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AGING CARDIOVASCULAR SYSTEM: GENETIC *and* EPIGENETIC DETERMINANTS *of* VASCULAR OUTCOMES *and* CARDIOMETABOLIC RISK

• *Eliana Portilla Fernández* •

The aging cardiovascular system: genetic and epigenetic determinants of vascular outcomes and cardiometabolic risk

Eliana Portilla Fernández

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The aging cardiovascular system: genetic and epigenetic determinants of vascular outcomes and cardiometabolic risk

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A mis papás, hermanos
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Chapter 2

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Chapter 3

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CHAPTER 1

General Introduction

1.1 Vascular aging and cardiovascular risk

1.1.1 General aspects

Cardiovascular disease (CVD) causes one-third of all deaths worldwide and accounts for trillions of dollars of health care expenditure (1). The high prevalence of CVD, arising predominantly from increasing life expectancy, highlights the necessity of understanding how aging influences vascular function. Although aging contributes to a wide range of disorders (2), CVD carries the greatest burden for the older population. In 2005, CVD was the underlying cause of death in 864,480 of the approximately 2.5 million total deaths in the U.S., and in adults aged ≥ 65 years CVD accounted for 82% of death causes(3). In 2009, the cost of CVD and stroke, including direct and indirect cost, exceeded $475 billion, making CVD the most expensive disease category in the U.S (3). Aging is thought to be a consequence of the continued exposure to risk factors, e.g., dyslipidemia, smoking, high blood pressure and diabetes mellitus, during which accumulation of damage increases the risk of developing vascular dysfunction and associated disease (4). Apart from the impact of several classical cardiovascular risk factors on the vasculature, chronological aging remains the single most important determinant of cardiovascular problems. The causative mechanisms by which chronological aging mediates its impact, independently from classical risk factors, remain to be elucidated.

Alterations in the structure and function of arteries accompany aging, and contribute to increased risks of developing CVD (5). Vascular aging is described as a gradual process involving biochemical, enzymatic, and cellular changes of the vasculature and modification of the signals that modulate them (6). Morphological changes in the vasculature consist of outward remodeling, increased media-to-lumen (M/L) ratio, calcification, and reduced elastin and increased collagen in the extracellular matrix (ECM). These changes lead to elasticity loss, which increases wall stiffness, leading to the pathological raise in blood pressure and overall cardiovascular risk during aging (6-8).

1.1.2 Role of endothelium, nitric oxide and reactive oxygen species

Physiologically, aging associates with an impairment in endothelial function that disrupts arterial homeostasis. Endothelial dysfunction favors an over-production of reactive oxygen species (ROS), an increase of lipid oxidation, pro-inflammatory pathways, and a shift toward a provasoconstrictor phenotype, all of which predisposes to CVD and adverse events (9). At the cellular level, these factors also contribute to a loss of the DNA integrity, triggering a cell survival response featured by apoptosis, cellular senescence and increased autophagy (10). The diminished bioavailability of nitric oxide (NO), a key mediator of vasorelaxation and inhibitor of medial smooth muscle cell proliferation, hallmarks age-dependent endothelial dysfunction (11, 12). Reduced NO bioavailability can occur due to a decreased synthesis or increased degradation of NO. Under normal conditions, endothelial nitric oxide synthase (eNOS) produces NO from l-arginine in the presence of the cofactor tetrahydrobiopterin (BH_4) (13). With aging, there is an increased production of superoxide (O_2^-) that is associated with alterations in eNOS function, referred to as eNOS uncoupling (14). Uncoupling of eNOS features activation of the enzyme in the absence of BH_4. This leads to O_2^- instead of NO production. Additionally, aging may increase NO scavenging (15) due to an over-production of ROS, mediated, in part, by chronic low-grade inflammation, constituting a vicious cycle that depletes NO (16). Pro-inflammatory cytokines and adhesion molecules associates with NOX (NADPH oxidase)– and mitochondrial-produced ROS, such as O_2^- and H_2O_2. The increased superoxide reduces NO availability as the molecules react to form $ONOO^-$. This process further increases inflammatory signaling through NFκB (nuclear factor κB) activation and induces MMP (matrix metalloproteinase)-9 that contribute to the alterations observed in the ECM and the consequent

arterial stiffening (17). As a consequence the endothelium is exposed to a higher hemodynamic load, leading to further endothelial damage. In addition, decreased NO bioavailability may lead to higher arterial stiffness via an increase in smooth muscle tone (18). Thus, endothelial dysfunction and vascular stiffness are closely interconnected mechanisms of age-dependent impaired vascular function (19).

1.2. The search for mechanisms that precipitate vascular aging

1.2.1 DNA damage and vascular aging

The study of metabolites logically implicates also evaluation of their (extra) cellular targets. This is the case for ROS, which react with macromolecules, leading amongst others to DNA damage and activation of DNA repair mechanisms. During aging, oxidized DNA molecules increase, implying that aging is associated with accumulation of DNA lesions (20). Over the past decade the paradigm that accumulating DNA damage might be responsible, at least in part, of aging has become an accepted paradigm (21). When DNA is not properly repaired, RNA transcription can be stalled, which jeopardizes proper protein production. This forces the cell to increase autophagy of damaged cell parts and recycling of proteins (22). Another aspect of unrepaired DNA damage is that it can force cells into cell senescence, a dormant state during which the cell does not proliferate and may not execute its physiological role properly (23). Senescent cells have a secretory phenotype, referred to as senescence-associated secretory phenotype (SASP), This SASP is featured by release of cytokines that are typically found to be associated with vascular aging and cardiovascular risk due to the earlier described inflammatory effects (24). Thus both faulty RNA transcription and senescence might contribute to CVD. Indeed defective DNA repair in all body cells in mice as well as in humans is associated with accelerated appearance of vascular aging features through several of the earlier described mechanisms (12). However, the relationship between local DNA damage in endothelial cells and vascular aging has not been investigated. The mouse models for accelerated vascular aging are attractive tools to solve this question, as well as for pathophysiological explorations and drug development studies. A large variety of processes might be involved in vascular aging. Hence, an important question when starting with mouse models of vascular aging is what processes are relevant for humans, To answer this question some a priori knowledge of human candidate mechanism is very helpful, if not indispensable.

1.2.2 Human studies of vascular aging

Candidate mechanisms can be generated with several types of human studies. A good starting point is literature study, especially in the form of systematic reviews. In addition, genetic epidemiological studies that map associations between genetic variations and age-related CVD in humans are believed to be a powerful tool (25). The use of genetic variation studies alone might not provide sufficient information because genetic variety does not 'describe' the interaction between environment and disease risk. The incorporation of gene-environment interaction analyses may raise particular concerns including lack of reproducibility, especially in complex traits studies (26). The molecular pathways underlying morphological and physiological age-related changes in the vasculature, some of which are described above, are the result of the complex interaction of the environment with key gene regulation and protein production mechanisms. This interaction is affected by genetic variations, modifications of the epigenetic landscape, metabolic changes and accumulating damage to genomic material. The interaction involves a myriad of cellular processes, such as metabolism, autophagy/repair and cellular function, all of which

can be cell type-specific and thus contribute to specific age-related diseases. However, which of these changes occur, and to what specific aging-related problem they lead, remains to be charted. Supplementation with epigenetic studies could be very helpful in further pinpointing of relevant candidate CVD mechanisms as they also include the interaction with environment. Despite the advances in the field of CVD research, current scientific knowledge does not completely explain the complex pathophysiology underlying vascular aging and related outcomes.

1.3 Vascular aging study toolbox

1.3.1 Experimental models of accelerated aging

Aging is a process that gradually increases the disease burden, ultimately leading to the organism's death. In humans, the presence of individual genetic and environmental variations evoke differences in the rate of aging between individuals. Within the individual, organs can age at a different rate. This 'segmental aging' might be attributable to differential exposure of the individual organs and cell types to risk factors that confer their specific contribution to age-related disease via molecular and metabolic pathways. DNA damage through risk factor-related genotoxic substances is believed to be a major mechanism for a number of tissues, amongst which the cardiovascular system (10). An important piece of evidence is the observation of segmental aging in mouse models that display accelerated aging due to mutations of genes coding for proteins that are needed for DNA repair. Accordingly, a large numbers of mice models to study the mechanisms of aging have been developed (27-29). Mouse models of accelerated aging have contributed greatly to unravel important mechanistic insights into the processes of deterioration seen in normal physiological aging as well as into the premature ageing process in progeroid syndromes. In addition, mice models of accelerated aging have become a very attractive tool to investigate intervention strategies for healthy aging, because of their short lifespan, their relatively simple creation by single gene deletion, and their strong phenotypic overlap with normal aging lesions (30). One of these models is the *Ercc1Δ/–* mouse. *Ercc1Δ/–* mice harbor a deletion mutation (Δ) in exon 7 of the *Ercc1* gene, and one null allele for the *Ercc1* gene, causing impaired function of the ERCC1 (Excision Repair Cross Complementation group 1) protein and progressive accumulation of DNA damage (31). ERCC1 is an essential component in the pathway of DNA nucleotide excision repair (NER), which removes a wide class of helix-distorting DNA lesions induced by UV, chemicals and oxidative stress. Apart from that, ERCC1 is involved in other DNA repair systems such as double strand break and cross link repair (32). Mutations in proteins of the NER pathway have shown severe effects on human health as evidenced in several human progeroid syndromes such as Cockayne syndrome, trichothiodystrophy and Xpf-Ercc1 syndrome (33).

Ercc1Δ/– mice are short-lived (24-28 weeks) and within 12 weeks from birth develop neurodegeneration, osteoporosis, many features of aging in liver, kidney, heart, muscle and the hematopoietic system. Also the vascular system ages fast in these mice. In 8-week old *Ercc1Δ/–* mice an increased blood pressure was observed, which appeared to become smaller at 12 weeks of age (12) (34). Thus, the blood pressure increase might be biphasic, as is seen also in aging human (35). Also, increased vascular stiffness and loss of macro- and microvascular dilator function was observed (12). The vasodilator dysfunction in *Ercc1Δ/–* mice is explained by reduced NO-cGMP signaling, partly due to decreased eNOS expression (12). Many of these features are very similar to what was previously found in natural rodent and human aging. Therefore, Ercc1 mutant mice are a potential tool to efficiently investigate vascular aging. It is unclear whether the vascular aging features in *Ercc1Δ/–* mice are driven by general systemic processes due to widespread DNA damage or by local processes due to vascular DNA damage. The creation of Cre-lox-driven *Ercc1*

knockout mice (36) allows the generation of endothelial cell-specific *Ercc1* knockout mice.

1.3.2 Systematic reviews

Systematic reviews (SR) are an important tool to validate the role of genes on cardiometabolic risk factors, contributors of vascular aging outcomes. Systematic reviews make use of data from existing literature that is selected with a well-defined, critical search procedure and explored with a strict statistical protocol (37).

1.3.3 Genetic association studies of complex diseases

Since the completion of the human genome project, GWAS have been considered to hold promise for unraveling the genetic etiology of complex traits (38). The identification of genetic risk factors has yielded valuable knowledge of physiological, biochemical, and functional changes underlying human traits and disease(39). In clinical practice, the findings from GWAS have been valuable in the identification of novel targets and strategies in prevention and therapy. For example, targets identified from GWAS on lipids have been subject of pharmacology research and included in randomized control trials. *PCSK9* gene is the most established one, as it has been proved that the gain-of-function and loss-of-function variants in the *PCSK9* gene increase (40) and decrease (41) the risk of CAD and myocardial infarction, respectively. Current therapeutic concepts have exploited the use of monoclonal antibodies to inhibit the effect of *PCSK9* in the circulation, as well as the inclusion of RNAi- and other small molecule-based approaches are also in the development and evaluation (42).

The human genome contains the genetic information that provides the building blocks, gene-segment of DNA for the manufacture of all proteins needed for cell function activity. Differences in the sequence of DNA bases in each gene or gene-regulating genome parts among individuals can be found as single nucleotide polymorphisms (SNPs), insertions and deletions (indels), and other structural variants and are collectively called genetic variation. SNPs are the most common form of genetic variation and they are encountered at a frequency of 1/1000 base pairs. SNPs constitute approximately 90% of the isolated variations in the human genome (43). SNPs in the gene coding sequence can result in changes in the amino acid structure and therefore play a crucial role in disease pathophysiology. Genetic studies have traditionally been conducted using candidate gene studies and family-based linkage studies to identify diseases-associated genes. Candidate genes studies rely on our partial understanding of genes with known biological relevance in the mechanism of the disease (trait) being investigated (44). However, by looking at genes that are expected to be important based on current understanding some key players might be missed. Family-based linkage studies have been performed to identify regions of the genome where a disease-causal gene is located (45). However, this approach fails to identify genes associated with complex disorders in the general population because the family-base genetic studies often reveals rare variations, resulting in low power and lack of replication in large cohorts. Application of GWAS is an hypothesis-free approach designed to identify genetic variants associated with common diseases without relying on prior knowledge (39). In the recent decade, with the development of new high-throughput genotyping and next generation sequencing platforms, GWAS have evolved into a powerful tool for investigating the genetic architecture of many complex traits and diseases (46-50) (Figure 1). The completion of the Human Genome Project and the International HapMap project have allowed to map patterns of genetic variation in several population groups, and to select a set of genetic variants that are representative for human haplotypes, groups of alleles that are co-inherited based on linkage disequilibrium (LD)(51). LD is defined as the difference between the observed frequency of a particular combination of alleles at two loci and the frequency expected for random asso-

ciation (52). Parallel technological advances in array technologies, partly prompted by the HapMap project, have allowed the interrogation of hundreds of thousands SNPs in a single experiment. In addition, the incorporation of genotype imputation (53), the reduction of nearby SNPs in LD to a single representative SNP, optimizes the statistical power in association studies. Consequently, the implementation of the 1000 G imputation reference panel improved the genomic coverage providing the most detailed map of human variation, and has allowed the identification of novel genetic variants associated with a particular trait (54).

For GWAS to successfully identify variants influencing trait variation or disease risk, there must be multiple common loci (>1% of the population), of which each locus exerts substantial additive genetic effects on the overall trait variance or disease susceptibility (55). In this sense, statistical power of individual GWAS may be limited by sample size, small effect sizes, causal allele frequency and marker allele frequency of the genetic variants (55). Therefore, GWAS require the inclusion of large sample sizes and the use of a more rigorous threshold based on multiple testing correction to avoid false positives. Meta-analysis of GWAS data provides the opportunity to increase power, to identify new risk genetic variants and to get a further insight into molecular mechanisms underlying human traits.

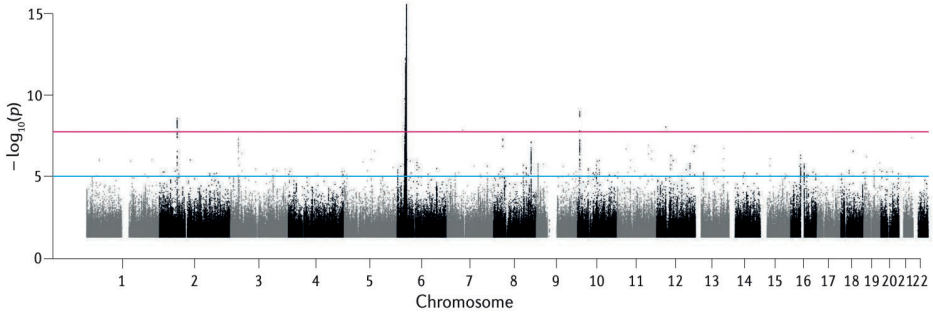

Figure 1. Manhattan plot showing the statistical association between SNPs and a trait of interest. Each SNP is represented by a dot. Genomic coordinates (Chr 1-22) are displayed along the X-axis and the negative logarithm of the association p-value for each SNP displayed on the Y-axis. Dash line shows significance threshold.

1.3.4 Epigenetic studies and complex disorders

Beyond environmental factors and genetic susceptibility, scientists now believe there is a third powerful influencer of our health outcomes, called epigenetics. Epigenetics is the science of how our environment chronically shapes our genetic program through targeted, endogenous gene-regulating chemical modification of genomic macromolecules. The role of epigenetic determinants is increasingly recognized as a potential important link between environmental exposure and disease risk (56). Thus, epigenetic determinants may serve as a benchmark to capture both genetic and environmental influences (57). Moreover, epigenetics may account for the missing heritability determinants of complex diseases and epigenetic pathways may offer a new perspective in the etiology and treatment of atherosclerosis, hypertension, chronic inflammation, diabetes and CVD (57). Several prominent risk factors for cardiovascular traits, including blood pressure, dyslipidemia, inflammation and glycemic traits are suggested to be regulated by epigenetic mechanisms. This knowledge can potentially help to further unravel our understanding of underlying mechanisms leading to vascular dysfunction and cardiovascular outcomes.

Epigenetic information is found across the human genome and provides instructions on how, where, and when the genetic information should be used by the body. Our DNA is made

from repeating units of nucleotides, Adenine, Guanine, Cytosine and Thymine. While genetic modifications lead to a change in the base sequence of DNA, epigenetic changes do not involve a change in the primary DNA sequence or to base pairing. Rather, epigenetic changes are heritable changes in gene function (active versus inactive genes) without a change in the DNA sequence. DNA methylation, histone modification, and non-coding RNA are three major types of epigenetic marks (58). DNA methylation (Figure 2) refers to the addition of a methyl group to cytosine at CpG dinucleotides that further influences the function of DNA as it activates or represses gene transcription. Posttranslational histone modification is another type of epigenetic mark that influences gene expression, mainly by altering chromatin structure as to alter accessibility of transcription factors. Noncoding RNAs (ncRNAs) have recently emerged as key regulators of gene expression and important players in physiological complexity of biological functions in humans (59). Many ncRNAs, especially long ncRNAS, can interact with chromatin-modifying proteins and recruit their catalytic activity to specific sites in the genome, thereby modifying chromatin states and influencing gene expression (60). The genome-wide distribution of these marks and regulators refers as "the epigenome" (61).

