patients, and play a role in the prevention and recurrence of future disease. This article documents the advancing capacity of personalized sequencing, reviews its impact on disease-oriented scientific discovery and anticipates its role in the future of medicine.

INTRODUCTION

In the 10 years since the official completion of the Human Genome Project (HGP)[1] technological advances in the speed and scale of sequencing analysis have maintained an accelerating pace. The tools produced by these advances now enable holistic analysis of individual human genomes at a cost and within a timeframe to allow practical and productive application to research questions and, more recently, personalized clinical evaluations.

Targeted, single gene sequence analysis of individual patients has been a clinically applicable diagnostic tool since before completion of the Human Genome Project. However, such testing has always been limited to thoroughly characterized genes, for which a phenotype is recognizable and clinically certified testing is available. Current next-generation sequencing technologies, including whole-exome sequencing (WES) and whole-genome sequencing (WGS) now allow analyses beyond a handful of genes, to include a more comprehensive genetic analysis. These sequencing tools are being actively applied to well-studied, but as yet unconquered diseases such as cancer, where significant advances in understanding the pathophysiology, diagnostics, treatment and surveillance are likely to greatly benefit patients; they are also being applied to the analyses of unsolved diseases in children and adults, and more recently to the analyses of healthy individuals. It is expected that genetic information will play an ever increasingly important role in helping us to better predict, diagnose and treat diseases. Here, we present a perspective on how next-generation sequencing may change pharmacogenomics and medicine as a whole through discovery and treatment of many types of disease and personalized pharmacological intervention.

Cancer genome sequencing

Many of the most innovative tools of biomedical investigation have been based on understanding the various presentations of cancer. Accordingly, soon after the completion of the HGP and the advent of new sequencing technologies, genome sequencing was applied to the analysis of cancer. Cancer has long been recognized as being caused by acquisition of multiple genetic mutations, which are thought to 'drive' cells toward uncontrollable growth. Studies have described driver gene versus passenger gene mutations in many forms of cancer[2]. Driver gene mutations are classically defined as mutations that, when they occur in a cell, confer a selective growth advantage and drive the cell's progression to malignancy[3]. Some driver mutations are inherited at birth (e.g., *APC* and mutations) whereas others are acquired somatically and may be heavily influenced by environmental exposure.

Personalized sequencing impacts cancer in several ways. The first is cancer cell DNA sequencing. One of the first genomic studies to apply WGS to cancer involved analysis of the DNA of an acute myeloid leukemia patient in which both tumor and normal cells were sequenced.[4] Ten mutations were identified in the tumor DNA and not in the normal DNA. Two of these had been previously described as linked to acute myeloid leukemia and the remaining eight were novel. This proof of principle study put forward WES/WGS as a tool to discover novel mutations and potential therapeutic targets. This effort has now been expanded on a very large scale. One large project is The Cancer Genome Atlas (TCGA), which is systematically analyzing WGS and WES of more than 20 types of human cancer.[5] One of the biggest outcomes of these efforts is the discovery that most cancers are very different from one other, although common mutated pathways can often be observed. For example, in ovarian cancer patients mutations in the *BRCA1* and the *BRCA2* pathways, affecting homologous recombination, are frequently observed. Moreover, cancer from different tissues of origin can often have the same types of mutations. For example, the *EGFR* gene, previously known to be commonly mutated in breast cancer patients, is

often amplified or mutated in other cancer types. As such, cancers are now being classified based on their genetic changes rather than their tissue of origin.

Another active area in the pathophysiology of cancer is the clonal evolution theory of cancer. In 1976 Peter Nowell posited that cancer develops as differently mutated clonal cells out-compete each other, with the expectation that less fit variant clones die, leaving one clone to comprise the majority of a tumor.[6] However, genomic analyses in recent years have demonstrated that for many cases, there is a significant level of genetic diversity within single tumors, suggesting that tumors are more mosaic, rather than being dominated by a predominant clone.[7] These observations have raised the profile of several aspects of tumoral genetic heterogeneity, and the role heterogeneity plays in diagnosis and treatment of cancer. For example, intratumoral, intermetastatic, intrametastatic and interpatient tumor heterogeneity each impact our efforts to achieve early diagnosis and successful therapeutic intervention.[3]

New methods have emerged that use DNA sequencing to monitor cancer progression. Tumor DNA sequencing is rapidly expanding its capacity to produce a clinically relevant tumor profile. This is currently focused on somatic DNA variations, but there is growing effort to analyze RNA expression and DNA methylation patterns. Such information can help determine which signaling pathways are active in tumor cells, which may not have been suggested by histological assessment alone, and thereby suggests therapeutic avenues that would not be uncovered by conventional methods.[8]

Cancer sequencing treatment implications

Personalized tumor DNA sequencing can directly impact treatment by identifying mutations that can suggest therapeutic treatments. In some cases the information from DNA sequencing can identify a known cancer target or pathway for which an existing pharmacological treatment is available (often initially used for a cancer involving a different tissue) and sometimes even new potential targets are uncovered. For example, researchers recently found through WES, a loss of function mutation in *TSC1* in approximately 5% of advanced bladder cancers. This specific mutation correlated with tumor sensitivity to everolimus, suggesting that this subgroup of bladder cancer patients might benefit from everolimus therapy.[9] Other examples of genome sequencing based clinical interventions include utilization of EGFR kinase inhibitors in cancers with *EGFR* gene mutations (found in many different types of cancers), and BRAF inhibitors in tumors with *BRAF* mutations (often found in melanomas).[10, 11] In these situations application of pharmacogenomic principles to individual tumors is critical to determine their susceptibility to these specific drug therapies, as only a fraction of patients will respond to these targeted therapies and treating patients prior to confirming their tumor's sensitivity would expose patients to drug side effects

while allowing their cancers to advance.[3] For example, identification of KRAS alterations in codons 12 or 13, which occurs in approximately 30% of colon cancer patients, suggest some toxicity risk and no particular treatment benefit with EGFR specific antibodies.[12]

Despite these advances with clear impact on current patient care, tumor somatic mutation assessment has impacted clinical intervention for a limited number of cancers. Currently, less than 10% of oncology drugs approved by the US FDA have documented molecular

predictors of efficacy, and there is tremendous potential for progress in this area.[8]

Even as sequencing has led to these advances in cancer therapy, new targets are emerging, such as within the pathways of tumor suppressor genes, which individually can be difficult to impact therapeutically. For example, *BRCA1* and *BRCA2* gene defects impact

downstream DNA repair pathways and make cells more susceptible to drugs that inhibit repair of DNA damage, such as PARP, and clinical trials with this strategy are in underway.[13] Individual cancer genomes have been noted to contain highly variable numbers (often 30–70 mutations) in coded proteins. Each of these changes is foreign to the native immune system and exploiting these changes has been suggested to lead to the development of highly tumor-specific antigens as a powerful tool for cancer directed therapies.[8] This is one example of advances in the development of molecularly targeted cancer therapies enabled by genomic sequencing. This is especially important in the context of the substantial problem of cancer drug resistance, in which resistance causing events in tumors appear to be selected for in a Darwinian fashion.[14, 15] The mechanism of cancer drug resistance can be influenced by genetic and histological background of the tumor as well as previously applied treatments.[14, 16] One of the strategies to combat drug resistance may eventually include using simultaneous drug combinations.[14, 17]

Cancer pharmacogenomics

Another major area of impact of cancer genome sequencing is germline sequencing. Germline sequencing enables the estimation of underlying patient risk arising from known alterations causing characterized syndromes of cancer predisposition, such as familial adenomatous polyposis or Li Fraumeni syndrome, which facilitates implementation of prophylactic interventions and screening protocols to optimize early detection. Familial predisposition is estimated to account for up to 10% of melanoma, breast, colon and gastric cancers and up to 25% of ovarian cancer. Among these, testing is available to identify known predisposing genes in approximately 2–3% of colon cancer, 3–5% of gastric cancer, 5–10% of breast cancer, up to 10% of melanoma and up to 25% of endometrial cancer.[18-23] Germline testing can potentially impact an estimated 40,000 new cases of these types of cancer alone,[24] in addition to the thousands of family members who benefit from germline sequencing by finding they do not carry the genetic predisposition. While germline sequencing currently impacts a minority of cancer, it is clear that significant potential remains for personal sequencing to discover novel genetic etiologies that account for the thousands of familial and individual cases of cancer for which a molecular etiology remains unclear. For example, WGS and WES analysis of individuals with pancreatic cancer identified segregating variants of the *ATM* gene, implicating it as a pancreatic cancer predisposition gene.[25] WGS of patients with multiple adenomas and/or colorectal adenocarcinoma (CRC) found mutations in *POLE* and *POLD*, identifying them as CRC susceptibility genes.[26]

These same technologies are being applied to address pharmacogenomic issues, such as the etiology of chemotherapeutic failure, clinical side effects, drug metabolism or drug resistance. The identification of a germline variant of *TPMT* was found to result in life-threatening toxicity in patients treated with mercaptopurine (a treatment for acute lymphoblastic leukemia). This led the FDA to recommend genotyping of patients prior to treatment, and reduce the dosage for those with appropriate genotypes.[27] The FDA currently recommends genotyping prior to treatment with other chemotherapeutics, such as irinotecan for CRC.[28] Patient-specific drug metabolism can also lead to inadequate dosing, as in the case of tamoxifen for estrogen receptor positive breast carcinoma. Tamoxifen is metabolized into multiple metabolites, including endoxifen, which is central to treatment efficacy. Germline patient sequencing found variants in the *CYP2D6* gene, which are associated with lower serum concentrations of endoxifen due to decreased enzyme activity and lead to risk of drug failure due to inadequate dosing.[29] In addition to the clear potential for cancers to mutate and develop resistance to particular chemotherapeutic interventions,[8] germline sequence analysis has found patients whose tumors are inherently resistant. For example, a study of chronic myeloid leukemia patients found a deletion of BCL2-like (also known as

BIM) in patients whose cancer treatment was resistant to tyrosine kinase inhibitors. Further analyses confirmed presence of the BIM deletion in the germline of the patients resistant to treatment, and that the proapoptotic domain affected by the deletion was the mechanism of the patients' inherent resistance to tyrosine kinase inhibitor therapeutics.[28] Each of these circumstances demonstrates the increasingly important role of pretreatment, germline personalized sequencing in successful cancer intervention.

Although the benefit of genetic information is clear, the overall impact of these advances on patient care currently remains limited. To date, approximately 40 FDA approved oncology drugs (Table 1) have been updated to include clinically relevant pharmacogenomics information in their package inserts.[30] Similarly, only approximately 40 known cancer genes have FDA approved drugs, some with multiple drugs per gene target. However, greater than 30 additional cancer genes have experimental drugs under development, which will greatly enhance the impact of genome sequencing in the future.[31]

Cancer sequencing & surveillance

Following intervention for a diagnosed malignancy, such as CRC, personalized sequencing will likely also play a role in guiding ongoing surveillance for recurrence. With one WGS costing the approximate equivalent of one to two colonoscopies,[32] post-treatment identification of known genetics susceptibilities can inform individualized management and follow-up protocols. An area of promise for future monitoring of remission and recurrence is that of cell-free DNA (cfDNA) sequencing through which it is possible to detect cancer mutations in body fluids such as blood, urine and stool, which enables monitoring of response to treatment and tumor evolution.[33] Developed and clinically applied in the realm of prenatal diagnostics, the analysis of cell-free fetal DNA, as collected from maternal plasma collection, has recently lead to noninvasive WGS of a fetus at 18.5 weeks gestation, enabled in part by the capacity to distinguish maternal from fetal DNA sequence.[34] The observation of significant levels of tumor DNA in the blood of patients with cancer has led to the idea of monitoring tumor cfDNA levels as a marker of disease.[35] The presence of tumor specific mutations offers an additional opportunity for cfDNA analysis, with post-treatment sequencing of cfDNA having the potential to identify those mutations unique to the eradicated primary tumor, as a marker of remission and sentinel indicator of recurrence.[35] In light of the advances made in cfDNA analysis in the prenatal realm to the point of WGS of an actively growing fetus, it seems reasonable to envision a future ability to obtain WGS of a patient tumor at an early stage of recurrence, as a noninvasive form of surveillance that potentiates early re-intervention. It is crucial to emphasize here that surveillance strategies require tests with high sensitivity and specificity, the establishment of which will require large randomized control trials. This is necessary to avoid the potential harm from false positives or ambiguous results. Overall, this could lead personalized sequencing to become an integral part of the full spectrum of clinical cancer care, including risk assessment, prevention, disease screening and diagnostics, personalized pharmacogenomic-based therapy, and post-therapy surveillance (Figure 1).

Sequencing the unknown: rare diseases

Multiple genome sequencing studies have already uncovered novel relationships for genetic variants with monogenetic Mendelian disorders and complex diseases.[36-41] Approximately 7000 well-defined Mendelian disorders are currently known, of which the corresponding allelic variants underlying fewer than half of these monogenic disorders have been discovered, and the etiology of many monogenic diseases is still unknown.[41, 42] Furthermore, genome sequencing enables us to decipher the causes and even guide treatment of an evergrowing number of "mystery" diseases, of which many cluster in families

Table 1. US FDA-approved oncology drugs with package inserts containing pharmacogenetics and pharmacogenomics information.

Drug	Pharmacogenomic biomarker(s)
Ado-trastuzumab emtansine	ERBB2
Afatinib	EGFR
Anastrozole	ESR1, PGR
Arsenic trioxide	PML/RARA
Bosutinib	BCR/ABL1
Brentuximab vedotin	TNFRSF8
Busulfan	Ph chromosome
Capecitabine	DPYD
Cetuximab	EGFR, KRAS
Cisplatin	TPMT
Crizotinib	ALK
Dabrafenib	BRAF, G6PD
Dasatinib	BCR/ABL1
Denileukin diftitox	IL2RA
Erlotinib	EGFR
Everolimus	ERBB2, ESR1
Exemestane	ESR1
Fluorouracil	DPYD
Fulvestrant	ESR1
Ibritumomab tiuxetan	MS4A1
Imatinib	KIT, BCR/ABL1, PDGFRB, FIP1L1/PDGFRA
Irinotecan	UGT1A1
Lapatinib	ERBB2
Letrozole	ESR1, PGR
Mercaptopurine	TPMT
Nilotinib	BCR/ABL1, UGT1A1
Obinutuzumab	MS4A1
Ofatumumab	MS4A1
Omacetaxine	BCR/ABL1
Panitumumab	EGFR, KRAS
Pazopanib	UGT1A1
Pertuzumab	ERBB2
Ponatinib	BCR-ABL T315I
Rasburicase	G6PD
Rituximab	MS4A1
Tamoxifen	ESR1, PGR, F5, F2
Thioguanine	TPMT
Tositumomab	MS4A1
Trametinib	BRAF
Trastuzumab	ERBB2
Tretinoin	PML/RARA
Vemurafenib	BRAF

but can also involve individual probands, such as Charcot–Marie–Tooth neuropathy, Miller's syndrome, and dopa (3,4-dihydroxyphenylalanine)-responsive dystonia.[36, 40, 41] Sequencing has shown the potential

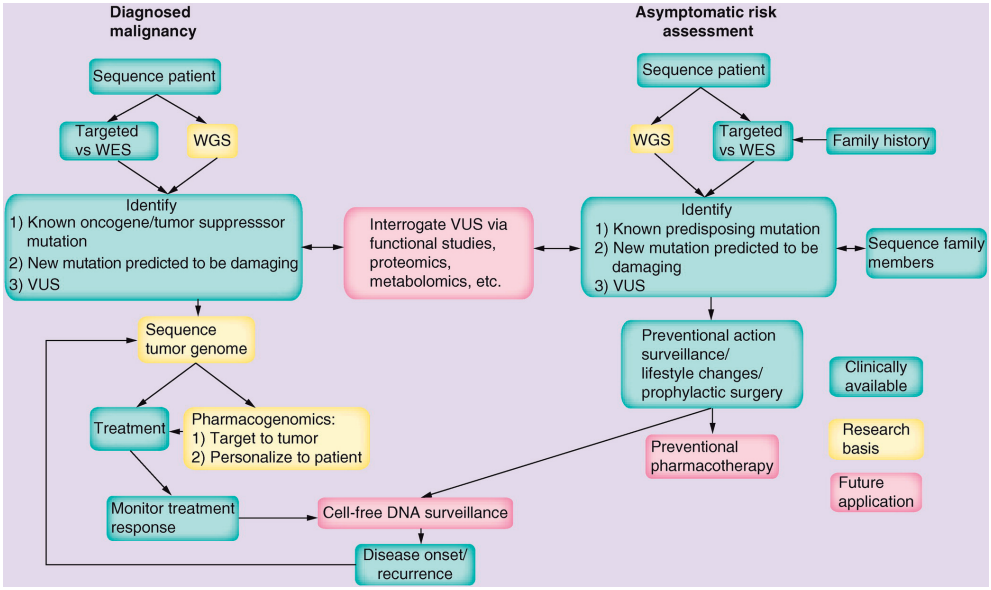

Figure 1. Personalized sequencing in cancer: current and future medical applications. This includes applications that are currently clinically available, those applied in research protocols and those envisioned for the future, such as preventional management. WES: Whole-exome sequencing; WGS: Whole-genome sequencing; VUS: Variants of unknown significance.

to provide a solution in cases where there is an initial inability to make a clinical diagnosis of the disease and in rare cases has been shown to subsequently direct a course of treatment.[43]

A notable example was reported by Worthey et al. A male infant presented with proctitis. This progressed to pancolitis, which was concerning for a Crohn's disease-like affliction. The severity of disease was suggestive of underlying immune dysfunction, however, substantial clinical evaluation was unable to determine a definitive diagnosis. Utilization of WES in this patient identified over 16,000 variants which, after further analysis observed a novel mutation in the X-linked inhibitor of the apoptosis gene, not previously connected with Crohn's disease but known to be involved in the proinflammatory response.[44] Functional analyses confirmed the deleterious nature of the mutation and the diagnosis of X-linked inhibitor of apoptosis deficiency. Indicated treatment was hematopoetic cell transplant, after which the patient experienced resolution of the symptoms of colitis.[44] Another example is described by Bainbridge et al., in two fraternal twins afflicted by clinical symptoms of dystonia whose diagnostic evaluation was unrevealing until one of the twins experienced symptomatic improvement with L-dopa treatment, at which time they were diagnosed with dopa-responsive dystonia (DRD), based on this clinical response. Even with L-dopa treatment, the patients continued to experience a combination of mild tremor, dystonic posturing, unsteady gait, dysphonia and bradykinesia.[36] The twins' DNA, as well as their parents and an unaffected sibling, were subjected to WGS and, after shared mutation analysis, filtering and genetic annotation, three genes with significant nonsynonymous mutations were found, one of which, *SPR*, had been previously associated with DRD. *SPR* encodes an enzyme important to the generation of BH4, a cofactor for dopamine and serotonin. Functional studies confirmed the deleterious impact of the compound heterozygous *SPR* mutations found in the patients, and their treatment was modified to include a serotonin precursor, which is recommended in patients with DRD due to *SPR* mutations.

Two weeks after therapeutic modification, the patients both experienced symptomatic improvement including increased ability to participate in athletic activities at school.[36] Though a minority of cases result in successful treatment interventions, even a diagnosis without a current therapy provides a family with important information regarding a patient's prognosis, medical management and allows for informed family planning.

Challenges of sequence interpretation

Despite these clear successes, many challenges make these successes less frequent than is desirable. In one of the above examples, multiple individuals in addition to the probands underwent WGS to facilitate filtering of the thousands of identified variants, which are not relevant to the clinical question in the proband. However in many clinical scenarios, only a single proband is under evaluation and, even with sequencing of both of the proband's parents, thousands of variants will segregate in a fashion that makes it difficult to unequivocally identify the causative variant. Filtering of identified variants is also dependent on the clarity of the phenotype, as candidate gene lists are developed based on known disease gene associations. If a patient's phenotype is too broadly defined or nonspecific, then the identification of likely candidate genes, from thousands of sequence variants, is significantly complicated. The clarity of a patient's phenotype may also be difficult to describe in the setting of monogenic conditions with decreased penetrance or variable expressivity. Finally, once a promising candidate is distilled from the filtering process, there is no standardized approach to functionally verify that the causative genetic mutation has been ascertained. In the examples noted, in vitro functional analyses were performed to obtain supportive evidence, though the true confirmation of the diagnosis was observed in the patients' response to genomic sequence based treatment. Unfortunately, there remain many genetic diseases without a known treatment, for which these means of confirmation is unavailable.

Sequencing & the potential for discovery

Nevertheless, the application of WES/WGS appears well suited to the elucidation of genetic diseases of Mendelian inheritance, as outlined in Figure 2. It is a powerful approach to the discovery of novel causative genes underlying Mendelian disorders where conventional strategies have failed. Even in conditions where conventional approaches are expected to find the genetic etiology, WES/WGS provides a means to accelerate discovery.[45] WES in particular is anticipated to accelerate the discovery of genes causing rare Mendelian disorders as: many known alleles of these conditions disrupt protein-coding sequences; a large fraction of rare protein impacting variants are predicted to have deleterious effects; and the exome represents an enriched genomic subset in which to search for these alterations with large effect sizes providing the opportunity to capture nearly all of the protein-coding gene rare alleles present in a sample.[45] This includes diagnostic application to pediatric patients with rare diseases, like the examples already described. There is also potential to impact other inherited disease, such as the wide range of inherited cardiovascular diseases. Nonsyndromic cardiomyopathies such as dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia and left ventricular noncompaction, among others, have been attributed to mutations in over 40 genes.[46] Among these known cardiomyopathy genes, it is estimated that the specific genetic cause is identified in as many as 65% of familial hypertrophic cardiomyopathy cases, 50% of arrhythmogenic right ventricular dysplasia cases and 30% of DCM cases; however, lower identification rates are achieved in sporadic cases. Of the remaining undiagnosed cases of familial cardiomyopathy, application of WES/WGS has identified rare variants, for example, in the DCM gene *TTN*, with 25% of familial cases demonstrating

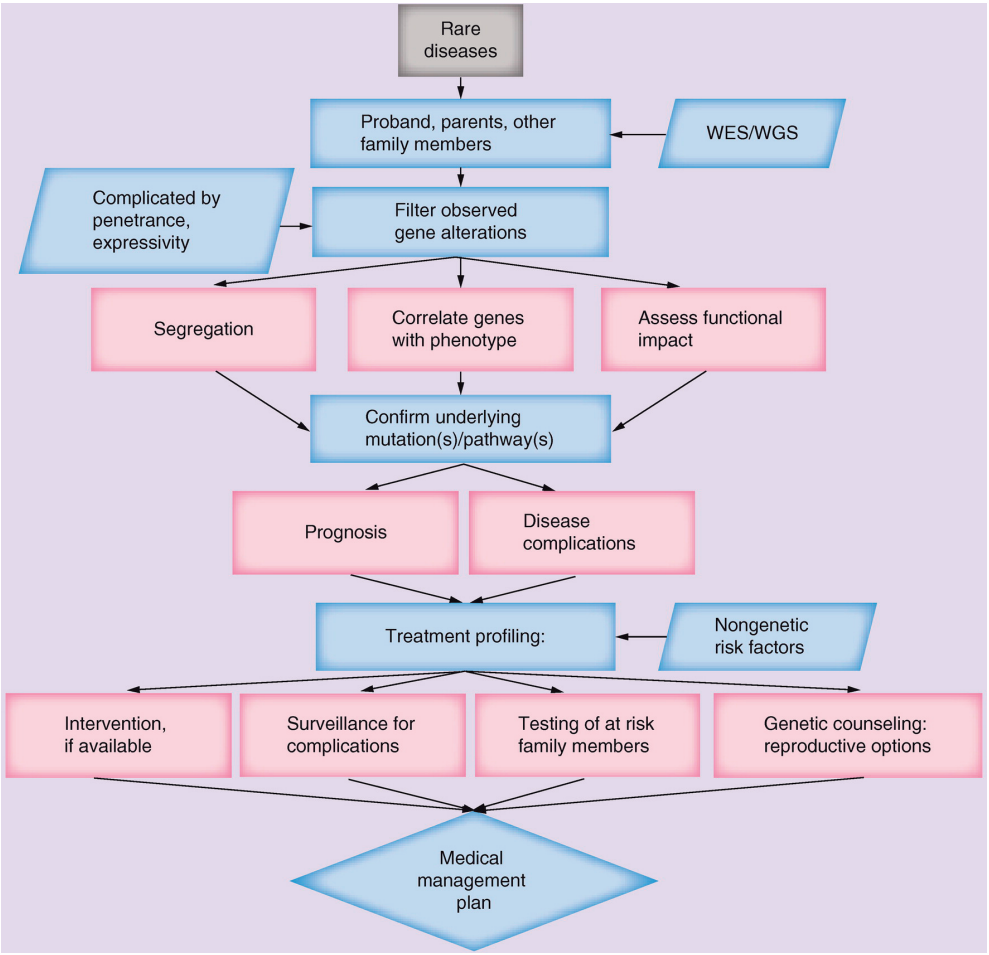

Figure 2. Investigation of rare diseases: potential and pitfalls. As the obstacles facing WES/WGS in the diagnosis of rare diseases are overcome, they can ultimately guide medical management. WES: Whole-exome sequencing; WGS: Whole-genome sequencing.

potentially causative variants.[46] With such an extensive, and growing, collection of genes accounting for the various inherited cardiomyopathies, many of which overlap between categories, personalized sequencing is a promising tool for streamlining of diagnostics, via both WES/WGS as well as targeted sequencing panels. It also demonstrates ongoing promise in delineating the underlying rare variants responsible for those cases of heritable cardiomyopathy yet to be elucidated.

Clinical personalized sequencing

The process of performing WES began to be offered on a clinical basis in 2011. There are currently a number of CLIA-certified laboratories that offer WES as a diagnostic test for patients with a phenotype for which an underlying molecular etiology has not yet been defined. While WES can be performed on the patient only, various sites offer, and recommend, testing of family trios, including the patient

and both parents, to facilitate subsequent sequence interpretation. In all cases of clinically available WES, identified mutations thought to be of clinical relevance are confirmed via Sanger sequencing prior to being reported. The output of clinical WES includes disruptive mutations to which the patient's phenotype is attributable, mutations which appear unrelated to the observed phenotype, and variants of unknown significance (VUS).

There can also be mutations and incidental findings discovered during WGS and WES studies that predispose to conditions unrelated to the original indication, the reporting of which has been addressed by the American College of Medical Genetics and Genomics (ACMG) and remains a subject of ongoing evaluation.[47] Though early estimates of the success of WES/WGS in diagnosing rare disease have been as high as 50%,[48] recent studies suggest the success rate to be closer to 20–30%,[49, 50] with chief obstacles being efficient and accurate clinical interpretation of the genomic variants[51] and the fact that many genes have yet to be associated with a specific disorder, an obstacle WES itself will help to overcome. Nevertheless, it is probably just a matter of time until pharmacogenomic sequencing studies experience similar successful discoveries in the realm of rare Mendelian diseases.