Recent emphasis has been placed upon CpG island methylation of cytosine to methylcytosine in the promoter region of specific genes, but the overall or global methylation of the entire genome is of interest as well. The assessment of the total methylcytosine content in a DNA sample can be conducted in the genome overall, in order to determine whether changes to the global status of DNA methylation can be a biomarker of disease and of treatment effects (62). Several methods exist to measure global methylation levels. Most methylation sites within the genome are found in repeat sequences and transposable elements, such as Alu and long-interspersed nuclear element (LINE-1). These methylation sites correlate with total genomic methylation content (63, 64). Such elements have

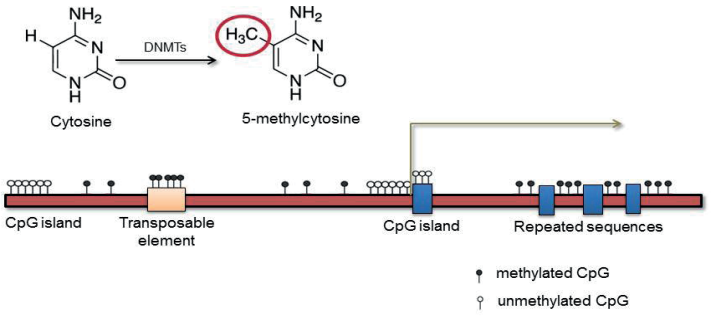

Figure 2. Typical mammalian DNA methylation landscape. The 5-position of cytosine is covalently methylated by DNA cytosine methyltransferases (DNMTs). The genome is depleted of CpGs and some regions are methylated (black lollipops). CpG islands are rich in CpGs and can be normally found unmethylated (white lollipops) in gene promoters, irrespective of gene expression status.

served as a useful proxy for global DNA methylation because they are commonly heavily methylated in normal tissue and are widespread throughout the genome (65) (66). Other methods (e.g., Luminometric Methylation Assay, LUMA and the [3H]-methyl acceptance based method) that assess global genomic DNA methylation are primarily based on the digestion of genomic DNA by restriction enzymes (such as HpaII and MspI) (67).

Epigenome Wide Association Studies

The interrogation of DNA methylation status at different positions in the genome may have

wide varied effects on gene regulation and expression. The implementation of epigenome-wide association studies (EWAS), which are the large scale, systematic, epigenomic equivalent of GWAS, alongside with the development of microarray technologies, has allowed the interrogation of DNA methylation sites at single-nucleotide resolution (68). Currently, Illumina Infinium Methylation450 bead Chip is one of the most widely used platforms and has been praised for its cost-effectiveness, the high number of sites it can test, and its overall good accuracy (69).

The epigenome-wide profiling of CpG sites located in relevant regions throughout the genome may provide more insight into the effects of DNA methylation status on gene expression depending on its position towards coding genes. Moreover, the profiling of CpG islands at epigenome-wide level provides a better understanding of gene regulation and allows the evaluation of phenotypic variation that is attributable to inter-individual epigenomic variation (68). Moreover, the implementation of additional analytical approaches in EWAS data may unravel important biological processes through the characterization of differentially methylated regions (DMRs). DMR are regions of the genome at which adjacent CpG sites show differential methylation levels. Accounting for this correlation structure may increase our power to detect changes in DNA methylation. The combination of information from multiple nearby methylation sites may aid biological inference as well as increase the power to detect associations with human traits (70). Therefore, the implementation of DMR analysis could lead to the identification of epigenetic patterns that best determines differences in DNA methylation at genomic-region level and could be useful in early detection and diagnosis of human diseases (71).

1.3.5 Study populations

Rotterdam Study

The Rotterdam Study (RS) was designed in the mid-1980s as a response to the demographic changes that were leading to an increase of the proportion of elderly people in most populations (72). The study was designed to identify the health and disease determinants of several outcomes that are frequent in the elderly: coronary heart disease (CAD), heart failure and stroke, Parkinson's disease, Alzheimer disease and other dementias, depression and anxiety disorders, macular degeneration and glaucoma, COPD, emphysema, liver diseases, diabetes mellitus, osteoporosis, dermatological diseases and cancer. RS is a prospective study, population-based cohort study ongoing since 1990 including population from the well-defined Ommoord district in the city of Rotterdam. Initially, the study included 7983 individuals 55 years aged or older. In 2000, 3011 additional participants who had become 55 years or moved into the study district were included to the cohort. In 2006, the RSIII cohort was established including 3932 subjects aged 45-54 years. As of 2008, the Rotterdam Study cohort comprises a total of 14,926 subjects aged 45 years All individuals comprised in this study were of European and African descent. An overview of baseline and follow-up visits is shown in Figure 3. The study has conducted extensive clinical examinations, repeated every 3–4 years, to investigate the causes and risk factors associated with a variety of diseases (73, 74).

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium was formed to facilitate genome-wide association study (GWAs) meta-analyses and replication opportunities among multiple large and well-phenotyped longitudinal cohort studies (75). With the emerging field of epigenetics, the consortia started new efforts to facilitate epigenome-wide association studies (EWAs) meta-analyses of different outcomes. Initiatives like CHARGE have enabled the boost of power in genetic studies and thereby increasing the probability of identifying new genetic and epigenetic variants. Furthermore, it has brought the

development of epidemiology research to another level giving new opportunities for methodological advances and reliable strategies for discovery and replication of (epi) genetic variants of great importance for human health. Among these projects the CHARGE Subclinical & CHD and CHARGE epigenetics working groups were set up in 2016 to run the first GWAS on abdominal aortic diameter and the first EWAS on common carotid intima media thickness. The following cohorts contributed to the GWAS effort: RS, Kooperative Gesundheitsforschung in der Region Augsburg (KORA), Cardiovascular Health Study (CHS), Framingham Heart Study (FHS), Multi-Ethnic Study of Atherosclerosis (MESA), Study of Health in Pomerania (SHIP-2 and SHIP-T) and PBIO1. The following cohorts contributed to the EWAS on cIMT effort: RS, FHS, KORA, SHIP, Lothian Birth Cohorts (LBC), CHS, Young Finns Study (YFS) and the Medical Research Council (MRC) National Survey of Health and Development (NSHD) (MRC1946). The cohorts that contributed to the EWAs on statin use are: the Avon Longitudinal Study of Parents and Children (ALSPAC), Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung (ESTHER), KORA-F4, the London Life Science Population study (LOLIPOP), RS, and SHIP-Trend.

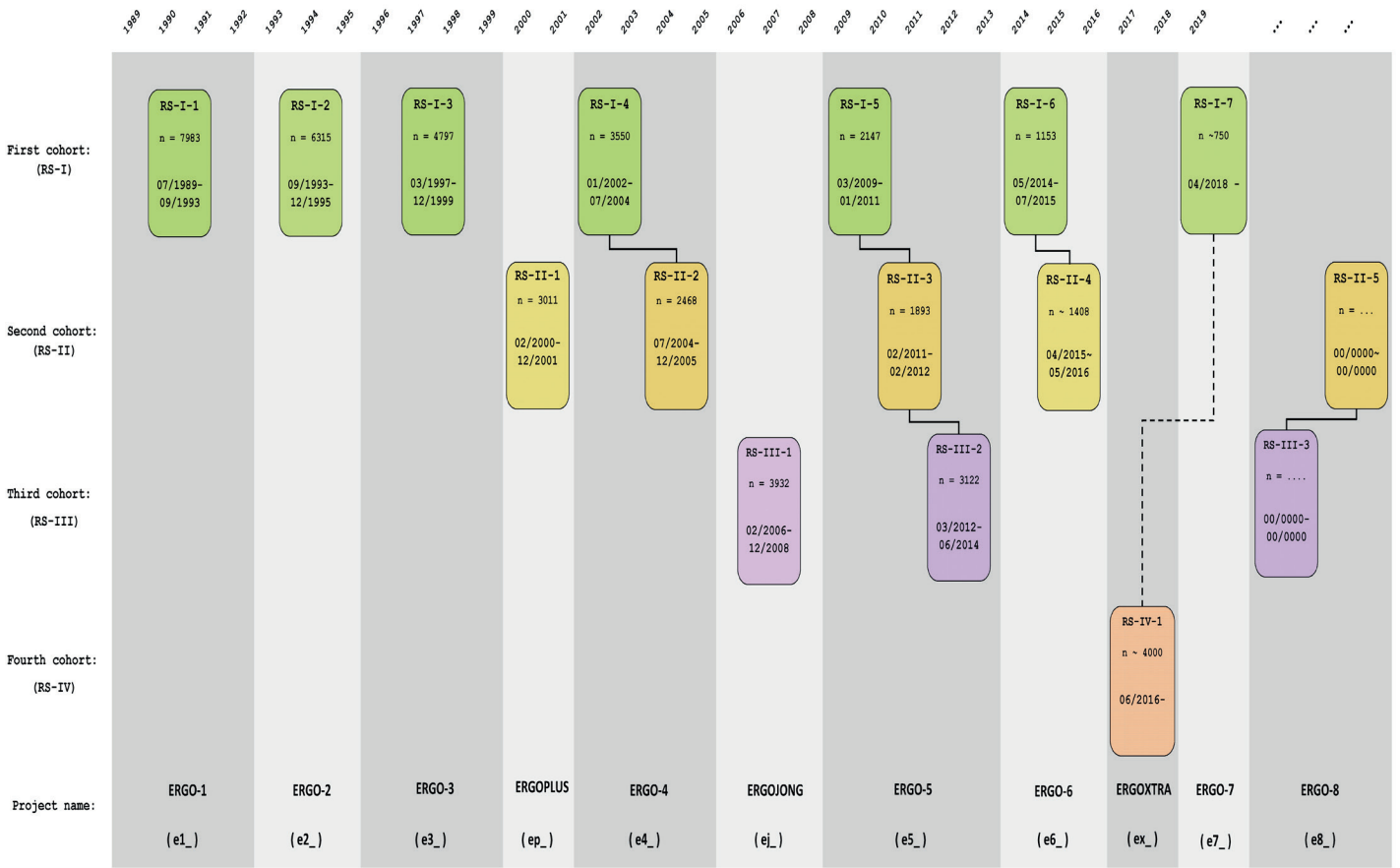

Figure 3. Overview of the sub cohorts and examination visits of the Rotterdam Study, and imaging visits of the Rotterdam Scan Study.

AIM OF THIS THESIS AND OUTLINE

In this thesis, we aimed to untangle novel mechanisms underlying the aging of the vasculature. Among the multiple 'unknowns' in the field of cardiovascular physiopathology, we addressed the effect of local DNA damage in endothelial cells on vascular aging. We also studied the role of dysfunctional autophagy in cardiometabolic traits, which remains an open question in cardiovascular and cardiometabolic health research. Moreover, we characterized novel mechanisms of aortic diameter and arterial thickness through genetic and epigenetic studies. To accomplish this aim, we implemented a multidisciplinary approach, referred to as the "vascular aging study toolbox", which combines animal models and (big) data from human studies as a source of target mechanisms and a fundament for validation of the models (Figure 4).

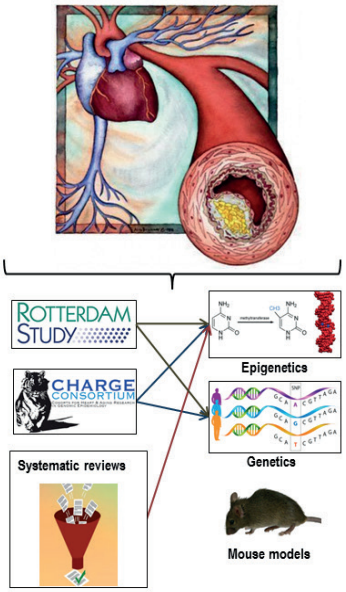

Figure 4. Components of the study tools covered in this thesis

Chapter 2 focuses on the characterization of novel genes and mechanisms associated with an impaired vascular function and changes in the vasculature. **Chapter 2.1** outlines the role of DNA damage in vascular aging, and describes the present mechanisms by which genomic instability interferes with regulation of the vascular tone. In addition, we present potential remedies against vascular aging induced by genomic instability In **Chapter 2.2** we describe the cardiovascular effects of local endothelial DNA repair defects, using a mouse model with loss of *ERCC1* DNA repair in vascular endothelial cells. **Chapter 2.3** explores the potential role of autophagy in cardiovascular diseases and intermediate vascular traits; through a comprehensive evaluation of both genetic and epigenetic variations in autophagy-related genes. We implemented a multidirectional approach using several molecular epidemiology tools, including genetic association analysis with genome wide association studies and exome sequencing data and differential DNA methylation analysis. **Chapter 2.4** describes two novel loci, *LDLRAD4* and *PCSK5*, associated with abdominal aortic diameter. *LDLRAD4* gene acts as a negative regulator of *TGF-β*, a growth factor important in aortic dilation. *PCSK5* is important in collagen deposition and may be relevant to aortic dilation biology. The association of these genes was identified through the implementation of meta-analysis

of Genome-Wide association and exome array studies. In addition, we characterized the potential causal association between risk factors for aortic dilation and aortic diameter using Mendelian randomization methods. **Chapter 2.5** dissects the role of epigenetic modifications in single CpGs and differentially methylated regions on common intima media thickness. We describe the association of a CpG site in *AHRR* gene with cIMT. Through the identification of differentially methylated regions, we highlight novel target regions with biological relevance in the etiology of cIMT, including inflammation and lipid metabolism pathways. Furthermore, we showed the potential mediation effect of this CpG in the smoking-cIMT association. **Chapter 3** of this thesis is devoted to explore the association of epigenetic signatures with several cardiometabolic-related traits. Overall, we have systematically reviewed all the evidence on this topic. In addition, we have incorporated the scanning of epigenetic profiling in individuals with intima media thickness data. In **Chapters 3.1, 3.2, 3.3** we systematically reviewed the association of epigenetics with blood pressure, inflammation, and cardiovascular diseases. We included population-based studies that evaluated the main epigenetic measurements: global DNA methylation; CpG sites, identified from Epigenome-Wide Association Studies and histone modifications. **Chapter 3.4.** explores the potential impact of statins use on DNA methylation, gene expression and how it is implicated in the pathogenesis of type 2 diabetes. Moreover, we investigated whether DNA methylation may be a mechanism linking statin use with diabetes risk.

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CHAPTER 2

Determinants of impaired vascular function and vascular aging-related outcomes

2.1.

DNA Damage: A Main Determinant of Vascular Aging

2.2.

Local endothelial DNA repair defect causes aging-resembling endothelial-specific dysfunction

2.3.

Dissecting the association of autophagy-related genes with cardiovascular diseases and intermediate vascular traits: a population-based approach

2.4.

Genetic and clinical determinants of abdominal aortic diameter: Genome-wide association studies, exome array data and Mendelian randomization study

2.5.

Meta-analysis of epigenome-wide association studies of carotid intima media thickness

CHAPTER 2.1

DNA Damage: A Main Determinant of Vascular Aging

Bautista-Niño PK, **Portilla-Fernandez E**, Vaughan DE, Danser AH, Roks AJ. Int J Mol Sci. 2016;18;17(5).

Abstract

Vascular aging plays a central role in health problems and mortality in older people. Apart from the impact of several classical cardiovascular risk factors on the vasculature, chronological aging remains the single most important determinant of cardiovascular problems. The causative mechanisms by which chronological aging mediates its impact, independently from classical risk factors, remain to be elucidated. In recent years evidence has accumulated that unrepaired DNA damage may play an important role. Observations in animal models and in humans indicate that under conditions during which DNA damage accumulates in an accelerated rate, functional decline of the vasculature takes place in a similar but more rapid or more exaggerated way than occurs in the absence of such conditions. Also epidemiological studies suggest a relationship between DNA maintenance and age-related cardiovascular disease. Accordingly, mouse models of defective DNA repair are means to study the mechanisms involved in biological aging of the vasculature. We here review the evidence of the role of DNA damage in vascular aging, and present mechanisms by which genomic instability interferes with regulation of the vascular tone. In addition, we present potential remedies against vascular aging induced by genomic instability. Central to this review is the role of diverse types of DNA damage (telomeric, non-telomeric and mitochondrial), of cellular changes (apoptosis, senescence, autophagy), mediators of senescence and cell growth (plasminogen activator inhibitor-1 (PAI-1), cyclin-dependent kinase inhibitors, senescence-associated secretory phenotype (SASP)/senescence-messaging secretome (SMS), insulin and insulin-like growth factor 1 (IGF-1) signaling), the adenosine monophosphate-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR)-nuclear factor kappa B (NFκB) axis, reactive oxygen species (ROS) vs. endothelial nitric oxide synthase (eNOS)-cyclic guanosine monophosphate (cGMP) signaling, phosphodiesterase (PDE) 1 and 5, transcription factor NF-E2-related factor-2 (Nrf2), and diet restriction.