Sequencing the "healthy"

Future steps will involve integration of established disease variants into clinical decision-making for asymptomatic, healthy individuals. Several pilot projects have been published where sequenced genomes from single individuals were annotated for known genetic risk factors.[52-54] The Varimed database, which contains published knowledge on hundreds of thousands of genetic variations in relation to thousands of traits, formed the reference for annotation with the Risk-OGram algorithm.[53] Other examples of annotation databases are: Online Mendelian Inheritance in Man (OMIM),[55, 56] the Human Gene Mutation Database (HGMD),[57, 58] NCBI ClinVar[55, 59] the European Genome-phenome Archive,[60] dbGaP[61, 62] and the GWAS catalog[63, 64]. Ashley et al. provided the first example where a patient with a family history of vascular disease and early sudden death was clinically assessed, including the patient's full genome sequence, to provide risk prediction for coronary artery disease and screening for causes of sudden cardiac death.[52] The 'Snyderome' paper revealed that genome sequencing can be used to assess various medical risks, direct the monitoring of specific diseases (in this study, aplastic anemia and Type 2 diabetes) and successfully guide lifestyle interventions and pharmacotherapy.[53] The subject carried a *TERT* mutation, predicted to be damaging, which has been associated with aplastic anemia.[65] However, measurements of telomere length suggested little or no decrease in telomere length and a modest increase in numbers of cells with short telomeres. Importantly, the patient and his mother share the same mutation but neither exhibit symptoms of aplastic anemia, indicating that this mutation does not always result in disease and is likely context specific in its effects. This illustrates that previously reported statistically significant associations of genetic variants with diseases may have imperfect positive predictive values. The subject was predicted to have significantly elevated risk levels for hypertriglyceridemia and diabetes, including associated variants in *GCKR* (homozygous),[66]*KCNJ11* (homozygous)[67] and *TCF7* (heterozygous)[68]. Consistent with the elevated hypertriglyceridemia risk, triglycerides were found to be high (321 mg/dl) at the beginning of the study and these levels were reduced (81–116 mg/dl) after regularly taking simvastatin (20 mg/day). Although the subject lacked many known factors associated with diabetes (nonsmoker, normal BMI) and for that reason usually would not have been screened, monitoring of glucose levels and glycated hemoglobin revealed the onset of Type 2 diabetes during study follow-up as diagnosed by the subject's physician. Interestingly, the participant possessed two genotypes in the *LPIN1* and *SLC22A1* genes associated with favorable responses to two diabetic drugs

(rosiglitazone and metformin). Nonetheless, after dramatic changes in diet, exercise and ingestion of low doses of acetylsalicylic acid, gradual decreases in glucose and glycated hemoglobin levels were observed and no auxiliary pharmacological agents were prescribed.

Pharmacogenomic sequencing

In addition to drugs relevant to diabetes, the subject described by Chen et al. had pharmacogenomic variants such as that of *VKORC1* (C/T) associated with a low maintenance dose of warfarin and CYP2C19, which has been associated with increased risk of bleeding on standard doses of clopidogrel. There were also variants associated with slow metabolism of codeine, increased risk of neurological adverse events and Stevens–Johnson syndrome with carbamazepine and increased risk of adverse effects with methotrexate, among others.[53] The subject described by Ashley et al. similarly carried the *VKORC1* variant (C/T) for low warfarin maintenance dose, and variants in *CYP4F2* associated with reduced warfarin dosing, *ADRB1* suggesting favorable response to atenolol, *HMGCR* associated with favorable response to statins, and *CDKN2A/B* suggesting reduced likelihood of response to metformin and troglitazone, among others.[52] In each case, these findings could impact the choice or dosing of medications in these individuals, should any of the impacted drugs be indicated in future medical management. In both cases, the pharmacogenomics variants were annotated based on the Pharmacogenomics Knowledge Base (PharmGKB), a publicly available web-based knowledge base.[69] It contains data from approximately 2,500 variants, from which approximately 650 are specifically related to drug response phenotypes, each of which are assigned levels of evidence through literature review by database curators.[52] It represents one of the most up to date sources of human genetic variation as relevant to drug response. There are a number of databases accumulating pharmacogenomic information, including PharmaADME,[70, 71] the human cytochrome P450 (CYP) allele nomenclature website,[70, 72] the human arylamine N-acetyltransferase (NAT) gene nomenclature website,[70, 73] Pharmacogenetics of Membrane Transporters (PMT) database,[70, 74] Transporter Database (TP-search),[70, 75] the UDP-glucuronosyltransferase (UGT) Allele Nomenclature Page,[70, 76] and PACdb,[77, 78] among others. The information compiled by these and other sources is anticipated to play an ever growing role in guiding patient care in conjunction with personal sequencing. While these pilot studies were performed in ostensibly healthy individuals, similar sequence analysis has clear potential relevance in individuals for whom any one of the above mentioned drugs may be indicated for a known medical condition.

The described pilots in single individuals should be replicated in greater numbers, potentially leading the way towards more specific upstream screening for risk factors and diseases. Additional genetic variants are known and have been validated to be of potential clinical relevance, such as the Val174Ala allele in the *SLCO1B1* gene for statin-induced myopathy.[79, 80] *HLA B*5701* has been associated with slow or nonprogression of HIV infection and with hypersensitivity reactions to abacavir.[81-83] Therefore, most treatment guidelines recommend that upon considering administration of abacavir, patients should be tested for the presence of this allele, and that those who are positive should not receive the drug. Since the widespread introduction of *HLA B*5701* testing, the incidence of hypersensitivity reactions in those receiving abacavir has dropped substantially.[84] Recently, it was shown in a large cohort that *HLA B*5701*-positive patients were more likely to achieve viral suppression than negative patients on a nonabacavir regimen and less likely to experience viral rebound.[85] Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines have been published regarding the use of *CYP2D6* and *CYP2C19* genotyping test results to modify patient dosing of tricyclic antidepressants, such as amitriptyline and nortriptyline.[86] The effect of an individual patient's CYP genotype on metabolism of these tricyclic agents can be taken

into consideration at initial dosing, in an effort to maximize the efficacy in utilizing these medications for such indications as depression, obsessive compulsive disorder, migraine prophylaxis and neuropathic pain management while minimizing the associated anticholinergic, CNS and cardiac adverse effects.[86] With these and other examples of pharmacogenomic indications, the FDA currently includes pharmacogenomic information in the drug labels of approximately 100 approved drugs (Table 2), in addition to the 40 oncology drugs mentioned previously (Table 1).

Information derived from personalized genome sequencing could point to undeveloped or concealed monogenic/oligogenic phenotypes where lifestyle and pharmacological interventions may minimize disease risks and future complications. Additionally, genome sequencing may provide evidence supporting the elevated risk for, or diagnosis of, complex disorders such as Alzheimer's disease and prostate cancer, where genome sequencing has contributed novel findings.[37-39] Where multiple pharmacological options are available, the pharmacogenomics profile may guide more effective and less deleterious treatment decisions. To this end are more pharmacogenomic studies needed, as the associated variants form the basis for prediction of treatment response. It may become of increasing interest to invest in drug-response sequencing studies within clinical trials. Though it may seem unattractive to the pharmaceutical industry to identify those individuals genetically prone to adverse effects, this information can provide opportunities for the development of drugs with application to a broader population and drugs uniquely effective for significant individual cohorts, both in terms of tolerance and drug metabolism related individualized dosing.

Personal sequencing & family history

The patient interest and demand for personal sequencing seems poised to grow, for example in those individuals with a known family history of a particular condition as well as healthy, proactive individuals curious to learn about their genomic health and corresponding disease risks. Indeed on-demand genetic testing has already been commercially available for some time, including personal sequencing. As such, we anticipate increasing examples, such as those described above, of sequence analysis in individuals with subclinical or nondisease status, some having traditional risk factors (such as family history), which could contribute to improved prediction of who will not and who will ultimately develop disease and allow preventive measures to potentially avert disease in some (Figure 3).

Table 2. US FDA-approved drugs with package inserts containing pharmacogenetics and pharmacogenomics information.

Drug	Disease type	Pharmacogenomic biomarker(s)
Abacavir	Infectious diseases	HLA-B
Amitriptyline	Psychiatry	CYP2D6
Aripiprazole	Psychiatry	CYP2D6
Atomoxetine	Psychiatry	CYP2D6
Atorvastatin	Endocrinology	LDLR
Azathioprine	Rheumatology	TPMT
Belimumab	Autoimmune diseases	BTG3
Boceprevir	Infectious diseases	IFNL3
Carbamazepine	Neurology	HLA-B, HLA-A
Carglumic acid	Metabolic disorders	NAGS
Carisoprodol	Rheumatology	CYP2C19

Table 2. US FDA-approved drugs with package inserts containing pharmacogenetics and pharmacogenomics information. (continued)

Drug	Disease type	Pharmacogenomic biomarker(s)
Carvedilol	Cardiology	CYP2D6
Celecoxib	Rheumatology	CYP2C9
Cevimeline	Dermatology	CYP2D6
Chloroquine	Infectious diseases	G6PD
Chlorpropamide	Endocrinology	G6PD
Citalopram	Psychiatry	CYP2C19, CYP2D6
Clobazam	Neurology	CYP2C19
Clomipramine	Psychiatry	CYP2D6
Clopidogrel	Cardiology	CYP2C19
Clozapine	Psychiatry	CYP2D6
Codeine	Anesthesiology	CYP2D6
Dapsone	Dermatology, infectious diseases	G6PD
Desipramine	Psychiatry	CYP2D6
Dexlansoprazole	Gastroenterology	CYP2C19, CYP1A2
Dextromethorphan and quinidine	Neurology	CYP2D6
Diazepam	Psychiatry	CYP2C19
Doxepin	Psychiatry	CYP2D6
Drospirenone and ethinyl estradiol	Neurology	CYP2C19
Eltrombopag	Hematology	F5, SERPINC1
Esomeprazole	Gastroenterology	CYP2C19
Fluorouracil	Dermatology	DPYD
Fluoxetine	Psychiatry	CYP2D6
Flurbiprofen	Rheumatology	CYP2C9
Fluvoxamine	Psychiatry	CYP2D6
Galantamine	Neurology	CYP2D6
Glimepiride	Endocrinology	G6PD
Glipizide	Endocrinology	G6PD
Glyburide	Endocrinology	G6PD
Iloperidone	Psychiatry	CYP2D6
Imipramine	Psychiatry	CYP2D6
Indacaterol	Pulmonary	UGT1A1
Isosorbide and hydralazine	Cardiology	NAT1–2
Ivacaftor	Pulmonary	CFTR
Lansoprazole	Gastroenterology	CYP2C19
Lenalidomide	Hematology	del (5q)
Lomitapide	Endocrinology	LDLR
Mafenide	Infectious diseases	G6PD
Maraviroc	Infectious diseases	CCR5
Methylene blue	Hematology	G6PD
Metoclopramide	Gastroentrology	CYB5R1–4
Metoprolol	Cardiology	CYP2D6
Mipomersen	Endocrinology	LDLR
Modafinil	Psychiatry	CYP2D6
Mycophenolic acid	Transplantation	HPRT1
Nalidixic acid	Infectious diseases	G6PD
Nefazodone	Psychiatry	CYP2D6

Table 2. US FDA-approved drugs with package inserts containing pharmacogenetics and pharmacogenomics information. (continued)

Drug	Disease type	Pharmacogenomic biomarker(s)
Nitrofurantoin	Infectious diseases	G6PD
Nortriptyline	Psychiatry	CYP2D6
Omeprazole	Gastroenterology	CYP2C19
Pantoprazole	Gastroenterology	CYP2C19
Paroxetine	Psychiatry	CYP2D6
PEG-3350	Gastroenterology	G6PD
Peginterferon alfa-2b	Infectious diseases	IFNL3
Pegloticase	Rheumatology	G6PD
Perphenazine	Psychiatry	CYP2D6
Phenytoin	Neurology	HLA-B
Pimozide	Psychiatry	CYP2D6
Prasugrel	Cardiology	CYP2C19
Pravastatin	Endocrinology	LDLR
Primaquine	Infectious diseases	G6PD
Propafenone	Cardiology	CYP2D6
Propranolol	Cardiology	CYP2D6
Protriptyline	Psychiatry	CYP2D6
Quinidine	Cardiology	CYP2D6
Quinine sulfate	Infectious diseases	G6PD
Rabeprazole	Gastroenterology	CYP2C19
Rifampin, isoniazid and pyrazinamide	Infectious diseases	NAT1–2
Risperidone	Psychiatry	CYP2D6
Rosuvastatin	Endocrinology	LDLR
Simeprevir	Infectious diseases	IFNL3
Sodium nitrite	Antidotal therapy	G6PD
Sofosbuvir	Infectious diseases	IFNL3
Succimer	Hematology	G6PD
Sulfamethoxazole and trimethoprim	Infectious diseases	G6PD
Telaprevir	Infectious diseases	IFNL3
Terbinafine	Infectious diseases	CYP2D6
Tetrabenazine	Neurology	CYP2D6
Thioridazine	Psychiatry	CYP2D6
Ticagrelor	Cardiology	CYP2C19
Tolterodine	Genitourinary	CYP2D6
Tramadol	Analgesic	CYP2D6
Trimipramine	Psychiatry	CYP2D6
Valproic acid	Neurology	POLG, NAGS, CPS1, ASS1, OTC, ASL, ABL2
Velaglucerase alfa	Metabolic disorders	GBA
Venlafaxine	Psychiatry	CYP2D6
Voriconazole	Infectious diseases	CYP2C19
Vortioxetine	Neurology	CYP2D6
Warfarin	Cardiology, hematology	CYP2C9, VKORC1, PROC

F2: Prothrombin; F5: Factor V Leiden. Data taken from [104].

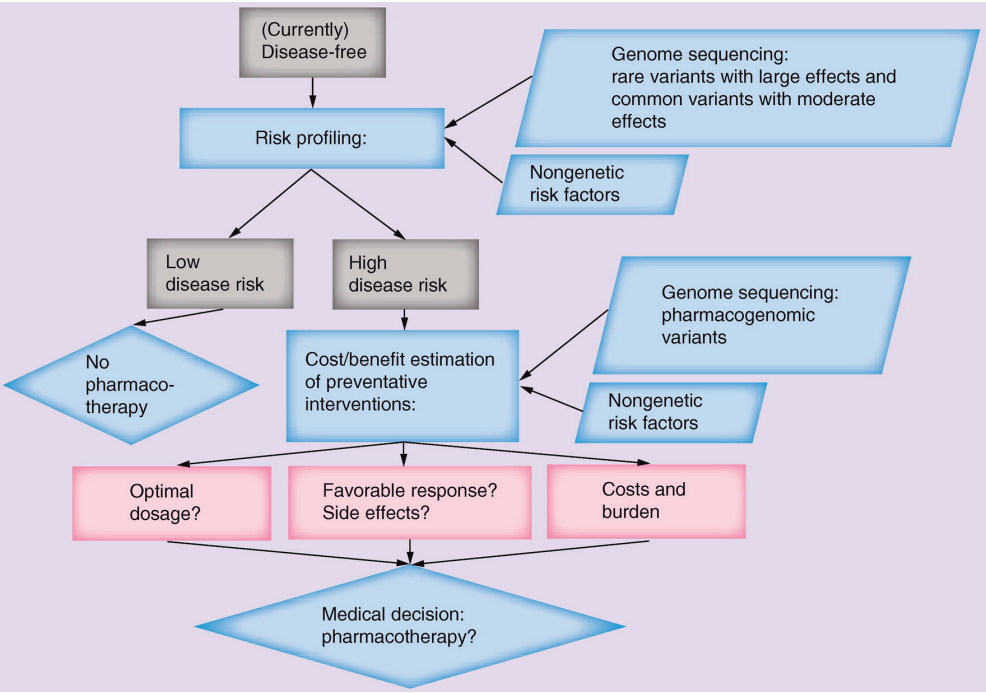

Figure 3. Profiling rare diseases. Issues and pipeline for personal genome sequencing in disease risk profiling of the "healthy" state.

Understandably, there remains a significant amount of trepidation in the clinical community regarding genome sequencing in individuals as an adjunctive screen for risk of various types of disease. However, information obtained via a family history has long been an accepted and critical part of an individual's clinical evaluation as a potent predictor of risk for certain diseases. WES/WGS represents a potentially more accurate means of determining what portion of the family history is specifically relevant to a particular individual's health, and should be seen as a powerful supplement to the clinical family history. Limitations of personalized sequencing Nevertheless, WES and WGS are often performed with relatively low read depth, which results in data of insufficient quality to be directly used in clinical practice. Consequently, they can return thousands or tens of thousands of false-positive variants, necessitating validation by a separate platform such as Sanger sequencing or targeted amplicon sequencing with high read depth. As an example, for the Snyderome a variety of technologies and platforms manufactured by different distributors were applied to achieve deep sequencing. WGS by Complete Genomics (CA, USA; 35 nt paired end; 150-fold total coverage) and Illumina (CA, USA; 100 nt paired end; 120-fold total coverage). WES by Agilent (CA, USA), Illumina (CA, USA) and Nimblegen (WI, USA) at 80- to 100-fold coverage; crossvalidation with Illumina Omni1-Quad genotyping arrays (99.3% sensitivity); stringent data quality control and calling criteria; RNA sequencing by Illumina HiSeq with high depth; Sanger sequencing of randomly selected variants (36/36 single nucleotide variations validated; 14/15 indels validated).

In the majority of cases of clinically available WES, identified mutations thought to be of clinical relevance are confirmed via an independent platform prior to being reported with Sanger sequencing being the predominantly applied confirmatory platform. Sanger sequencing is held by many as the gold

standard,[87] while some have suggested conventional Sanger may no longer be the gold standard.[88] This appears in part related to the observation that some variants identified by WES/WGS are not confirmable by Sanger sequencing.[89-91] It should be noted that no platform is perfect as each has its own systematic weaknesses.[88, 92] Nevertheless, while one clinical laboratory has begun to forgo Sanger confirmation for WES/WGS variants identified above a specified quality threshold, Sanger sequencing remains a relevant technique for validation of variants found by WES/WGS, as the systematic errors associated with each are different.[93]

It is also important to realize that previously reported associations of genetic variants with disease may have suboptimal positive- and negative-predictive values. Some variants have been evaluated in this context with sufficient sample sizes in independent studies, but for many associations these statistics remain to be adequately determined. More research is needed to support high-quality evidence-based genomic medicine.

Sequencing & VUS: benefit-to-harm ratio

As application of personal sequencing expands, WES/WGS will yield an abundance of data including VUS.[94, 95] Inadequate in silico prediction algorithms and incomplete penetrance are among the factors complicating clinical interpretation of these findings.[43] A 'binning system' has been proposed by which genetic variants can be 'triaged' in the clinical diagnostic setting to help address the field's limited, though growing, understanding about most genetic variants, to facilitate focused attention on those variants demonstrated to have clinical implications.[96] Additional in vitro investigations, such as RNA expression and proteomic analyses, will be needed to confidently disregard a VUS or establish its association with a condition. As more sequencing data are becoming available, variants previously designated disease causing, benign or of uncertain significance are being reclassified. This theoretically adds to healthcare costs and the practical and mental burden of patients tested; these aspects need to be taken into consideration and require further investigation.

Ethical discussions surrounding DNA sequencing are ongoing and confidentiality of patient health information has become a serious issue.[97] Patients, but also the general public, should be properly educated about genome sequencing, its applications and limitations, enabling them to make informed health decisions. Informed consent and data sharing agreements should include clauses specifying to whom a patient wishes to grant access to his or her genomic data, while taking into consideration the individuals for whom the patient's genomic data represents actionable clinical information. While these issues remain to be resolved, we are convinced that correct application of information resulting from personal sequencing will prove to be cost effective with a favorable benefit-to-harm ratio.

Sequencing patients: integration into clinical care

The genome is believed to be relatively stable throughout life and it will gradually become more attractive to retain data resulting from genome sequencing for future use. The cost–effectiveness of personalized exome and genome sequencing will improve with time, as the costs of sequence data generation, processing and storage decline and we will learn how to best utilize this information. For clinical care, it may become relevant to integrate genomic information into medical health records. Any information to be integrated into the electronic medical record (EMR) needs to be of high quality and accuracy, and the current quality of genomic sequence does not meet that level of rigor. An interim strategy would be to establish a clinical research database in which the full genome sequence and downstream analyses are stored and selected results from established, clinically related follow-up tests

would get into the EMR (Figure 4). The selection criteria would require regular updating per advancing scientific understanding and the patients' sequences would need to be regularly re-evaluated, gradually enriching the genomic information in the EMR. A pharmacogenomic example could be that automated alerts would notify the treating physician of genetically determined sensitivity to certain medications or suboptimal metabolism requiring drug dose adjustment, such as the Snyderome related variants impacting clopidogrel and warfarin efficacy.[53]

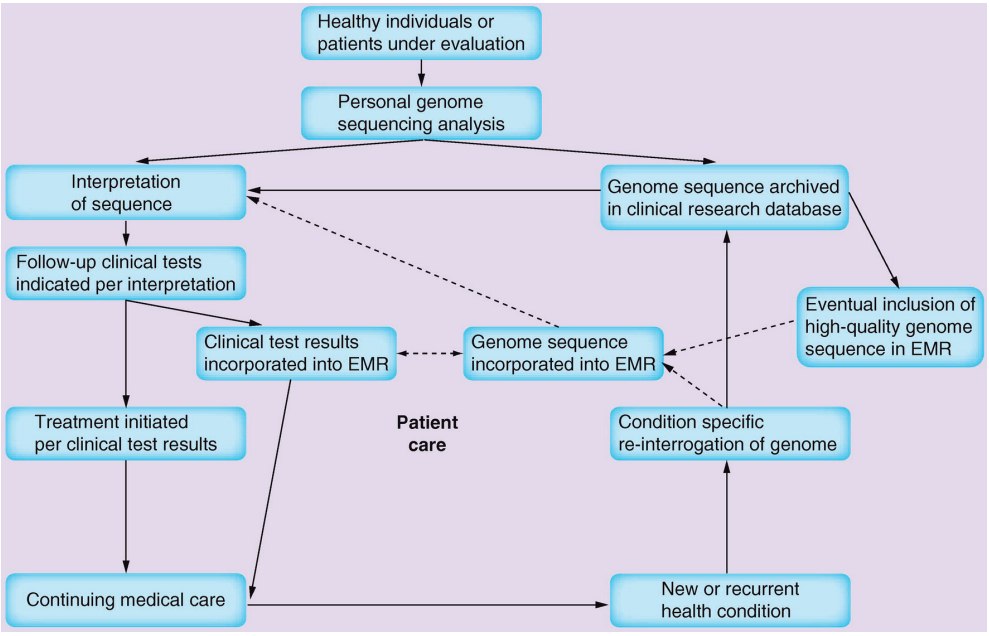

Figure 4. Incorporation of the personal genome sequence into healthcare. As genome sequence quality improves, future direct incorporation into the EMR will be possible, as indicated by the dashed arrows. EMR: Electronic medical record.

Studies have also been designed for genotype-driven scans of the interaction of numerous non-genetic factors. A group of investigators lead by Atul Butte devised a design where a set of well-established genetic variants are screened for interactions with a great variety of non-genetic factors, termed 'environment-wide association study'.[98-100] One of the published studies found an association between SNP genotypes and β-carotenes, where β-carotenes seem to negate certain detrimental genetic effects and prevent genetically predisposed persons from getting Type 2 diabetes.[99] Whether β-carotene administration to persons with risk genotypes for Type 2 diabetes could actually prevent them from getting the disease remains to be proven. In the future it may even be possible to isolate cells from patients known to carry detrimental alleles and test responses to different compounds in vitro, which may support eventual medical decision-making.

Obstacles to EMR implementation

While personalized sequencing provides us with a wealth of genomic data including single nucleotide variations or SNPs and structural variations, it is key to keep in mind the current limitations of the tech-

nology, such as read depth and data quality, when interpreting the results. Confirmation by another reliable platform with high read depth remains necessary. Moreover, there is additive value in combining genomic sequencing information with RNA sequencing, transcriptomic, proteomic and metabolomic data. These omics analyses yield independent information about dynamic changes in health and disease states and are critical to correct interpretation of genomic variation and its clinical application.[53, 101, 102]

Many of these clinical applications for personal sequencing remain in the near and distant future, as we continue to unravel the mysteries of the human genome.[95] As our understanding grows through discovery and validation studies the accuracy of medical genetic sequencing will improve. This will increase the need for carefully curated databases with wellcorroborated genetic variants and reference genomes to support sequence interpretation. Analytical validation and evaluation studies with adequate study sample sizes and performed in different population groups are necessary to translate findings from the research realm into clinically validated tests. While the clinical applications of personal sequencing and validated medically actionable variants remains limited, their growing application to the practice of medicine is anticipated to accelerate.

CONCLUSION

Personalized sequencing has fast evolved to become a tool broadly applied in the study of disease and of increasing value in medical application to the diagnosis and treatment of disease. There is a growing need for improved methods, both in silico and in vitro, to predict the clinical impact of VUS identified in the process of large-scale sequencing. The quality of the currently generated WES and WGS is also in need of improvement, if it is to be incorporated in the future into an individual's EMR as a reference.

Even with these obstacles, personalized sequencing has already begun to demonstrate its applicability to the practice of medicine. With it we have begun to better understand the etiologies of rare diseases and long studied diseases, such as cancer. Successes have been most evident in the field of rare Mendelian disorders where a single variant is sought to explain the phenotype in a patient, and can guide disease management by establishing the diagnosis. It demonstrates utility in refining and expanding our current diagnostic capacity such as through individual tumor DNA sequencing. Germline sequencing has also produced actionable information able to indicate lifestyle changes for an individual at risk for diabetes, as well as guiding pharmacologic interventions for the treatment of cancer, attuned to both tolerance of the patient and effectiveness of therapy, in a step towards personalized medicine. The application of personalized sequencing to clinically healthy individuals awaits the replication of recent studies in larger cohorts, but has the potential to be a medically valuable application in the not too distant future. As implementation of personalized sequencing on a large-scale is becoming progressively achievable, and accuracy of interpretation is significantly improved, we expect a transformation of healthcare in its current form.

FUTURE PERSPECTIVE

With the rapid advances that have taken place in personalized sequencing over the past 5 years, it is difficult to predict its overall impact on the field of medicine in the coming 5–10 years, though we expect its impact to be significant. For example, while current WES data quality and interpretation remain currently inadequate to the task, there exists the future possibility that personal sequencing technology might be applied in clinically healthy individuals, both with and without known predispositions to disease, as a

screening method to detect preclinical conditions, such as cancer, and facilitate pre-emptive treatment, with the potential to abrogate progression of otherwise clinically undetectable disease. It should be noted that, while we feel the current sequence quality and associated interpretative capacity require significant advancement prior to this type of clinical application, asymptomatic adult whole-exome screening tests are already being offered by CLIA-certified facilities (i.e., 'adult screening exome sequencing'),[103] emphasizing the urgent need for advancement in sequence quality and interpretive acuity. Where WES is currently available on a clinical basis, we would predict WGS to be clinically available in 5–10 years' time, as an evaluation that is routinely ordered by physicians. As broader utilization of EMRs takes hold in the practice of medicine and the quality of WGS continues to improve, we envision an individual's genomic sequence becoming archived as an accessible part of their EMR in as little as 10 years' time, for physicians to reference as a part of patient care.

In this era of medicine where medical practitioners include intensivists who treat diseases at their critical extremity and interventionalists who utilize invasive techniques, often at significant expense and morbidity, to ameliorate the complications of advanced sequelae of preventable disease, we envision a future in which personalized genomic sequencing enables the emergence of a new breed of medical practitioner: the preventional geneticist. Personalized sequencing portends a future for medicine where specialized healthcare providers, preventional geneticists, carefully interpreting and applying an individual's genomic profile can foresee their potential for various major diseases, such as diabetes and cancer. This information can then be used by preventional geneticists, possibly prior to clinical onset of these conditions, to institute surveillance, lifestyle changes and even preemptive pharmacogenomic-based therapeutics, with the potential to delay disease sequelae, and ultimately prevent disease onset in its entirety, defeating disease. Personalized sequencing represents a first major step toward this revolutionary future for medicine.

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EXECUTIVE SUMMARY

Background

– Personalized sequencing has advanced in scale, speed and affordability to become a powerful tool in the study of individuals and their diseases.

Cancer genome sequencing

– The diagnosis and management of various forms of cancer has benefited from two forms of personalized sequencing: tumor DNA and germline. These have enabled the beginning of individualized therapy of cancer.

Sequencing the unknown: rare diseases

– Rare and "mystery" diseases as targets for personalized sequencing have yielded etiologies for previously undiagnosed diseases, in rare cases capacitating effective treatment.

Sequencing the "healthy"

- At least one study analyzing the genomic sequence of a clinically healthy individual found a predisposition to diabetes, pharmacological interventions to which the individual would favorably respond, and allowed lifestyle changes to prevent the disease's onset.
- Several obstacles impede the current clinical application of personalized sequencing, one of which is the technological limitations of sequencing accuracy, where much progress is needed to allow transition to clinical medicine.
- Interpretation of the genomic sequence currently remains a significant impediment to clinical applications. While many approaches are under development, new in silico and in vitro strategies are critical to understanding genomic data with acuity sufficient for clinical decision-making.
- Variant(s) of unknown significance uncovered during sequencing will require additional interrogation via RNA expression, proteomic, metabolomic and other functional analyses, to facilitate accurate classification as benign and disease causing.

Sequencing patients: integration into clinical care

- An individual's personalized sequence may eventually become a valuable part of their electronic medical record, to be referenced periodically in the identification and management of disease.

Future perspective

- Personalized sequencing represents a major step toward a revolutionary future of disease treatment, prediction and prevention in the practice of medicine.

REFERENCES

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Collins FS, Green ED, Guttmacher AE, Guyer MS. A vision for the future of genomics research. Nature. 2003 Apr 24;422(6934):835-47.
2. Bozic I, Antal T, Ohtsuki H, Carter H, Kim D, Chen S, et al. Accumulation of driver and passenger mutations during tumor progression. Proc Natl Acad Sci U S A. 2010 Oct 26;107(43):18545-50.
3. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW. Cancer genome landscapes. Science. 2013 Mar 29;339(6127):1546-58.

•• **Overview of genome sequencing's impact on cancer research.**

4. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008 Nov 6;456(7218):66-72.

• **Early study comparing whole-genome sequencing (WGS) of patient's tumor cell and normal** cell DNA.

5. Garraway LA, Lander ES. Lessons from the cancer genome. Cell. 2013 Mar 28;153(1):17-37.
6. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976 Oct 1;194(4260):23-8.
7. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012 Mar 8;366(10):883-92.
8. McLeod HL. Cancer pharmacogenomics: early promise, but concerted effort needed. Science. 2013 Mar 29;339(6127):1563-6.
9. Kreso A, O'Brien CA, van Galen P, Gan OI, Notta F, Brown AM, et al. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. Science. 2013 Feb 1;339(6119):543-8.
10. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer. 2007 Mar;7(3):169-81.

11. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011 Jun 30;364(26):2507-16.
12. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol. 2008 Apr 1;26(10):1626-34.
13. Irshad S, Ashworth A, Tutt A. Therapeutic potential of PARP inhibitors for metastatic breast cancer. Expert Rev Anticancer Ther. 2011 Aug;11(8):1243-51.
14. Lord CJ, Ashworth A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. Nat Med. 2013 Nov;19(11):1381-8.
15. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012 Jan 19;481(7381):306-13.
16. Ashworth A, Lord CJ, Reis-Filho JS. Genetic interactions in cancer progression and treatment. Cell. 2011 Apr 1;145(1):30-8.
17. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, et al. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. Proc Natl Acad Sci U S A. 2008 Nov 4;105(44):17079-84.
18. Gabree M, Seidel M. Genetic testing by cancer site: skin. Cancer J. 2012 Jul-Aug;18(4):372-80.
19. Shannon KM, Chittenden A. Genetic testing by cancer site: breast. Cancer J. 2012 Jul-Aug;18(4):310-9.
20. Senter L. Genetic testing by cancer site: colon (nonpolyposis syndromes). Cancer J. 2012 Jul-Aug;18(4):334-7.
21. Jasperson KW. Genetic testing by cancer site: colon (polyposis syndromes). Cancer J. 2012 Jul-Aug;18(4):328-33.
22. Chun N, Ford JM. Genetic testing by cancer site: stomach. Cancer J. 2012 Jul-Aug;18(4):355-63.
23. Weissman SM, Weiss SM, Newlin AC. Genetic testing by cancer site: ovary. Cancer J. 2012 Jul-Aug;18(4):320-7.
24. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013 Jan;63(1):11-30.
25. Roberts NJ, Jiao Y, Yu J, Kopelovich L, Petersen GM, Bondy ML, et al. ATM mutations in patients with hereditary pancreatic cancer. Cancer Discov. 2012 Jan;2(1):41-6.
26. Palles C, Cazier JB, Howarth KM, Domingo E, Jones AM, Broderick P, et al. Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. Nat Genet. 2013 Feb;45(2):136-44.
27. Paugh SW, Stocco G, McCorkle JR, Diouf B, Crews KR, Evans WE. Cancer pharmacogenomics. Clin Pharmacol Ther. 2011 Sep;90(3):461-6.
28. Wheeler HE, Maitland ML, Dolan ME, Cox NJ, Ratain MJ. Cancer pharmacogenomics: strategies and challenges. Nat Rev Genet. 2013 Jan;14(1):23-34.

•• **Overview of pharmacogenomic applications to cancer treatment with relevant examples.**

29. Binkhorst L, van Gelder T, Mathijssen RH. Individualization of tamoxifen treatment for breast carcinoma. Clin Pharmacol Ther. 2012 Oct;92(4):431-3.
30. Weng L, Zhang L, Peng Y, Huang RS. Pharmacogenetics and pharmacogenomics: a bridge to individualized cancer therapy. Pharmacogenomics. 2013 Feb;14(3):315-24.
31. Wheeler DA, Wang L. From human genome to cancer genome: the first decade. Genome Res. 2013 Jul;23(7):1054-62.
32. Sharaf RN, Ladabaum U. Comparative effectiveness and cost-effectiveness of screening colonoscopy vs. sigmoidoscopy and alternative strategies. Am J Gastroenterol. 2013 Jan;108(1):120-32.
33. Dunn BK, Jegalian K, Greenwald P. Biomarkers for early detection and as surrogate endpoints in cancer prevention trials: issues and opportunities. Recent Results Cancer Res. 2011;188:21-47.
34. Kitzman JO, Snyder MW, Ventura M, Lewis AP, Qiu R, Simmons LE, et al. Noninvasive whole-genome sequencing of a human fetus. Sci Transl Med. 2012 Jun 6;4(137):137ra76.
35. Benesova L, Belsanova B, Suchanek S, Kopeckova M, Minarikova P, Lipska L, et al. Mutation-based detection and monitoring of cell-free tumor DNA in peripheral blood of cancer patients. Anal Biochem. 2013 Feb 15;433(2):227-34.
36. Bainbridge MN, Wiszniewski W, Murdock DR, Friedman J, Gonzaga-Jauregui C, Newsham I, et al. Whole-genome sequencing for optimized patient management. Sci Transl Med. 2011 Jun 15;3(87):87re3.

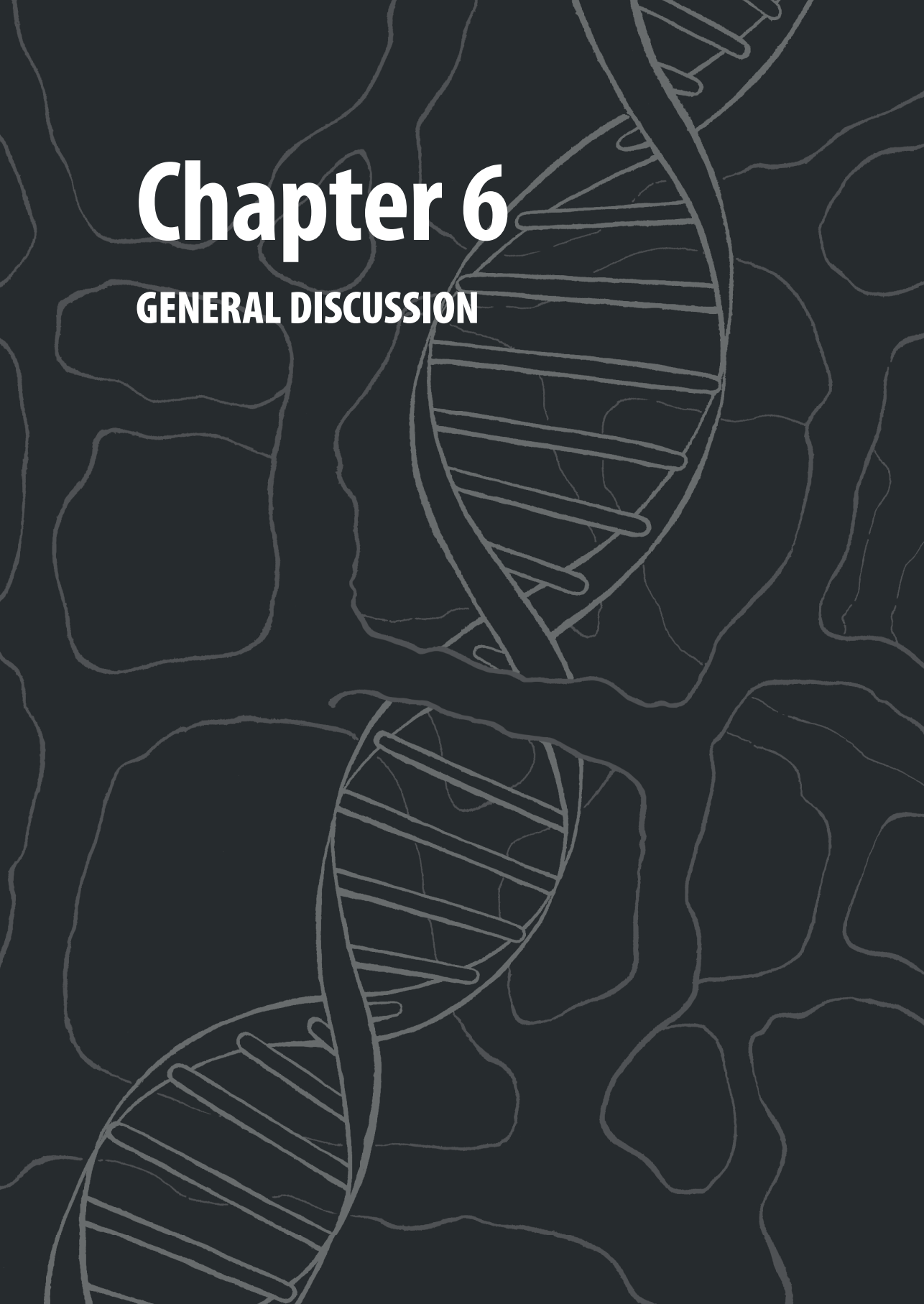
37. Gudmundsson J, Sulem P, Gudbjartsson DF, Masson G, Agnarsson BA, Benediktsdottir KR, et al. A study based on whole-genome sequencing yields a rare variant at 8q24 associated with prostate cancer. Nat Genet. 2012 Dec;44(12):1326-9.
38. Jonsson T, Atwal JK, Steinberg S, Snaedal J, Jonsson PV, Bjornsson S, et al. A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. Nature. 2012 Aug 2;488(7409):96-9.
39. Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, et al. Variant of TREM2 associated with the risk of Alzheimer's disease. N Engl J Med. 2013 Jan 10;368(2):107-16.
40. Lupski JR, Reid JG, Gonzaga-Jauregui C, Rio Deiros D, Chen DC, Nazareth L, et al. Whole-genome sequencing in a patient with Charcot-Marie-Tooth neuropathy. N Engl J Med. 2010 Apr 1;362(13):1181-91.
41. Ng SB, Buckingham KJ, Lee C, Bigham AW, Tabor HK, Dent KM, et al. Exome sequencing identifies the cause of a mendelian disorder. Nat Genet. 2010 Jan;42(1):30-5.
42. Peltonen L, Perola M, Naukkarinen J, Palotie A. Lessons from studying monogenic disease for common disease. Hum Mol Genet. 2006 Apr 15;15 Spec No 1:R67-74.
43. Kohane IS. (Mis)treating the pharmacogenetic incidentalome. Nat Rev Drug Discov. 2012 Feb;11(2):89-90.
44. Worthey EA, Mayer AN, Syverson GD, Helbling D, Bonacci BB, Decker B, et al. Making a definitive diagnosis: successful clinical application of whole exome sequencing in a child with intractable inflammatory bowel disease. Genet Med. 2011 Mar;13(3):255-62.
- **Early example of whole-exome sequencing (WES) application to diagnose a rare disease** and guide effective medical treatment.
45. Bamshad MJ, Ng SB, Bigham AW, Tabor HK, Emond MJ, Nickerson DA, et al. Exome sequencing as a tool for Mendelian disease gene discovery. Nat Rev Genet. 2011 Nov;12(11):745-55.
46. Norton N, Li D, Hershberger RE. Next-generation sequencing to identify genetic causes of cardiomyopathies. Curr Opin Cardiol. 2012 May;27(3):214-20.
47. Green RC, Berg JS, Grody WW, Kalia SS, Korf BR, Martin CL, et al. ACMG Recommendations for Reporting of Incidental Findings in Clinical Exome and Genome Sequencing. American College of Medical Genetics and Genomics. 2013.
- **Current clinical guidelines for communication of results, including incidental findings, of WES and WGS.**
48. Need AC, Shashi V, Hitomi Y, Schoch K, Shianna KV, McDonald MT, et al. Clinical application of exome sequencing in undiagnosed genetic conditions. J Med Genet. 2012 Jun;49(6):353-61.
49. Yang Y, Muzny DM, Reid JG, Bainbridge MN, Willis A, Ward PA, et al. Clinical whole-exome sequencing for the diagnosis of mendelian disorders. N Engl J Med. 2013 Oct 17;369(16):1502-11.
50. Atwal PS, Brennan ML, Cox R, Niaki M, Platt J, Homeyer M, et al. Clinical whole-exome sequencing: are we there yet? Genet Med. 2014 Sep;16(9):717-9.
51. Dewey FE, Grove ME, Pan C, Goldstein BA, Bernstein JA, Chaib H, et al. Clinical interpretation and implications of whole-genome sequencing. JAMA. 2014 Mar 12;311(10):1035-45.
52. Ashley EA, Butte AJ, Wheeler MT, Chen R, Klein TE, Dewey FE, et al. Clinical assessment incorporating a personal genome. Lancet. 2010 May 1;375(9725):1525-35.
•• **First sequencing of a healthy individual's genome to predict risk of disease and identify pharmacogenomic profile.**
53. Chen R, Mias GI, Li-Pook-Than J, Jiang L, Lam HY, Miriami E, et al. Personal omics profiling reveals dynamic molecular and medical phenotypes. Cell. 2012 Mar 16;148(6):1293-307.
•• **First example of personalized sequencing combined with omics analyses, which predicted onset of diabetes in an individual and enabled successful intervention.**
54. Gupta R, Ratan A, Rajesh C, Chen R, Kim HL, Burhans R, et al. Sequencing and analysis of a South Asian-Indian personal genome. BMC Genomics. 2012;13:440.
55. Peterson TA, Doughty E, Kann MG. Towards precision medicine: advances in computational approaches for the analysis of human variants. J Mol Biol. 2013 Nov 1;425(21):4047-63.
56. Online Mendelian Inheritance in Man. Available from: www.omim.org.

57. Stenson PD, Mort M, Ball EV, Shaw K, Phillips A, Cooper DN. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Hum Genet. 2014 Jan;133(1):1-9.
58. The Human Gene Mutation Database. Available from: www.hgmd.org.
59. ClinVar. Available from: www.ncbi.nlm.nih.gov/clinvar.
60. European Genome-Phenome Archive. Available from: www.ebi.ac.uk/ega.
61. Mailman MD, Feolo M, Jin Y, Kimura M, Tryka K, Bagoutdinov R, et al. The NCBI dbGaP database of genotypes and phenotypes. Nat Genet. 2007 Oct;39(10):1181-6.
62. dbGap. Available from: www.ncbi.nlm.nih.gov/gap.
63. Hindorff LA, Sethupathy P, Junkins HA, Ramos EM, Mehta JP, Collins FS, et al. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. Proc Natl Acad Sci U S A. 2009 Jun 9;106(23):9362-7.
64. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, et al. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic Acids Res. 2014 Jan;42(Database issue):D1001-6.
65. Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, et al. Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. N Engl J Med. 2005 Apr 7;352(14):1413-24.
66. Vaxillaire M, Cavalcanti-Proenca C, Dechaume A, Tichet J, Marre M, Balkau B, et al. The common P446L polymorphism in GCKR inversely modulates fasting glucose and triglyceride levels and reduces type 2 diabetes risk in the DESIR prospective general French population. Diabetes. 2008 Aug;57(8):2253-7.
67. Hani EH, Boutin P, Durand E, Inoue H, Permutt MA, Velho G, et al. Missense mutations in the pancreatic islet beta cell inwardly rectifying K+ channel gene (KIR6.2/BIR): a meta-analysis suggests a role in the polygenic basis of Type II diabetes mellitus in Caucasians. Diabetologia. 1998 Dec;41(12):1511-5.
68. Erlich HA, Valdes AM, Julier C, Mirel D, Noble JA. Evidence for association of the TCF7 locus with type I diabetes. Genes Immun. 2009 Dec;10 Suppl 1:S54-9.
69. PharmGKB: Pharmacogenomics. Knowledge. Implementation.; Available from: www.pharmgkb.org.
70. Sim SC, Altman RB, Ingelman-Sundberg M. Databases in the area of pharmacogenetics. Hum Mutat. 2011 May;32(5):526-31.
71. PharmaADME. Available from: www.pharmaadme.org.
72. The Human Cytochrome P450 (CYP) Allele Nomenclature Database. Available from: www.cypalleles.ki.se.
73. University of Louisville School of Medicine: Department of Pharmacology and Toxicology. Available from: http://n-acetyltransferasenomenclature.louisville.edu.
74. Pharmacogenetics of Membrane Transporters Database. Available from: http://pharmacogenetics.ucsf.edu.
75. TP-Search. Available from: www.tp-search.jp.
76. UGT1A and UGT2B haplotypes and SNPs tables. Available from: www.pharmacogenomics.pha.ulaval.ca/sgc/ugt_alleles.
77. Gamazon ER, Duan S, Zhang W, Huang RS, Kistner EO, Dolan ME, et al. PACdb: a database for cell-based pharmacogenomics. Pharmacogenet Genomics. 2010 Apr;20(4):269-73.
78. PACdb. Available from: www.pacdb.org.
79. Link E, Parish S, Armitage J, Bowman L, Heath S, Matsuda F, et al. SLCO1B1 variants and statin-induced myopathy--a genomewide study. N Engl J Med. 2008 Aug 21;359(8):789-99.
80. Feng Q, Wilke RA, Baye TM. Individualized risk for statin-induced myopathy: current knowledge, emerging challenges and potential solutions. Pharmacogenomics. 2012 Apr;13(5):579-94.
81. Mallal S, Nolan D, Witt C, Masel G, Martin AM, Moore C, et al. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet. 2002 Mar 2;359(9308):727-32.
82. Hughes AR, Mosteller M, Bansal AT, Davies K, Haneline SA, Lai EH, et al. Association of genetic variations in HLA-B region with hypersensitivity to abacavir in some, but not all, populations. Pharmacogenomics. 2004 Mar;5(2):203-11.
83. Mallal S, Phillips E, Carosi G, Molina JM, Workman C, Tomazic J, et al. HLA-B*5701 screening for hypersensitivity to abacavir. N Engl J Med. 2008 Feb 7;358(6):568-79.

84. Waters LJ, Mandalia S, Gazzard B, Nelson M. Prospective HLA-B*5701 screening and abacavir hypersensitivity: a single centre experience. AIDS. 2007 Nov 30;21(18):2533-4.
85. HLA B*5701 status, disease progression, and response to antiretroviral therapy. AIDS. 2013 Oct 23;27(16):2587-92.
86. Hicks JK, Swen JJ, Thorn CF, Sangkuhl K, Kharasch ED, Ellingrod VL, et al. Clinical Pharmacogenetics Implementation Consortium guideline for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressants. Clin Pharmacol Ther. 2013 May;93(5):402-8.
87. MacConaill LE. Existing and emerging technologies for tumor genomic profiling. J Clin Oncol. 2013 May 20;31(15):1815-24.
88. Ulahannan D, Kovac MB, Mulholland PJ, Cazier JB, Tomlinson I. Technical and implementation issues in using next-generation sequencing of cancers in clinical practice. Br J Cancer. 2013 Aug 20;109(4):827-35.
89. Linderman MD, Brandt T, Edelmann L, Jabado O, Kasai Y, Kornreich R, et al. Analytical validation of whole exome and whole genome sequencing for clinical applications. BMC Med Genomics. 2014;7:20.
90. McCourt CM, McArt DG, Mills K, Catherwood MA, Maxwell P, Waugh DJ, et al. Validation of next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. PLoS One. 2013;8(7):e69604.
91. Chin EL, da Silva C, Hegde M. Assessment of clinical analytical sensitivity and specificity of next-generation sequencing for detection of simple and complex mutations. BMC Genet. 2013;14:6.
92. Esteban-Jurado C, Garre P, Vila M, Lozano JJ, Pristoupilova A, Beltran S, et al. New genes emerging for colorectal cancer predisposition. World J Gastroenterol. 2014 Feb 28;20(8):1961-71.
93. Strom SP, Lee H, Das K, Vilain E, Nelson SF, Grody WW, et al. Assessing the necessity of confirmatory testing for exome-sequencing results in a clinical molecular diagnostic laboratory. Genet Med. 2014 Jul;16(7):510-5.
94. Henrikson NB, Burke W, Veenstra DL. Ancillary risk information and pharmacogenetic tests: social and policy implications. Pharmacogenomics J. 2008 Apr;8(2):85-9.
95. Westbrook MJ, Wright MF, Van Driest SL, McGregor TL, Denny JC, Zuvich RL, et al. Mapping the incidentalome: estimating incidental findings generated through clinical pharmacogenomics testing. Genet Med. 2013 May;15(5):325-31.
96. Berg JS, Khoury MJ, Evans JP. Deploying whole genome sequencing in clinical practice and public health: meeting the challenge one bin at a time. Genet Med. 2011 Jun;13(6):499-504.
97. Gymrek M, McGuire AL, Golan D, Halperin E, Erlich Y. Identifying personal genomes by surname inference. Science. 2013 Jan 18;339(6117):321-4.
98. Patel CJ, Chen R, Butte AJ. Data-driven integration of epidemiological and toxicological data to select candidate interacting genes and environmental factors in association with disease. Bioinformatics. 2012 Jun 15;28(12):i121-6.
99. Patel CJ, Bhattacharya J, Butte AJ. An Environment-Wide Association Study (EWAS) on type 2 diabetes mellitus. PLoS One. 2010;5(5):e10746.
100. Patel CJ, Cullen MR, Ioannidis JP, Butte AJ. Systematic evaluation of environmental factors: persistent pollutants and nutrients correlated with serum lipid levels. Int J Epidemiol. 2012 Jun;41(3):828-43.
101. Snyder M, Weissman S, Gerstein M. Personal phenotypes to go with personal genomes. Mol Syst Biol. 2009;5:273.
102. Snyder M, Du J, Gerstein M. Personal genome sequencing: current approaches and challenges. Genes Dev. 2010 Mar 1;24(5):423-31.
103. Medical Genetics Test Details. Available from: www.bcm.edu/research/medical-genetics-labs/test_detail.cfm?testcode=1605.
104. Table of Pharmacogenomic Biomarkers in Drug Labeling. Available from: www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm.
- **Online resource providing pharmacogenomics information in all US FDA-approved druglabels.**

Chapter 6

GENERAL DISCUSSION



In this thesis, various genetic and metabolic factors were investigated to characterize the determinants underlying skeletal endocrine and degenerative diseases. These included alterations in bone leading to increased fracture risk in osteoporosis and diabetic bone disease. The main objective of this thesis was to identify novel risk factors including genetic markers and to further characterize known determinants of fracture risk. This chapter places the main findings under a unifying perspective together with a discussion and suggestions for future research.

EPIDEMIOLOGICAL ASPECTS OF ENDOCRINE BONE DISEASES

Fracture is a "complex" clinical event, or phenotype, with environmental and genetic factors influencing risk of fracture. Fractures can be classified according to skeletal site, shape, etiological mechanism and clinical expression. Table 1 shows an overview of various fracture definitions used. Here, it has to be noted that the definition of fracture is not uniform and difficult to standardize, and is based on different criteria in different studies. The studies in this thesis include mainly two phenotypes: 1) the all-type of fracture phenotype, further stratified according to fracture site, and 2) radiographic vertebral fractures, further characterized by shape. These fracture definitions were selected, because the all-type of fracture phenotype and radiographic vertebral fractures maximize sample size, and the latter can be diagnosed on population imaging by radiographs.

RADIOLOGICAL ASPECTS OF VERTEBRAL FRACTURES AND ENDOCRINE BONE DISEASES

Currently, clinical scores available for osteoporosis only predict incident non-vertebral fracture risk. It would be desirable to devise a clinical risk score for vertebral fractures as well. Risk factors for incident vertebral fractures have previously been identified, such as: prevalent vertebral fractures, age, gender, height, weight, smoking history, height loss[1] and use of a walking aid.[2, 3] Further studies may identify additional risk factors for osteoporotic vertebral fractures.