Keywords: vascular; aging; endothelium; genomic instability; DNA damage; senescence; PAI-1; eNOS; phosphodiesterase; dietary restriction

Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide, responsible for killing 17.3 million persons per year (1). The onset of CVD is triggered by vascular endothelial alterations characterized by an impaired endothelium-dependent vasodilation, the overproduction of pro-inflammatory and prothrombotic molecules, and oxidative stress (2). Age is the strongest independent predictor for CVD in risk scores in middle-aged persons, and an important determinant for cardiovascular health in the population aged 65 or older (3,4). Aging is characterized by the complex interaction of cellular and molecular mechanisms that leads to a collection of functional problems. Focusing on the vasculature, such problems are closely associated with each other, and include worsened vasodilation, increased arterial stiffness and overt remodeling of the extracellular matrix, diffuse intimal thickening and a dysfunctional endothelium (4). The mechanisms through which age actually contributes to cardiovascular risk remain the subject of speculation. From a classical perspective, modifiable risk factors promote and modulate molecular mechanisms that, as time progresses, culminate in an imbalance in the production vs. scavenging of ROS (i.e., superoxide anions, hydrogen peroxide and hydroxyl radicals), increasing ROS levels, and, as a consequence, reducing the bioavailability of nitric oxide (NO) (5,6). NO is crucial in the maintenance of vascular homeostasis, including in the regulation of vascular dilation, the modulation of cell growth and the prevention of thrombosis (7). In the absence of a healthy endothelium, these factors gradually increase the pathologic phenotype of the vasculature up to the point that cardiovascular events occur. While this paradigm explains vascular aging in view of classical risk factors as causative mechanisms, a recently proposed alternative view on vascular aging has emerged that presents new mechanistic alternatives for understanding the process of vascular aging (8). In this novel paradigm, causal mechanisms for the process of aging itself, most notably genomic instability, including telomere attrition, drive the detrimental changes occurring increasingly with (biological) aging (Figure 1). The involvement of these causal factors of aging in general have been discussed elsewhere (9). In the present review we summarize the evidence that supports the role of genomic instability in vascular aging. In addition, we present mechanisms through which genomic instability generates the functional changes that are typical for the aging vasculature.

2. Genomic Instability and Aging: A Short Outline of the Basic Principles

2.1. DNA Repair Systems

The maintenance of genomic integrity is critical for the prevention of aging of organisms. To safeguard genomic integrity, cells are equipped with several genomic maintenance systems that sense and repair DNA damage (10,11). The sources of DNA damage are very diverse and range from intrinsic molecular reactions within DNA molecules such as hydrolysis, attacks by endogenous metabolic products, and ROS, to damage by exogenous physical and chemical entities such as chemotherapy and UVB light (12). To account for the different types of DNA damage, cells are equipped with multiple DNA repair pathways. Each repair system is responsible for a specific subset of lesions, although partial overlap can occur depending on the type of DNA lesion that needs to be repaired. At least six DNA repair pathways can be listed in mammalian cells: (1) the direct reversal pathway, which executes the direct reversal of chemical modifications of nucleotides; (2) mismatch repair (MMR), which repairs

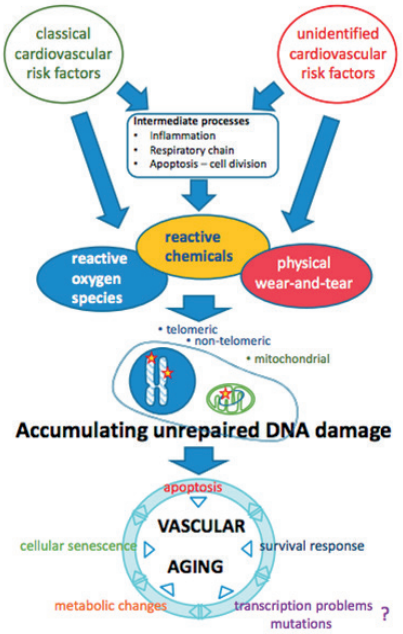

Figure 1. Etiology of vascular aging based on genomic instability as a causal factor. Classical and unidentified risk factors contribute to various types of DNA lesions. Unrepaired lesions accumulating during life lead to a growing set of pathophysiological changes that, either independently or in mutual interaction, lead to progressive vascular aging. The putative role of transcriptional problems or mutations herein needs to be established. The survival response may have beneficial (increased Nrf2-regulated antioxidants) as well as detrimental (decreased IGF-1 signaling, pro-inflammatory status) effects (see text and Ref. [8]).

base pair mismatches; (3) base excision repair (BER), repairing mainly oxidized and alkylation lesions in the nucleus and mitochondria, as well as single-strand breaks; (4) nucleotide excision repair (NER), to correct transcription-disturbing bulky adducts; (5) homologous recombination (HR); and (6) non-homologous end joining (NHEJ), which correct single- and double-strand breaks (10,13). Telomere maintenance requires further specialized proteins (14). Hypothetically, the classical cardiovascular risk factors initiate ROS-induced DNA damage and thus contribute to genomic instability-related vascular aging (Figure 1). Although some factors that lead to (vascular) genomic instability have been identified, the road to identification of all relevant contributors is still long (Figure 1) (8,15).

2.2. Aging: The Interplay between Genomic Damage, the Survival Response and Cellular Senescence

Unrepaired genomic damage enables the generation of harmful mutations that can be transferred to new cells during proliferation. This puts complex organisms at the potential risk of rapidly developing dysfunctional tissues or even tumors. As a protective measure, accumulating unrepaired DNA damage triggers a switch in biological pathways from a phenotype supporting growth to one favoring maintenance of the organism, a switch often referred to as the "survival response" (16). However, the switch is believed to contribute to the typical changes that occur during aging, as demonstrated in humans and animals with defective DNA maintenance (16). To avoid the harmful consequences of genomic instability, such as cancer, complex organisms

have developed protective cellular mechanisms, namely apoptosis and cellular senescence. Whereas apoptosis embodies the loss of (dysfunctional) tissue due to programmed cell death, which might account for loss of organ function, cellular senescence has a more intricate relationship with aging tissues. Senescent cells undergo cell cycle arrest and thus can no longer replicate, although they remain metabolically active and often acquire a SASP, an immunogenic phenotype consisting of interleukins, pro-inflammatory cytokines, and growth factors (17). It is believed that this results in an increased susceptibility to age-associated disease, including cancer and cardiovascular disease (17). As a consequence of cellular senescence, the organisms age and become susceptible to age-associated diseases. Paradoxically, the accumulation of senescent cells with age, which is believed to result from an inefficient clearance by the immune system, might also help delay tissue dysfunction through cell loss. Recently, however, it was shown that removal of senescent cells expressing the cyclin-dependent kinase inhibitor p16INK4A in genetically modified mice (*INK-ATTAC* mice) leads to a prolonged life and health span (18), supporting a fundamental role for cellular senescence in aging. The mechanisms through which removal of senescent cells leads to these effects remain to be elucidated.

3. Genomic Instability as a Causal Factor in Vascular Aging: Evidence in Humans

There is ample evidence that genomic instability is involved in vascular aging in humans. The following section highlights the observations that have accumulated until the present.

3.1. Cardiovascular Disease in Progeria Syndromes

The role of DNA damage in aging is further highlighted in human progeria syndromes. Human syndromes of progeria arise from mutations in genes involved in genomic maintenance in at least 75% of the known cases (19). Progeria syndromes provide a unique opportunity to study aging, but it should be noted that they are not a complete phenocopy, e.g., progeria patients show phenotypes that are rare during normal aging, such as clavicular agenesis in Hutchinson-Gilford progeria syndrome or the intensified risk of cancer in Werner syndrome (20). The relation of progeria to normal aging remains debatable. Despite this continuing debate, it is intriguing to observe that several progeria syndromes manifest severe, juvenile cardiovascular disease. Werner syndrome (WS) is characterized by the premature onset of clinical signs of aging, such as cancer, osteoporosis and cardiovascular disease (diabetes mellitus type II and atherosclerosis) (21). WS is caused by a *WRN* (Werner) gene mutation. WRN encodes a DNA helicase protein, Escherichia coli recQ-like helicase L2 (RECQL2), which is involved in DNA recombination, replication, repair and transcription, and also in telomere maintenance (22). WS patients develop a considerable burden of atherosclerotic plaques in the coronary arteries and the aorta; calcification of the aortic valve is also frequently observed. Consequently, most WS patients die during middle age (average life expectancy is 46 years) due to myocardial infarction and stroke (21). A related disease called Bloom syndrome, a consequence of mutation of the RecQ helicase gene *BLM*, features telangiectasias (dilated blood vessels in the skin), but the function of blood vessels has not been extensively investigated, although the occurrence of diabetes in these patients might be an important confounder in such investigations (23). Hutchinson-Gilford progeria syndrome (HGPS), perhaps the best-known progeroid disorder, is characterized by hair loss, pain in the joints, wrinkled skin, and cardiovascular problems (24). HGPS is caused, in most patients, by a point mutation in the lamin A gene (*LMNA*), which encodes the A-type nuclear lamins. The mutant lamin A, called progerin, remains fixed to the nuclear envelope causing various cellular changes, such as irregular nuclear shape and disorganization of heterochromatin, that lead to abnormal regulation of gene expression, therefore

inducing premature aging. Death occurs around the age of 13 years mostly due to myocardial infarction or cerebrovascular events; however, in contrast to typical human aging or WS, atherosclerosis is very rare. Instead a major loss of vascular smooth muscle cells (VSMCs) in both big and small arteries is observed (25). Interestingly, accumulation of prelamin A was observed in medial VSMCs and in atherosclerotic lesions from normally aged individuals. Moreover, prelamin A colocalized with β-galactosidase-positive VSMCs, i.e., senescent VSMCs, and thus prelamin A was proposed as a marker of vascular aging in the general population (26). Excision repair cross-complementation group 1 (ERCC1)-xeroderma pigmentosum (XP) F is a structure-specific protein complex serving as an endonuclease that participates in the repair of several types of DNA lesions, mainly bulky, helix-distorting lesions that are repaired by the NER pathway, but also double-strand breaks and interstrand cross-links (27–29). Progeroid syndromes arising from *ERCC1-XPF* mutations, often unique cases as each of the mutations found until now has been encountered in individual patients, have been repeatedly reported as being characterized by hypertension (30). This is further accompanied by frailty, loss of subcutaneous fat, liver dysfunction, vision and hearing loss, renal insufficiency, bone marrow degeneration, and kyphosis (31). Although the hypertension observed in this syndrome might point at accelerated vascular aging, this still needs to be confirmed, certainly if one takes into consideration the presence of renal insufficiency in the patients suffering from this type of syndrome. For other progeroid syndromes related to mutations in genomic DNA repair enzymes, data concerning vascular function are not available. it is uncertain whether this is an indication for the absence of vascular aging. Rather, more prominent problems in other organ systems or a focus on increased susceptibility to cancer might mask the presence of cardiovascular problems. In general, the patients are very frail, and cases are rare. Extensive cardiovascular characterization of such patients is, therefore, a very challenging task, and perhaps even not without risk for the patients themselves.

3.2. Indicators of a Role of Genomic Instability in the General Population

The role of genomic instability in disorders of the vasculature or the consequences thereof is a question that becomes increasingly important for the general population. If, indeed, this mechanism is central in age-related cardiovascular disease, there are major implications for prediction and detection and prevention. Research on the role of genomic instability in cardiovascular risk prediction opens a new window into expanding our understanding of the pathophysiology and causative risk factors in age-related diseases (8). The use of emerging markers of DNA damage, identified in vascular and cardiac ischemic cells, has provided evidence for this role (32). Part of the evidence comes from studies assessing the effect of ionizing radiation. An increased amount of circulating cell-free DNA and mitochondrial DNA (mtDNA) fragments has been observed in subjects exposed to low levels of ionizing radiation, suggesting the possible role of circulating DNA as a relevant biomarker of cellular damage (33). In turn, it has been established that there is an association between radiation exposure and indicators of accelerated vascular aging, coronary artery disease and stroke in occupationally exposed groups. Andreassi et al. observed that long-term, low level radiation exposure is positively correlated to early atherosclerosis, as identified by increased subclinical cIMT (carotid intima media thickness), and to telomere shortening, an indicator for genomic instability (34). This study also concluded that subjects with the Thr241Met polymorphism in the *XRCC3* gene (gene coding for X-ray repair cross-complementing protein 3) have a greater susceptibility to radiation-induced vascular effects. Data of the Life Span study showed that people who had received an acute single dose of 1–2 Sv (sievert) had a significantly increased risk of mortality from myocardial infarction after 40 years of radiation exposure (35). Other evidence is provided by observation of DNA damage markers in vascular tissue and circulat-

ing cells. Several groups observed elevated levels of oxidative DNA damage in human atherosclerotic plaques compared to non-atherosclerotic vessels or in circulating cells of persons with arterial disease (36,37). Likewise, several proteins involved in DNA repair including DNA-dependent protein kinase (DNA-PK), poly (ADP-ribose) polymerase 1 (PARP-1), p53, and apyrimidinic endonuclease 1/redox factor 1 (APE-1/Ref1), were up-regulated in plaques of carotid endarterectomy specimens compared with non-atherosclerotic arteries (36). On the other hand, genetic association studies have shown a significant association of single nucleotide polymorphisms (SNPs) in NER-related genes with age-related vascular phenotypes. In the population of the AortaGen Consortium, comprising 20,634 participants from nine cohort studies, Durik et al. identified an association of the SNP rs2029298 (p-value: 1.04 × 10−4) in the Damage-Specific DNA Binding Protein 2 (*DDB2*) gene with carotid-femoral pulse wave velocity, a measure of vascular stiffness (38). In addition, suggestive associations were found for eight SNPs located within or near *ERCC5*, *ERCC6*, general transcription factor IIH (*GTF2H)* subunit 1 and 3 (*GTF2H3, GTF2H1*), and *ERCC2* (38). Verschuren et al. showed, in data from the GENDER (GENetic DEterminants of Restenosis) and PROSPER (Patient-centered Research Into Outcomes Stroke Patients Prefer and Effectiveness Research) studies comprising 6110 coronary artery disease (CAD) patients in total, that genetic variations in the *NHEJ* repair system are associated with risk for CAD (39). In addition, several smaller studies have shown associations between polymorphisms in single DNA repair genes and risk of coronary artery disease, as reviewed elsewhere (40). Interesting to note is also the finding that statins were found to improve DNA damage detection, which might be a mechanism leading to the improvement of atherosclerosis next to the reduction of lipids and oxidative stress (41,42).

3.3. Telomere Shortening

Human chromosomes are normally capped by telomeres that protect the end-segment of chromosomes between cell divisions. Since telomeres do not fully replicate during mitosis, they gradually become shorter as individuals age (43). Defects in telomerase activity, abnormalities in DNA polymerase to synthesize terminal ends of the DNA, and the inhibition of the sheltering component telomeric repeat-binding factor 2 (TRF2) leads to an accelerated velocity of telomere shortening between cell divisions, which induces cellular senescence when the telomere reaches a critical length (43). Telomere shortening promotes chromosome end fusion, chromosomal abnormalities and aneuploidy, suggesting that loss of chromosome end protection is correlated to genome instability (44). Studies using knockout mouse models have established that the targeted deletion of *53BP1* and *TRF2* genes is one of the main mechanisms involved in double-strand breaks and an increase of non-reciprocal translocations caused by telomere shortening (45). In addition, RNA template of telomerase *(TERC)−/−* and high mobility group box 1 (*HMGB1)−/−* mice exhibit a reduced/null telomerase activity and telomere dysfunction, triggering aging-like cellular changes (46,47). Population-based studies suggest that telomere shortening plays a role in the onset of vascular aging-related phenotypes. Individuals with a shorter mean telomere length exhibit a two-fold risk of abdominal aortic aneurysm compared to those with a higher telomere length (odds ratio = 2.30, p = 0.005) (48,49). Moreover, an association between telomere shortening and the following CVD risk factors has been found: atherosclerosis, arterial stiffness (as measured by carotid-femoral pulse wave velocity), smoking, insulin resistance, type 1 and type 2 diabetes, obesity, hypertension and up-regulation of the renin–angiotensin–aldosterone system (50–57). Likewise, an increased level of telomere shortening, via increased oxidative DNA damage, has been identified in circulating endothelial progenitor cells (EPC) in CAD patients with metabolic syndrome (58). These observations suggest that oxidative stress-induced telomere shortening in EPC may accelerate vascular dysfunction, promoting the onset and progression of CAD due to a lack of vascular repair (58). Despite the fact that the asso-

ciation of telomere shortening with aging and vascular-related disorders has been demonstrated, its potential use as a biomarker of age-related diseases remains unclear (59–61).

3.4. Cellular Senescence and Its Regulators

The Role of Senescent Cells and Plasminogen Activator Inhibitor-1 (PAI-1)-Related Signaling Pathways in Vascular Aging

As mentioned previously, genomic instability causes increased cellular senescence, which is an important candidate mechanism bridging the gap between DNA damage and vascular aging (8). Senescent cells and tissues exhibit a distinctive pattern of protein expression, including increased plasminogen activator inhibitor-1 (PAI-1) as a part of the senescence-associated secretome (Figure 2) (62). In addition to contributing to the molecular fingerprint of senescence, PAI-1 is essential and even sufficient for the induction of replicative senescence in vitro and is a critical downstream target of the tumor-suppressor p53 (63,64). The contribution of PAI-1 to cellular senescence is broadly relevant in the organism as a whole, and age-dependent increases in plasma PAI-1 levels have been identified in wild-type mice as they age, in murine models of accelerated aging (*Klotho* and *BubR1H/H*), and in humans (18,65,66). Partial and complete PAI-1 deficiency in Klotho-deficient animals (*kl/kl*) prevents telomere shortening and extends median survival up to 4.2-fold with substantial protection against aging-like phenotypes in various organs (67). Furthermore, both genetic as well as pharmacological inhibition of *PAI-1* protects against development of aortic arteriosclerosis in mice treated with long-term nitric oxide synthase inhibition (68). Metabolism also plays a fundamental role in the biology of (vascular) aging and iIGF1 are widely endorsed as critical contributors to senescence and aging in several experimental models (e.g., flies, worms, and mammals) (69). In observational human studies, PAI-1 levels were increased in obesity and insulin resistance and independently predicted the future development of type 2 diabetes mellitus (T2DM) (70,71). Potential anti-aging interventions have focused on caloric restriction and on drugs with metabolic effects, including metformin and resveratrol, all of which reduce *PAI-1* expression (72–75). Conversely, PAI-1 production is enhanced by insulin, free fatty acids, and glucose (76–78). Taken together, these data suggest that *PAI-1* and insulin exhibit a coordinated regulatory reciprocity, and in this context *PAI-1* represents a high-yield translational target linking metabolism and biological aging, including aging of the vasculature (Figures 2 and 3)

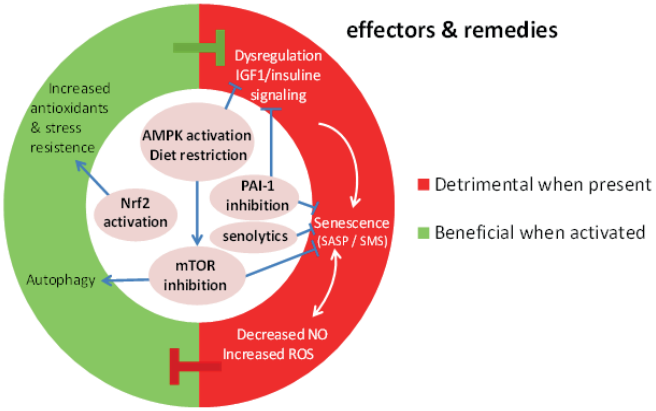

Figure 2. Molecular effectors of genomic instability that contribute to vascular aging, and the potential remedies (center of the chart) against that currently under development. Senescence, im-

balanced NO vs. ROS production, inflammation and changes in insulin signaling are detrimental when present while autophagy, apoptosis and stress resistance have a beneficial contribution to vascular aging. IGF-1 putatively has a detrimental effect, although this needs further scrutiny (Ref. (8)). Pointed arrows indicate stimulatory processes, while blunted arrows indicate inhibitory processes.

3.5. Cyclin-Dependent Kinase Inhibitor 2 (CDNK2) A and B

Further exploring the role of genomic instability–induced cellular senescence in vascular aging, gene variations in senescence regulators have been associated with age-related vascular disease in humans. Several studies have provided insight about the risk association of the 9p21 locus with aging-related cardiovascular diseases such as atherosclerosis and aortic aneurysm (79). This chromosomal region codes for two cyclin-dependent kinase inhibitor genes, *CDKN2A*, comprising codes for *p16INK4A* and *p14ARF* (*p19ARF* in mice), and *CDKN2B*, coding for p15INK4B. These CDKs are key molecules involved in the regulation of cellular replication, among others in vascular cells (80). Genetic polymorphisms in this chromosomal region have indicated that 9p21 variation has a significant influence in the genetic expression of *CDKN2A* and *CDKN2B* in VSMCs, which could increase the susceptibility to CAD (81). In addition, differential expression of *CDNK2A* and *CDNK2B* has been observed in senescent cells in vitro and in aging tissues of rodents and humans (82). Thus, the measurement of the expression of these genes has led to their use as a potential biomarker of biological age (83). Most of the studies have determined the role of *CDNK2A* and *CDNK2B* in aging by focusing on human tumors, concluding that the deletion and silencing of the *CDKN2A–CDKN2B* locus are among the most frequent genetic events found in human cancer cells (84). Thus, *CDNK2A* and B play a central role in diseases of aging.

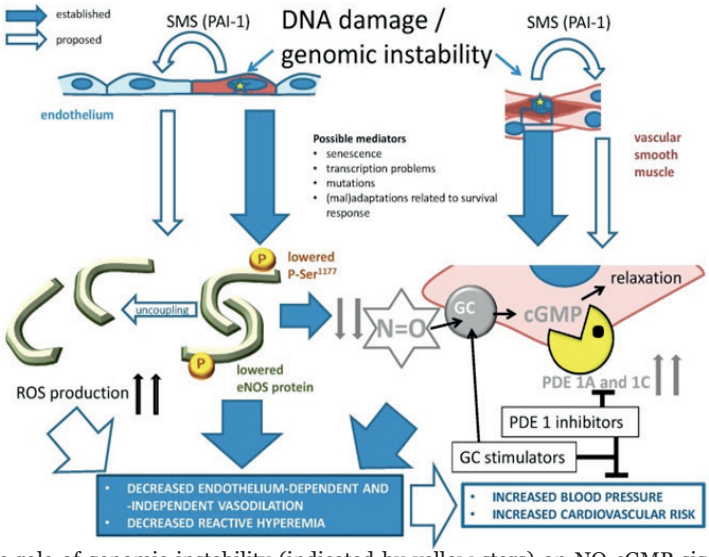

Figure 3. The role of genomic instability (indicated by yellow stars) on NO-cGMP signaling, and its consequences for age-related cardiovascular disease. Large, blue closed arrows indicate established relationships: genomic instability primarily leads to endothelial eNOS dysfunction in endothelial cells and to increased cGMP metabolism by PDE1A and 1C (Section 4.5.1). Large, blue open arrows refer to proposed mechanisms that were not fully explored: cellular senescence caused by unrepaired DNA could affect healthy cells through SASP/SMS, in which PAI-1 potentially plays a central role (Sections 3.4.1 and 5.2.2.). The affected cells in turn might worsen vascular function through changes in eNOS-cGMP signaling. PDE1 subtype inhibitors and guanylyl cyclase (GC) stimulators are promising drugs

to at least acutely improve vascular function. Their value for prevention of genomic instability and vascular aging needs to be assessed. PDE1A and 1C have a putative role in atherosclerosis, arteriosclerosis, reduced blood flow and hypertension (see Sections 4.5.1 and 5.4). Their expression is strongly related to cellular senescence, and genetic variables of the PDE1A gene affect blood pressure and vascular hypertrophy (Section 4.5.1). Thus, both PDE1 subtypes appear to be central in vascular aging-related disease. Small, thick arrows pointing up or down indicate up- and down-regulation respectively. Blunt arrows indicate inhibition, pointed thine arrow indicate stimulation.

4. Genomic Instability as a Causal Factor in Vascular Aging: Evidence from Animal Models

4.1. Telomerase-Deficient (TERC−/− and TERT−/−) Mice

Telomerase-deficient mouse models have been developed by knocking out *TERC, TERC−/−* mice, or the telomerase reverse transcriptase (*TERT−/−* mice). Homozygous *TERT−/−* and *TERC−/−* mice display short telomeres and a similar phenotype, but the *TERC−/−* mice have been studied more comprehensively. The telomeres of the *TERC−/−* mice shorten at a rate of ~5 kb in every subsequent generation (G). In conscious *TERC−/−* mice, higher systolic blood pressures were observed in mice from G1 compared with wild-type mice, whereas in G3 mice, both systolic and diastolic blood pressures were increased compared with wild-type and G1 mice (85). The differences in blood pressure between *TERC−/−* and wild-type mice appear to be caused by an increased production of endothelin-1 in the *TERC−/−* (85).

4.2. Mouse Models of Genomic Instability Associated with Human Progeria

Different mouse models of WS have been developed with either a complete knockout of the WRN protein, the transgenic expression of human *WRN* lacking helicase activity, or the in-frame deletion of the helicase domain. Depending on the model studied, the extent of the aging phenotype varies. The models lacking RecQ helicase activity show increased genomic instability and have increased visceral fat, high fasting triglycerides and cholesterol levels, insulin resistance and hyperglycemia (86). Telomere shortening appears to be pivotal in the development of some of these metabolic changes (87), which are relevant analogues for human cardiovascular risk factors. In these models no vascular problems were reported, except, perhaps, decreased wound healing, which might implicate worsened angiogenesis.

A mouse model of *HGPS* expressing human progerin showed aberrations that were largely restricted to the vascular system. Recapitulating the vascular phenotype seen in patients with HGPS, these mice exhibited an increasing loss of VSMCs in the lamina media of large arteries (88). Those changes were accompanied by a reduced vasodilator response to the NO donor sodium nitroprusside. Interestingly, the endothelium is initially undamaged, but with progression of the vessel damage, some loss in the endothelium is observed in 12-month-old mice (88).

Mice lacking proper function of the versatile DNA repair enzyme ERCC1 show many features of accelerated aging. *Ercc1−/−* mice display a severe aging phenotype featuring frailty, osteoporosis, neurodegeneration, atrophic epidermis, sarcopenia, liver and kidney dysfunction and bone marrow degeneration (89). *Ercc1−/−* mice only live four weeks, while mice with reduced ERCC1 function due to a combined exon 7 deletion allele and a null allele (*Ercc1d/−, Ercc1−/Δ7 or Ercc1Δ/−*) live longer (up to 30 weeks). The *Ercc1Δ/−* mice are healthy up to an age of eight weeks, when they start developing a gradual aging phenotype. In our previous study we found that *Ercc1Δ/−* mice had an increased systolic blood pressure compared to wild-type mice (38). They also display a decreased vasodilator response in their microvasculature (38). Microvascular function was assessed by hind leg reactive hyperemia using a laser Doppler technique, which measures superficial resistance vessels and possibly represents both endothelium-dependent and endothelium-inde-

pendent relaxations (90–92). Those microvascular changes resemble the ones seen in aged rodents as well as in humans, and, strikingly, these changes in humans are at least partly independent of classical cardiovascular risk factors (90,93). Another important characteristic of the human vascular aging phenotype that was recapitulated in the *Ercc1Δ/–* mice includes greater stiffness, as measured by pressure-diameter relationships in the carotid arteries (38). While aortic tissue from *Ercc1Δ/–* contains increased amounts of senescent cells, the contribution of cellular senescence to vascular dysfunction remains uncertain (38).

Other genetic models add to the evidence, linking vascular senescence with vascular pathology and disease. Mice carrying the human XPD *R722W* mutation, so-called *XpdTTD* mice, show signs of accelerated vascular aging. In humans, the mutation in *XPD* causes trichothiodystrophy (TTD) which is characterized by postnatal growth failure, UV sensitivity, neurological degeneration, cachexia, osteoporosis and a shortened life span (94). The *XpdTTD* mice show a similar phenotype, but the onset and severity of progeria is slower compared to *Ercc1Δ/–* mice. We assessed vascular function in *XpdTTD* mice at 26 and 52 weeks and observed significantly reduced vasodilator responses to acetylcholine in aortic rings at 52 weeks compared to 26-week-old *XpdTTD* and wild-type mice (38).

4.3. Mitochondrial DNA Maintenance Defects

ApoE-deficient mice lacking protein kinase ATM (ataxia telangiectasia mutated), a protein pivotal in DNA damage detection, showed accelerated development of atherosclerosis (95). This was attributed to increased mtDNA damage leading to malignant metabolic changes. Further exploring the involvement of DNA (mtDNA), polymerase gamma (POLG) performs DNA synthesis inside the mitochondria, and thus mutations in *POLG* cause mitochondrial disorders. A mouse model with an mtDNA mutator phenotype, conferred by a homozygous mutation in *POLG*, has been used to study the role of mitochondrial function and aging. In early adulthood the *POLG* mutant mice develop many features of premature aging such as weight loss, reduced subcutaneous fat, kyphosis, osteoporosis, cardiomyopathy and a reduced life span (96). Oxidative stress and respiratory chain dysfunction due to the accumulation of mtDNA point mutations in protein-coding genes of the respiratory chain complexes are considered to contribute to the premature aging phenotype of the *POLG* mutator mice. Using a double *POLG/ApoE* low-density lipoprotein (LDL) receptor knock-out, it was shown that instability of mtDNA might contribute to atherosclerosis. *POLG–/–/ApoE–/–* mice had increased atherosclerosis in the brachiocephalic artery and descending aorta as compared to *POLG+/+/ApoE–/–* controls. The *POLG–/–/ApoE–/–* mice also exhibited reduced body weight, reduced fat mass, hyperlipidemia and apoptosis of VSMCs (97). Apart from vascular effects, *POLG* mutant mice develop cardiac hypertrophy and dilatation, impairment of systolic and diastolic function, and increased cardiac fibrosis within 13 months of age (98). Overexpression of catalase in the mitochondria of these mice attenuated the malignant cardiac phenotype, providing evidence for the role of oxidative stress in the development of cardiomyopathy due to mtDNA instability.

4.4. BubR1 Knockout

The spindle assembly checkpoint protein BubR1 has an important role in the maintenance of genomic stability by ensuring the correct microtubule-kinetochore attachment and segregation of chromatids during mitosis (99). Mice with reduced expression of *BubR1* (10% of normal levels) develop progressive aneuploidy and exhibit a vascular aging phenotype characterized by reduced arterial elasticity, a reduced number of VSMCs, loss of endothelial-dependent relaxation, and increased production of superoxide anions. Apart from problems reminiscent of cardiovascular aging, *BubR1* mice also show a variety of progeroid symptoms (100,101). Also, naturally aged wild-type

mice have decreased BubR1 expression in different tissues, suggesting that *BubR1* may be a regulator in normal aging (18,27,100,101). Clearance of p16INK4A-positive senescent cells with the INK-ATTAC strategy in *BubR1* progeroid mice ameliorates several of the progeroid hallmarks. However, the cardiovascular problems are not rescued, which corresponds with the observation that these features are p16INK4A-independent in this model (102). Importantly, vascular aging in *ERCC1*-defective mice appears to be associated with p53- and p21Cip1 (or p21Waf1)-related senescence (8,38), and this might further explain the ineffectiveness of removing p16-positive cells to improve cardiovascular function in *BubR1* mice. Interestingly, it was recently shown that clearance of p16INK4A-positive senescent cells in non-progeroid mice increases the life span and reduces cardiac stress sensitivity (18,102). This indicates that cellular senescence is indeed involved in deleterious cardiovascular phenotypes, involving both p16INK4A as well as p53- and p21-related senescence in a differential way.

4.5. Vascular Functional/Pharmacological Changes Due to Genomic Instability

4.5.1. NO-cGMP Signaling

NO is a key participant in many physiological pathways such as vasodilation, neurotransmission, and macrophage-mediated immunity. In the vascular endothelium, NO is produced from the substrate L-arginine by the enzyme eNOS (or nitric oxide synthase type III). The eNOS is activated by increased cytoplasmic Ca2+ levels, as induced, among others, by binding of vasodilatory (neuro)hormones to their G protein-coupled receptors (103). Evidence provided up to date suggests that there is a reciprocal relationship between defective eNOS activity and genomic instability. During vascular aging, there is an increased production of ROS (104). This ROS can be partly produced by eNOS, when the enzyme is in a so-called uncoupled state due to a reduced expression of the cofactor tetrahydrobiopterin (BH4), as has been shown in aging rats (105). ROS coming from this and other sources, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or mitochondria, react with NO to form harmful free radicals, including peroxynitrite (ONOO–) and N2O3. The overproduction of ROS not only leads to a gradual reduction of NO bioavailability in the vasculature, but in addition can cause single-strand DNA breaks, 7,8-dihydro-8-oxoguanine and other oxidative lesions (106).

The aberrant eNOS function is closely associated with dysfunction as observed in aged and diseased blood vessels. In *eNOS–/–* mice, systemic hypertension, altered vascular remodeling, dysfunctional angiogenesis and a prothrombotic phenotype have been observed (107–111). In human atherosclerosis, *eNOS* mRNA expression was shown to be decreased in endothelial cells of advanced atherosclerotic plaques, which is accompanied by overt DNA damage (36). In addition, eNOS uncoupling has been reported in patients with endothelial dysfunction as a consequence of diabetes, hypertension, hypercholesterolemia and smoking, linking the mechanism to classical risk factors (112).

Apart from a potential role of eNOS dysfunction in the production of DNA lesions, genomic instability itself causes dysfunction of NO signaling. Organ bath studies and molecular analyses in *Ercc1d/–* mechanistically explained the decreased vasodilator responses (38). These experiments showed that NO-mediated responses, *eNOS* expression, and *eNOS* activation through phosphorylation serine 1177 were decreased. Increased generation of ROS, a central mechanism in age-related decreased NO-dependent vasodilation, was partly responsible for the diminished vasodilation in *Ercc1d/–* mice since anti-oxidants such as N-acetylcysteïne and BH4 improved vasodilation (38). Therefore, faulty *eNOS* activation and genomic instability appear to form a vicious circle leading to progressive endothelial dysfunction and accelerated vascular aging (Figure 3).

Downstream of NO production, the *ERCC1* functional mutation causes a pronounced defect

in cGMP responsiveness. In the vascular function assessment of the *Ercc1d/–* mice we found a strong reduction in the relaxations to the NO donor SNP compared to wild-type mice. The responses were completely dependent on soluble guanylyl cyclase (sGC) activity. No differences in sGC activity or protein levels were found between *Ercc1d/–* and wild-type mice. When we measured the vasodilator responses of the aortic rings to SNP in the presence of vinpocetine, a phosphodiesterase (PDE) type 1 inhibitor, or Sildenafil, a *PDE5* inhibitor, the dilator responses were increased. However, while in wild-type mice the greatest improvement was given by Sildenafil, in *Ercc1d/–* mice it was given by vinpocetine (113). This finding suggests that in *Ercc1d/–* mice, a mouse model for accelerated aging due to genomic instability, *PDE1* has a stronger role than *PDE5* in regulating cGMP signaling and vasomotor function (113); at least, it undergoes stronger regulation, because cellular senescence was shown to be strongly associated with increased expression of both *PDE1A* and C subtypes, and to a lesser extent with *PDE5*, in human cultured VSMCs. Supportive for a role in humans, *PDE1A* gene variations in human cohorts were associated with diastolic blood pressure and intima media thickness (113). Together with previous observations showing associations of *PDE1C* with atherosclerosis, this places cGMP metabolism alongside these enzymes in the center of human vascular aging (114,115) (Figure 3).