Vertebral fractures may go misdiagnosed as the clinical presentation can be aspecific. Moreover, as two thirds of vertebral fractures do not give clinical symptoms, these may be only detected on radiological imaging.[4] Nevertheless, vertebral fractures increase the risk of new vertebral fracture up to five-fold and the risk of other fragility fractures two- to four-fold.[4, 5] Drugs available for the treatment of osteoporosis are highly effective, with the most potent bisphosphonate zoledronic acid reducing the risk of vertebral fractures by 76% and of non-vertebral fractures by 24%.[6]

Therefore, accurate diagnosis is crucial, however, at present, a gold standard is lacking. The Dutch national multi-disciplinary osteoporosis guideline of 2011 recommends performing DXA-BMD measurements and Vertebral Fracture Assessments (DXA-VFA or lateral radiographs) in persons: 1) aged 50 years and over with clinical suspicion of vertebral fractures; 2) having recent non-vertebral fracture; 3) with diseases or medication use with potential bone loss; and 4) aged 60 years and over with multiple risk factors.[7] A clear and correct fracture definition is crucial, because vertebral fractures form an integral part of clinical decision making to initiate anti-osteoporotic drugs. In addition, switching to the expensive osteoanabolic agent teriparatide for two years is indicated in patients who have sustained three fractures of which at least two are located in the spine while treated with other osteoporosis drugs.[7]

Various morphometric methods are currently used to diagnose vertebral fractures and we compared the algorithm based qualitative (ABQ) method with quantitative morphometry (QM) in a dataset of

Table 1. Description of various fracture-related definitions (alphabetical order).

Fracture terminology	Definition
All-type or any-type	Break in any bone of the skeleton resulting from the application of excessive force. In most studies focusing on osteoporosis, fractures by high-trauma and of the skull, toes and fingers are excluded from the definition.
Asymptomatic	Fracture which does not present with any clinical signs. Versus clinical fractures.
Atypical	Refers to fractures that occur at an unusual site for osteoporosis, and principally concerns Atypical Femoral Fractures (AFF) located in the subtrochanteric region and diaphysis of the femur. They have been reported in patients taking bisphosphonates or denosumab, but they also occur in patients with no exposure to these drugs.
Avulsion	A fragment of bone tears away from the main mass by traumatic traction.
Biconcave	Vertebral fracture with middle height loss.
Burst	Severe vertebral fracture with compression of the posterior height due to a high-energy axial load.
Clinical	Coming to clinical attention and diagnosed by a physician and confirmed by radiological imaging.
Colles	Fracture of the distal radius in the forearm with dorsal (posterior) and radial displacement of the wrist and hand. Most common causal mechanism is falling onto a hard surface and breaking of the fall with outstretched arms.
Comminuted	Breaking of the bone in more than two pieces.
Complicated	Fracture in which the broken bone fragment(s) cause damage to neighboring structures.
Compression	Collapse of the vertebral body
Crush	Vertebral fracture with posterior height loss.
Fragility	Occurs spontaneously or after minimal trauma, such as a fall from standing height. This definition arises because a normal human being ought to be able to fall from standing height without breaking any bones, and a fracture therefore suggests weakness of the skeleton. There are three fracture sites said to be typical of fragility fractures: vertebral fractures, fractures of the neck of the femur, and Colles fracture of the wrist.
Hip	Refers to fracture of the femur bone.
Malunion	Healing of a fracture in an abnormal (nonanatomic) position
Nonunion	Fractured bone fails to heal completely and a space remains between the fragments
Osteoporotic	Occurring as a consequence of deterioration of bone quantity and qualities, where decreased BMD (World Health Organization definition: 2.5 standard deviations or more below the mean peak bone mass of average young, healthy adults as measured by dual-energy X-ray absorptiometry[DXA]) and deteriorated microarchitecture may be present. Major osteoporotic fractures comprise clinical vertebral, hip, forearm or proximal humerus fractures. Fractures of the skull, hands and toes are often excluded, as they are often associated with trauma and not with osteoporosis.
Pathologic	Caused by disease that led to weakness of the bone structure.
Radiographic	Diagnosed on imaging studies. Particularly applies to vertebral fractures, as a majority of these fractures are asymptomatic and thus can only be comprehensively captured by radiological surveys.
Spiral	The bone has been twisted apart and the line of break is helical; also called torsion fracture.
Stress	Caused by the repetitive application of force resulting in microcracks in bone which may eventually progress to larger fractures; often by overuse and sometimes in combination with osteoporosis.
Traumatic versus low-trauma or non-traumatic	Traumatic fractures are caused by a significant external force, such as traffic accidents or falls from heights; low-trauma or non-traumatic fractures occur spontaneously or after minimal trauma, such as a fall from standing height, and are considered fragility fractures.
Unstable	Fracture with a high risk to displace.
Vertebral or spinal versus non-vertebral	Occurring in the spinal column versus not occurring in the spine. This traditional distinction arose due to clinical and epidemiological differences in diagnostic methodology and underlying mechanisms in a substantial number of cases.
Wedge	Vertebral fracture with anterior height loss.
Wrist	Commonly refers to fractures of the distal radius bone.

2,827 subjects of the Rotterdam Study (RS-III cohort). QM-based methods evaluate vertebral height,[8] while the ABQ method[9] mainly judges endplate integrity, regardless of vertebral height reduction (Table 2). Our study showed that osteoporotic vertebral fractures prevalence rates are significantly different when applying either software-assisted QM or ABQ. Both QM and ABQ identify a considerable number of deformities that were assessed as normal by the other: 16.9% were assessed as QM deformities but not ABQ fractures; 1.2% were judged fractured according to ABQ but not QM. Further work is needed to reveal which of the discordant cases are actually clinically relevant: association studies evaluating the predictive ability of the different definitions with different relevant outcomes like future non-vertebral and vertebral fractures, and mortality are desirable. Merely measuring vertebral heights in clinical practice (which is the current recommendation in the Dutch osteoporosis guideline),[7] frequently leads to misdiagnosis of fracture in non-osteoporotic conditions including Scheuermann's disease, as illustrated by our case examples. Simultaneous assessment of vertebral heights together with endplate integrity may correctly differentiate these cases. One of the major advantages of software-assisted QM is the detailedness of the data recording. If more evidence supporting the ABQ method will be put forward, it will be worthwhile to explore if endplate integrity can be captured in software-assisted assessments based on computer-based morphometric recognition or if necessary also assess qualitative parameters. In addition to improvement of the radiological vertebral fracture definition by itself, clearer criteria for non-fracture deformities differential diagnosis, are necessary. Also, the relevance of more quantitative measures that could be derived from the raw X-ray data can be investigated, such as the kyphosis or lordosis angle or vertebral wedging, intervertebral disc space, and spondylolisthesis.

Table 2. Comparison of vertebral fracture definitions across quantitative morphometry (QM) and algorithm based qualitative (ABQ) methods.

	Algorithm based qualitative (ABQ)	Quantitative morphometry (QM)[b]
Vertebral body height reduction	+ or -	+
Endplate depression	+[a]	-

[a]The algorithm based qualitative (ABQ) method offers an approach for the differentiation of non-fracture deformities (e.g., degenerative changes in spine osteoarthritis such as vertebral wedging and intervertebral disc space narrowing) from osteoporotic vertebral fractures.
[b]One kind of quantitative morphometry-based method is SpineAnalyzer®.

The extensive imaging-based phenotyping effort of the Rotterdam Study has over 100,000 radiographs of the spine, hips, hands and knees available. All spine radiographs from 11,344 participants from the 4-yearly visits were assessed for vertebral fractures with a comprehensive range of quantitative and qualitative methods (Figure 1). Assessments have been performed for osteoporotic vertebral fractures according to QM with the McCloskey-Kanis method, ABQ methods, and semi-automated morphometry using SpineAnalyzer software. It is also an excellent setting to expand our knowledge about other spine diseases through readings that have been done for Scheuermann's disease, various degenerative traits for osteoarthritis, etc. For instance, Rotterdam Study results revealed a prevalence of 4.0% of radiographic Scheuermann's disease in Dutch individuals aged 45 years and over, indicating that the disorder is underappreciated.

TBS seems a promising osteoporosis quantitative imaging parameter, to some extent independent of DXA-BMD. DXA-BMD is a measure of bone quantity, while TBS provides information on the biomechanics and microarchitecture which reflects trabecular structure. Studies with larger sample sizes and aiming at additional phenotype associations are underway in the Rotterdam Study. TBS is less expensive

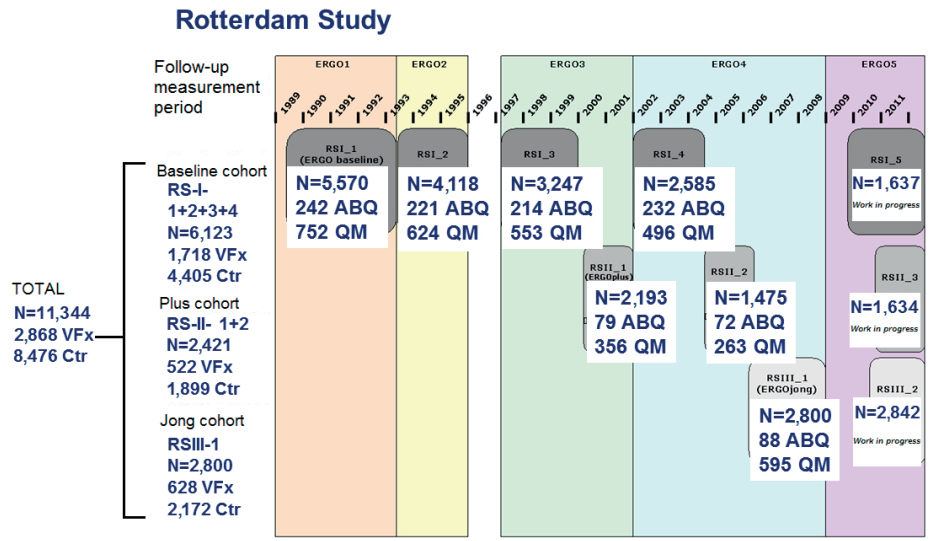


Figure 1. Diagram of the radiographic osteoporotic vertebral fracture data for the examination cycles of the Rotterdam Study (RS). These are part of a series of over 100,000 radiographs of the spine, hips, hands and knees. Five visit cycles have been conducted until now for the first original cohort RS-I and are indicated by RS-I-1 to RS-I-5. RS-II-1 and RS-II-2 relate to the extension of the cohort and RS-III-1 denotes the baseline examination of the second extension cohort. The total sample sizes per cohort counting the participants that have had a radiographic examination for assessment of their vertebral fracture status at least once are depicted on the left, comprising N=11,344 individuals of which 2,868 participants with vertebral fractures and 8,476 controls. The center shows total sample sizes and osteoporotic vertebral fracture status per assessment method for the separate examination cycles. Abbreviations: N=total sample size; VFx=vertebral fracture cases; Ctr=controls without vertebral fractures; ABQ=vertebral fracture case according to the algorithm based qualitative method; QM=vertebral fracture case according to quantitative morphometry.

and better accessible than CT or MRI imaging for wide-spread implementation. The very first TBS reports showed applications for the prediction of fracture risk in osteoporosis,[10-12] have added value in those individuals with bone density outside of the osteoporosis range[13] and monitoring of treatment effects,[14,15] and similarly, TBS may find an application in other conditions such as primary hyperparathyroidism,[16] hypercortisolism,[17] rheumatoid arthritis,[18] and diabetes-related bone disease[19]. A major advantage is that it can be easily derived from DXA scans, similar to VFA, which is already being applied in the clinic as recommended in the Dutch national guideline[7].

DIABETES, DEGENERATION AND BONE

Intuitively, a higher BMD should be protective against fracture. For instance, an individual with 5% higher femoral neck BMD would have a 10% decrease in fracture risk. Intriguingly, the contrary seems true in lumbar disc degeneration (LDD) and type 2 diabetes. Our studies showed that subjects with LDD have systematically higher BMD at the lumbar spine, femoral neck, skull, and consequently, at the total body measurement. In spite of this systematically higher BMD, persons with LDD are at higher risk of osteoporotic fractures, particularly males in whom LDD seems more severe. Second, De Liefde et al. were

among the first to show, using Rotterdam Study data, that individuals with type 2 diabetes have 69% higher fracture risk than those without diabetes despite having higher BMD at the femoral neck and lumbar spine.[20] Schwartz and colleagues established that the World Health Organization's fracture risk assessment tool (FRAX) underestimates osteoporotic fracture risk in individuals with diabetes;[21] this is why diabetes as a risk factor should be considered for inclusion in future iterations of FRAX.[22]

Abnormal alterations of bone may be mediated by processes such as glycemic load and inflammation. The main biomarker to clinically monitor glycemic load is glycated hemoglobin (Hb_{A1C}). Individuals with higher levels of Hb_{A1C} and CRP have been found to suffer from more cardiovascular disease,[23] and as we have shown also more bone complications. From these results, we were among the first to postulate that an inefficient redistribution of bone mass, accumulation of microcracks and cortical porosity reflecting impaired bone repair give rise to fragility in apparently "strong" bones in inadequately controlled diabetes. A study using high-resolution peripheral quantitative computed tomography (HR-pQCT), reported that the cortical porosity in type 2 diabetic patients was up to twice that of controls at the radius.[24] Subsequently, Patsch et al. showed in a four-group comparison of type 2 diabetes patients with and without fragility fractures to controls with and without fractures that cortical porosity is specific to those type 2 diabetes patients that fracture.[25] Moreover, an innovative investigation utilizing in vivo microindentation testing of the tibia showed that patients with type 2 diabetes have reduced serum markers of bone turnover and lower bone material strength than controls.[26] In this same study the average Hb_{A1C} level over the previous ten years was negatively correlated with bone material strength.[26] As hip structural analysis gives limited information, it would be desirable to investigate these phenomena on a larger population scale applying (pQ)CT and magnetic resonance imaging (MRI) in future work.

These data indicate that the first line of action for fracture prevention in diabetes is targeting adequate glycemic control. However, results from a randomized trial did not find changes in fracture or fall risk between standard glycemia and intensive glycemia.[27] Nevertheless, the study group may represent mostly less severe diabetes and average follow-up was less than 4 years. Hence, inference of long-term effects, i.e., from long-standing control and diminishing carryover of pretreatment glycemic exposure, is not yet possible. In sum, there is a need for adequate imaging and laboratory biomarkers to aid diagnosis and monitoring of diabetic bone disease. Additionally, this contention opens the possibility for alternative treatments that consider different pathophysiologic mechanisms than those present in typical osteoporosis processes, such as osteo-anabolic therapies like teriparatide, to overcome low bone formation and accumulation of microcracks.[28] More studies including randomized controlled trials in this area are needed.

GENETIC ASPECTS OF ENDOCRINE BONE DISEASES

Genome-wide screening as achieved in GWAS has advantages over the candidate gene approach. The traditional candidate gene approach is largely limited by its reliance on the a priori knowledge about the physiological, biochemical or functional aspects of possible candidates.[29] On the other hand, genome-wide genotyping is unbiased in the sense that by surveying the whole genome in a hypothesis-free manner, involvement of unexpected candidates or even loci with unknown function could be revealed.[30] Nonetheless, meta-analysis can unite the best of both worlds, including follow-up of top loci and genes prioritized by GWAS in candidate gene studies or use of existent GWAS for look-ups of functional biological hypotheses, as performed in a few of the studies in this thesis.

Family history has been shown to be an adequate predictor of risk across varied health conditions as heart disease, colorectal cancer, breast cancer, ovarian cancer, atopy or asthma, type 2 diabetes, among many others.[31] Similarly, a positive family history is one of the most important risk factors for osteoporosis and fractures.[32] The risk ratio (RR) for any fracture is 1.17, and for hip fracture is 1.49 (95% CI: 1.17-1.89). Parental hip fracture has been incorporated as a risk factor in the FRAX clinical score. Hence, the underlying possibilities provided by genomics make genetic studies appealing.

Compared to other quantitative traits that have been subject of GWAS, investigating BMD seems very prolific (Figure 2), while GWAS for dichotomous disease as a direct outcome including osteoporotic fractures, have yielded relatively lower numbers of loci discovered (Figure 3). Identifying the specific genetic determinants contributing to the risk of fracture has been difficult due to its multifactorial nature and occurrence late in life. High phenotype heterogeneity and ascertainment bias reduce the power to detect association. Studying correlated endophenotypes, such as in this case DXA-BMD, have shown to be a good alternative to study the genetic basis of osteoporosis. Endophenotypes may be nearer to the coding DNA in the chain of events at the basis of multifactorial diseases, and, homogeneous determination of endophenotypes may be simpler than defining certain diseases. Indeed, our hypothesis-free genome-wide screens have shown that the most prominent and consistently replicating genetic loci associated with fracture risk are also associated with BMD, which serves as proof of BMD being a very powerful endophenotype for fracture prediction. An underlying fragility component mediated through genetic predisposition seems to form the basis for fracture risk.

From the graphs it becomes clear that, first a trait-specific minimum sample-size threshold needs to be reached, and thereafter, the number of loci discovered increases along with growing sample sizes as study power improves.[33] Mega-sized biobanks, such as 23andMe and UK Biobank, including hundreds of thousands participants with GWAS of adequate quality are increasingly becoming available to in include in meta-analyses.[34, 35] A drawback is that phenotype data may be of variable quality and detailedness, however there is a trade-off where the huge numbers may boost study power tremendously and overcome measurement error to a certain extent. In addition, the success rate of unraveling underlying genetic mechanisms may be influenced by the complexity of the genetic architecture of the trait of

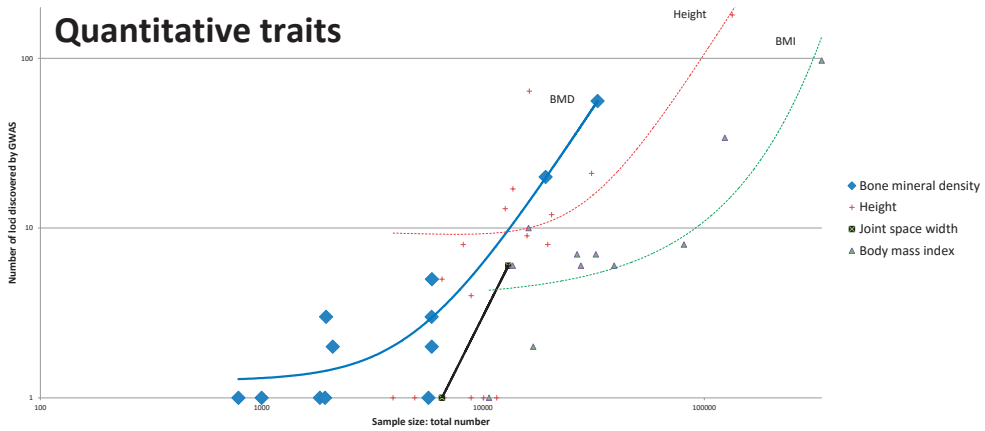


Figure 2. The number of associated loci discovered by GWAS at genome-wide significance ($P<5x10^{-8}$) on the Y-axis by the total number of study participants included in the discovery stage for different complex quantitative traits.

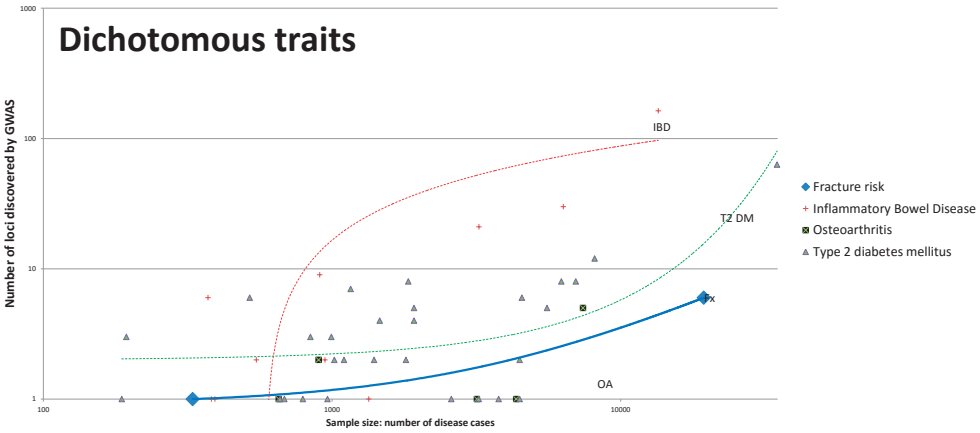


Figure 3. The number of associated loci discovered by GWAS at genome-wide significance ($P<5x10^{-8}$) on the Y-axis by the number of study participants with the disease (cases) included in the discovery stage for different complex diseases.

interest, including imperfect penetrance, allelic heterogeneity, and gene-environment and epigenetic effects.

At the beginning of the GWAS era, the field was dominated by the common disease-common variant hypothesis, which states that common diseases are caused by common genetic variants.[36] Yet, the list of rare genetic variants influencing common disease is growing.[37] In between these two categories are SNPs with minor allele frequency (MAF) of 0.5-5%. In our study of fracture risk, evidence was presented that the study of rare CNVs (defined as MAF<0.5% in some studies) deserves follow-up. The proportion of fracture cases with at least one deletion was significantly higher compared to controls and a 210 kb deletion located on chromosome 6p25.1 was associated with fracture risk (OR=32.58). Also, the first effort in our GEFOS and GENOMOS consortium encompassing a sequencing-based GWAS meta-analysis has discovered *EN1* as a determinant of bone density and fracture (OR=0.85).[38] Likewise, deCODE investigators have discovered common sequence variants in *PTCH1*[39] (MAF= 11.4–22.6%) and less frequent (MAF=0.14%–0.18%) variants in *LGR4*[40] associated with BMD and fractures (OR=1.09 and OR=3.12). Discovery of rare variants is hindered by the large sample sizes required to attain sufficient study power, where research consortia prove their worth through ever-increasing sized meta-analyses. Larger imputation reference panels and sequencing-based genotyping are becoming progressively available, facilitating examination of lower-frequency SNPs and other type of genetic variants such as indels and larger deletions.[41] Furthermore, genetic variance estimation with imputed variants found that the missing heritability for human height and body mass index is negligible;[42] this might apply to other (quantitative) traits such as BMD as well, for studies are needed. Until now, rare variant association studies have found variants with large effects that each explains only a tiny proportion of the phenotypic variance, because the heritability explained is dependent on the effect size and allele frequency.[43] Therefore, arguments can be found to study common and rare variants in the occurrence of common diseases,[43] as confirmed by our experiences. Additionally, the osteoporosis field has started to explore non-coding variation and epigenetics for for instance microRNA,[44] long non-coding RNA,[45] and DNA methylation[46].

I believe that there are skeletal-site specific effects for fracture risk, for example cortical versus trabecular bone, which would justify separate GWAS efforts for specific fracture types. This thinking comes

from the observations that heritability of BMD varies across skeletal sites due to a mixture of shared and specific genetic and environmental influences as quantified by the genetic correlations,[47] which supports the findings that some genetic loci display skeletal-site specific effects.[48] Furthermore, it has been hypothesized that using stricter phenotype definitions and taking into account fracture mechanisms, while preserving sample size, may increase study power. Efforts for clinical vertebral, hip and wrist fractures are underway, but struggle with attaining sufficient study samples to enable discoveries. Therefore, the all-type of fracture GWAS approach seems the starting point to attain maximum sample size for power to perform the first screening for genetic variants that contribute to osteoporotic fracture risk in general. Until now, sample sizes for the subtypes of fractures have been insufficient to perform explorative studies, comparable to those by Kemp, Medina-Gomez et al. for BMD,[47] into genetic correlations of fracture risk. This information could guide the joint selection and exclusion of sub-phenotypes for GWAS. Other even more specific subjects of clinical studies could be atypical (femoral) fractures or fracture healing, which could yield insight into differences in natural healing mechanisms and efficacy of medical treatment between patients.

CLINICAL ASPECTS OF ENDOCRINE BONE DISEASES

The occurrence of fracture is without doubt the most important clinical outcome in osteoporosis. Although fracture risk prediction tools such as FRAX® and Garvan Fracture Risk Calculator may predict which patients will sustain a fracture, these algorithms still underestimate observed fracture risk in at least half of patients.[49] The number of associated conditions with corresponding biomarkers and medications with reported adverse effects on skeletal health continues to expand.[50] A high predicted risk justifies preventative treatment. Clinical measurement of BMD by dual energy X-ray absorptiometry (DXA) is currently the most widespread method to diagnose osteoporosis and evaluate the risk of fracture. Sensitivity and specificity for incident osteoporotic fractures are limited with an area under the ROC curve of 0.63,[51,52] as most fractures occur in mildly to moderately decreased BMD, i.e., osteopenia, or even at normal BMD values.[53] A moderate proportion (0.19) of variance in risk in the all-type of fracture GWAS meta-analysis could be explained by genome-wide common SNPs and the proportion of variance explained by the independent genome-wide replicating SNPs was 0.02. More genetic and non-genetic fracture determinants remain to be discovered.[54]

Furthermore, GWAS holds the potential to identify novel therapeutic targets and genetic biomarkers that will be useful for drug discovery.[55] We have identified multiple brand-new candidate loci and genes associated with BMD and fracture risk and postulate that these could be potential novel drug targets for osteoporosis. Several of the current drug targets for osteoporosis are detected in our unbiased GWAS approach (Table 3). Theoretically, novel understanding of underlying osteoporosis biology may also be hypothesis-generating to explore new indications for existing drugs which are already applied in other conditions if a common genetic basis would be found. In such cases many phases of costly drug development could be shortened. Nelson et al. estimated that selecting genetically supported targets could double the success rate in clinical drug development.[56] Further, among our top fracture associations were signals in the vicinity of the genes encoding sclerostin (SOST) and DKK1, known Wnt signaling antagonists, and RSPO3 and low-density lipoprotein receptor-related protein 5 (LRP5), activators and regulators of canonical Wnt signaling.[57] This is an exciting era in which novel promising drugs are becoming available, for example romosozumab,[58] a monoclonal antibody that binds to SOST. However, it has been difficult to obtain specific Wnt signaling modulators because of the ubiquitous expression

of genes across tissue types and the importance of Wnt signaling in diverse physiological processes, thereby increasing the likelihood of systemic side-effects.[59] Anti-DKK1 monoclonal antibodies BHQ880 are currently under investigation as a potential therapeutic agent for multiple myeloma.[60] No human trials in osteoporosis have been published to date; in animal models, anti-DKK1 antibody increases BMD in mice[61] and macaques[62] and promotes fracture healing through activation of beta-catenin signaling[63]. Perhaps potentially most exciting, the rest of our all-type of fracture loci, 18p11.21 (*FAM210A*), 7q21.3 (*SLC25A13*), 7q31.31 (*CPED1*) and 21q22.2 (*FLJ45139*), repeatedly appear among the top hits in different GWAS for fracture-related traits, such as DXA-BMD and heel bone properties.[64] These may thus indicate until now unknown biological pathways deserving functional follow-up. A good example of this is the follow-up of *WNT16*, which was prioritized from the findings of BMD GWAS,[65-67] in functional biological studies. *WNT16* is highly expressed in cortical bone but for unknown reasons is only moderately expressed in trabecular bone,[68] and *Wnt16*$^{-/-}$ mice have a substantial loss of cortical bone, whereas mice with *Wnt16* overexpression display an increase in trabecular bone.[69]

Table 3. Drugs applied to the field of osteoporosis with corresponding signals from genome-wide association studies (GWAS).