4.5.2. NF-E2-Related Factor-2 (Nrf2) and Antioxidant Pathways

NF-E2-related factor-2 (Nrf2) is a transcription factor activated in the vasculature to modulate the up-regulation of antioxidant genes, whose protein products are involved in the clearance of ROS and electrophilic molecules (116). Consequently, Nrf2-dependent signaling pathways are activated as an adaptive mechanism in response to increased production of ROS, attenuating vascular oxidative stress and the damage caused by several stressors (117–119). A dysfunction in Nrf2 action increases age-related cellular oxidative stress and cellular damage in aged vessels (120). Nrf2 has a reduced function in senescence, whereas its silencing leads to premature senescence (121). Remarkably, despite the fact that *Nrf2* deletion leads to slower cell growth and shorter life span of individual cells in murine embryonic fibroblast cultures, it paradoxically induces immortalization in such cultures due to an early loss of p53 and p53-dependent gene expression (122). This shows that *Nrf2* loss-of-function has a dual pro-aging and oncogenic effect, centering the transcription factor in age-related disease. In addition, the effects of ROS associated with classical risk factors (smoking, hyperglycemia) in cardiomyocytes and mouse hearts are worsened after depletion of Nrf2, suggesting that Nrf2 protects against cardiac damage induced by mechanisms that can contribute to genomic instability (117,123–125). *Xpg–/–* and *SIRT6–/–* mice, experimental models that exhibit progeroid phenotypes on the basis of genomic instability, have shown an accelerated cellular senescence, increased ROS levels and dysregulated redox metabolism. The Nrf2-regulated antioxidant pathways are up-regulated in the cerebellum and mesenchymal stem cells of these mice (126,127), which illustrates the physiological importance of Nrf2 as a line of defense against genomic instability caused by ROS. Importantly, Nrf2 is also involved in the regulation of production of NO vs. ROS through its role in eNOS uncoupling and activation (128). This evidence suggests that Nrf2 provides a protective pathway during genomic instability, although the specific relation to vascular aging as caused by genomic instability has not been investigated yet.

5. Perspectives

5.1. Directions for Future Studies Establishing the Role of Genomic Instability

Although the evidence summarized above strongly indicates a major role of genomic instability in vascular aging, important questions remain to be solved. Firstly, there is the question as to whether the accelerated vascular aging features that are observed are due to local vascular processes, or whether they are the consequence of the general accelerated deteri-

oration in mouse models of genomic instability. Tissue-specific inactivation of DNA repair enzymes is a means to explore this question. To this end, endothelial cell- and VSMC-specific Cre recombinase strains and mouse strains with Plox sites in DNA repair genes of interest are available, but still need to be combined. Furthermore, the mechanisms that are the interface between genomic instability and derailment of vascular signaling systems need to be resolved. Cellular senescence has been mentioned as an option, but apoptosis is also a candidate mechanism. The role of cellular senescence vs. vascular cell apoptosis in vascular aging remains an important question. Although previously discussed mouse studies provide compelling evidence, the role of cellular senescence in vivo in human aging remains unclear, mostly due to the absence of specific biomarkers that can provide information about the state of cells in tissues (129). Restricting this to vascular aging, no conclusive evidence for a causal role of cellular senescence in vascular aging, let alone that induced by genomic instability, has been published yet. The aforementioned results in *INK-ATTAC* models on a wild-type mouse background, and in *ERCC1*-defective mice, are, however, highly indicative (18,38). Models combining constructs to eliminate senescent cells in a background of vascular-specific genomic instability are putative tools to further establish this mechanism.

Another mechanism could be stem cell exhaustion, which requires comprehensive analysis of vascular cell progenitors in mice with increased genomic instability. In *ERCC1*-deficient mice, reduced hematopoietic progenitor cell reserves have been observed (130). Since hematopoietic cells generate vascular progenitor cells (131), there is indeed a motive to explore this possibility. Whether genomic instability in nuclear DNA outside of telomeres mediates vascular dysfunction through mutations or transcriptional dysfunction as caused by DNA lesions is a most important question that remains to be explored (Figure 1). Since DNA lesions have been repeatedly reported (see above), this is a very realistic option.

5.2. Towards New Interventions in Vascular Aging Caused by Genomic Instability

The awareness that genomic instability and cellular senescence arising thereof play a key role in general and in vascular aging opens new possibilities to prevent age-related cardiovascular disease. In particular, life-extending therapies that have been identified thus far are candidate interventions to decelerate vascular aging. In addition, interventions that prevent vascular genomic instability or readily improve NO-cG MP signaling are eligible for such purposes. We delineate the various options here.

5.2.1. Mtor, Rapamycin and Autophagy

During aging, increasing dysfunction related to a progressive failure of maintenance and repair pathways takes place as aberrant macromolecules, dysfunctional organelles and DNA damage may accumulate in cells and tissues (132). Therefore, cellular maintenance mechanisms are crucial to preserve normal cellular functions. Autophagy, one of the main cellular preservation processes, is involved in the degradation of long-lived proteins and dysfunctional organelles as well as in the maintenance of the cell in case of failure of macromolecule repair (133). With age the rate of autophagy and protein degradation declines (134,135). Importantly, genetic ablation of *ATG7*, an important mediator of autophagy, causes an accelerated appearance of vascular aging hallmarks in mice (136). Autophagy-modifying drugs, such as rapamycin, inhibit the mammalian target of rapamycin complex 1 (mTORC1) and control the activation of autophagy-related signaling pathways. Rapamycin (also known as sirolimus) increases longevity and delays pathological lesions in mice (137). Furthermore, the therapeutic use of rapamycin or related drugs prevents age-related diseases such as cancer and cardiovascular diseases in animal models (138,139). In addition, the pleiotropic anti-atherosclerotic effects of rapamycin have allowed the implementation of rapamycin-based therapies to prevent

or delay the pathogenesis of atherosclerosis (140). Further, it has been reported that rapamycin improves endothelium-dependent vasodilation in old rodents (141). A potential beneficial effect of mTOR inhibition on vascular aging independent from autophagy regulation was proposed (142). This effect would be based on the regulation of a signaling network, consisting of mTOR, adenosine monophosphate-activated protein kinase (AMPK), and sirtuin (SIRT)-1. In this model, mTOR inhibition, in concert with SIRT-1 and AMPK activation, would counteract age-related vascular dysfunction thanks to modulation of the common transcription factors NFκB, FoxO and p53, that, when integrated, determine stress resistance, inflammation, ROS production, NO signaling, genomic instability and cellular senescence. Apart from this link to genomic instability and senescence, it has been found in a mouse model of Hutchinson-Gilford progeria that AMPK activation and mTOR inhibition occurs in conjunction with activation of autophagy (143). Thus, models of genomic instability appear to implicate the proposed mTOR–AMPK signaling interaction, with a link to the regulation of autophagy (Figure 2). However, the effect on vascular aging as based on genomic instability remains to be explored.

Discouraging the use of mTOR inhibition is the fact that rapamycin significantly attenuates both endothelial function and the expression of eNOS in human endothelial cell lines in vitro, although it does not cause endothelial cell death (144). Studies with mTOR-inhibiting drugs, among others applied on coronary stents in patients with advanced arterial aging, have reported deleterious effects of such drugs on various variables of endothelial (dys)function, although conflicting results are abundant (145–149). It is unclear whether the conflicting results are dependent on the concentration of the mTOR inhibitor to which the endothelial cells are exposed, which presumably is very high in the case of drug-eluting stents. Although in cultured endothelial cells the increasing anti-inflammatory effect of increasing concentrations of mTOR inhibitors parallels the increasing cytostatic effect (150), this issue needs further inspection. We have also shown that rapamycin actually induces *PAI-1* expression in cultured endothelial cells and in vivo in mice (151), so the net benefit of this drug in preventing senescence may be mixed at best.

In summary, mTOR inhibition, on the one hand, seems to be an attractive hypothetical option to reduce vascular aging in relation to genomic instability, but the idea should be approached cautiously.

5.2.2. Senolytics and Inhibitors of Senescent Cell Signaling

Cellular senescence and the overproduction of SASP-associated proteins, also referred to as the senescence-messaging secretome (SMS), contributes to local and systemic dysfunction and disease. Therefore, the implementation of "senolytic" therapies has been approached as an intervention to specifically target senescent cells (Figure 2), eliminate them, and thus diminish the contribution of SASP and SMS (152). The use of senolytic drugs including dasatinib and quercetin has been effective in eliminating senescent primary mouse embryonic fibroblasts and senescent human fat cell progenitors. In vivo, the combination of these drugs reduced senescent cells in normal aged, radiation-exposed mice, and in *Ercc1–/Δ* mice (152). In addition, this study showed that periodic drug administration extended the health span in *Ercc1–/Δ* mice and delayed age-related symptoms and pathology, osteoporosis, and loss of intervertebral disc proteoglycans. Despite the evidence suggesting that interventions that reduce the number of senescent cells could mitigate age-related tissue dysfunction, the burden of cell senescence biomarkers and SASP needs to be further studied and validated in humans. Therefore, the implementation of new therapies to reduce senescent cell number and SASP must be characterized.

Pioneer results from our group showed for the first time that lation of the SMS can actually prevent the development of senescence in *kl/kl* mice, a mouse model of accelerated aging (67). We observed that forced decrease of *PAI-1* attenuated levels of the SMS factors insulin-like growth factor-binding protein 3 (IGFBP3) and interleukin-6 in plasma of *kl/kl* mice to levels seen in wild-type (WT) mice. In addition, telomere integrity was partial-

ly protected in numerous tissues. Furthermore, the nuclear accumulation of the senescence marker p16INK4A was prevented. Similar observations were made in another aging-related model (68). It is important to note that *IGFBP3* is also strongly affected in DNA repair-defective progeroid models, as are other components of the *IGF-1* growth factor signaling pathway, placing this pathway in the center of genomic instability-related (vascular) aging (8,153). Moreover, this link raises the exciting possibility that *PAI-1* might be involved in genomic instability-related vascular aging (Figure 2). As a still remote possibility, *PAI-1* might act as part of the SMS from cells that become senescent due to unrepaired DNA damage, thus transmitting a harmful signal to cells in which the genomic integrity is still warranted (Figure 3). Application of genetic or pharmacological inhibition of *PAI-1* in models of genomic instability is therefore an attractive approach to test this hypothesis.

5.3. Dietary Restriction

In search of treatment perspectives, it is of course important to consider more general anti-aging and longevity-increasing interventions. Apart from the previously discussed possibility to employ rapamycin against vascular aging, dietary restriction (DR) is perhaps the most important and well-known option. DR is a reduction of intake of food to the level that it results in low-normal levels of energy intake while avoiding malnutrition (154). Claims of an effect of diet restriction on longevity date back as far as 3000 years. Studies that have taken place over many decades over the last century indeed confirm such an effect in various species, including yeast, worms, flies, spiders, rotifers, fish and rodents, demonstrating that DR is the most effective intervention to slow down aging and extend life expectancy (155–161).

DR is known also to protect against age-related cardiovascular disease. Two main mechanisms can be involved: (1) reduction of the intake of harmful food, such as carbohydrates and polysaturated fats (162,163); or (2) slowing down of the aging process itself. It has been shown that chronic DR improves the aging-related rise of blood pressure and vascular wall remodeling, as shown in rodents (164,165). This effect can be attributed to the improvement of vascular relaxation, a consequence of decreased ROS and increased NO bioavailability. In addition, DR has been been to attenuate cardiovascular disease in nonhuman primates (74,166).

It is not clear what the main mechanisms of the anti-aging effect of DR are. However, the reduction of genomic instability is a possibility. In a previous review (8) we discussed that effects on oxidative stress-induced DNA and macromolecular damage are a putative mechanism. Reports have shown a possible effect of specific nutrient restriction and of caloric restriction on markers of DNA damage and DNA repair capacity, and a plethora of publications regarding the association between food consumption and telomere length is available (167–170). This observation pleads for evaluation of the effects of DR on the general and vascular aspects of aging in models of genomic instability. Alternatively, effects on IGF-1/growth hormone (GH) signaling, SIRT-1 and nutrient-sensing pathways might be at play (8) (Figure 2). Since IGF-1/GH signaling is suppressed both after DR and in mouse models of genomic instability, this pathway apparently shares a common function in DR and the survival response in progeroid mice. Mouse models in which GH signaling is intentionally knocked out display increased longevity, and share features of the genetic program with genomic instability models (153). Therefore, IGF-1/GH suppression is a point of convergence between DR, genomic instability and longevity. Whether this convergence takes place after genomic instability to improve survival, contributes to improved genomic integrity, as proposed above, or both remains to be elucidated. The effect of dietary restriction therefore needs to be explored in models of genomic instability, importantly those involving evaluation of vascular aging. The role of altered GH vs. IGF-1/insulin therein on vascular function needs special attention as these pathways appear to have opposite effects, as previously explained (8).

We here propose that the aforementioned relationship with mTOR and AMPK might also

be important in DR effects (Figure 2). There is evidence that DR deactivates the mTOR-dependent signaling pathways, slowing aging and delaying aging-related diseases (171). This suggests that DR and rapamycin can act together but have different effects on several pathways related to an increased longevity in young mice; therefore, the combination of both therapies could cause and exponential rise of lifespan in mice (172). It would be interesting to investigate if such an interaction also exists for the attenuation of vascular aging.

5.4. PDE Inhibition

As mentioned before, there appears to be a pivotal role in vascular aging for *PDE1* (Figure 3), and possibly also *PDE5* (113). At least, the inhibition of both PDE subtypes can acutely counteract diminished vasodilator responses caused by genomic instability (113). Whether chronic treatment will also slow down vascular aging remains to be explored. An attractive aspect of *PDE1* and 5 as drug targets is that there are several experimental and clinically approved drug candidates that might overcome the increased PDE activity. One is the selective *PDE1* inhibitor IC86340, but unfortunately this drug appears not to be available anymore (173,174). Other *PDE1* inhibitors are under development (175). Further, there is the possibility to inhibit *PDE5*, or both *PDE1* and 5. Sildenafil is a *PDE5* inhibitor which also blocks *PDE1* at high doses (176). Sildenafil was found to reduce both diastolic and systolic blood pressure in untreated hypertensive patients. However, due to Sildenafil's short duration of action, research is focusing on new inhibitors such as tadalafil (177). Vinpocetine is a PDE inhibitor with a preferential affinity for *PDE1* over *PDE5*. Vinpocetine is an Food and Drug Adminstration (FDA)-approved nutriceutical and a registered drug in Eastern Europe, used to enhance cerebral bloodflow and improve memory (178). *PDE1* inhibitors were also developed for the treatment of cognitive impairment associated with schizophrenia (175). Such treatment inhibits injury-induced hypertrophy in human and rodent vessels, and decreases atherosclerosis in ApoE knockout mice (179,180). Therefore *PDE1* inhibition is an attractive option for treating age-associated cardiovascular diseases. Until now, vinpocetine never found widespread application, for reasons that are unclear.

5.5. Reconsideration of Antioxidant Therapies

As explained above, ROS have been identified as a source of DNA damage, and therefore ROS scavenging is a potential treatment modality. Clinical studies applying ROS scavengers (antioxidants) have, however, not resulted in benefits for patients suffering from cardiovascular diseases (181). Although this might be due to the fact that such interventions might require the onset of intervention early in life, there is also a shortcoming in that the drugs might not reach the right place at the right time or even hamper healthy cellular signaling that is performed by ROS (182). A better targeted interaction of antioxidant enzymes and ROS might overcome the latter shortcomings of exogenously applied ROS scavengers.

Nrf2 has been proposed as a "master regulator" of cytoprotective mechanisms and it could be associated with increased longevity and attenuating age-related diseases in mice (183). Therefore, *Nrf2* gene regulation and the enhancement of the endogenous antioxidant capacity (Figure 2) could be an important therapeutic target to diminish the production of ROS, reducing DNA damage and their effects on vascular aging. Certainly, several drugs have been developed and tested to stimulate the bioavailability of NO through the regulation of the Nrf2/antioxidant response element (Nrf2/ARE). The combined action of NO and Nrf2/ARE signaling could improve vascular function and confer protection against vascular diseases (184). On the other hand, several alternatives to increase Nrf2 have been currently explored, including calorie restriction, ozone therapy, hyperbaric oxygen and physical exercise (185).

6. Summary

There is ample evidence that genomic instability is involved in vascular aging. Nuclear DNA lesions, among which is telomere erosion, and mitochondrial DNA damage are strongly associated with several main features of vascular aging, such as diminished vasodilator capacity and increased vasoconstriction, increased blood pressure, increased vascular stiffness and atherosclerosis. Pivotal cellular biological changes involved in these pathological features comprise cellular senescence, apoptosis, autophagy, stem cell exhaustion and altered proliferative capacity of vascular cells. The role of gene mutation and of compromised transcription remains unknown (Figure 1). Potential mediating signaling pathways involved include components of the survival response (Figure 1), notably antioxidants under regulation of *Nrf2* (beneficial), increased inflammatory status (detrimental) and decreased IGF-1/GH signaling (detrimental), as well as the interplay between mTOR, AMPK and NFκB, SIRT-1, and PAI-1, p53- and p21- and p16-related signaling. Proposed remedies against genomic instability–related vascular aging include *PAI-1* inhibition, mTOR inhibition, DR, senolytics, *PDE1* and 5 inhibitors and stimulators of *Nrf2*.

Abbreviations

AMPK adenosine monophosphate-activated protein kinase
APE-1/Ref1 Apurinic/apyrimidinic endonuclease 1/redox factor 1
ApoE ApoE, Apolipoprotein E
ARE antioxidant response element
ATM ataxia telangiectasia mutated
BER base excision repair
CAD coronary artery disease
CDNK2 cyclin-dependent kinase inhibitor 2
cGMP cyclic guanosine monophosphate
cIMT carotid intima media thickness
CVD cardiovascular diseases
DDB2 Damage-Specific DNA Binding Protein 2
DNA-PK DNA-dependent protein kinase
DR dietary restriction
eNOS endothelial nitric oxide synthase
EPC endothelial progenitor cells
Ercc1 excision repair cross-complementation group 1
GH growth hormone
GTF2H general transcription factor IIH
HGPS Hutchinson-Gilford progeria syndrome
HMBG-1 high mobility group box 1
hMSC human mesenchymal stem cells
HR homologous recombination
IGF1 insulin-like growth factor 1
IGFBP3 insulin-like growth factor-binding protein 3
INK-ATTAC mice genetically modified mice in which cells expressing the cyclin-dependent kinase inhibitor p16INK4A are being removed by apoptosis due to caspase 8 activation
LMNA lamin A gene
MMR mismatch repair

MtDNA	mitochondrial DNA
mTOR(C1)	mammalian target of rapamycin (complex 1)
NADPH	nicotinamide adenine dinucleotide phosphate
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NfkB	nuclear factor kappa B
NO	nitric oxide
Nrf2	transcription factor NF-E2-related factor-2
PAI-1	plasminogen activator inhibitor-1
PARP-1	poly [ADP-ribose] polymerase 1
PDE	phosphodiesterase
POLG	polymerase gamma
RecQ	Escherichia coli recQ-like helicase
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
(s)GC	(soluble) guanylyl cyclase
SIRT-1	sirtuin-1
SMS	senescence-messaging secretome
SNP	single nucleotide polymorphism
T2DM	type 2 diabetes mellitus
TERC	RNA template of telomerase
TERT	telomerase reverse transcriptase
TRF2	telomeric repeat-binding factor 2
TTD	trichothiodystrophy
VSMC	vascular smooth muscle cell
WRN	Werner gene
WS	Werner Syndrome
XP	xeroderma pigmentosum
XRCC3	gene coding for x-ray repair cross-complementing protein 3

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CHAPTER 2.2

Local endothelial DNA repair defect causes aging-resembling endothelial-specific dysfunction

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Abstract

We previously identified genomic instability as a causative factor in vascular aging-related outcomes. We found that loss of endothelium-dependent and -independent vasorelaxation is accelerated in mice with systemic DNA repair dysfunction, which results in an aging phenotype resembling human aging. We aim to determine if these changes are due to local or systemic effects of the inability to repair DNA damage. We investigated cardiovascular function in a mouse model with loss of ERCC1 DNA repair in vascular endothelial cells (EC-KO) at the age of 3 and 5 months. The in vivo measurements of vascular function revealed a progressive decrease in microvascular dilation of the skin, lung perfusion, aortic distensibility, together with cardiac stroke volume and output at the age of 5 months in EC-KO vs. WT. Between 5.5 and 6 months of age EC-KO suddenly died, which was associated with renal papillary and tubular necrosis. Ex vivo vasodilatory function in aorta and iliac artery was decreased at 5 months. Coronary artery dilator function was decreased at 3 months and to a higher degree at 5 months of age. Nitric oxide mediated endothelium-dependent vasodilation was abolished in aorta and coronary artery, whereas endothelium-derived hyperpolarization and responses to nitric oxide donor sodium nitroprusside were intact. Systolic blood pressure was increased at 3 months, but normal at 5 months. In summary, DNA repair defect in the endothelium produces features of age-related endothelial dysfunction; these effects of local endothelial DNA damage can be largely attributed to loss of endothelial-derived NO.

Key words: Aging, DNA damage, endothelium-dependent dilation, endothelial dysfunction, nitric oxide.

Introduction

Despite the currently available prevention and treatment options, cardiovascular diseases (CVD) continue to be a main cause of morbidity and mortality worldwide. Even when traditional risk factors are absent or controlled, cardiovascular problems remain a major health issue as reflected by the independent risk factor age.(1) Aging, which is not synonymous to age, is a natural but very complex process leading to the decline and ensuing loss of organ function. The accumulation of damaged DNA is considered as one of the primary causes driving the process of aging, and involves various processes.(2) Firstly, cells with unrepaired DNA damage may enter into apoptosis or senescence; apoptosis can lead to atrophy and organ function decline due to the loss of cells or tissue, and senescence-related mechanisms trigger the acquisition of a senescence-associated secretory phenotype (SASP) that affects surrounding cells and triggers age-related traits.(3) In parallel, accumulating DNA damage triggers also a so-called 'survival response' that switches the organism's physiological status from one that promotes growth to one that suppresses growth and focuses on maintenance in an attempt to delay aging and extend lifespan.(4) In humans, the presence of individual genetic and environmental variations evoke differences in the rate of aging between individuals, but also between organs within an individual. This differential pace of aging is also observed in mouse models of accelerated aging as provoked by DNA repair defects.(4)

Several mouse models have been generated that, by deletion of one DNA repair gene, show striking similarities to human aging.(4) One of these models is the *Ercc1Δ/–* mouse with a deletion mutation (Δ) in exon 7 of the *Ercc1* gene, causing impaired function of the ERCC1 (Excision Repair Cross Complementation group 1) protein and progressive accumulation of DNA damage.(5)

ERCC1 is an essential component in the pathway of DNA nucleotide excision repair (NER), which removes a wide class of helix-distorting DNA lesions induced by UV, chemicals and oxidative stress. Apart from that, ERCC1 is involved in other DNA repair systems such as double strand break and cross link repair (6). Mutations in proteins of the NER pathway have shown severe effects on human health as evidenced in several human progeroid syndromes such as Cockayne syndrome, trichothiodystrophy and Xpf-Ercc1 syndrome (7).

Ercc1Δ/– mice are short-lived (24-28 weeks) and within 12 weeks from birth develop neurodegeneration, osteoporosis, many features of aging in liver, kidney, heart, muscle and the hematopoietic system. In 8-week old *Ercc1Δ/–* mice an increased blood pressure was observed, which appeared to become smaller at 12 weeks of age (8, 9). Thus, the blood pressure increase might be biphasic. Also, increased vascular stiffness and loss of macro- and microvascular dilator function was observed.(8) The vasodilator dysfunction in *Ercc1Δ/–* mice is explained by reduced NO-cGMP signaling, partly due to decreased endothelial nitric oxide synthase (eNOS) expression.(8) Many of these features are very similar to what was previously found in natural rodent and human aging.

Segmental progeria observed in *Ercc1 Δ/–* mice implies that affected organs might suffer from the impact of local and/or systemic DNA damage. To address the question if a local endothelial DNA repair defect is critical for the specific changes in vascular function as observed in *Ercc1Δ/–* mice, we investigated cardiovascular function in a mouse model with specific loss of *Ercc1* in vascular endothelial cells.

Methods

Animals

We evaluated the effect of endothelial genomic instability on cardiovascular function in a mouse model with endothelium-specific deletion of *Ercc1* (*Tie-2Cre+ Ercc1fl/-* mouse model). To target the endothelium, various Cre re-

combinase models are available. Tie2Cre models have been used most widely. VE-Cadherin- (CD144) Cre models have been suggested as being perhaps the models in which the endothelium is targeted most uniformly (10). However, the only example known to us in which a direct comparison is made between the models does not reveal a difference, at least, when used for fate mapping purposes of EC in adult organs (11). Both Tie2 and VE-Cadherin are not only expressed in endothelial cells, but also in hematopoietic (stem) cells (HSC), potentially affecting leucocyte populations of HSC-derived lineage (12-15). Interestingly, Tie2 is known to be expressed in lineages forming monocytes that have a pro-angiogenic function(14). Thus, leucocytes specifically devoted to endothelial maintenance would be undermined, possibly preventing also the recovery of the endothelium if *Ercc1* deletion indeed leads to dysfunction of the mature endothelium. Therefore, we preferred the *Tie2* promotor region as the sequence driving Cre-recombinase. To explore the consequences of Tie2Cre-driven *Ercc1* deletion in HSC we have examined the blood of the relevant mouse strains (see below).

The Cre-loxP system was used to generate a conditional mouse model expressing Cre-recombinase under the control of the vascular endothelial cell receptor tyrosine kinase (*Tie2*) promoter (Tie2Cre). *Tie2Cre+/-* female mice were crossed with *Ercc1+/-* male mice to generate *Tie2Cre+/- Ercc1+/-* mice in a C57BL/6J background. The females were then crossed with *Ercc1fl/fl* male mice in a FVB/N background to produce *Tie2Cre+ Ercc1fl/-* mice in a C57BL6/FVB F1 hybrid background (16). These *Tie2Cre+ Ercc1fl/-* mice were homozygous for *Ercc1*, after deletion of the floxed allele in endothelial cells expressing Cre-recombinase. These mice are referred throughout this manuscript as endothelial cell-knock out mice (EC-KO). Littermates (genotypes: *Tie2Cre+ Ercc1fl/+, Tie2Cre- Ercc1fl/+, Tie2Cre- Ercc1fl/-*) were used as controls. These mice are referred to as WT mice in the paper. Mice were kept in individually ventilated cages, in a 12 h light/dark cycle and fed normal chow and water *ad libitum*.

EC-KO mice were dying suddenly when they were around 5.5-6 months-old, because of this measurements were performed at 3 and 5 months. EC-KO mice unexpectedly died at the age of 5.5 – 6 months (100% of the cases). Consequently, we decided to evaluate mice at 3 and 5 months of age. Mice under profound anaesthesia were euthanized by exsanguination from the vena porta. All animal procedures were performed at the Erasmus MC facility for animal experiments following the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All animal studies were approved by the Animal Care Committee of Erasmus University Medical Center Rotterdam (protocol number 118-13-03).

Pathological examination, tissue collection and blood analysis

All sudden deaths of EC-KO occurred at night, except for 1 case. This mouse was submitted to whole body fixation in formalin for 48 hours and pathological examination. Tissues were processed by paraffin-embedding techniques, sectioned, and stained with Hematoxylin and Eosin. Slides were examined by a board-certified veterinary pathologist. For mice sacrificed at 3 and 5 months of age, blood was collected under anesthesia from the vena porta and analyzed for cell counts. Vascular, cardiac, renal and lung tissue were collected for further study. For scanning electron microscopy (EM) abdominal aorta was fixated in 4% formaldehyde / 2% glutaraldehyde. The lumen was exposed after longitudinal opening of the aorta, and scanning EM recordings were made after platinum sputtering.

Blood vessel permeability

We performed Evans Blue dye method in mice, as described (17), to determine the presence of vascular leakage in kidneys . Shortly, we injected 100 uL of 1% Evans Blue dye (0.133 gr of Evans Blue in 10 ml of PBS with Ca2+ and Mg2+, prepared under sterile con-

ditions) through femoral cannulation. After 4 hours of incubation, blood was drawn (100-200ul). Blood sampling was followed by whole animal PBS perfusion through the femoral infusion cannula after opening of the right atrium. Perfusion was performed with a peristaltic rotation pump until no blood residue remained in the atrium. Kidneys were collected, weighted and transferred to sample tubes with 500 µl of formamide. The sample tubes were incubated in a heat block at 55°C for 24 hours to extract Evans Blue from the tissue. After incubation, the formamide/Evans Blue mixture was centrifuged to pellet any remaining tissue fragments. Absorbance of each sample was measured at 610 nm (VersaMax™ Microplate Reader). Absorbance values from a standard curve with known concentrations of Evans Blue in formamide, with pure formamide as a background blank, and the total weight of each sample, were used to calculate the amount of Evans Blue per mg of renal tissue.

Cardiac function

Cardiac geometry and function were measured by performing 2-D guided short axis M-mode transthoracic echocardiography (Vevo770 High-Resolution Imaging System, VisualSonics) equipped with a 35-MHz probe. Left ventricular (LV) external and internal diameters were traced, and heart rate, LV mass and fractional shortening were subsequently calculated using the VisualSonics Cardiac Measurements Package. Anesthesia was induced with 2,5% and maintained with 2,0% isoflurane, the animals were breathing freely and intubation was not required, while body temperature was kept at 37°C.

Blood pressure measurement

Blood pressure (BP) was measured non-invasively in conscious mice using the tail cuff technique (CODA High-Throughput device, Kent Scientific). BP was measured on 5 consecutive days and each session consisted of 30 measurement cycles for each mouse. The first 4 days were taken as acclimatization sessions. BP values reported here correspond to the average of all valid measurements recorded at day 5.

Aortic strain and stiffness

Using the data on systolic and diastolic aortic diameters acquired by transthoracic echocardiography, we calculated aortic dilatation by subtracting the diastolic aortic diameter from the systolic aortic diameter.

Microvascular vasodilator function and lung perfusion *in vivo*

We assessed in vivo vasodilator function using Laser Doppler perfusion imaging, after three to seven days of blood pressure measurement. Reactive hyperemia, defined as the increase of the hindleg perfusion after temporary occlusion of the blood flow, was calculated. Blood flow was measured in the left hindleg one day after removing the leg's hair using a hair removal cream. The hindleg was kept still with help of a fixation device. After recording baseline perfusion for 5 minutes, blood flow was occluded for 2 minutes with a tourniquet. To record hyperemia and the return of the blood flow to the post-occlusion baseline, blood flow was monitored for 10 minutes after releasing the tourniquet. During all measurements mice were under 2.8% isoflurane anesthesia, and temperature was constantly monitored and maintained between 36.4-37.0 °C. For each mouse we calculated the maximum response to occlusion and the area under the curve relative to the post-occlusion baseline. Only the area above the baseline was considered. Values below the baseline were set at 0.

In a separate set of 5 month-old WT vs. EC-KO (n= 4 vs 7), lung perfusion was measured by microCT imaging. µCT scans were performed and reconstructed at the Applied Molecular

Imaging Erasmus MC facility (AMIEf) by using the Quantum FX (PerkinElmer). Mice were anesthetized with 2.5% isoflurane in O2 and received an IV injection with eXIA160 (Binitio Biomedical Inc., Canada) contrast agent. The injected amount of agent followed the dose recommended by the manufacturers. First, a pre-contrast scan was made as a baseline. The animal was taped to the imaging bed with a catheter placed in the tail vein to ensure minimal displacement and prevent misalignment during post-processing. After acquisition of the pre-contrast scan, the contrast agent was slowly infused (150-200 uL in 1 minute), after which a second scan was made. Mice were scanned using intrinsic cardio-respiratory gating to reduce artifacts caused by breathing. CT acquisition parameters: 90kv, 160μA, field of view 20mm, 40 um resolution with an acquisition time of 4,5 min. Scans were quantified using Analyze 11.0 software (AnalyzeDirect). By using the image calculator option the pre-contrast image was subtracted from the post-contrast image. This resulted into an image of iodine only, which was subjected to further filtering with a median filter (kernel size 3 x 3 x 3). During semi-automatic segmentation of the lungs, the large and midsize blood vessels were excluded. On the resulting lung image segmentation, we calculated the average intensity value as a measure for average lung perfusion, with a minimum of 40% of total lung volume.

Ex vivo vascular assessment

Immediately after sacrifice thoracic aorta, iliac and left anterior descending coronary arteries were carefully dissected from mice and kept in cold Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7,CaCl2 2.5, MgSO4 1.2 , KH2PO4 1.2, NaHCO3 25 and glucose 8.3 in distilled water; pH 7.4). Vessel rings of 1.5-2 mm length were mounted in small wire myograph organ baths (Danish Myograph Technology, Aarhus, Denmark) containing 6 mL of Krebs-Henseleit buffer oxygenated with 95% O2 and 5% CO2. After warming, the tension was normalized by stretching the vessels in steps until 90% of the estimated diameter at which the effective transmural pressure of 100 mmHg is reached. Thereafter, the viability of the vessels was tested by inducing contractions with 30 and 100 mmol/L KCl. After the maximum response to KCl had been reached vessels were washed. To evaluate vasodilatory responses, aortic and iliac segments were first pre-constricted with 30 nmol/L of the thromboxane A2 analogue U46619, resulting in a preconstriction corresponding with 50-100% of the response to 100 mmol/L KCl. After this, concentration-response curves (CRCs) were constructed with the endothelium-dependent vasodilator acetylcholine (ACh) at cumulative doses (10-10-10-5 mol/L). When the CRC to ACh was completed, we used the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-4} mol/L). Complete CRCs to SNP (10^{-10}-10^{-4} mol/L) were performed in parallel rings preconstricted with 30 nmol/L U46619.

The contribution of nitric oxide (NO) and prostaglandins in the aortic ACh responses was explored by performing the experiments in the presence of the endothelial nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester salt (L-NAME, 10-4 mol/L) and the cyclo-oxygenase (COX) inhibitor indomethacin (INDO, 10-5 mol/L). Inhibitors were added to the organ bath 20 minutes prior to U46619. N-acetyl-cysteine (NAC; 10-2 mol/L), a reactive oxygen species (ROS) scavenger, was used to test the involvement of ROS in the vasodilator responses to ACh.

In coronary arteries we investigated endothelium-dependent vasodilation by performing CRCs to ACh and to the Adenosine 5'-O-(2-thiodiphosphate) (ADPβS) and Uridine-5'- O- (3- thiotriphosphate) (UTPγS). The intracellular signalling tion caused by ADP and UTP, has not been studied in detail in the mouse heart. However, studies on blood pressure and cerebral arterioles in the eNOS-/- mice have shown that the two nucleotides do not cause vasodilation through the same mechanisms. It is thought that UTP-induced vasodilation exclusively involves endothelium-dependent hyperpolarization (EDH) (18), while

ADP acts through both NO and EDH on a 50%/50% basis.(19) CRCs to ADPβS and UTPγS were performed in coronary arteries precontracted with U46619 and then in coronary segments precontracted with 30 mM KCl. The latter was done to elucidate the contribution of EDH in ADPβS- and UTPγS-induced vasodilation because when arteries are precontracted with 30 mmol/L KCl, EDH cannot occur because the artery is too strongly depolarized. (20-22) VSMC dilatory function was tested by constructing CRCs to the NO donor SNP. In iliac rings, after washing out KCl 100 mmol/L, we investigated contractile responses to angiotensin II (AngII, 10-10-10-7 mol/L), endothelin-1 (ET-1 10-10-10-6 mol/L) and phenylephrine (PE, 10-9-10-5 mol/L). The involvement of Ang II type 2 (AT2) receptors in AngII responses was tested by adding PD123319 (10-7 mol/L) 30 minutes prior to starting the CRCs.