Drugs	Company	*Candidate genes* and protein products	GWAS Loci	GWAS References
Raloxifene	Daiichi Sankyo	*ESR1* estrogen receptor alpha	6q25.1	[48, 64, 70, 71] GEFOS-2
Bazedoxifene	Pfizer			
Denosumab	Amgen	*TNFRSF11A* receptor activator of nuclear factor kappa B; *TNFRSF11B* osteoprotegerin	18q21.33; 8q24.12	[47, 48, 70-74] GEFOS-2
Romosozumab	Amgen	*SOST* sclerostin; *LRP5* low-density lipoprotein receptor-related protein 5; *MEF2C* myocyte enhancer factor 2C	17q21.31; 11q13.2; 5q14.3	[47, 48, 70, 72, 74-76] GEFOS-2
BHQ880	Novartis	*DKK1*Dickkopf-1; *LRP5* low-density lipoprotein receptor-related protein 5	10q21.1; 11q13.2	[47, 48, 64, 72, 74] GEFOS-2

GEFOS-2 denotes the BMD and all-type of fracture GWAS meta-analysis described in Chapter 4.1.

FUTURE RESEARCH DIRECTIONS

1. Phenotypes

More research would be desirable to gain insight into the pathophysiology and acquire a wider and optimized diagnostic and therapeutic arsenal for endocrine and musculoskeletal diseases, including osteoporosis. Deeper phenotyping to better understand underlying disease mechanisms is necessary. In parallel, increasing sample size is clearly a key factor for the identification of more genetic factors of complex diseases, which is evidently illustrated by Figures 2 and 3. Larger-scale integrative and collaborative systematic screening of genetic and non-genetic factors, together with development of more advanced genotyping including sequencing, bioinformatic and statistical analysis techniques is the way forward. The work described in this thesis has contributed to these aspects of osteoporosis research, but further studies are needed.

There may be value in examining longitudinal follow-up assessments within a cohort throughout time, such as for example change in levels of BMD or height. Eventually, as ingeniously demonstrated with the Snyderome,[77] monitoring many different types of measurements of high quality throughout time even in a single individual can be powerful. On the longer term, this longitudinal -omics study design may be

transferrable to cohort studies, including whole genome sequencing data as a backbone of thousands of individuals with millions of datapoints comprising different -omics such as transcriptomics and metabolomics repeated during study follow-up. At present, analytical and computing power are limiting factors. Statistical methods for GWAS of longitudinal BMD have been explored in work by Sikorska et al.,[78] with proposals for statistical methodology to apply for expedite computations[79, 80]. In the specific case of BMD, a life-course approach may be appropriate where for peak bone mass attained at younger ages the emphasis should be on genetic studies because of the high heritability (h^2~50%–85%),[81] whereas for bone loss non-genetic factors become more prominent especially with advanced age (h^2~0%–70%)[82] and possibly the focus should be more on metabolic and environmental factors.

2. Imaging

Histomorphometry is the gold standard for assessing bone, because it is the only method for the direct analysis of bone cells and their activities.[83] Yet, even in the clinical setting bone biopsies are rarely used to diagnose and manage patients with osteoporosis, because of their invasiveness.[84] Molecular imaging, the in vivo characterization and measurement of biologic processes at the cellular and molecular level, is being hailed as the next great advance for imaging.[85] Technical improvements are necessary for human application and minimization of invasiveness and radiation exposure are prerequisites for the introduction into large-scale population imaging studies in the future to aid the analysis of a large variety of musculoskeletal disorders including osteoporosis. In the meantime, alternative innovations may provide fair results to minimally invasively measure bone material properties of human bone in vivo, such as microindentation by the Osteoprobe®,[86, 87] for which the very first results look promising in osteoporotic fractures[88, 89] and type 2 diabetes[26]. Such techniques need to be further validated in clinical studies.

Aforementioned DXA innovations such as TBS and "3D-DXA"[90] with a C-arm can accomplish 3D reconstructions. TBS may be recognized as an independent endophenotype and may have potential to guide clinical decision making similar to DXA-BMD in the future, which again would justify investigations into the determinants of TBS. Nonetheless, there is still a need for additional and more refined radiological imaging investigations for osteoporosis, such as assessments based on CT or MRI, where MRI does not require radiation. However, CT is more costly and requires more radiation than DXA and conventional radiography; MRI, is even more costly and time consuming, produces a limited spatial resolution, and, importantly, there is no signal from cortical bone with conventional MRI pulse sequences. This may be overcome with novel ultrashort or zero echo time (UTE/ZTE) MRI techniques. A concession is QCT, which utilizes low dose scan protocols on a standard CT scanner or (HR-)pQCT by a dedicated extremity scanner.[91] This allows more sophisticated analysis of cortical and trabecular bone, the imaging of trabecular structure and the application of finite element analysis (FEA) to biomechanically estimate bone strength.[92-94] pQCT is also used in exploratory analyses for muscle.[95] However, medical evidence is still too limited to warrant implementation in clinical practice at this point.[96, 97] In the future, diagnostics and therapeutics may separately target cortical versus trabecular bone compartments. Currently applied teriparatide works osteoanabolic for trabecular surfaces, but at the same time it increases cortical porosity.[98]

In population imaging, images of areas of interest or even of the whole body are acquired and analyzed in hundreds to thousands of participants in population-based cohort studies. This approach increases our understanding of natural variation and the natural history of diseases, and may point us to novel risk factors and biomarkers. In the Rotterdam Study, radiography, ultrasound, CT and MRI images are available of for example brain, abdominal organs, cardiovascular and locomotor systems in a total

of almost 15,000 individuals.[99] Population imaging groups will need to join forces in research consortia to pool sample sizes. By banding together, knowledge and experiences could be exchanged more intensively, furthermore, common research databases systems can be set-up containing de-identified participant data including image biobanks. In that case, close to perfect data harmonization would become conceivable through central measurements by expert investigators employing a single clearly defined protocol.

3. Genomics

However, some of the measurement methods currently available are simply too expensive or invasive to apply on a population level at present. Yet, current limits are being challenged, with the very first successful large-scale applications of whole-genome sequencing and deep imputation using sequencing-based reference panels.[38] The Haplotype Reference Consortium (HRC) is creating a large reference panel of human haplotypes by combining together sequencing data from multiple cohorts. However, the genome may be too distant in the cascade from the disease of interest to detect clinically relevant patterns, therefore, screening the transcriptome, epigenome, metabolome, proteome and even microbiome at certain time points may prove necessary. This, again, can be followed-up by typing only a selection of markers, possibly by custom content on microarrays, in a select replication sample of succinctly phenotyped individuals or several distinct clinical states in fewer individuals. This may change with the continuing drop in costs of technologies in the long term, particularly next-generation sequencing. Improved quality and increased density of genotyping and imputations will increase confidence in genetic information and will further enhance examination of structural variation and rarer variants.

Oftentimes the function of genes contained in the associated loci are not (completely) known. Functional follow-up studies are needed, yet, the development of animal knock-out-models may take years. Establishment of multi-disciplinary research consortia world-wide may be beneficial to efficiently take GWAS discoveries to functional follow-up in a harmonized research pipeline. Also, publicly available databases are being launched to enhance interpretation of genomic sequence information, promoting mutual data sharing between expert consortia, professional organizations, health care providers, and patients.[100] Moreover, the GWAS association signal in the radiographic vertebral fracture GWAS did not lie within a gene, and the same was true for some of the signals in the BMD and all-type of fracture GWAS. An inventory of the GWAS catalog in 2009 revealed that 88% of the GWAS associations are in either intergenic or intronic regions,[101] regions of the genome we still understand little about, but to which GWAS has contributed by indicating regulatory sites. The Encyclopedia of DNA Elements (ENCODE) project, aiming to identify all functional elements in the human genome, has drastically enriched our comprehension about regions outside of the exome and showed that many GWAS SNPs overlap transcription-factor-occupied regions whereas or DNase I hypersensitive sites and are particularly enriched in the segmentation classes associated with enhancers and transcription start sites.[102] A striking finding is that obesity-associated noncoding sequences within the *FTO* locus are associated with expression of the homeobox gene *IRX3* at megabase distances, but not with *FTO* itself;[103] this association seems to be driven by a topologically associated domain (TAD) structure encompassing the *FTO* and *IRXB* genes cluster.[104] Such genomic explorations remain to be performed for osteoporosis-related traits.

GWAS for various osteoporosis-related traits have shown that targeting quantitative endophenotypes with excellent measurement properties (root mean square standard deviation expressed as coefficient of variation of 1.01.2% for the spine and 1.12.2% for the femoral neck by DXA)[105] is efficient in the number of loci discovered. An exception may be when extreme phenotypes[76, 106] display threshold effects, then

fewer subjects may be needed with enrichment for highly penetrant variants,[107, 108] but still adequate sample sizes should be collected to achieve sufficient discovery power.[109] However, the tough start of the fracture GWAS may be rooted in the complex phenotype definition and heterogeneity of the trait and its underlying genetics. A better understanding of the genetic architecture seems necessary. More clarity is needed which fracture phenotypes should be studied together because they have a joint genetic etiology, and which do not and thus should be analyzed separately. Then robust selection criteria should be defined for an optimal fracture phenotype definition of interest. Research ideas include data enrichment for cases that have a known family history for osteoporosis, fractured at relatively young age or sustained multiple fractures, etc. Perhaps further exclusion criteria need to be established for cases that are thought to be caused by non-genetic mechanisms. Refinement and automatization of measurements may enhance the richness, quality and quantity of research data available. Combination into multivariate GWAS of multiple disease-related traits could further exploit the detection of pleiotropic effects[110] and novel statistical methods may be able to better utilize the richer phenotype information that will become available.[111, 112]

4. Drugs and pharmacogenetics

So far, therapies used to increase bone strength in individuals with osteoporosis are mainly antiresorptives.[113] Bisphosphonates are the most widely used first-line because of their effectiveness, reasonable safety, and a low cost price.[114] However, no single antiresorptive therapy is currently appropriate for all patients, as a subgroup of patients put on anti-fracture medication responds suboptimally, e.g., small gain in bone mass or new fractures occur in spite of treatment, or negative side-effects including as osteonecrosis of the jaw or Atypical Femoral Fractures (AFF).[115] To our knowledge no pharmacogenetic studies examining these phenomena in osteoporosis have been published to date. In the future, results from pharmacogenomics studies may aid in assigning the most effective therapy to specific patient groups and it has been hypothesized that genetic biomarkers can be identified to pinpoint those patients most vulnerable to side-effects of certain agents. Nevertheless, because interaction studies tend to involve more parameters, up to four times as many subjects are needed;[116] unless extremely large effects are in place, as we have witnessed for a few pharmacogenomic successes, such as anticoagulant dosing according to *VKORC1* haplotypes and HLA-B*5701 screening for the risk of hypersensitivity reaction to abacavir in HIV.[117] Until now in genetic osteoporosis research, solely candidate gene studies have appeared investigating genetically-based variation in treatment response to raloxifene, teriparatide and bisphosphonates.[118] One of the reasons for this is that the coverage of pharmacogenomics variants is limited on current GWAS genotyping platforms,[119, 120] but this may improve with novel microarrays becoming available.

5. Risk prediction and personalized medicine

Finally, our clinical practice is in need for improved prediction of fracture risk and more effective prevention and treatment options. Populations at increased risk of fractures should be identified in time and subgroups of patients may require different approaches. To be able to initiate precision medicine for osteoporosis and other conditions associated with fractures, we will require better performing yet feasible and cost-effective investigations. Eventually, applying an integrative personal omics profile (iPOP)[77] approach to osteoporosis may be the Holy Grail. Clinical risk assessments and treatment evaluations may be enhanced with integrated diagnostics utilizing novel biomarkers incorporating possibly (molecular) imaging, minimally invasive measurements of bone material properties, genomic, transcriptomic, and

metabolomic information.[121] Standardized storage and longitudinal follow-up of these parameters integrated into the electronic medical record will be required. We should not be afraid to acquire these data in clinic, transfer them to the research bench to get to understand the meaning and clinical relevance of this data, and then bring this new knowledge back to the bedside to actually help our patients. Knowledge is required about what findings are normal and what is abnormal, and how to interpret what is clinically relevant. Then, medical education about this methodology is needed to have this implemented in practice. Choices for osteoporosis therapy have and are still expanding; GWAS findings may point to even more potential drug targets remaining to be explored. Hence, it will become increasingly important to apply the optimal individualized treatment strategy as our patients deserve the right drug at the right time.

REFERENCES

1. Hillier TA, Lui LY, Kado DM, LeBlanc ES, Vesco KK, Bauer DC, et al. Height loss in older women: risk of hip fracture and mortality independent of vertebral fractures. J Bone Miner Res. 2012 Jan;27(1):153-9.
2. van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2002 Jun;17(6):1051-6.
3. van der Klift M, de Laet CE, McCloskey EV, Johnell O, Kanis JA, Hofman A, et al. Risk factors for incident vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res. 2004 Jul;19(7):1172-80.
4. Szulc P, Bouxsein ML. Overview of osteoporosis: Epidemiology and clinical management. Vertebral Fracture Initiative Resource Document. 2011;PART I:1-65.
5. Cauley JA, Hochberg MC, Lui LY, Palermo L, Ensrud KE, Hillier TA, et al. Long-term risk of incident vertebral fractures. JAMA. 2007 Dec 19;298(23):2761-7.
6. Liu M, Guo L, Pei Y, Li N, Jin M, Ma L, et al. Efficacy of zoledronic acid in treatment of osteoporosis in men and women-a meta-analysis. Int J Clin Exp Med. 2015;8(3):3855-61.
7. The Dutch Institute for Health Care Improvement CBO. Guideline Osteoporosis and fracture prevention (third revision)2011.
8. Brett A, Miller CG, Hayes CW, Krasnow J, Ozanian T, Abrams K, et al. Development of a clinical workflow tool to enhance the detection of vertebral fractures: accuracy and precision evaluation. Spine (Phila Pa 1976). 2009 Oct 15;34(22):2437-43.
9. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. Osteoporos Int. 2004 Nov;15(11):887-96.
10. Briot K, Paternotte S, Kolta S, Eastell R, Reid DM, Felsenberg D, et al. Added value of trabecular bone score to bone mineral density for prediction of osteoporotic fractures in postmenopausal women: the OPUS study. Bone. 2013 Nov;57(1):232-6.
11. Hans D, Goertzen AL, Krieg MA, Leslie WD. Bone microarchitecture assessed by TBS predicts osteoporotic fractures independent of bone density: the Manitoba study. J Bone Miner Res. 2011 Nov;26(11):2762-9.
12. Iki M, Tamaki J, Kadowaki E, Sato Y, Dongmei N, Winzenrieth R, et al. Trabecular Bone Score (TBS) Predicts Vertebral Fractures in Japanese Women Over 10 Years Independently of Bone Density and Prevalent Vertebral Deformity: The Japanese Population-Based Osteoporosis (JPOS) Cohort Study. J Bone Miner Res. 2014 Feb;29(2):399-407.
13. Boutroy S, Hans D, Sornay-Rendu E, Vilayphiou N, Winzenrieth R, Chapurlat R. Trabecular bone score improves fracture risk prediction in non-osteoporotic women: the OFELY study. Osteoporos Int. 2013 Jan;24(1):77-85.
14. Krieg MA, Aubry-Rozier B, Hans D, Leslie WD. Effects of anti-resorptive agents on trabecular bone score (TBS) in older women. Osteoporos Int. 2013 Mar;24(3):1073-8.
15. Popp AW, Guler S, Lamy O, Senn C, Buffat H, Perrelet R, et al. Effects of zoledronate versus placebo on spine bone mineral density and microarchitecture assessed by the trabecular bone score in postmenopausal women with osteoporosis: a three-year study. J Bone Miner Res. 2013 Mar;28(3):449-54.

16. Eller-Vainicher C, Filopanti M, Palmieri S, Ulivieri FM, Morelli V, Zhukouskaya VV, et al. Bone quality, as measured by trabecular bone score, in patients with primary hyperparathyroidism. Eur J Endocrinol. 2013 Aug;169(2):155-62.
17. Eller-Vainicher C, Morelli V, Ulivieri FM, Palmieri S, Zhukouskaya VV, Cairoli E, et al. Bone quality, as measured by trabecular bone score in patients with adrenal incidentalomas with and without subclinical hypercortisolism. J Bone Miner Res. 2012 Oct;27(10):2223-30.
18. Breban S, Briot K, Kolta S, Paternotte S, Ghazi M, Fechtenbaum J, et al. Identification of rheumatoid arthritis patients with vertebral fractures using bone mineral density and trabecular bone score. J Clin Densitom. 2012 Jul-Sep;15(3):260-6.
19. Leslie WD, Aubry-Rozier B, Lamy O, Hans D. TBS (trabecular bone score) and diabetes-related fracture risk. J Clin Endocrinol Metab. 2013 Feb;98(2):602-9.
20. de Liefde II, van der Klift M, de Laet CE, van Daele PL, Hofman A, Pols HA. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporos Int. 2005 Dec;16(12):1713-20.
21. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011 Jun 1;305(21):2184-92.
22. Giangregorio LM, Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, et al. FRAX underestimates fracture risk in patients with diabetes. J Bone Miner Res. 2012 Feb;27(2):301-8.
23. Xu L, Chan WM, Hui YF, Lam TH. Association between HbA1c and cardiovascular disease mortality in older Hong Kong Chinese with diabetes. Diabet Med. 2012 Mar;29(3):393-8.
24. Burghardt AJ, Issever AS, Schwartz AV, Davis KA, Masharani U, Majumdar S, et al. High-resolution peripheral quantitative computed tomographic imaging of cortical and trabecular bone microarchitecture in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2010 Nov;95(11):5045-55.
25. Patsch JM, Burghardt AJ, Yap SP, Baum T, Schwartz AV, Joseph GB, et al. Increased cortical porosity in type 2 diabetic postmenopausal women with fragility fractures. J Bone Miner Res. 2013 Feb;28(2):313-24.
26. Farr JN, Drake MT, Amin S, Melton LJ, 3rd, McCready LK, Khosla S. In Vivo assessment of bone quality in post-menopausal women with type 2 diabetes. J Bone Miner Res. 2013 Oct 1.
27. Schwartz AV, Margolis KL, Sellmeyer DE, Vittinghoff E, Ambrosius WT, Bonds DE, et al. Intensive glycemic control is not associated with fractures or falls in the ACCORD randomized trial. Diabetes Care. 2012 Jul;35(7):1525-31.
28. Gonnelli S, Caffarelli C, Giordano N, Nuti R. The prevention of fragility fractures in diabetic patients. Aging Clin Exp Res. 2015 Apr;27(2):115-24.
29. Zhu M, Zhao S. Candidate gene identification approach: progress and challenges. Int J Biol Sci. 2007;3(7):420-7.
30. Manolio TA, Brooks LD, Collins FS. A HapMap harvest of insights into the genetics of common disease. J Clin Invest. 2008 May;118(5):1590-605.
31. Guttmacher AE, Collins FS, Carmona RH. The family history--more important than ever. N Engl J Med. 2004 Nov 25;351(22):2333-6.
32. Kanis JA, Johansson H, Oden A, Johnell O, De Laet C, Eisman JA, et al. A family history of fracture and fracture risk: a meta-analysis. Bone. 2004 Nov;35(5):1029-37.
33. Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. Am J Hum Genet. 2012 Jan 13;90(1):7-24.
34. Servick K. Can 23andMe have it all? Science. 2015 Sep 25;349(6255):1472-4, 6-7.
35. Thompson SG, Willeit P. UK Biobank comes of age. Lancet. 2015 Aug 8;386(9993):509-10.
36. Hirschhorn JN. Genomewide association studies--illuminating biologic pathways. N Engl J Med. 2009 Apr 23;360(17):1699-701.
37. Iles MM. What can genome-wide association studies tell us about the genetics of common disease? PLoS Genet. 2008 Feb;4(2):e33.
38. Zheng HF, Forgetta V, Hsu YH, Estrada K, Rosello-Diez A, Leo PJ, et al. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature. 2015 Oct 1;526(7571):112-7.

39. Styrkarsdottir U, Thorleifsson G, Gudjonsson SA, Sigurdsson A, Center JR, Lee SH, et al. Sequence variants in the PTCH1 gene associate with spine bone mineral density and osteoporotic fractures. Nat Commun. 2016;7:10129.
40. Styrkarsdottir U, Thorleifsson G, Sulem P, Gudbjartsson DF, Sigurdsson A, Jonasdottir A, et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. Nature. 2013 May 23;497(7450):517-20.
41. Zheng HF, Rong JJ, Liu M, Han F, Zhang XW, Richards JB, et al. Performance of genotype imputation for low frequency and rare variants from the 1000 genomes. PLoS One. 2015;10(1):e0116487.
42. Yang J, Bakshi A, Zhu Z, Hemani G, Vinkhuyzen AA, Lee SH, et al. Genetic variance estimation with imputed variants finds negligible missing heritability for human height and body mass index. Nat Genet. 2015 Oct;47(10):1114-20.
43. Gibson G. Rare and common variants: twenty arguments. Nat Rev Genet. 2011 Feb;13(2):135-45.
44. Krzeszinski JY, Wei W, Huynh H, Jin Z, Wang X, Chang TC, et al. miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2. Nature. 2014 Aug 28;512(7515):431-5.
45. Tong X, Gu PC, Xu SZ, Lin XJ. Long non-coding RNA-DANCR in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. Biosci Biotechnol Biochem. 2015 May;79(5):732-7.
46. Reppe S, Noer A, Grimholt RM, Halldorsson BV, Medina-Gomez C, Gautvik VT, et al. Methylation of bone SOST, its mRNA, and serum sclerostin levels correlate strongly with fracture risk in postmenopausal women. J Bone Miner Res. 2015 Feb;30(2):249-56.
47. Kemp JP, Medina-Gomez C, Estrada K, St Pourcain B, Heppe DH, Warrington NM, et al. Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment. PLoS Genet. 2014 Jun;10(6):e1004423.
48. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009 Nov;41(11):1199-206.
49. van Geel TA, Eisman JA, Geusens PP, van den Bergh JP, Center JR, Dinant GJ. The utility of absolute risk prediction using FRAX(R) and Garvan Fracture Risk Calculator in daily practice. Maturitas. 2014 Feb;77(2):174-9.
50. Davidge Pitts CJ, Kearns AE. Update on medications with adverse skeletal effects. Mayo Clin Proc. 2011 Apr;86(4):338-43; quiz 43.
51. Kanis JA, Oden A, Johnell O, Johansson H, De Laet C, Brown J, et al. The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women. Osteoporos Int. 2007 Aug;18(8):1033-46.
52. Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, Kanis JA. Independent clinical validation of a Canadian FRAX tool: fracture prediction and model calibration. J Bone Miner Res. 2010 Nov;25(11):2350-8.
53. Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone. 2004 Jan;34(1):195-202.
54. Silverman SL, Calderon AD. The utility and limitations of FRAX: A US perspective. Curr Osteoporos Rep. 2010 Dec;8(4):192-7.
55. Kingsmore SF, Lindquist IE, Mudge J, Gessler DD, Beavis WD. Genome-wide association studies: progress and potential for drug discovery and development. Nat Rev Drug Discov. 2008 Mar;7(3):221-30.
56. Nelson MR, Tipney H, Painter JL, Shen J, Nicoletti P, Shen Y, et al. The support of human genetic evidence for approved drug indications. Nat Genet. 2015 Aug;47(8):856-60.
57. Cruciat CM, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harb Perspect Biol. 2013 Mar;5(3):a015081.
58. McClung MR, Grauer A, Boonen S, Bolognese MA, Brown JP, Diez-Perez A, et al. Romosozumab in Postmenopausal Women with Low Bone Mineral Density. N Engl J Med. 2014 Jan 1.
59. Zimmerman ZF, Moon RT, Chien AJ. Targeting Wnt pathways in disease. Cold Spring Harb Perspect Biol. 2012 Nov;4(11).