Mechanical properties of the carotid vascular wall

Carotid arteries explanted from 5 months old mice were mounted in a pressure myograph (Danish Myograph Technology, Aarhus, Denmark) in calcium free buffer (in mmol/L: NaCl 120, KCl 5.9, EGTA 2, MgCl2 3.6, NaH2PO4 1.2, glucose 11.4, NaHCO3 26.3; pH 7.4). The intraluminal pressure of the carotid artery was increased stepwise by 10 mm Hg starting at 0 mm Hg and reaching 120 mm Hg. Lumen and vessel diameter were measured and used to calculate wall strain and stress (23).

Quantitative real-time PCR

Total RNA was isolated from aortic tissue and cDNA was prepared, which was amplified by real-time PCR on a StepOne thermocycler (Applied Biosystems). Each reaction was performed in duplicate with SYBR Green PCR Master Mix (Applied Biosystems). β-actin and HPRT-1 were used for normalization. The relative amount of genomic DNA in DNA samples was determined as follows: RQ = 2(-ΔΔCt). Sequences of the primers used are provided in supplementary Table S1.

Immunoblots

Frozen tissues were homogenized in ice cold RIPA buffer (50 mmol/L HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate and 1 mmol/L EDTA) containing protease and phosphatase inhibitors (1 mmol/L PMSF, 1 mmol/L NaVO4, 1 mmol/L NaF, 1 µg/mL aprotinin, 1 µg/mL pepstatin and 1 µg/mL leupeptin) using a stainless-steel ultraturrax (Polytron). Homogenized tissues were centrifuged and protein concentration was measured in the supernatants using the BCA method (Thermo Scientific, USA). For eNOS, membranes were blocked with 5% milk TBS-T; for pSer1177- eNOS membranes were blocked with 5% BSA TBS-T. After blocking, membranes were incubated overnight with the primary antibodies as follows: eNOS (Santa Cruz, SC-654 1:500 in 5% milk TBS-T) and pSer1177- eNOS (Santa Cruz, SC-21871-R 1:500 in 5% BSA TBS-T). We used an HRP (Horseradish peroxidase)- conjugated antibody (Bio-Rad 1:2000 in 1% milk-TBS-T) to detect the primary antibodies. For visualization we used an enhanced chemiluminiscent substrate for detection of HRP (Pierce ECL Immuno-Blotting Substrate, Thermo Scientific). All protein expression levels were normalized to actin.

Statistical methods

Data are presented as mean and standard error of the mean, unless otherwise indicated. Statistical analysis between the groups of single values was performed by unpaired two-tailed t-test. Differences in dose-response curves were tested by general linear model for repeated measures (sphericity assumed). Differences were considered significant at $p < 0.05$ (two-tailed).

Results

General health features

There were no general signs of developmental problems in EC-KO, and body weights were normal up until 5 months of age (Figure 1A). Blood cell analyses revealed no significant changes (supplementary Table S2). However, EC-KO had a strongly reduced lifespan, with a median of 24.6 weeks (supplementary Figure S1). Shortly before death, immobility and rapid breathing was observed in 2 of the EC-KO mice. Most deaths occurred at night, except for one mouse (22.6 weeks) which underwent full body pathological examination. Organs included were brain, heart, skeletal muscle, aorta, sciatic nerve, liver, spleen, lung and kidney. Again, no signs of developmental problems were observed. Except for kidney, the examined organs did not show morphological or histological aberrations. In 3 out of 5 EC-KO vs. 1 out of 6 WT of 5 months of age the kidney revealed red blood cells in the tubules (Figure 1B-C), indicating microvascular leakage in EC-KO. These data suggest that endothelial DNA repair deficiency strongly impairs the permeability of the renal medullary microvasculature.

Renal blood vessel permeability

To corroborate the increased permeability of the renal vasculature we used an in vivo Evans Blue tissue penetration assay, comparing the vessel leakage between EC-KO and WT mice (5 months old mice) in kidneys. The difference in vessel permeability was quantified spectrophotometrically by measuring the Evans Blue that was captured per gram of tissue. Our results show an increase in dye leakage from the kidney vessels of EC-KO mice when compared with WT mice (Figure 1D, p-value=0.05).

In vivo microvascular function

To further examine peripheral microvascular function we performed laser Doppler reactive hyperemia studies. At 3 months of age there was no difference in reactive hyperemia in the hindlimb skin between EC-KO and WT (Figure 2A, B, E, F). At 5 months EC-KO showed decreased reactive hyperemia (Figures 2C-F). When passing from the age of 3 to 5 months reactive hyperemia tended to increase in WT mice, whereas it tended to decrease in EC-KO mice. In addition, lung perfusion was measured in WT (n=4) and EC-KO mice (n=7) showing significantly decreased lung perfusion in EC-KO mice (Figure 3A-C).

Ex vivo vasodilator responses in aorta and iliac arteries

To examine vasodilation in large arteries and reveal the mechanism of vasodilator dysfunction ex vivo organ bath experiments were performed. Aorta and iliac artery of EC-KO showed decreased endothelium-dependent relaxations to acetylcholine compared to WT at the age of 5 months, which was still absent at 3 months of age (Figure 4A, B). Vascular smooth muscle dilatory function to the NO donor SNP was intact at both ages (Figure 4C-D). In WT mice, approximately half of the ACh response was mediated by NO (response to ACh was reduced by ~50% in the presence of the eNOS inhibitor L-NAME) (Figure 5A). No apparent contribution of prostaglandins was observed since the COX inhibitor indomethacin did not further reduce vasodilator responses (Figure 5A).

Compared to WT mice, in EC-KO there was no contribution of the NO or prostaglandin pathways to the ACh responses (Figure 5B). In both WT and EC-KO a residual ACh response was observed that was similar in both groups of mice, suggesting that the contribution of endothelium-derived hyperpolarizing factors (EDHFs) is intact.

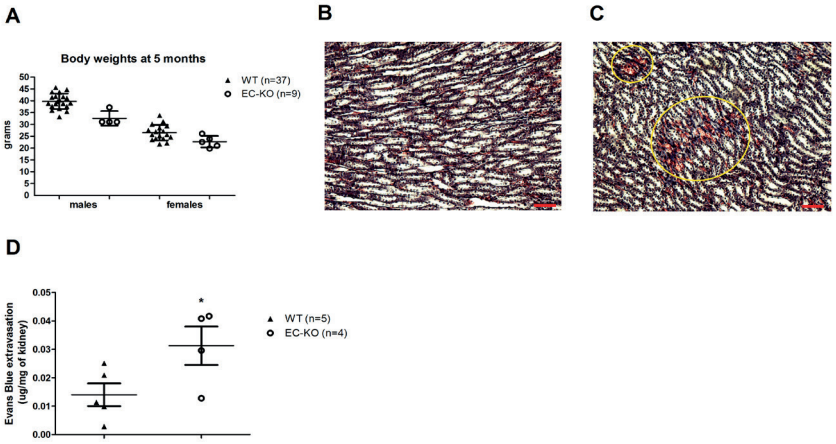


Figure 1. General health and pathology findings. . Body weights at 5 months (n= 9 EC-KO and 37 WT) (A). Mason's trichrome staining of kidney sections from a control mouse (B) and a EC-KO mouse (C) The area encircled shows extravasation of red blood cells in the proximity of the tubules. Kidney sections from 5 EC-KO and 6 WT mice were examined. Renal Evans Blue leakage (ug Evans Blue × mg of kidney tissue) 24 hours after intravenous injection, results are expressed as means ± SE, * p-value ≤ 0.05 compared to WT group (D).

The reduced NO availability and vasodilator dysfunction could be explained by the presence of high levels of oxidative stress, which might be rescued by ROS scavengers. The presence of NAC resulted in a leftward shift of the ACh dose effect curve in EC-KO mice (Figure 5C), but not in WT littermates, confirming an inhibitory role of ROS on vasodilations only in EC-KO.

Ex vivo vasodilator responses in coronary arteries

As a representative for mid-sized arteries important for direct blood supply in vital organs relevant for cardiovascular disease we examined coronary artery function. In coronary arteries endothelium-dependent relaxation to ACh was significantly decreased in EC-KO both at 3 months and 5 months of age (Figure 6A, p-value=0.009 and 0.0007, respectively). Endothelium-independent relaxations to SNP were unchanged (Figure 6B). To study other endothelium-dependent agonists than ACh and the mechanism of vasodilator dysfunction ADPβS (NO and EDH-dependent) and UTPγS (EDH-dependent) were employed. ADPβS relaxation curves were shifted rightward in EC-KO (Figure 6C, p-value=0.03) but responses to UTPγS unchanged (Figure 6D). Responses to ADPβS and UTPγS in arteries preconstricted with 30 mmol/L KCl were examined as to exclude EDH (20-22). Maximal dilations to ADPβS were decreased in EC-KO vs. WT (Figure 6E), whereas dilations to UTPγS were cancelled in both mouse strains (Figure 6F). This result confirms that UTPγS is entirelylyrely on EDH, and that NO-mediated responses are decreased in EC-KO.

Levels of eNOS, and scanning electron microscopy

Immunoblot analysis showed a tendency towards reduced baseline pulmonary eNOS protein level in EC-KO vs. WT, but this did not reach statistical significance (Figure 7A). Also, the ratio of eNOS-activating phosphorylation of the serine residue at position 1177 (pSer1177-eNOS) to total eNOS protein was not different at baseline (Figure 7A). The luminal surface of the abdominal aorta from 5 months-old animals was investigated for endothelial denudation (5 WT vs. 5 EC-KO). Scanning EM of the luminal surface also did not reveal a loss of endothelial cells (Figure 7B). Also, wall thickness measured microscopi-

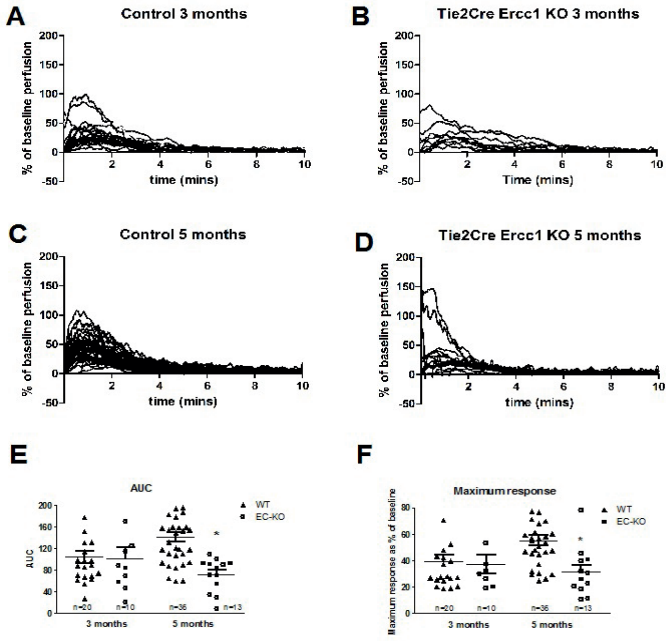


Figure 2. In vivo vasodilator function was assessed using Laser Doppler perfusion imaging. Functional differences between skin reperfusion after 2 minutes of occlusion between WT (A) and EC-KO (B) at 3 months; and between WT (C) and EC-KO (D) at 5 months. Calculated area under the curve (E), and average maximum response (F) for the observed differences in skin reperfusion. * = $p < 0.05$ (t test EC-KO vs WT). At 3 months, 10 EC-KO and 20 WT mice were examined. At 5 months 13 EC-KO and 36 WT mice were examined.

cally in HE-stained aortic microsections was similar between EC-KO and WT (Figure 7C).

Blood pressure

Systolic blood pressure (SBP) was higher in EC-KO at 3 months (138 mm Hg in EC-KO vs 125 mm Hg in WT mice) whereas no differences were observed at 5 months (p-value SBP=0.72, p-value DBP=0.86). No differences were observed in DBP at 3 or 5 months (Table 1).

Cardiac function

The change in SBP followed a biphasic course, being slightly elevated at 3 months and returned to normal at a time when endothelium-dependent vasodilation was markedly reduced (5 months). Therefore, we investigated cardiac function, measuring volume variables, as a possible explanation. Since male and female mice have a different heart volume and weight, both two-way ANOVA including genotype and sex, and heart weight-indexed volume variables were used as statistical corrections. No differences between EC-KO and WT were observed at 3 months. However, stroke volume (SV) and cardiac output (CO) were significantly decreased in 5 month-old EC-KO compared to WT (Table 1). In agreement, SV and CO indexed for heart weight were also decreased. When indexed for body weight, SV and CO were equal between EC-KO and WT (Table 1).

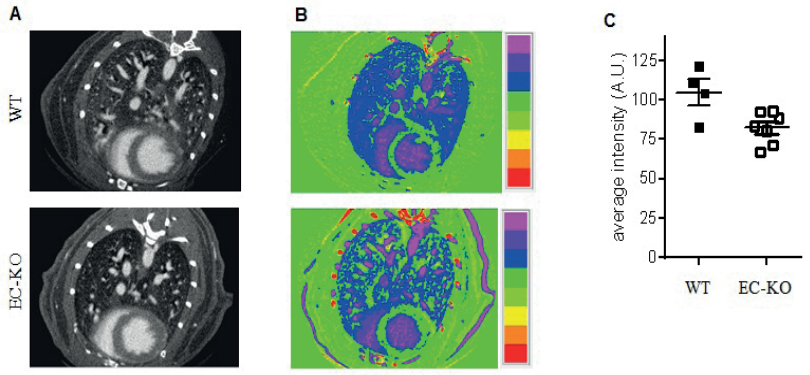


Figure 3. µCT-based contrast-aided perfusion. µCT-based contrast images of WT and EC-KO lungs (A). µCT-based contrast-aided perfusion images of WT and EC-KO lungs, showing average intensity of perfusion after subtraction of the contrast to non-contrast images as the color bar indicates (B). Scatter plots depicting average intensity as a measure for lung perfusion, which is significantly reduced in EC-KO compared to WT. * = $p < 0.05$ (t test EC-KO vs WT) (C). 7 EC-KO and 4 WT mice were included for these measurements.

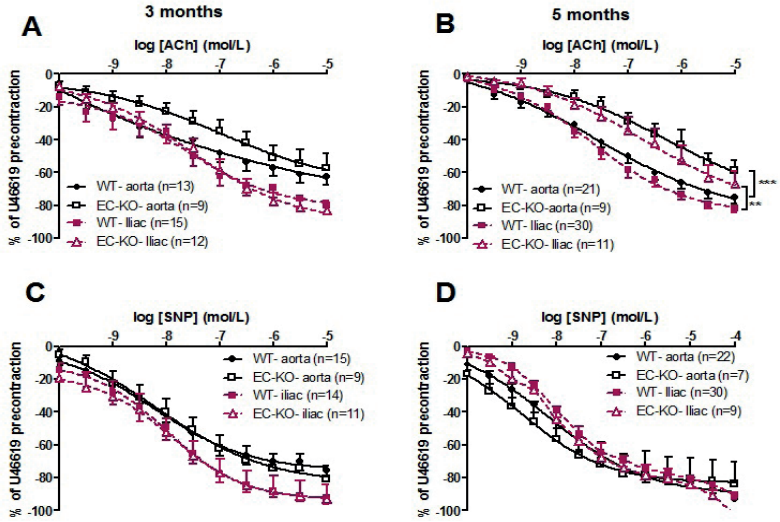


Figure 4. Endothelium-dependent and independent relaxations in isolated aortic and iliac rings measured ex vivo in organ bath set-ups. ACh-induced vasodilation of EC-KO and WT in aorta and iliac artery at 3 months (A), and at 5 months (B). Endothelial-independent relaxations induced by SNP in aortic and iliac rings at 3 months (C) and at 5 months (D). ** = $p<0.001$; *** = $p<0.0001$ (general linear model for repeated measures, sphericity assumed; EC-KO- aorta vs WT-aorta and EC-KO- iliac vs WT-iliac). At 3 months arteries from 9-12 EC-KO and 12-15 WT were studied. At 5 months arteries from 7-11 EC-KO and 21-30 WT were studied.

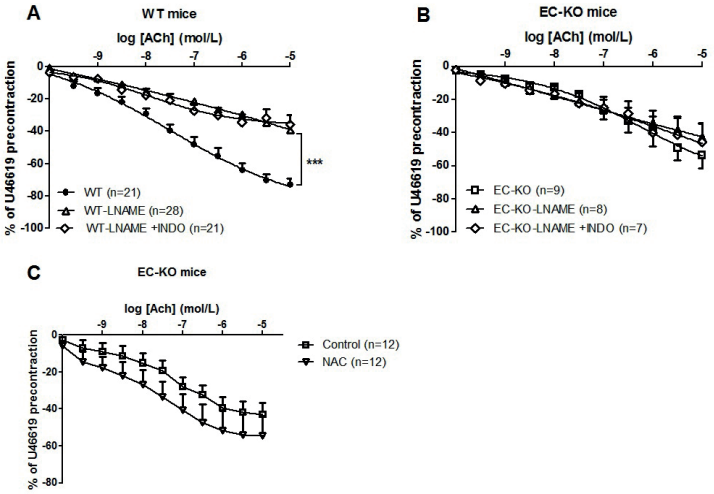


Figure 5. Contribution of NO, prostaglandins and oxidative stress to the endothelium-dependent vasodilations. At 5 months we evaluated the contribution of NO and prostaglandins to ACh-induced vasodilation in WT (A) and EC-KO (B). Vasodilator response in the presence of ROS scavengers (NAC) in EC-KO mice (C). *** = p<0.0001 (general linear model for repeated measures, sphericity assumed; the responses to ACh alone were compared to the responses to ACh after preincubation with L-NAME). Aorta arteries from 7-12 EC-KO and 21-28 WT were studied.