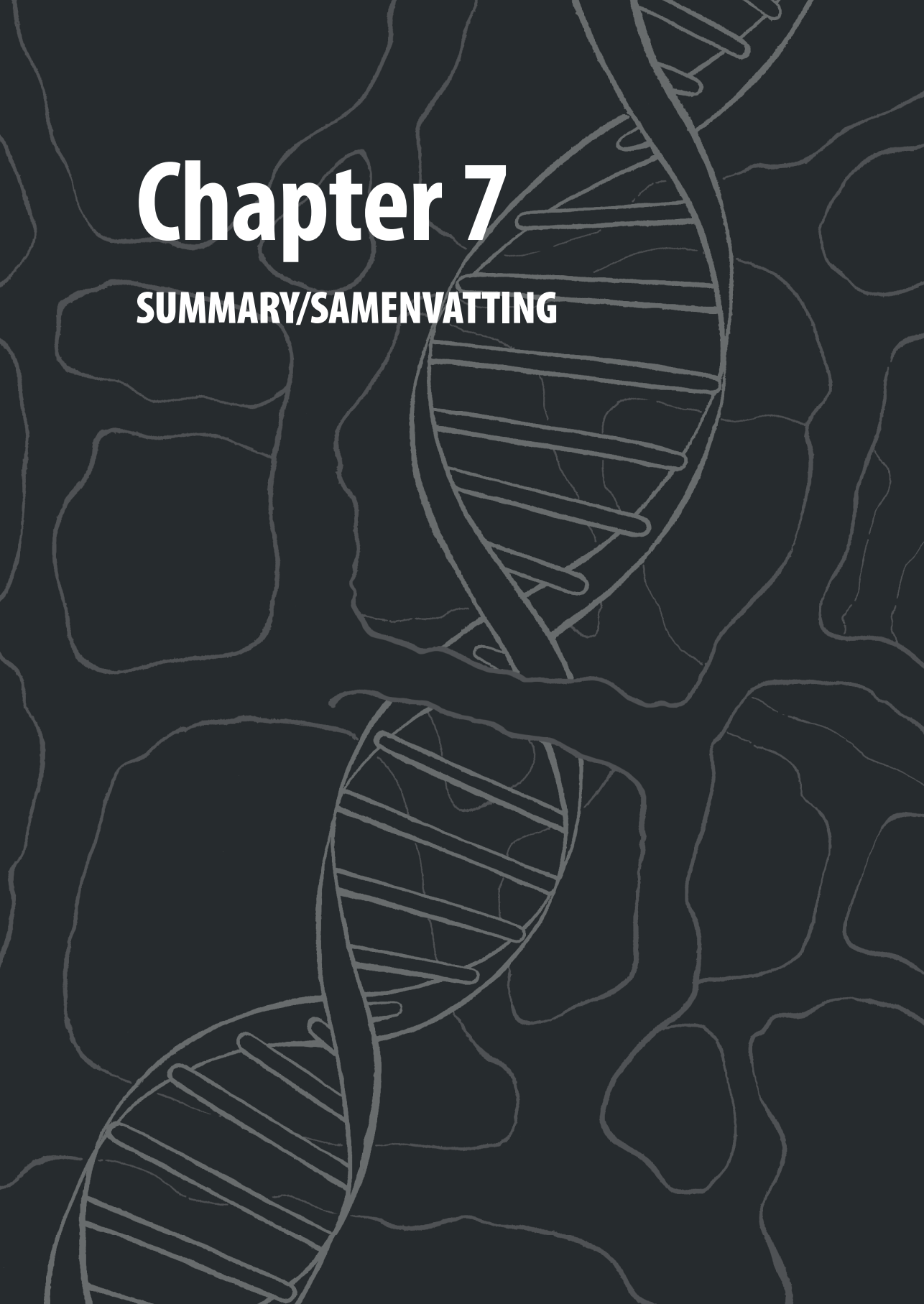
60. Iyer SP, Beck JT, Stewart AK, Shah J, Kelly KR, Isaacs R, et al. A Phase IB multicentre dose-determination study of BHQ880 in combination with anti-myeloma therapy and zoledronic acid in patients with relapsed or refractory multiple myeloma and prior skeletal-related events. Br J Haematol. 2014 Nov;167(3):366-75.
61. Li X, Grisanti M, Fan W, Asuncion FJ, Tan HL, Dwyer D, et al. Dickkopf-1 regulates bone formation in young growing rodents and upon traumatic injury. J Bone Miner Res. 2011 Nov;26(11):2610-21.
62. Glantschnig H, Scott K, Hampton R, Wei N, McCracken P, Nantermet P, et al. A rate-limiting role for Dickkopf-1 in bone formation and the remediation of bone loss in mouse and primate models of postmenopausal osteoporosis by an experimental therapeutic antibody. J Pharmacol Exp Ther. 2011 Aug;338(2):568-78.
63. Jin H, Wang B, Li J, Xie W, Mao Q, Li S, et al. Anti-DKK1 antibody promotes bone fracture healing through activation of beta-catenin signaling. Bone. 2015 Feb;71:63-75.
64. Moayyeri A, Hsu YH, Karasik D, Estrada K, Xiao SM, Nielson C, et al. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Hum Mol Genet. 2014 Jun 1;23(11):3054-68.
65. Medina-Gomez C, Kemp JP, Estrada K, Eriksson J, Liu J, Reppe S, et al. Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. PLoS Genet. 2012 Jul;8(7):e1002718.
66. Zheng HF, Tobias JH, Duncan E, Evans DM, Eriksson J, Paternoster L, et al. WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. PLoS Genet. 2012 Jul;8(7):e1002745.
67. Koller DL, Zheng HF, Karasik D, Yerges-Armstrong L, Liu CT, McGuigan F, et al. Meta-analysis of genome-wide studies identifies WNT16 and ESR1 SNPs associated with bone mineral density in premenopausal women. J Bone Miner Res. 2013 Mar;28(3):547-58.
68. Moverare-Skrtic S, Henning P, Liu X, Nagano K, Saito H, Borjesson AE, et al. Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. Nat Med. 2014 Nov;20(11):1279-88.
69. Moverare-Skrtic S, Wu J, Henning P, Gustafsson KL, Sjogren K, Windahl SH, et al. The bone-sparing effects of estrogen and WNT16 are independent of each other. Proc Natl Acad Sci U S A. 2015 Dec 1;112(48):14972-7.
70. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. Multiple genetic loci for bone mineral density and fractures. N Engl J Med. 2008 May 29;358(22):2355-65.
71. Paternoster L, Lorentzon M, Lehtimaki T, Eriksson J, Kahonen M, Raitakari O, et al. Genetic determinants of trabecular and cortical volumetric bone mineral densities and bone microstructure. PLoS Genet. 2013;9(2):e1003247.
72. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008 May 3;371(9623):1505-12.
73. Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, et al. An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet. 2010 Jun;6(6):e1000977.
74. Zhang L, Choi HJ, Estrada K, Leo PJ, Li J, Pei YF, et al. Multistage genome-wide association meta-analyses identified two new loci for bone mineral density. Hum Mol Genet. 2014 Apr 1;23(7):1923-33.
75. Zheng HF, Duncan EL, Yerges-Armstrong LM, Eriksson J, Bergstrom U, Leo PJ, et al. Meta-analysis of genome-wide studies identifies MEF2C SNPs associated with bone mineral density at forearm. J Med Genet. 2013 Jul;50(7):473-8.
76. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011 Apr;7(4):e1001372.
77. Chen R, Mias GI, Li-Pook-Than J, Jiang L, Lam HY, Miriami E, et al. Personal omics profiling reveals dynamic molecular and medical phenotypes. Cell. 2012 Mar 16;148(6):1293-307.
78. Sikorska K, Rivadeneira F, Groenen PJ, Hofman A, Uitterlinden AG, Eilers PH, et al. Fast linear mixed model computations for genome-wide association studies with longitudinal data. Stat Med. 2013 Jan 15;32(1):165-80.
79. Sikorska K, Montazeri NM, Uitterlinden A, Rivadeneira F, Eilers PH, Lesaffre E. GWAS with longitudinal phenotypes: performance of approximate procedures. Eur J Hum Genet. 2015 Feb 25.

80. Sikorska K, Lesaffre E, Groenen PF, Eilers PH. GWAS on your notebook: fast semi-parallel linear and logistic regression for genome-wide association studies. BMC Bioinformatics. 2013;14:166.
81. McGuigan FE, Murray L, Gallagher A, Davey-Smith G, Neville CE, Van't Hof R, et al. Genetic and environmental determinants of peak bone mass in young men and women. J Bone Miner Res. 2002 Jul;17(7):1273-9.
82. Moayyeri A, Hammond CJ, Hart DJ, Spector TD. Effects of age on genetic influence on bone loss over 17 years in women: the Healthy Ageing Twin Study (HATS). J Bone Miner Res. 2012 Oct;27(10):2170-8.
83. Iwaniec UT, Wronski TJ, Turner RT. Histological analysis of bone. Methods Mol Biol. 2008;447:325-41.
84. Malluche HH, Mawad H, Monier-Faugere MC. Bone biopsy in patients with osteoporosis. Curr Osteoporos Rep. 2007 Dec;5(4):146-52.
85. Hoffman JM, Gambhir SS. Molecular imaging: the vision and opportunity for radiology in the future. Radiology. 2007 Jul;244(1):39-47.
86. Bridges D, Randall C, Hansma PK. A new device for performing reference point indentation without a reference probe. Rev Sci Instrum. 2012 Apr;83(4):044301.
87. Randall C, Bridges D, Guerri R, Nogues X, Puig L, Torres E, et al. Applications of a New Handheld Reference Point Indentation Instrument Measuring Bone Material Strength. J Med Device. 2013 Dec;7(4):410051-6.
88. Diez-Perez A, Guerri R, Nogues X, Caceres E, Pena MJ, Mellibovsky L, et al. Microindentation for in vivo measurement of bone tissue mechanical properties in humans. J Bone Miner Res. 2010 Aug;25(8):1877-85.
89. Guerri-Fernandez RC, Nogues X, Quesada Gomez JM, Torres Del Pliego E, Puig L, Garcia-Giralt N, et al. Microindentation for in vivo measurement of bone tissue material properties in atypical femoral fracture patients and controls. J Bone Miner Res. 2013 Jan;28(1):162-8.
90. Kolta S, Quiligotti S, Ruyssen-Witrand A, Amido A, Mitton D, Bras AL, et al. In vivo 3D reconstruction of human vertebrae with the three-dimensional X-ray absorptiometry (3D-XA) method. Osteoporos Int. 2008 Feb;19(2):185-92.
91. Adams JE. Quantitative computed tomography. Eur J Radiol. 2009 Sep;71(3):415-24.
92. Boutroy S, Van Rietbergen B, Sornay-Rendu E, Munoz F, Bouxsein ML, Delmas PD. Finite element analysis based on in vivo HR-pQCT images of the distal radius is associated with wrist fracture in postmenopausal women. J Bone Miner Res. 2008 Mar;23(3):392-9.
93. Amin S, Kopperdhal DL, Melton LJ, 3rd, Achenbach SJ, Therneau TM, Riggs BL, et al. Association of hip strength estimates by finite-element analysis with fractures in women and men. J Bone Miner Res. 2011 Jul;26(7):1593-600.
94. Chevalier Y, Quek E, Borah B, Gross G, Stewart J, Lang T, et al. Biomechanical effects of teriparatide in women with osteoporosis treated previously with alendronate and risedronate: results from quantitative computed tomography-based finite element analysis of the vertebral body. Bone. 2010 Jan;46(1):41-8.
95. Erlandson MC, Lorbergs AL, Mathur S, Cheung AM. Muscle analysis using pQCT, DXA and MRI. Eur J Radiol. 2016 Mar 4.
96. Engelke K, Adams JE, Armbrecht G, Augat P, Bogado CE, Bouxsein ML, et al. Clinical use of quantitative computed tomography and peripheral quantitative computed tomography in the management of osteoporosis in adults: the 2007 ISCD Official Positions. J Clin Densitom. 2008 Jan-Mar;11(1):123-62.
97. Zemel B, Bass S, Binkley T, Ducher G, Macdonald H, McKay H, et al. Peripheral quantitative computed tomography in children and adolescents: the 2007 ISCD Pediatric Official Positions. J Clin Densitom. 2008 Jan-Mar;11(1):59-74.
98. Burr DB. Cortical bone: a target for fracture prevention? Lancet. 2010 May 15;375(9727):1672-3.
99. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, et al. The Rotterdam Study: 2016 objectives and design update. Eur J Epidemiol. 2015 Aug;30(8):661-708.
100. Rehm HL, Berg JS, Brooks LD, Bustamante CD, Evans JP, Landrum MJ, et al. ClinGen--the Clinical Genome Resource. N Engl J Med. 2015 Jun 4;372(23):2235-42.
101. Hindorff LA, Sethupathy P, Junkins HA, Ramos EM, Mehta JP, Collins FS, et al. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. Proc Natl Acad Sci U S A. 2009 Jun 9;106(23):9362-7.
102. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012 Sep 6;489(7414):57-74.

103. Smemo S, Tena JJ, Kim KH, Gamazon ER, Sakabe NJ, Gomez-Marin C, et al. Obesity-associated variants within FTO form long-range functional connections with IRX3. Nature. 2014 Mar 20;507(7492):371-5.
104. Hunt LE, Noyvert B, Bhaw-Rosun L, Sesay AK, Paternoster L, Nohr EA, et al. Complete re-sequencing of a 2Mb topological domain encompassing the FTO/IRXB genes identifies a novel obesity-associated region upstream of IRX5. Genome Med. 2015;7:126.
105. Baim S, Wilson CR, Lewiecki EM, Luckey MM, Downs RW, Jr., Lentle BC. Precision assessment and radiation safety for dual-energy X-ray absorptiometry: position paper of the International Society for Clinical Densitometry. J Clin Densitom. 2005 Winter;8(4):371-8.
106. Kung AW, Xiao SM, Cherny S, Li GH, Gao Y, Tso G, et al. Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies. Am J Hum Genet. 2010 Feb 12;86(2):229-39.
107. Romeo S, Pennacchio LA, Fu Y, Boerwinkle E, Tybjaerg-Hansen A, Hobbs HH, et al. Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. Nat Genet. 2007 Apr;39(4):513-6.
108. Emond MJ, Louie T, Emerson J, Zhao W, Mathias RA, Knowles MR, et al. Exome sequencing of extreme phenotypes identifies DCTN4 as a modifier of chronic Pseudomonas aeruginosa infection in cystic fibrosis. Nat Genet. 2012 Aug;44(8):886-9.
109. Berndt SI, Gustafsson S, Magi R, Ganna A, Wheeler E, Feitosa MF, et al. Genome-wide meta-analysis identifies 11 new loci for anthropometric traits and provides insights into genetic architecture. Nat Genet. 2013 May;45(5):501-12.
110. Cross-Disorder Group of the Psychiatric Genomics C, Genetic Risk Outcome of Psychosis C. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. Lancet. 2013 Apr 20;381(9875):1371-9.
111. Lee SH, Yang J, Goddard ME, Visscher PM, Wray NR. Estimation of pleiotropy between complex diseases using single-nucleotide polymorphism-derived genomic relationships and restricted maximum likelihood. Bioinformatics. 2012 Oct 1;28(19):2540-2.
112. Cross-Disorder Group of the Psychiatric Genomics C, Lee SH, Ripke S, Neale BM, Faraone SV, Purcell SM, et al. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. Nat Genet. 2013 Sep;45(9):984-94.
113. Chen JS, Sambrook PN. Antiresorptive therapies for osteoporosis: a clinical overview. Nat Rev Endocrinol. 2012 Feb;8(2):81-91.
114. Lems WF, den Heijer M. Established and forthcoming drugs for the treatment of osteoporosis. Neth J Med. 2013 May;71(4):188-93.
115. McClung M, Harris ST, Miller PD, Bauer DC, Davison KS, Dian L, et al. Bisphosphonate therapy for osteoporosis: benefits, risks, and drug holiday. Am J Med. 2013 Jan;126(1):13-20.
116. Smith PG, Day NE. The design of case-control studies: the influence of confounding and interaction effects. Int J Epidemiol. 1984 Sep;13(3):356-65.
117. Hakonarson H. Ask the experts: pharmacogenomics and genome-wide association studies. Pharmacogenomics. 2013 Mar;14(4):365-8.
118. Rojo Venegas K, Aguilera Gomez M, Eisman JA, Garcia Sanchez A, Faus Dader MJ, Calleja Hernandez MA. Pharmacogenetics of osteoporosis-related bone fractures: moving towards the harmonization and validation of polymorphism diagnostic tools. Pharmacogenomics. 2010 Sep;11(9):1287-303.
119. Peters EJ, McLeod HL. Ability of whole-genome SNP arrays to capture 'must have' pharmacogenomic variants. Pharmacogenomics. 2008 Nov;9(11):1573-7.
120. Motsinger-Reif AA, Jorgenson E, Relling MV, Kroetz DL, Weinshilboum R, Cox NJ, et al. Genome-wide association studies in pharmacogenomics: successes and lessons. Pharmacogenet Genomics. 2013 Aug;23(8):383-94.
121. Krestin GP, Grenier PA, Hricak H, Jackson VP, Khong PL, Miller JC, et al. Integrated diagnostics: proceedings from the 9th biennial symposium of the International Society for Strategic Studies in Radiology. Eur Radiol. 2012 Nov;22(11):2283-94.

Chapter 7

SUMMARY/SAMENVATTING



SUMMARY

An abnormally low bone mineral density and/or a disrupted bone microarchitecture predispose to increased fracture risk, as is the case in osteoporosis and diabetes-related bone disease. These disease entities are known to have a multifactorial etiology and have a high population prevalence, particularly in the elderly. In this thesis, different aspects of musculoskeletal diseases were investigated, i.e., epidemiology, radiology, and genetics.

Chapter 2 describes several epidemiological studies of osteoporotic fractures performed in the Rotterdam Study. In chapter 2.1 we found that in women, short term use of loop diuretics is associated with an increased level of free deoxypyridinoline, most likely reflecting increased bone resorption by osteoclasts. In chapter 2.2 we learned that cases with lumbar disc degeneration have a systemically higher bone mineral density, but this does not protect them from getting vertebral and non-vertebral fractures. In chapter 2.3 we explored the association between C-reactive protein levels and increased fracture risk. The following two sub-chapters focus on the epidemiology of diabetes-related bone disease. In chapter 2.4 we studied the literature available for the association between bone mineral density and type 2 diabetes mellitus. Our meta-analysis of these observational studies confirmed that bone mineral density is elevated at multiple skeletal sites in patients with type 2 diabetes mellitus compared to individuals without diabetes. As our meta-regression indicated that glycated hemoglobin (HbA_{1C}) modifies this relationship, we decided to explore this further in the Rotterdam Study in chapter 2.5. Here we found that the group with type 2 diabetes mellitus and poor glucose control had a higher fracture risk than the group with type 2 diabetes mellitus and good glucose control or the group without diabetes. Intriguingly, at the same time the group with type 2 diabetes mellitus and poor glucose control had a higher bone mineral density, narrower neck width and thicker cortices at the femoral neck. Chapter 2.6 contains a review article on diabetic bone complications. Chapter 2.7 is a review describing osteoporotic vertebral fractures as part of systemic disease.

Chapter 3 brings a comparative appraisal of radiological scoring methods for osteoporotic vertebral fractures. In chapter 3.1 we review different radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings. When applying two of the most commonly used radiological scoring methods to the Rotterdam Study we found that prevalences vary widely between these two methods, as described in chapter 3.2. The data in chapter 3.3 demonstrate that trabecular bone scores are strongly associated with prevalent vertebral fractures in women, and that this measure provides information independent of bone mineral density analyzed by dual energy X-ray absorptiometry. Chapter 3.4 illustrates that the data acquired in vertebral fracture morphometry analyses provide multiple quantitative parameters, which could be relevant for different musculoskeletal disorders. In chapter 3.5 we evaluated the radiological criteria and disease prevalence in the Rotterdam Study. In chapter 3.6 we assessed how many of Scheuermann's disease cases would be incorrectly diagnosed as osteoporotic vertebral fractures, where we found that the algorithm-based qualitative method performs better than quantitative morphometry. Chapter 3.7 contains case series, which emphasizes the need to differentiate osteoporotic vertebral fractures from Scheuermann's disease in daily clinical practice.

Chapter 4 presents various genetic epidemiological studies for fracture risk, with the majority being large-scale projects executed within the framework of the genetic factors for osteoporosis (GEFOS) and genetic markers for osteoporosis (GENOMOS) consortia. In Chapter 4.1 we identified 56 genetic loci for femoral neck bone mineral density and lumbar spine bone mineral density measured by dual energy X-ray absorptiometry through a world-wide genome-wide association study meta-analysis of 83,894 individuals. Furthermore, additional analyses in 133,460 participants revealed that fourteen of

these bone mineral density loci are also associated with fracture risk. In chapter 4.2 we directly applied a hypothesis-free genome-wide approach for fracture risk, meta-analyzing data from 102,873 persons. This effort highlighted 35 genetic signals as associated with fracture risk. Interestingly, half of these loci (i.e., eighteen) were among the previously discovered bone mineral density loci, while other gene regions have been implicated in neurological and hormonal processes. This seems to illustrate the complex interplay of factors contributing to an increased fracture risk. In the end, ten loci replicated at genome-wide significant level. In chapter 4.3 a genome-wide copy number association study of osteoporotic fractures highlighted the 6p25.1 locus. A rare (minor allele frequency [MAF]=0.1%) 210 kb deletion in 6p25 was associated with increased fracture risk in the Rotterdam Study and further replicated in other array-based studies In chapter 4.4 we did the first genome-wide association study for radiographic vertebral fractures in the Rotterdam Study finding a marker on chromosome 16q24 as genome-wide significantly associated. Although the 16q24 locus has been found associated with bone mineral density and vertebral defects at birth before, our association with vertebral fracture risk could not be replicated by de-novo genotyping across 15 studies worldwide. In chapter 4.5 we tested single nucleotide polymorphisms in the *TRPV4* gene for association with osteoporotic fracture risk in human Rotterdam Study participants, as these genetic variants were found to have male-specific skeletal effects on osteoblast – osteoclast uncoupling in mice. In spite of finding an association in our Rotterdam Study as well, analyses of three more studies did not yield the same results. Chapter 4.6 shows the case of a young woman with severe osteoporosis and vertebral fractures due to osteoporosis pseudoglioma syndrome/familial exudative vitreoretinopathy due to compound heterozygous missense mutations in *LRP5*, which illustrates that genetic screening should be considered in pregnancy associated osteoporosis. The review in chapter 4.7 presents the current state of knowledge on the genetic basis of osteoporotic vertebral fractures and, additionally, of structural vertebral deformities resembling osteoporotic vertebral fractures but which may have their own genetic basis.

Chapter 5 is a future perspective on personalized sequencing and the future of medicine in general.

Finally, in chapter 6 a general discussion is presented of the studies presented in this thesis, and findings are placed in a broader context. Additionally, future directions are proposed at the end of chapter 6.

SAMENVATTING

Een abnormaal lage botmineraaldichtheid en/of een verstoorde bot microarchitectuur predisponeren voor een verhoogd risico op botbreuken, zoals het geval is bij osteoporose en diabetes-gerelateerde botziekte. Deze ziektebeelden hebben een multifactoriële etiologie hebben en hebben een hoge prevalentie in de algemene bevolking, met name bij ouderen. In dit proefschrift worden verschillende aspecten van musculoskeletale aandoeningen onderzocht, namelijk de epidemiologie, radiologie, en genetica.

Hoofdstuk 2 beschrijft een aantal epidemiologische studies van osteoporotische fracturen uitgevoerd in de Rotterdam Studie. In hoofdstuk 2.1 vonden we dat in vrouwen, kortdurend gebruik van lisdiuretica is geassocieerd met een verhoogd niveau van vrij deoxypyridinoline, wat waarschijnlijk weer het gevolg is van toegenomen botresorptie door osteoclasten. In hoofdstuk 2.2 hebben we gedemonstreerd dat mensen met lumbale degeneratie een systemisch hogere botdichtheid hebben, maar dit resulteert bij hen niet in bescherming tegen het krijgen van wervel- en niet-wervelfracturen. In hoofdstuk 2.3 hebben we de associatie tussen C-reactief proteïne en een verhoogd risico op fracturen onderzocht. De volgende twee sub-hoofdstukken richten zich op de epidemiologie van diabetes-gerelateerde botziekte. Hoofdstuk 2.4 is een literatuurstudie naar de associatie tussen botdichtheid en type 2 diabetes mellitus. Onze meta-analyse van observationele studies bevestigde dat de botmineraaldichtheid op meerdere plaatsen van het skelet verhoogd is bij patiënten met type 2 diabetes mellitus in vergelijking met mensen zonder diabetes. Uit onze meta-regressie bleek dat geglycolyseerd hemoglobine (HbA_{1C}) hierin een rol speelt, en wij hebben dit verder onderzocht in de Rotterdam Studie in hoofdstuk 2.5. Hier vonden we dat de groep met type 2 diabetes mellitus en een slechte glycemische controle een hoger risico heeft op botbreuken dan de groep met type 2 diabetes mellitus en een goede glycemische controle of de groep zonder diabetes. Intrigerend was ook dat de groep met type 2 diabetes mellitus met slechte glucosecontrole een hogere botdichtheid had, smallere femurhals breedte en een dikker cortex. Hoofdstuk 2.6 bevat een overzichtsartikel over diabetische botcomplicaties. Hoofdstuk 2.7 is een beschrijvend overzicht over osteoporotische wervelfracturen als onderdeel van systemische ziekte.

Hoofdstuk 3 geeft een vergelijkend overzicht van de radiologische scoren methoden voor osteoporotische wervelfracturen. In hoofdstuk 3.1 bespreken we verschillende radiologische scoren methoden van osteoporotische wervelfracturen voor klinisch onderzoek en de klinische praktijk. Bij de toepassing van twee van de meest gebruikte radiologische scoringsmethoden in de Rotterdam Studie vonden we dat prevalenties enorm verschilden tussen deze twee methoden, zoals beschreven in hoofdstuk 3.2. De gegevens in hoofdstuk 3.3 tonen dat de trabeculaire botscore sterk is geassocieerd met prevalente vertebrale fracturen bij vrouwen, en dat deze indicator informatie onafhankelijk van botdichtheid van dual energy X-ray absorptiometrie oplevert. Hoofdstuk 3.4 illustreert dat de bij vertebrale morfometrie verkregen data meerdere kwantitatieve parameters geeft die relevant kunnen zijn voor verschillende musculoskeletale aandoeningen. In hoofdstuk 3.5 onderzochten we de radiologische criteria en de prevalentie van de ziekte van Scheuermann in de Rotterdam Studie. In hoofdstuk 3.6 onderzochten we hoeveel ziekte van Scheuermann gevallen verkeerd worden gediagnosticeerd als osteoporotische wervelbreuken, waar we vonden dat de algoritme gebaseerde kwalitatieve methode beter presteert dan kwantitatieve morfometrie. Hoofdstuk 3.7 bevat een case serie, die de noodzaak om osteoporotische wervelfracturen te onderscheiden van de ziekte van Scheuermann in de dagelijkse klinische praktijk benadrukt.

Hoofdstuk 4 presenteert verschillende genetische epidemiologische studies naar risico op botbreuken, waarvan de meesten grootschalige projecten zijn, uitgevoerd in het kader van de genetische

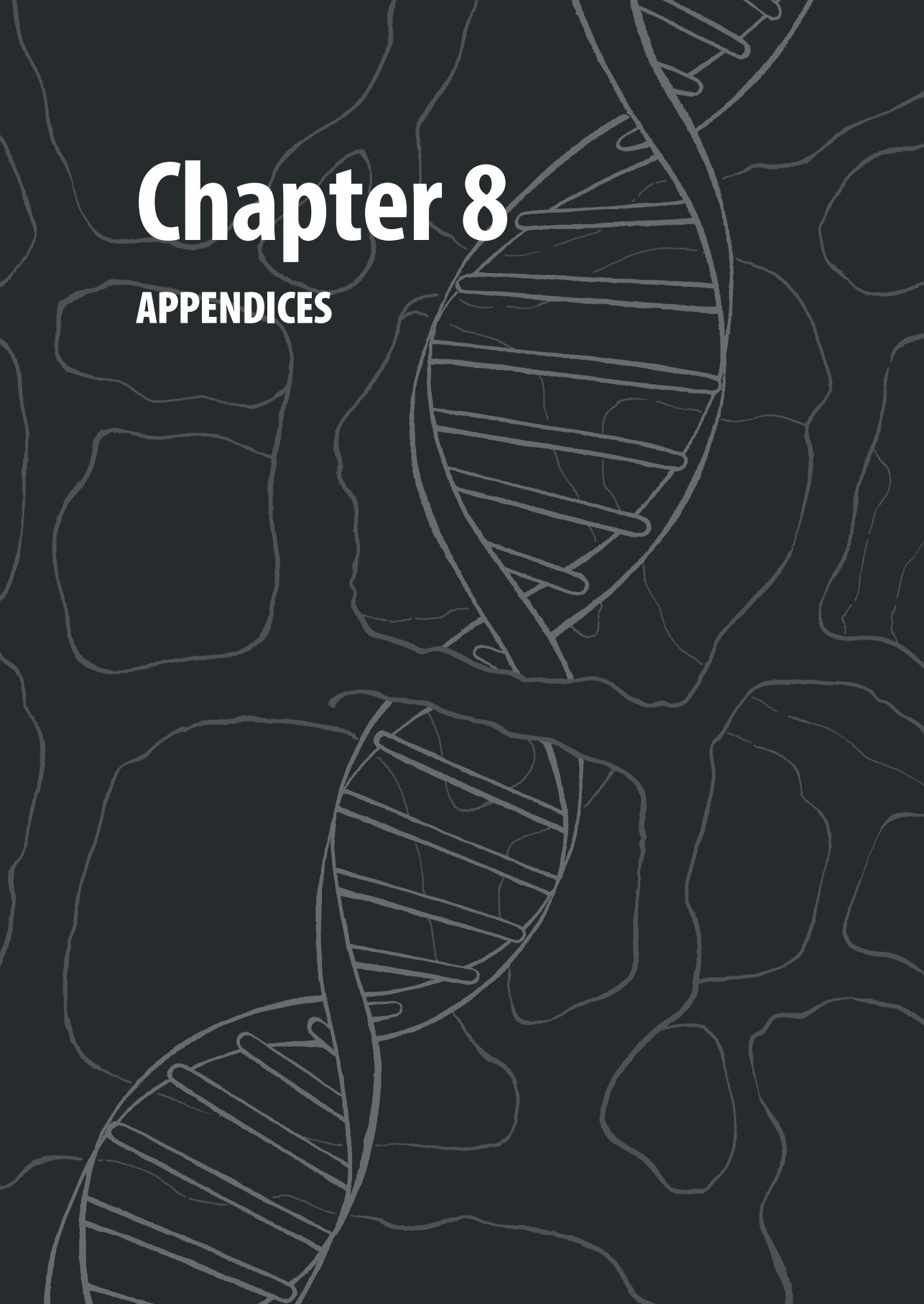
factoren voor osteoporose (GEFOS) en genetische merkers voor osteoporose (GENOMOS) consortia. In hoofdstuk 4.1 identificeerden we 56 genetische loci voor femurhals botmineraaldichtheid en de lumbale wervelkolom botmineraaldichtheid gemeten met dual energy X-ray absorptiometrie door middel van een wereldwijde genoom-wijde associatie studie meta-analyse van 83.894 personen. Uit aanvullende analyses in 133.460 deelnemers is gebleken dat veertien van deze botmineraaldichtheid loci ook geassocieerd zijn met risico op botbreuken. In hoofdstuk 4.2 pasten we direct een hypothesevrije genoom-brede analyse toe voor het risico op botbreuken, door middel van meta-analyse van gegevens van 102.873 personen. Deze studie leverde 35 genetische signalen op in associatie met het risico op botbreuken. Interessant is dat de helft van deze loci (18) behoorden tot de eerder ontdekte botmineraaldichtheid loci, terwijl andere gengebieden zijn betrokken bij neurologische en hormonale processen. Dit lijkt de complexe interactie van factoren die bijdragen aan een verhoogd risico op botbreuken te illustreren. Uiteindelijk repliceerden tien signalen op genoom-wijde significantie. De in hoofdstuk 4.3 beschreven genoom-wijde kopie nummervariatie studie van osteoporotische fracturen wees op het 6p25.1 locus. Een zeldzame (minor allel frequentie [MAF]=0,1%) 210 kb deletie in 6p25 werd geassocieerd met een verhoogd risico op botbreuken in de Rotterdam Studie en verder gerepliceerd in andere array-gebaseerde studies. In hoofdstuk 4.4 hebben we de eerste genoom-brede associatie studie voor radiografische wervelfracturen in de Rotterdam Studie, waarin een marker op chromosoom 16q24 genoom-breed significant geassocieerd bleek te zijn. Hoewel het 16q24 locus eerder geassocieerd was bevonden met botdichtheid en congenitale wervelkolom afwijkingen, kon de associatie met wervelfractuur risico niet worden gerepliceerd door de-novo genotypering in 15 studies over de hele wereld. In hoofdstuk 4.5 testten we single nucleotide polymorfismen in het *TRPV4* gen voor associatie met osteoporotische fracturen in Rotterdam Studie deelnemers, omdat deze genetische varianten in mannen skeletale effecten op de osteoblast bleken te hebben – namelijk osteoclasten ontkoppeling bij muizen. Ondanks het vinden van de associatie in de Rotterdam Studie, leverden analyses van drie andere studies niet dezelfde resultaten op. Hoofdstuk 4.6 toont de beschrijving van een jonge vrouw met ernstige osteoporose en wervelfracturen te wijten aan het osteoporose pseudoglioom syndroom / familiale exsudatieve vitreoretinopathie door compound heterozygote missense mutaties in *LRP5*, dat aantoont dat genetische screening in zwangerschap-gerelateerde osteoporose moet worden overwogen. De review in hoofdstuk 4.7 presenteert de huidige stand van kennis over de genetische basis van osteoporotische wervelfracturen, en van de structurele werveldeformaties die kunnen lijken op osteoporotische wervelfracturen, maar die hun eigen genetische basis kunnen hebben.

Hoofdstuk 5 is een toekomstperspectief over persoonlijke sequencing en de toekomst van de geneeskunde in het algemeen.

Tenslotte wordt in hoofdstuk 6 een algemene discussie gegeven van de studies in dit proefschrift, en de bevindingen worden in een bredere context geplaatst. Daarnaast worden toekomstige richtingen voorgesteld aan het einde van hoofdstuk 6.

Chapter 8

APPENDICES



LIST OF PUBLICATIONS

1. Nielson CM, Liu CT, Smith AV, Ackert-Bicknell CL, Reppe S, Johanna J, Wassel C, Register TC, **Oei L,** Alonso Lopez N, Oei EH, Parimi N, Samelson EJ, Nalls MA, Zmuda J, Lang T, Bouxsein M, Latourelle J, Claussnitzer M, Siggeirsdottir K, Srikanth P, Lorentzen E, Vandenput L, Langefeld C, Raffield L, Terry G, Cox AJ, Allison MA, Criqui MH, Bowden D, Ikram MA, Mellström D, Karlsson MK, Carr J, Budoff M, Phillips C, Cupples LA, Chou WC, Myers RH, Ralston SH, Gautvik KM, Cawthon PM, Cummings S, Karasik D, Rivadeneira F, Gudnason V, Orwoll ES, Harris TB, Ohlsson C, Kiel DP, Hsu YH. "Novel genetic variants are associated with increased vertebral volumetric bmd, reduced vertebral fracture risk, and increased expression of SCL1A3 and EPHB2." Journal of Bone Mineral Research. 2016 Aug 1. doi: 10.1002/jbmr.2913. [Epub ahead of print]. PMID: 27476799
2. **Oei L**, van den Meiracker AH, Oei EHG, Moelker A, van Leenders GJLH, Rikken B, Verhagen P, Clahsen-van Groningen MC, van der Wiel HE. "Reninoma: a rare cause of curable hypertension and hypokalemia." American Journal of Medicine. 2016 Apr 18. PMID: 27103051
3. McCloskey EV, Odén A, Harvey NC, Leslie WD, Hans D, Johansson H, Barkmann R, Boutroy S, Brown J, Chapurlat R, Elders PJ, Fujita Y, Glüer CC, Goltzman D, Iki M, Karlsson M, Kindmark A, Kotowicz M, Kurumatani N, Kwok T, Lamy O, Leung J, Lippuner K, Ljunggren Ö, Lorentzon M, Mellström D, Merlijn T, **Oei L**, Ohlsson C, Pasco JA, Rivadeneira F, Rosengren B, Sornay-Rendu E, Szulc P, Tamaki J, Kanis JA. "A meta-analysis of trabecular bone score in fracture risk prediction and its relationship to FRAX." Journal of Bone Mineral Research. 2016 May;31(5):940-8. PMID: 26498132
4. **Oei L**, Zillikens MC, Rivadeneira F, Oei EHG. "Osteoporotic vertebral fractures as part of systemic disease." Journal of Clinical Densitometry. 2016 Jan;19(1):70-80. (Invited Review). PMID: 26376171.
5. **Oei L**, Zillikens MC, Rivadeneira F, Oei EHG. "Genetics of osteoporotic vertebral fractures." Journal of Clinical Densitometry. 2016 Jan;19(1):23-8. (Invited Review). PMID: 26376172.
6. Zheng HF*, Forgetta V*, Hsu YH*, Estrada K*, Rosello-Diez A*, Leo PJ*, Dahia CL*, Park-Min KH*, Tobias JH*, Kooperberg C*, Kleinman A, Styrkarsdottir U, Liu CT, Uggla C, Evans DS, Nielson CM, Walter K, Pettersson-Kymmer U, McCarthy S, Eriksson J, Kwan T, Jhamai M, Trajanoska K, Memari Y, Min J, Huang J, Danecek P, Wilmot B, Li R, Chou W, Mokry LE, Moayyeri A, Claussnitzer M, Cheng C, Cheung W, Medina-Gómez C, Ge B, Chen S, Choi K, **Oei L**, Fraser J, Kraaij R, Hibbs MA, Gregson CL, Paquette D, Hofman A, Wibom C, Tranah GJ, Marshall M, Gardiner BB, Cremin K, Auer P, Hsu L, Ring S, Tung JY, Thorleifsson G, Enneman AW, van Schoor NM, de Groot LCPGM, van der Velde N, Melin B, Kemp JP, Christiansen C, Sayers A, Zhou Y, Calderari S, van Rooij J, Carlson C, Peters U, Berlivet S, Dostie J, Uitterlinden AG, Williams SR, FarberC, Grinberg D, LaCroix AZ, Haessler J, Chasman DI, Giulianini F, Rose LM, Ridker PM, Eisman JA, Nguyen TV, Center JR, Nogues X, Garcia-Giralt N, Launer LL, Gudnason V, Mellström D, Vandenput L, Karlsson MK, Ljunggren O, Svensson O, Hallmans G, Rousseau F, Giroux S, Bussière J, Arp PP, Koromani F, Prince RL, Lewis JR, Langdahl BL, Hermann AP, Jensen JB, Kaptoge S, Khaw K, Reeve J, Formosa MM, Xuereb-Anastasi A, Åkesson K, McGuigan FE, Garg G, Olmos JM, ZarrabeitiaMT, Riancho JA, Ralston SH, Alonso N, Jiang X, Goltzman D, Pastinen T, Grundberg E, Gauguier D, Orwoll ES, Karasik D, Davey-Smith G, AOGC Consortium, Smith AV, Siggeirsdottir K, Harris TB, Zillikens MC, van Meurs JBJ, Thorsteinsdottir U, Maurano MT, Timpson NJ, Soranzo N, Durbin R, Wilson SG, Ntzani EE, Brown MA, Stefansson K, Hinds DA, Spector T, Cupples LA, Ohlsson C, Greenwood CMT, Jackson RD*, Rowe DW*, Loomis CA*, Evans DM*, Ackert-Bicknell CL*, Joyner AL*, Duncan EL*, Kiel DP*, Rivadeneira F*, Richards JB for the GEFOS and UKK Consortia. "Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture." Nature. 2015 Oct 1;526(7571):112-7. PMID: 26367794.
7. Muka T, Trajanoska K, Kiefte-de Jong JC, **Oei L**, Uitterlinden AG, Hofman A, Dehghan A, Zillikens MC, Franco OH, Rivadeneira F. "The association between metabolic syndrome, bone mineral density, hip bone geometry and fracture risk: the Rotterdam Study." PLoS One. 2015 Jun 12;10(6):e0129116. PMID: 26066649
8. Armbrecht G, Felsenberg D, Ganswindt M, Lunt M, Kaptoge SK, Abendroth K, Aroso A, Banzer D, Bhalla AK, Dequeker J, Eastell R, Hoszowski K, Lyritis G, Delmas PD, Masaryk P, Miazgowski T, Cannata J, Nuti R, **Oei L**, Poor G, Redlund-Johnell I, Reid DM, Reisinger W, Schatz H, Todd CJ, Woolf AD, Javaid K, Rivadeneira F, Silman AJ, Cooper C, O'Neill TW, Reeve J; European Vertebral Osteoporosis Study (EVOS) and European Prospective Osteoporosis Study (EPOS) Groups. "Vertebral Scheuermann's disease in Europe: prevalence, geographic

variation and radiological correlates in men and women aged 50 and over." Osteoporosis International. 2015 Oct;26(10):2509-19. PMID: 26021761

9. Veldhuis-Vlug AG, **Oei L**, Souverein PC, Tanck MW, Rivadeneira F, Zillikens MC, Kamphuisen PW, Maitland-van der Zee AH, de Groot MC, Hofman A, Uitterlinden AG, Fliers E, de Boer A, Bisschop PH. "Association of polymorphisms in the beta-2 adrenergic receptor gene with fracture risk and bone mineral density." Osteoporosis International. 2015 Jul;26(7):2019-27. PMID: 25910744
10. **Oei L**, Rivadeneira F, Zillikens MC, Oei EHG. "Diabetes, diabetic complications, and fracture risk." (Invited Review). Current Osteoporosis Reports. 2015 Apr;13(2):106-15. PMID: 25648962
11. Esplin E*, **Oei L***, Snyder MP. "Personalized sequencing and the future of medicine: discovery, diagnosis and defeat of disease." (Invited Review). Pharmacogenomics. 2014 Nov;15(14):1771-1790. PMID: 25493570
12. Kemp JP*, Medina-Gomez C*, Estrada K, St Pourcain B, Heppe DHM, Timpson NJ, **Oei L**, Ring SM, Kruithof CJ, Wolber LE, Williams FMK, Zillikens MC, Hofman A, Uitterlinden AG, Davey Smith G, Jaddoe VWV, Tobias JH**, Rivadeneira F**, Evans DM**. "Phenotypic dissection of bone mineral density facilitates the identification of skeletal site specificity in the genetic regulation of bone mass attainment." PLoS Genetics. 2014 Jun 19;10(6):e1004423. PMID: 24945404
13. Campos-Obando N, Castano-Betancourt MC, **Oei L**, Franco OH, Stricker BHCh, Brusselle GG, Lahousse L, Hofman A, Tiemeier H, Rivadeneira F, Uitterlinden AG, Zillikens MC. "Bone mineral density and chronic lung disease mortality: the rotterdam study." Journal of Clinical Endocrinology and Metabolism. 2014 May;99(5):1834-42. PMID: 24606086
14. Moayyeri A*, Hsu YH*, Karasik D*, Estrada K*, Xiao SM*, Nielson C, Srikanth P, Giroux S, Wilson SG, Zheng HF, Smith AV, Pye SR, Leo PJ, Teumer A, Hwang JY, Ohlsson C, McGuigan F, Minster RL, Hayward C, Olmos JM, Lyytikäinen LP, Lewis JR, Swart KMA, Masi L, Oldmeadow C, Holliday EG, Cheng S, van Schoor NM, Harvey NC, Kruk M, Del Greco F, Igl W, Trummer O, Grigoriou E, Luben R, Liu CT, Zhou Y, **Oei L**, Medina-Gomez C, Zmuda J, Tranah G, Brown SJ, Williams FM, Soranzo N, Jakobsdottir J, Siggeirsdottir K, Holliday KL, Hannemann A, Go MJ, Garcia M, Polasek O, Laaksonen M, Zhu K, Enneman AW, McEvoy M, Peel R, Sham PC, Jaworski M, Johansson A, Hicks AA, Pludowski P, Scott R, Dhonukshe-Rutten RAM, van der Velde N, Kähönen M, Viikari JS, Sievänen H, Raitakari OT, González-Macías J, Hernández JL, Mellström D, Ljunggren O, Cho YS, Völker U, Nauck M, Homuth G, Völzke H, Haring R, Brown MA, McCloskey E, Nicholson GC, Eastell R, Eisman JA, Jones G, Reid IR, Dennison EM, Wark J, Boonen S, Vanderschueren D, Wu FCW, Aspelund T, Richards JB, Bauer D, Hofman A, Khaw KT, Dedoussis G, Obermayer-Pietsch B, Gyllensten U, Pramstaller PP, Lorenc RS, Cooper C, Kung AWC, Lips P, Alen M, Attia J, Brandi ML, de Groot LCPGM, Lehtimäki T, Riancho JA, Campbell H, Liu Y, Harris TB, Akesson K, Karlsson M, Lee JY, Wallaschofski H, Duncan EL, O'Neill TW, Gudnason V, Spector TD, Rousseau F, Orwoll E, Cummings S, Wareham NJ, Rivadeneira F, Uitterlinden AG, Prince RL*, Kiel DP*, Reeve J*, Kaptoge SK*."Genetic determinants of heel bone properties: Genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium." Human Molecular Genetics. 2014 Jun 1;23(11):3054-68. PMID: 24430505
15. **Oei L**, Hsu YH, Styrkarsdottir U, Nandakumar K, Eussen BH, de Klein A, Peters MJ, Halldorsson B, Liu CT, Alonso N, Kaptoge SK, Thorleifsson G, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Kruk M, Lewis JR, Patel MS, Scollen S, Svensson O, Trompet S, van Schoor NM, Zhu K, Buckley BM, Cooper C, Ford I, Goltzman I, González-Macías J, Langdahl BL, Leslie WD, Lips P, Lorenc RS, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Slagboom PE, Garcia-Ibarbia C, Ingvarsson T, Johannsdottir H, Luben R, Medina-Gómez MC, Arp P, Nandakumar K, Palsson STh, Sigurdsson G, van Meurs JBJ, Zhou Y, Hofman A, Jukema JW, Pols HAP, Prince RL, Cupples LA, Marshall CR, Pinto D, Sato D, Scherer SW, Reeve J, Thorsteinsdottir U, Karasik D, Richards JB, Stefansson K, Uitterlinden AG, Ralston SH, Ioannidis JPA, Kiel DP, Rivadeneira F, Estrada K. "A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus." Journal of Medical Genetics. 2014 Feb;51(2):122-31. PMID: 24343915
16. **Oei L**, Campos-Obando N, Dehghan A, Oei EHG, Stolk L, van Meurs JBJ, Hofman A, Pols HAP, Uitterlinden AG, Franco OH, Zillikens MC, Rivadeneira F. "Dissecting the relationship between high-sensitivity serum C-reactive protein and increased fracture risk: the Rotterdam Study." Osteoporosis International. 2013 Dec 13. PMID: 24337661

17. **Oei L**, Estrada K, Duncan EL, Christiansen C, Liu CT, Langdahl BL, Obermayer-Pietsch B, Riancho JA, Prince RL, van Schoor NM, McCloskey EV, Hsu YH, Evangelou E, Ntzani EE, Evans DM, Alonso N, Husted LB, Valero C, Hernandez JL, Lewis JR, Kaptoge SK, Zhu K, Cupples LA, Medina-Gómez MC, Vandenput L, Kim GS, Lee SH, Castaño-Betancourt MC, Oei EHG, Martinez J, Anna Daroszewska A, van der Klift M, Mellström D, Herrera L, Karlsson MK, Hofman A, Ljunggren O, Pols HAP, Stolk L, van Meurs JBJ, Ioannidis JPA, Zillikens MC, Lips P, Karasik D, Uitterlinden AG, Styrkarsdottir U, Brown MA, Koh J, Richards JB, Reeve J, Ohlsson C, Ralston SR, Kiel DP, Rivadeneira F. "Genome-wide association study for radiographic vertebral fractures: a potential role for the 16q24 BMD locus." Bone. 2014 Feb;59:20-7. PMID: 24516880
18. Campos-Obando N, **Oei L**, Simon MEH, Zillikens MC. "Osteoporotic vertebral fractures during pregnancy: be aware of a potential underlying genetic cause." Journal of Clinical Endocrinology and Metabolism. 2014 Apr;99(4):1107-11. PMID: 24423337
19. van der Eerden BCJ, **Oei L**, Hoenderop JGJ, van Schoor NM, Pettersson U, Koedam M, Uitterlinden AG, Hofman A, Suzuki M, Ohlsson C, Rivadeneira F, Bindels RJM, van Leeuwen JPTM. "TRPV4 deficiency causes sexual dimorphism in bone metabolism and osteoporotic fracture risk." Bone. 2013 Dec;57(2):443-54. PMID: 24084385
20. Breda SJ, **Oei L**, Oei EHG, Zillikens MC. "Clinical lesson. Osteoporotic vertebral fractures or Scheuermann's disease." Dutch Journal of Medicine. 2013;157(45):A6479. PMID: 24191924
21. **Oei L**, Ly F, El Saddy S, Makurthou AA, Hofman A, van Rooij FJA, Uitterlinden AG, Zillikens MC, Rivadeneira F, Oei EHG. "Multi-functionality of computer-aided quantitative vertebral fracture morphometry analyses." Quantitative Imaging in Medicine and Surgery. 2013 Oct;3(5):249-255. PMID: 24273742
22. Castaño-Betancourt MC, **Oei L**, Rivadeneira F, de Schepper EIT, Hofman A, Bierma-Zeinstra S, Pols HAP, Uitterlinden AG, van Meurs JBJ. "Association of lumbar disc degeneration with osteoporotic fractures; the Rotterdam Study And Meta-Analysis From Systematic Review." Bone. 2013 Nov;57(1):284-9. PMID: 23958823
23. Makurthou AA, **Oei L**, El Saddy S, Breda SJ, Castaño-Betancourt MC, Hofman A, van Meurs JBJ, Uitterlinden AG, Rivadeneira F, Oei EHG. "Scheuermann's disease: evaluation of radiological criteria and population prevalence." Spine (Phila Pa 1976). 2013 Sep 1;38(19):1690-4. PMID: 24509552
24. Ruiter R, **Oei L**, Visser LE, Peltenburg HG, Hofman A, Zillikens MC, Uitterlinden AG, Rivadeneira F, Stricker BHCh. "The effect of thiazide and loop diuretics on urinary levels of the osteoclastic bone resorption marker free deoxypyridinoline." Journal of Clinical Pharmacy and Therapeutics. 2013 Jun;38(3):225-9. PMID: 23442167
25. **Oei L**, Zillikens MC, Dehghan A, Buitendijk GHS, Castaño-Betancourt MC, Estrada K, Stolk L, Oei EHG, van Meurs JBJ, Janssen JAMJL, Hofman A, van Leeuwen JPTM, Witteman JC, Pols HAP, Uitterlinden AG, Klaver CCW, Franco OH, Rivadeneira F. "High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study." Diabetes Care. 2013 Jun;36(6):1619-28. PMID: 23315602
26. **Oei L**, Ly F, Breda SJ, Zillikens MC, Uitterlinden AG, Krestin GP, Rivadeneira F, Oei EHG. "Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings." European Radiology. 2013 Feb;23(2):476-86. PMID: 22892721
27. Liu CT*, Estrada K*, Yerges-Armstrong LM, Amin N, Evangelou E, Li G, Minster RL, Carless MA, Kammerer CM, **Oei L**, Zhou Y, Alonso N, Dailiana Z, Eriksson J, García-Giralt N, Giroux S, Husted LB, Khusainova RI, Koromila T, Kung AW, Lewis JR, Masi L, Mencej-Bedrac S, Nogues X, Patel MS, Prezelj J, Richards JB, Sham PC, Spector T, Vandenput L, Xiao SM, Zheng HF, Zhu K, Balcells S, Brandi ML, Frost M, Goltzman D, González-Macías J, Karlsson M, Khusnutdinova EK, Kollia P, Langdahl BL, Ljunggren O, Lorentzon M, Marc J, Mellström D, Ohlsson C, Olmos JM, Ralston SH, Riancho JA, Rousseau F, Urreizti R, Van Hul W, Zarrabeitia MT, Castano-Betancourt M, Demissie S, Grundberg E, Herrera L, Kwan T, Medina-Gómez C, Pastinen T, Sigurdsson G, Thorleifsson G, Van Meurs JB, Blangero J, Hofman A, Liu Y, Mitchell BD, O'Connell JR, Oostra BA, Rotter JI, Stefansson K, Streeten EA, Styrkarsdottir U, Thorsteinsdottir U, Tylavsky FA, Uitterlinden A, Cauley JA, Harris TB, Ioannidis JP, Psaty BM, Robbins JA, Zillikens MC, Vanduijn CM, Prince RL, Karasik D*, Rivadeneira F*, Kiel DP*, Cupples LA*, Hsu YH*. "Assessment of gene-by-sex interaction effect on bone mineral density." Journal of Bone Mineral Research. 2012 Oct;27(10):2051-64. PMID: 22692763
28. Estrada K*, Styrkarsdottir U*, Evangelou E*, Hsu YH*, Duncan EL*, Ntzani EE*, **Oei L***, Albagha OM, Amin N, Kemp JP, Koller DL, Li G, Liu CT, Minster RL, Moayyeri A, Vandenput L, Willner D, Xiao SM, Yerges-Armstrong LM,

Zheng HF, Alonso N, Eriksson J, Kammerer CM, Kaptoge SK, Leo PJ, Thorleifsson G, Wilson SG, Wilson JF, Aalto V, Alen M, Aragaki AK, Aspelund T, Center JR, Dailiana Z, Duggan DJ, Garcia M, Garcia-Giralt N, Giroux S, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Khusainova R, Kim GS, Kooperberg C, Koromila T, Kruk M, Laaksonen M, Lacroix AZ, Lee SH, Leung PC, Lewis JR, Masi L, Mencej-Bedrac S, Nguyen TV, Nogues X, Patel MS, Prezelj J, Rose LM, Scollen S, Siggeirsdottir K, Smith AV, Svensson O, Trompet S, Trummer O, van Schoor NM, Woo J, Zhu K, Balcells S, Brandi ML, Buckley BM, Cheng S, Christiansen C, Cooper C, Dedoussis G, Ford I, Frost M, Goltzman D, González-Macías J, Kähönen M, Karlsson M, Khusnutdinova E, Koh JM, Kollia P, Langdahl BL, Leslie WD, Lips P, Ljunggren Ö, Lorenc RS, Marc J, Mellström D, Obermayer-Pietsch B, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Ridker PM, Rousseau F, Slagboom PE, Tang NL, Urreizti R, Van Hul W, Viikari J, Zarrabeitia MT, Aulchenko YS, Castano-Betancourt M, Grundberg E, Herrera L, Ingvarsson T, Johannsdottir H, Kwan T, Li R, Luben R, Medina-Gómez C, Palsson ST, Reppe S, Rotter JI, Sigurdsson G, van Meurs JBJ, Verlaan D, Williams FM, Wood AR, Zhou Y, Gautvik KM, Pastinen T, Raychaudhuri S, Cauley JA, Chasman DI, Clark GR, Cummings SR, Danoy P, Dennison EM, Eastell R, Eisman JA, Gudnason V, Hofman A, Jackson RD, Jones G, Jukema JW, Khaw KT, Lehtimäki T, Liu Y, Lorentzon M, McCloskey E, Mitchell BD, Nandakumar K, Nicholson GC, Oostra BA, Peacock M, Pols HA, Prince RL, Raitakari O, Reid IR, Robbins J, Sambrook PN, Sham PC, Shuldiner AR, Tylavsky FA, van Duijn CM, Wareham NJ, Cupples LA, Econs MJ, Evans DM, Harris TB, Kung AW, Psaty BM, Reeve J, Spector TD, Streeten EA, Zillikens MC, Thorsteinsdottir U, Ohlsson C, Karasik D, Richards JB, Brown MA, Stefansson K, Uitterlinden AG**, Ralston SH**, Ioannidis JPA**, Kiel DP**, Rivadeneira F**. "Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture." Nature Genetics. 2012 Apr 15;44(5):491-501. PMID: 22504420

29. Ma L*, **Oei L***, Jiang L, Estrada K, Chen H, Wang Z, Yu Q, Zillikens MC, Gao X, Rivadeneira F. "Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies." European Journal of Epidemiology. 2012 May;27(5):319-32. PMID: 22451239
30. De Vries-van der Weij J, de Haan W, Hu L, Kuif M, **Oei HL**, van der Hoorn JW, Havekes LM, Princen HM, Romijn JA, Smit JW, Rensen PC. "Bexarotene induces dyslipidemia by increased VLDL production and CETP-mediated reduction of HDL; bexarotene and dyslipidemia." Endocrinology. 2009 May;150(5):2368-75. PMID: 19147676
31. **Oei L**, van den Meiracker AH, Oei EHG, Moelker A, van Leenders GJLH, Rikken B, Verhagen P, Clahsen-van Groningen MC, van der Wiel HE. (Invited Article). "Reninoom: een zeldzame oorzaak van hypertensie en hypokaliëmie". Focus Vasculair. 2016 September.
32. Campos-Obando N, **Oei L,** Zillikens MC. (Invited Article). "Clinical highlights of the 34th ASBMR meeting." Osteoporosis Journal. 2012 Dec.
33. **Oei L**, Estrada K, Medina-Gomez MC, Rivadeneira F. (Invited Article). "Update on the genetic basis of disorders of the musculoskeletal system: meeting report from the 3rd joint meeting of the European Calcified Tissue Society and the International Bone and Mineral Society." Bonekey Meeting Reports. 2011 Oct.
34. **Oei L**, Oei EHG. "Adjustment of fracture risk estimation is necessary in patients with type 2 diabetes mellitus." Dutch Journal of Medicine. 2011 Sep 27.

Submitted

35. Jainandunsing S*, **Oei L***. "A man with a pulsating abdominal mass". (Submitted)
36. **Oei L*,** Jainandunsing S*. "Hyponatremia caused by abdominal large vessel obstruction". (Submitted)

In preparation

37. Atanasovska B*, **Oei L***, Medina-Gomez C, Oei EHG, Campos-Obando N, Estrada K, Enneman A, van der Velde N, Hofman A, Aubry-Rozier B, Zillikens MC, Uitterlinden AG, Hans D, Rivadeneira F. "Prediction of vertebral fracture by trabecular bone score in elderly women of the Rotterdam Study." (In preparation)
38. **Oei L***, Breda SJ*, Koromani F, Schousboe JT, Clark EM, Ly F, van Meel ER, Dogterom EJ, de Kok LGC, van Meurs JBJ, Hofman A, Pols HAP, Waarsing E, Kiel DP, Bouxsein ML, Black DM, van Rooij FJA, Hunink MG, Uitterlinden AG, Zillikens MC, Krestin GP, Oei EHG**, Rivadeneira F**. "Osteoporotic vertebral fracture prevalences vary widely between radiological assessment methods: the Rotterdam Study." (In preparation)
39. **Oei L**, Breda SJ, Jiang G, El Saddy S, Makurthou AA, Ly F, Castaño-Betancourt MC, Hofman A, Waarsing E, Black DM, Schousboe JT, van Rooij FJA, van Meurs JBJ, Uitterlinden AG, Zillikens MC, Rivadeneira F, Oei EHG. "Iden-

tification of Scheuermann's disease with different radiological scoring methods for osteoporotic vertebral fractures: the Rotterdam Study." (In preparation)

40. **Oei L***, Zheng HF*, Ntzani EE*, Trajanoska K*, Morris J*, Nielson CM*, Estrada K*, Styrkarsdottir U*, Ridker PM*, Leong A, Ackert-Bicknell CL, van de Peppel J, Medina-Gómez C, Hsu YH, Duncan EL, Yang J, Esko T, Atanasovska B, Kaptoge S, Pettersson-Kymmer U, Nordström P, Garcia M, Aragaki AK, Enneman AW, Lehtimäki T, Trompet S, Eriksson J, Amin N, Kung AW, Tsilidis K, Thorleifsson G, Rose LM, Zmuda J, Liu CT, Smith AV, Srikanth P, Wilson SG, Clark G, Viikari J, Mihailov E, Moayyeri A, Li G, Kammerer CM, Lorentzon M, Rivera N, Xiao S, Pers T, Tranah GJ, Evans DS, Siggeirsdottir K, Oei EHG, Stefansson K, Leo PJ, Aalto V, Willner D,Wareham N, Minster RL, Bis J, Lyytikäinen LP, Brandi ML, Heckbert S, Cheung CL, Eriksson N, Cheng S, Sitlani C, Cooper C, Khusainova R, van Hul W, Dedousi G, McGuigan FE, Hocking LJ, Koh JM, Kollia P, van Schoor NM, Khusnutdinova E, Lips P, Langdahl BL, Grigouriou E, van Duijn CM, Boyle A, Snyder MP, Herrera L, Nogues X, Koller DL, Vandenput L, Cupples LA, Panagoula K, Zoe D, Aspelund T, Riancho JA, Mellström D, Obermayer-Pietsch B, Olmos JM, Reid DM, Raitakari O, Grinberg D, LaCroix AZ, Åkesson K, Khaw KT, Christiansen C, Formosa MM, Xuereb-Anastasi A, Jones G, Dailiana Z, Giroux S, Lacroix AZ, Frost M, Lorentzon M, McCloskey E, Robbins J, Liu Y, Breda SJ, Tang NL, Szulc P, Husted LB, Prince RL, Lewis JR, Nethander M, Rousseau F, Luben R, Cauley JA, Arnold A, Reppe S, Hibbs MA, Stolk L, Pasco J, Grundberg E, Gautvik KM, Yadav V, Choi K, van Leeuwen JPTM, Pols HAP, Hofman A, Lewis JR, Masi L, Shen J, van Meurs JBJ, Lee SH, Sham PC, Psaty BM, Harris TB, Reeve J, Jukema JW, Metspalu A, Kahonen M, van der Velde N, Brown MA, Ralston SH, Gudnason V, Ioannidis JP, Uitterlinden AG, Cummings SR, Spector TD, Karasik D, Zillikens MC, Visscher P, Michaëlsson K, Jackson RD**, Thorsteinsdottir U**, Chasman D**, Orwoll E**, Forgetta V**, Kiel DP**, Ohlsson C**, Richards JB**, Rivadeneira F**, for the GEFOS consortium. "Large-scale genome wide meta-analysis identifies genetic determinants for fracture risk." (In preparation)
41. Castaño-Betancourt MC, Campos-Obando N, Dehghan A, **Oei L**, Hoeven TA, Oei EHG, Rivadeneira F, Franco OH, Hofman A, Uitterlinden AG, Bierma-Zeinstra S, van Meurs JBJ. "Osteoarthritis as a risk factor for mortality: the Rotterdam Study." (In preparation)
42. Enneman AW, **Oei L**, Estrada K, Zillikens MC, Uitterlinden AG, van Meurs JBJ, van der Velde N, Rivadeneira F, In collaboration with the GEFOS-consortium. "The association between plasma homocysteine levels and fractures and bone mineral density: a Mendelian randomization approach." (In preparation)

*These authors contributed equally

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: H. Ling D.W. Oei
Erasmus MC Departments: Internal Medicine and Epidemiology
Research School: NIHES / MolMed Erasmus University
PhD period: 2010- 2016
Promotor(s): Prof. dr. A.G. Uitterlinden
Supervisor: Dr. F. Rivadeneira

1. PhD training		
	Year	**Workload**
General courses		
- Laboratory animal science (functionary, article 9)	2007	
- Radiation protection, competence level 5B	2007	
- BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2012	1.0 ECTS
- Biomedical English Writing and Communication	2012	4.0 ECTS
Specific courses		
NIHES:		
- Advances in Genome-Wide Association Studies (GE03)	2010	1.4 ECTS
- Principles of Research in Medicine (ESP01)	2010	0.7 ECTS
- Principles of Genetic Epidemiology (ESP43)	2010	0.7 ECTS
- Genomics in Molecular Medicine (ESP57)	2010	1.4 ECTS
- Master class: Advances in Genomics Research (ESP63)	2010	0.3 ECTS
- Genome Wide Association Analysis (ESP29)	2010	1.4 ECTS
- Study Design (CC01)	2010	4.3 ECTS
- Classical Methods for Data-analysis (CC02)	2010	5.7 ECTS
- Modern Statistical Methods (EP03)	2010	4.3 ECTS
- Courses for the Quantitive Researcher (EP17)	2010	1.4 ECTS
- Genetic Linkage Analysis: Model-free analysis (GE05)	2011	1.4 ECTS
- Mendelian Randomization (GE10)	2011	0.9 ECTS
- Missing Values in Clinical Research (EP16)	2011	0.7 ECTS
- Topics in Meta-analysis (ESP15)	2011	0.7 ECTS
- Clinical Decision Analysis (ESP04)	2011	0.7 ECTS
- Conceptual Foundation of Epidemiologic Study Design (ESP38)	2011	0.7 ECTS
- Cohort Studies (ESP39)	2011	0.7 ECTS
- Demography of Ageing (ESP59)	2011	0.7 ECTS
- Health Economics (ESP25)	2011	0.7 ECTS
- Genetic-epidemiologic Research Methods (GE02)	2011	5.7 ECTS
- Linux for scientists	2010, 2011	0.9 ECTS
- Introduction to Clinical and Public Health Genomics (EWP11)	2012	1.9 ECTS
- A first encounter with next-generation sequencing data (GE13)	2012	1.4 ECTS
Molmed:		
- SNPs and Human Diseases (GE08)	2010	1.4 ECTS
- Basic and translational endocrinology	2011	2 ECTS
- Annual Molmed Course	2011	0.7 ECTS
- Annual Molmed Day	2011	0.3 ECTS

- Workshop Browsing Genes and Genomes with UCSC	2011	0.4 ECTS
- Biobase course: principles of NGS data analysis and interpretation	2012	0.4 ECTS
Seminars and workshops		
- Joint Valorisation Workshop NCHA/CGC (Rotterdam, The Netherlands)	2010	March 10
- Clinical Translation of Bone Biology (Rhoon, The Netherlands)	2010	April 8
- Critical Appraisal of Osteoporosis Treatment (Delft, The Netherlands)	2010	April 13
- Imaging Workshop for MDs (MolMed) (Rotterdam, The Netherlands)	2010	May 17
- Endocrine Fellows Foundation/American Diabetes Association (EFF/ADA) Fellows Forum	2011	September 14-15
- Weekly scientific seminars at department of Epidemiology	2010-2012	
- Erasmus lectures on endocrinology	2010-2012	
- Erasmus course endocrinology	2009, 2014	
- Osteoporosis Symposium	2016	
Presentations		
Genome-wide Association In the Rotterdam Study Implicates the 16q24 Locus As Determinant of Osteoporotic Vertebral Fractures.		
- (oral) 32nd American Society for Bone Mineral Research Annual Meeting (Canada, Toronto).	2010	October 15-19
- (oral) 20th Dutch Society for Calcium and Bone Metabolism Annual Meeting (Zeist, The Netherlands).	2010	November 11-12
- (poster) Dutch Society for Human Genetics Meeting (Amsterdam, The Netherlands).	2010	November 19
- (poster) 15th Belgian Society of Internal Medicine (BVIG-SBMI) Annual Congress (Leuven, Belgium).	2010	December 3-4
- (poster) Erasmus MC Internal Medicine Annual Science Day, (Antwerp, Belgium).	2011	January 13-14
- (poster) 15th Molecular Medicine Day (Rotterdam, The Netherlands).	2011	February 3
- (poster) 13th European Congress of Endocrinology (Rotterdam, The Netherlands).	2011	April 30-May 4
Challenges on phenotype definition: the case osteoporotic vertebral fractures.		
- (poster) 6th CHARGE Consortium Investigators Meeting (Boston, United States).	2011	February 9-11
The first genome-wide association study for osteoporotic vertebral fractures.		
- (poster) Netherlands Consortium for Healthy Ageing outreach and kick-off meeting (Amersfoort, The Netherlands).	2011	March 14-15
Women with inadequately controlled type 2 diabetes are at increased risk of osteoporotic fractures despite higher bone mineral density: the Rotterdam Study.		
- (poster) 3rd Joint Meeting of the European Calcified Tissue Society and the International Bone and Mineral Society (Athens, Greece).	2011	May 7-11
- (plenary poster) 33rd American Society for Bone Mineral Research Annual Meeting (San Diego, United States).	2011	September 16-20
Large-scale meta-analyses of genome-wide association studies for fracture risk: the GEFOS consortium.		
- (oral) 33rd American Society for Bone Mineral Research Annual Meeting (San Diego, United States).	2011	September 16-20
- (poster) 12th International Congress of Human Genetics (Montreal, Canada)	2011	October 11-15
Review of radiological scoring methods of osteoporotic vertebral fractures for clinical and research settings.		
- (poster) 97th Annual Meeting of the Radiological Society of North America (Chicago, United States)	2011	November 27-December 2
Degree of glucose control and risk of fracture in type 2 diabetes: the Rotterdam Study		
- (poster) Erasmus MC Internal Medicine Annual Science Day, (Antwerp, Belgium).	2012	January 12-13
- (oral) Dutch Association of Endocrinology (NVE) Annual Meeting (Noordwijkerhout, The Netherlands)	2012	February 10-11

- (poster) Netherlands Consortium for Healthy Ageing outreach phase II Meeting (Amersfoort, The Netherlands).	2012	March 12-13
- (oral and poster) Meeting of the European Calcified Tissue Society (Stockholm, Sweden).	2012	May 19-23
- (poster) Dutch annual conference on Epidemiology (WEON) (Rotterdam, The Netherlands).	2012	June 14-15
Osteoporotic Vertebral Fracture Prevalences Vary Widely Between Radiological Scoring Methods: The Rotterdam Study		
- (poster) Dutch annual conference on Epidemiology (WEON) (Rotterdam, The Netherlands).	2012	June 14-15
- (oral) Dutch Society for Calcium and Bone Metabolism Meeting (Zeist, The Netherlands).	2012	November 1-2
- (oral) American Society for Bone Mineral Research Annual Meeting (Minneapolis, United States).	2012	October 12-15
Genetic Epidemiology of Diabetes and Bone Disease		
- (seminar) Stanford University, Department of Genetics (Stanford, United States).	2012	June 26
Brief Introduction to The Rotterdam Study		
- (invited presentation) University of California at San Francisco (San Francisco, United States).	2012	November 9
Genome-Wide Association Studies Meta-Analysis For Fracture Risk		
- (invited presentation) University of California at San Francisco (San Francisco, United States).	2012	November 9
Scheuermann's Disease: Evaluation of Radiological Criteria and Population Prevalence		
- (poster) World Congress on Osteoarthritis (Philadelphia, United States).	2013	April 18-21
Painful Vertebral Fractures During Pregnancy: Be Aware Of A Potentially Underlying Genetic Cause		
- (oral) Endocrine Society (San Francisco, United States)	2013	June 15-18
Dissecting the Relationship Between High-Sensitivity Serum C-Reactive Protein and Increased Fracture Risk: The Rotterdam Study		
- (oral) Endocrine Society (San Francisco, United States)	2013	June 15-18
- (oral poster) American Society for Bone Mineral Research Annual Meeting (Baltimore, United States).	2013	October 4-7
Large-scale Genetic Studies and Personalized Medicine in Osteoporotic Fractures		
- (invited seminar) University of California at San Francisco (San Francisco, United States).	2013	July 12
The genetic basis of cross-phenotype correlation with bone fracture risk: the GEFOS consortium		
- (poster) American Society for Bone Mineral Research Annual Meeting (Baltimore, United States).	2013	October 4-7
Differentiating Osteoporotic Vertebral Fractures from Scheuermann's Disease using Different Radiological Assessment Methods for Osteoporotic Vertebral Fractures: The Rotterdam Study		
- (oral) American Society for Bone Mineral Research Annual Meeting (Baltimore, United States).	2013	October 4-7
Prediction of vertebral fracture by Trabecular Bone Score in elderly women of The Rotterdam Study		
- (oral) American Society for Bone Mineral Research Annual Meeting (Baltimore, United States).	2013	October 4-7
Genes, Hormones and Bones: Genetic Epidemiology of Endocrine and Skeletal Disease		
- (invited seminar) Veterans Affairs Medical Center, University of California at San Francisco (San Francisco, United States).	2013	October 15

- (grand rounds) Stanford University, Department of Endocrinology (Stanford, United States).	2013	November 6
Type 2 Diabetes, Inflammation, Genetics and Bone Fractures		
- (invited seminar) University of California at San Francisco (San Francisco, United States).	2013	November 22
Large-scale Population Imaging to Investigate the Genetic Epidemiology of Radiographic Scheuermann's Disease: the Rotterdam Study		
- (poster) 99th Annual Meeting of the Radiological Society of North America (Chicago, United States).	2013	December 1-6
- (poster) World Congress on Osteoarthritis (Philadelphia, United States).	2013	April 18-21
Genome-Wide Association Studies (GWAS) meta-analysis for fracture risk points to loci related to hormonal and neurological pathways: the GEFOS Consortium		
- (poster) American Society for Bone Mineral Research Annual Meeting (Minneapolis, United States).	2012	October 12-15
- (oral) Dutch Society for Calcium and Bone Metabolism Meeting (Zeist, The Netherlands).	2012	November 1-2
- (oral) American Society of Human Genetics (San Francisco, United States).	2013	November 6-10
- (poster) Erasmus MC Internal Medicine Annual Science Day (Antwerp, Belgium).	2014	January 9-10
Reninoma: a Rare Cause of Curable Hypertension and Hypokalemia		
- (oral) Rotterdam Regional Clinical Conference of Internal Medicine (Rotterdam, The Netherlands).	2015	November 19
- (oral) Netherlands Internal Medicine Annual Meeting (Maastricht, The Netherlands).	2016	April 20-22
(Inter)national conferences		
- Dutch Association of Endocrinology (NVE) Annual Meeting, Noordwijkerhout, The Netherlands.	2010	January 29-30
- Dutch Internist Association Annual Meeting (Maastricht, The Netherlands).	2010	April 21-23
- CHARGE Consortium Investigators Meeting (Houston, United States).	2010	April 28-30
- European Symposium on Calcified Tissues (Glasgow, Great Britain).	2010	June 26-30
- GEFOS/GENOMOS Consortia Investigators Meeting (Glasgow, Great Britain).	2010	June 27
- American Society for Bone Mineral Research Annual Meeting (Toronto, Canada).	2010	October 15-19
- GEFOS/GENOMOS Consortia Investigators Meeting (Toronto, Canada).	2010	October 19
- Belgian Society of Internal Medicine (BVIG-SBMI) Annual Congress (Leuven, Belgium).	2010	December 3-4
- CHARGE Consortium Investigators Meeting (Boston, United States)	2011	February 9-11
- International Networking Conference: 'From DNA to phenotype' (Rotterdam, The Netherlands)	2011	March 9-11
- European Congress of Endocrinology (Rotterdam, The Netherlands).	2011	April 30 - May 4
- Joint Meeting of the European Calcified Tissue Society and the International Bone and Mineral Society (Athens, Greece).	2011	May 7-11
- GEFOS/GENOMOS Consortia Investigators Meeting (Athens, Greece).	2011	May 7
- American Society for Bone Mineral Research Annual Meeting (San Diego, United States).	2011	September 16-20
- GEFOS/GENOMOS Consortia Investigators Meeting (San Diego, United States)	2011	September 19
- Radiological Society of North America Annual Meeting (Chicago, United States)	2011	November 27 -December 02
- Dutch Association of Endocrinology (NVE) Annual Meeting (Noordwijkerhout, The Netherlands)	2012	February 10-11
- Meeting of the European Calcified Tissue Society, Stockholm, Sweden.	2012	May 19-23
- Dutch annual conference on Epidemiology (WEON) (Rotterdam, The Netherlands).	2012	June 14-15

- American Society for Bone Mineral Research Annual Meeting (Minneapolis, United States).	2012	October 12-15
- American Society of Human Genetics Annual Meeting (San Francisco, United States).	2012	November 6-10
- Radiological Society of North America Annual Meeting (Chicago, United States)	2012	November 25-30
- Personalized Medicine World Conference (Mountain View, United States).	2013	January 28-29
- Osteoarthritis Research Society International (Philadelphia, United States).	2013	April 18-21
- ENDO Endocrine Society Annual Meeting (San Francisco, United States).	2013	June 15-18
- American Society for Bone Mineral Research Annual Meeting (Baltimore, United States).	2013	October 4-7
- GEFOS/GENOMOS Consortia Investigators Meeting (Baltimore, United States).	2013	October 5
- Radiological Society of North America Annual Meeting (Chicago, United States)	2013	December 1-6
- Dutch Internist Association Annual Meeting (Maastricht, The Netherlands).	2014	April 23-25
- Dutch Internist Association Annual Meeting (Maastricht, The Netherlands).	2015	April 22-24
- European Calcified Tissue Society Meeting (Rotterdam, The Netherlands).	2015	April 25-28
- Dutch Internist Association Annual Meeting (Maastricht, The Netherlands).	2016	April 20-22
- European Congress of Internal Medicine Annual Meeting (Amsterdam, The Netherlands).	2016	September 2-4
Other		
Associate Editor for journals:		
- Gene	2013-2014	16 manuscripts
Peer-reviewing for journals:		
- European Journal of Epidemiology	2010, 2012	2 manuscripts
- Gene	2011, 2012, 2013	12 manuscripts
- Plos One	2012, 2013	3 manuscripts
- New England Journal of Medicine	2012	1 manuscript
- Calcified Tissue International	2012	2 manuscripts
- Osteoporosis International	2012, 2016	2 manuscripts
- Joint Bone Spine	2013	1 manuscript
- Journal of Bone and Mineral Research	2013, 2015	2 manuscripts
- Journal of Clinical Endocrinology and Metabolism	2014	1 manuscript
- BMC Musculoskeletal Disorders	2014	1 manuscript
- British Medical Journal	2015	1 manuscript
- Bone	2016	1 manuscript
Service positions for scientific organizations:		
- American Society for Bone and Mineral Research: Annual Meeting peer-reviewing of abstracts	2014, 2015	
- European Calcified Tissue Society: Annual Meeting session moderator	2015	
Grants and awards		
- American Society of Bone Mineral Research (ASBMR) Travel Grant	2013	
- Dutch Association of Endocrinology "Goodlife Healthcare Travel Grant"	2013	
- Simons Fund Foundation. Fellowship subsidy	2013	
- Erasmus University Trust Fund. Postdoctoral fellowship subsidy	2013	
- Dutch Society for Calcium and Bone Metabolism Best Presentation Award	2012	
- Endocrine Fellows Foundation/American Diabetes Association (EFF/ADA) Fellows Forum Meeting Support	2012	
- American Society of Bone Mineral Research (ASBMR) Travel Grant	2012	

- Endocrine Fellows Foundation/American Diabetes Association (EFF/ADA) Fellows Forum Meeting Support	2011	
- American Society of Bone Mineral Research (ASBMR) Travel Grant	2011	
- Gordon Research Conference Travel Support	2011	
- European Calcified Tissue Society Travel Grant	2011	
- Dutch Association of Endocrinology "Goodlife Healthcare Travel Grant"	2011	
- American Society of Bone Mineral Research (ASBMR) Young Investigator Award	2010	

2. Teaching

	Year	**Workload**
Lecturing		
Teacher for Erasmus University medical school:		
- journal club (diabetes)	2011	0.2 ECTS
- compulsory education (thyroid disease)	2011, 2012	0.6 ECTS
Supervising practicals and excursions, tutoring		
- Supervision of abstract writing (Student: Felisia Ly)	2011	
- Supervision of a student working team working on data cleaning of DXA scans in Erasmus Rotterdam Health Research (ERGO) and Generation R studies. (Students: Rodinde Bloot, Mette Offerhaus)	2010-2011	
- Supervision of a student working team working on digitization of radiographs in the ERGO study. (Students: Emma Dogterom, Laura de Kok, Mette Offerhaus, Felisia Ly, Hanna Ning, Florian Buisman, Nadia Rbia, Burak Kalin, Nuray Cakici, Stephan Breda, Bart Hazemeijer, Evelien van Meel, Nienke Bart)	2010-2011	
- Supervision of a student working team working on vertebral fracture assessment in the ERGO study. (Students: Rodinde Bloot, Laura de Kok, Mette Offerhaus, Felisia Ly, Hanna Ning, Stephan Breda, Bart Hazemeijer, Evelien van Meel, Nienke Bart, Khadija Moumni, Maarten Meijer, Sebastian Valk Bonila, Sander Verkade, Maria Tihaya, Lisanne van de Koevering)	2010-2012	
- SNP course practical	2012	
- Supervision of medical interns in internal medicine	2014-2016	
Supervising Master's theses		
- Medical student Salih El Saddy	2011	6 ECTS
- Medical student Ater Andrew Makurthou	2011	6 ECTS
- Medical student Stephan Breda	2012	6 ECTS
- Medical student Khadija Moumni	2013	Co-supervision
- Medical student Sema Ozdemir	2013	Co-supervision
- Medical student Sebastian Valk Bonilla	2013	Co-supervision
Other		
- GEnetic Factors for OSteoporosis (GEFOS) Consortium: general coordination, co-organizing meetings, set-up and coordination of vertebral fracture and all-type of fracture working groups	2010-2014	
- GENOMOS Consortium: general coordination, co-organizing meetings	2010-2014	
- CHARGE Consortium: participation in musculoskeletal working group	2010-2014	
- ERGO Rotterdam Study: coordination of musculoskeletal research findings that are potentially clinically relevant	2010-2014	
- Visiting scholar at dr. Michael Snyder's lab Stanford University, Department of Genetics	2013	
- Educational committee member IJsselland hospital	2015-2016	

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ABOUT THE AUTHOR

Hwee Ling Dian Widyarini Oei was born on October 27th, 1983 in Gouda, The Netherlands. Daughter of Yok Kong Oei and Liliana Oei-Kusumadewi; and eldest sister to Hwee Fang Dharma Suryani. She grew up in Bodegraven and attended grammar school at the Coornhert Gymnasium in Gouda, and graduated in 2003. Subsequently, she received her Medical Doctor training at Leiden University and at the same time she obtained Bachelor's and Master's degrees in Chinese Studies at Leiden University as well. During 2004-2005 she studied at Beijing University in China with a joint scholarship from the Chinese government and the Netherlands organization for international cooperation in higher education. After spending the last four months of her medical training at Leiden University Medical Center's Department of Internal Medicine and Endocrinology, she obtained her Doctor in Medicine degree in December 2009. In 2010 she started a PhD track at Erasmus University in Rotterdam under supervision of prof. dr. A.G. Uitterlinden and dr. F. Rivadeneira. In 2010 she was awarded the American Society of Bone Mineral Research Young Investigator Award and in 2012 she won the Dutch Bone and Mineral Society Best Presentation Award. In parallel, she completed an MSc in Health Sciences (specialization: Genetic Epidemiology) at Erasmus University Netherlands Institute for Health Sciences from 2010-2012, and visited prof. dr. M. Snyder's lab at Stanford University Department of Genetics for a year in 2013. Additionally, she assisted Dr. M.C. Zillikens as a resident in the outpatient clinic of Erasmus MC's Department of Internal Medicine, Endocrinology; and visited dr. J.Y. Wu's outpatient clinic of Stanford University's department of Medicine, Endocrinology, Gerontology and Metabolism. In 2014 she started her residency in internal medicine at IJsselland Hospital Department of Internal Medicine for the first two years, and continued her training at Erasmus MC in 2016. Ling is married to Edwin Hong Gwan Oei.