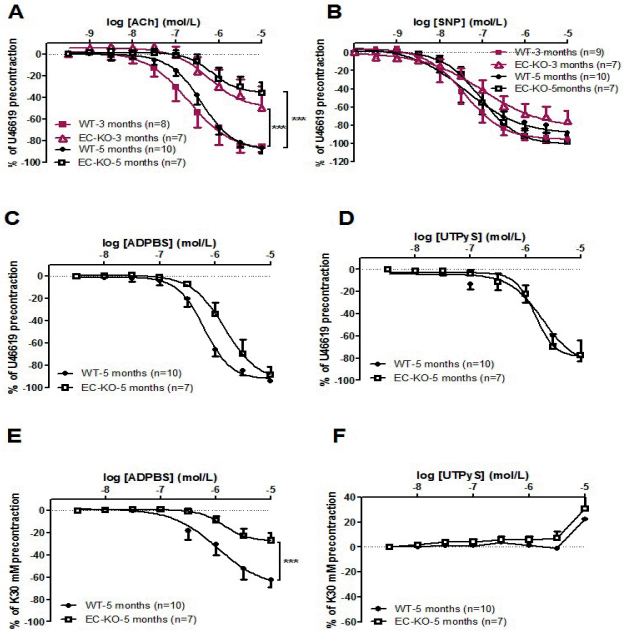


Figure 6. Vasodilation in coronary arteries measured ex vivo in small wire organ bath setups. Relaxations to ACh (A), SNP induced vasodilatation (B), ADPβS (C) and UTPγS (D) in coronary rings preconstricted with U46619. Relaxations to ADPβS (E) and UTPγS (F) in coronary rings preconstricted with KCl 30mM. *** = p<0.0001 (GLM-RM, EC-KO- 3 months vs

WT- 3 months and EC-KO- 5 months vs WT- 5 months). At 3 months coronary arteries from 7 EC-KO and 8-9 WT were studied. At 5 months arteries from 7 EC-KO and 10 WT were studied.

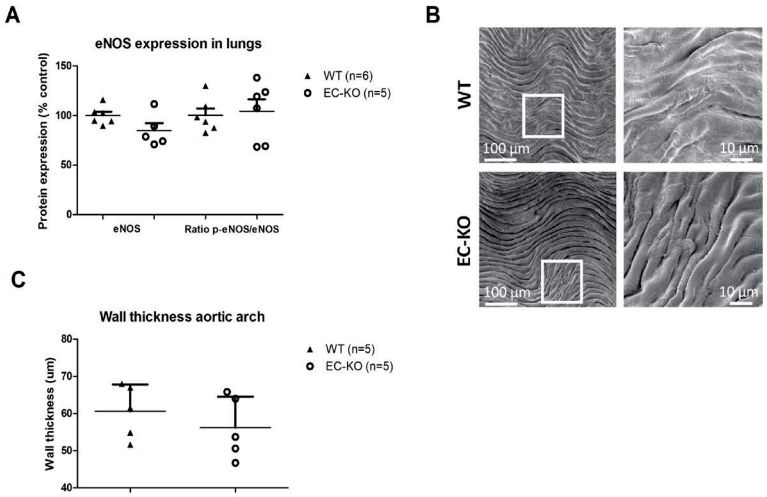


Figure 7. Levels of eNOS and scanning electron microscopy. Protein expression levels in lung of eNOS and p-eNOS (n=5 EC-KO and 6 WT) (A), representative scanning electron microscopy images of the endothelium (n=5 EC-KO and 5 WT); scale bars represent 100 µm and 10 µm (B). Wall thickness measured in HE-stained aortic microsections (n=5 EC-KO and 5 WT) (C).

Ex vivo vasoconstrictor responses

We tested contraction responses in iliac arteries to ET-1, Ang II and phenylephrine (supplementary Figure S2). Although at the age of 5 months phenylephrine responses were significantly lower in EC-KO, we saw no consistent differences between WT and EC-KO of 3 and 5 months of age.

Mechanical properties of the vascular wall

Since we previously found that arterial stiffness was increased in *Ercc1Δ/-* mice,(8) we measured aortic wall movement during cardiothoracic echography, and calculated distensibility of the aorta. At 3 months no differences were found between EC-KO and WT but at 5 months distensibility was decreased in EC-KO despite normal pulse pressures (Table 1). We further evaluated the mechanical properties of carotid arteries. No significant differences in lumen diameter or wall thickness under similar perfusion pressure increments were observed between EC-KO and WT. Likewise, no differences in media strain or stress were observed (supplementary Figure S3).

mRNA expression of SASP components

We previously found an increase of senescence markers in the VSMC of aortic wall of *Ercc1Δ/-* mice, whereas endothelial cells did not appear to show cellular senescence (8). We therefore performed qPCR for several SASP components to confirm the finding in the EC-KO. We found no significant differences in aortic mRNA levels of *p16, p19, p21, TNFα, IL-6* and *MMP13* (supplementary Figure S4).

Discussion

We investigated the role of endothelial specific DNA repair defectiveness on cardiovascular function in a mouse model with specific loss of *Ercc1* in vascular endothelial cells. We found that local endothelial genomic instability caused macrovascular and microvascular

Table 1. Cardiac function, blood pressure and aorta distensibility at 3 and 5 months

Parameter	3 months			5 months		
	EC-KO (n=10-19)	WT (n=11-21)	p-value	EC-KO (n=11-17)	WT (n=12-31)	p-value
Stroke volume (uL)	33 (5.5)	36 (5.1)	0.21[a]	32 (8.0)	36 (5.0)	0.01[a]
Stroke volume index (uL/mg myocardium)*	30 (4.5)	31 (4.1)	0.56[b]	25 (5.2)	30 (3.5)	0.03[b]
Stroke volume index (uL/g)$	1.4 (0.2)	1.4 (0.2)	0.81[b]	1.2 (0.2)	1.3 (0.2)	0.49[b]
Fractional shortening (%)	34 (5.1)	34 (5.7)	0.71[b]	33 (6.5)	33 (6.0)	0.94[b]
HR (bpm)	502 (37.4)	492 (37.0)	0.38[b]	494 (40.1)	498 (49.2)	0.78[b]
Cardiac output (ml/min)	17 (2.9)	18 (3.3)	0.24[a]	16 (4.1)	18 (3.6)	0.01[a]
Cardiac Index (ml/min/mg myocardium)*	15 (2.3)	15 (2.6)	0.92[b]	13 (2.4)	14 (1.7)	0.06[b]
Cardiac Index (ml/min/g) $	0.7 (0.1)	0.7 (0.1)	0.93[b]	0.6 (0.1)	0.6 (0.1)	0.46[b]
Heart weight (mg)	112 (12.8)	116 (17.1)	0.45[b]	125 (25.3)	122 (20.2)	0.77[b]
SBP (mmHg)	138 (14.6)	125 (11.9)	0.04[b]	128 (22.8)	125 (20.6)	0.72[b]
DBP (mmHg)	94 (15.2)	89 (15.6)	0.46[b]	88 (19.3)	86 (20.7)	0.86[b]
Aorta distensibility (mm)	0.3 (0.1)	0.3 (0.04)	0.69[b]	0.2 (0.1)	0.3 (0.1)	0.05[b]

Values are Mean (SD). HR, heart rate; bpm, beats per minute; SBP, systolic blood pressure; DBP, diastolic blood pressure. * Values are corrected for heart weight, expressed as per 100 mg of heart weight. $ Values are corrected for body weight, expressed as per gram of body weight. a Two-way ANNOVA. bt-test.

vasodilator dysfunction at least in part due to specific loss of endothelium-derived nitric oxide. Likely, there is a reduced NO availability in EC-KO due to scavenging by ROS, as confirmed with the use of NAC. A reduced eNOS expression does not seem to play a role. The preserved endothelial cell layer, as confirmed by scanning EM as well as normal EDHF-mediated vasodilation, excludes the loss of NO through reduced EC numbers as an explanation.

Notably, the rate of development of vasodilator dysfunction is location-specific as at 3 months of age this dysfunction is observed in coronary arteries, but not in iliac artery, aorta, or the skin microvasculature. This suggests a non-developmental origin of the vasomotor dysfunction and a non-homogenous regulation of eNOS across the various vascular beds under otherwise healthy conditions. Previously, we observed reduced eNOS expression and activation in *Ercc1Δ/-* mice.(8) One might propose that this is a non-cell autonomous effect of the DNA repair defect. However, eNOS reacts to aging in diverse ways, varying from increased expression to compensate for loss of vasodilator capacity due to NO scavenging by ROS in an early, compensated stage of endothelial aging, to decrease of eNOS and its activation in senescent cells.(24-26) Therefore, an alternative explanation might be that the stage of aging of the endothelium might be different in *Ercc1Δ/-*, with a mutation in *Ercc1* in all cells and tissues, versus EC-KO, with deletion of *Ercc1* on the endothelium specifically. The lack of senescence markers in EC-KO seems to support this possibility.

Apart from vasodilator dysfunction EC-KO showed a severely compromised microvascular barrier function in the kidney, as shown by both histological examination and Evans Blue permeability tests, an established method for this purpose (27). Therefore, the aging-mimicking effect of endothelial Ercc1 deletion is not restricted to loss of vasodilator function, but at least affects barrier function in the kidney as well. Through this mechanism DNA damage response might contribute to progressive kidney damage, in addition to decreased NO signaling, an important determinant of renal deterioration (28). In search of other features of vascular aging we have tested vascular stiffness. At the age of 5 months aortic distensibility measured by echography was reduced in EC-KO, suggesting increased vascular stiffness. Yet, this was not observed by *ex vivo* stress and strain measurements in carotid arteries. Since at this age stroke volume and cardiac output were reduced in EC-KO, and blood pressure was normalized again after first being increased at 3 months of age, we suspect that the observed decrease in aortic distensibility was due to the adaptation of cardiac function. Therefore, we conclude that vascular stiffness was not altered by the reduced endothelial NO. Indeed, eNOS KO mice of similar age as our EC-KO show a carotid compliance that is even marginally higher than in WT.(29) Therefore, we propose that the higher vascular stiffness observed earlier in Ercc1Δ/- mice cannot have been the results of endothelial dysfunction alone.(8)

Our study shows that the effect of DNA damage on NO signaling is very specifically evoked within the endothelium, possibly even cell-autonomously. Hence, the endothelial effect of DNA damage is not dependent on genomic lesions elsewhere in the body. Nevertheless, EC-KO share some features with Ercc1Δ/- mice, including a worsened renal morphology and a shortened lifespan.(30) In an earlier publication rescuing the liver of Ercc1 null mutant mice from genetic Ercc1 inactivation, it was shown that next to the liver the renal tissue is exceptionally vulnerable to loss of Ercc1 function, and it was suggested that renal problems might be an important cause of death in the 'rescued' mice.(31) Our present results indicate that endothelial DNA damage might contribute to the fatal renal problems.

Our results could not be correlated to markers of cellular senescence. This could be because cellular senescence might not be required for induction of vasodilator dysfunction. Alternatively, our analysis might not allow detection of EC-specific changes, since mRNA from

EC is strongly diluted by mRNA of VSMC in aortic tissue. In a previous study we showed that in *Ercc1Δ/-* mice senescent cells were VSMC, whereas senescent EC were not observed. (8) Senescent cells were very scarce, so it may be unsurprising that our assays were not sensitive enough to detect endothelial cell specific senescence. Recently, it was shown that injection of senescent cells homing to adipose tissue leads to further dissemination of cellular senescence and the SASP.(32) We cannot rule out that a similar phenomenon occurs in the endothelium, and that a small amount of senescent endothelial cells are needed to convey a dysfunctional phenotype to neighboring endothelial cells. In general, the knowledge regarding this question is rather minimal, which can be attributed to the complicated detection of cellular senescence and local inflammation in the endothelium, and the lack of studies regarding cell-cell communication in senescent EC. Nevertheless, it has been suggested previously that SASP in few EC might cause a more generalized dysfunction of circulatory function, much in a way that SASP of tumor fibroblasts might promote cancer progression.(3) Clearly, future studies are required to further explore the molecular mechanisms linking EC-restricted DNA damage to endothelial dysfunction and premature death in our mouse model.

In summary, we found that local endothelial genomic instability causes sudden death, clear microvascular complications in the skin, kidney and possibly also in the lung, coronary vasodilator dysfunction associated with lower cardiac stroke volume, and macrovascular dysfunction. Early signs of increased vascular stiffness are present in the aorta. Measurement of NO signaling and the striking resemblance with eNOS KO mice indicate that the loss of this signaling pathway might play a major role in the observed phenotype. Interventions that normalize this signaling pathway, such as pharmacotherapy with guanylyl cyclase stimulators or activators and specific phosphodiesterase inhibitors are warranted. Further, we have shown that diet restriction is another potential treatment option, as this rescues loss of vasodilator function in Ercc1Δ/- mice as well as in humans(5, 9, 33). Therefore, studies exploring the impact of dietary restriction on DNA damage-induced vascular aging and the involved rescue mechanisms can be of great value.

Clinical Perspectives

- We previously identified genomic instability as a causative factor in vascular aging-related outcomes. The endothelial dysfunction triggered by a systemic DNA repair dysfunction results in an vascular aging phenotype resembling that in humans. We here found that important features of endothelial dysfunction, an important part of the vascular aging phenotype, can be aroused by defective DNA repair exclusively in the endothelium. There are no clues that senescence or endothelial denudation plays a role herein. This is important knowledge in view of the development of senolytic drugs for clinical use.
- The coronary artery is the most rapidly affected vessel species in our study. This might have implications for coronary disease.
- Renal vascular leakage and necrosis were observed, which might have implications for aging related renal dysfunction.
- Microvascular function is disturbed by the endothelial DNA repair defect. This might have implications for end organ dysfunction in aging as caused by microvascular problems.
- Future clinical studies to DNA damage and repair in human vasculature is warranted.

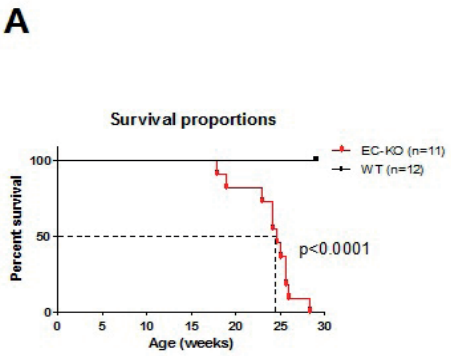
Supplementary data

Gene name	Forward primer	Reverse primer
p16	CCCAACGCCCCGAACT	GCAGAAGAGCTGCTACGTGAA
p19	GCCGCACCGGAATCCT	TTGAGCAGAAGAGCTGCTACGT
p21	GTCCAATCCTGGTGATGTCC	GTTTTCGGCCCTGAGATGT
TNFα	AGGGTCTGGGCCATAGAACT	CAGCCTCTTCTCATTCCT
IL-1α	TCAACCAAACTATATATATCAGGATGTGG	CGAGTAGGCATACATGT-CAAATTTTAC
IL-6	GACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
MMP13	ACTTCTACCCATTTGATGGACCTT	AAGCTCATGGGCAGCAACA
GAPDH	TGCACCACCAACTGCTTA	TGGATGCAGGGATGATGTTC

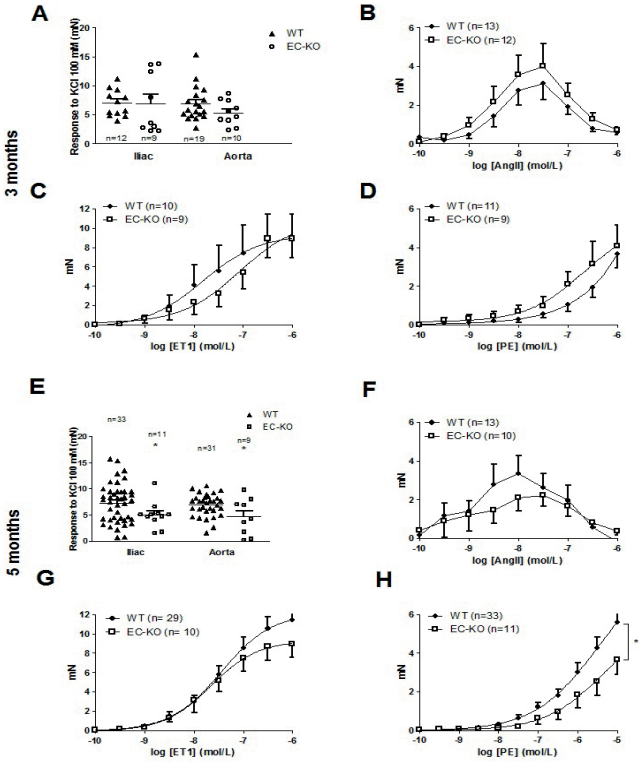
Supplementary Table S2. Blood cell counts at 3 and 5 months.

Parameter	3 months			5 months		
	EC-KO (n=10)	WT (n=17)	p-value	EC-KO (n=8)	WT (n=17-19)	p-value
% Leucocytes	7 (3.0)	6 (2.9)	0.70	7 (2.8)	9 (5.2)	0.22
% Lymphocytes	63 (7.6)	60 (8.0)	0.43	63 (6.5)	56 (16.6)	0.30
% Monocytes	4 (0.5)	4 (0.8)	0.10	4 (1.1)	4 (1.1)	0.24
% Granulocytes	33 (7.5)	36 (7.5)	0.33	34 (5.7)	35 (12.0)	0.80
Erythrocytes	10 (0.8)	10 (0.7)	0.35	10 (0.6)	10 (0.5)	0.71
Hemoglobin	9 (0.8)	9 (0.6)	0.87	10 (0.6)	9 (0.6)	0.15
Hematocrit	0.5 (0.04)	0.5 (0.03)	0.77	0.5 (0.03)	0.5 (0.03)	0.20
Platelets	383 (217)	343 (250)	0.67	451 (325)	497 (313)	0.74

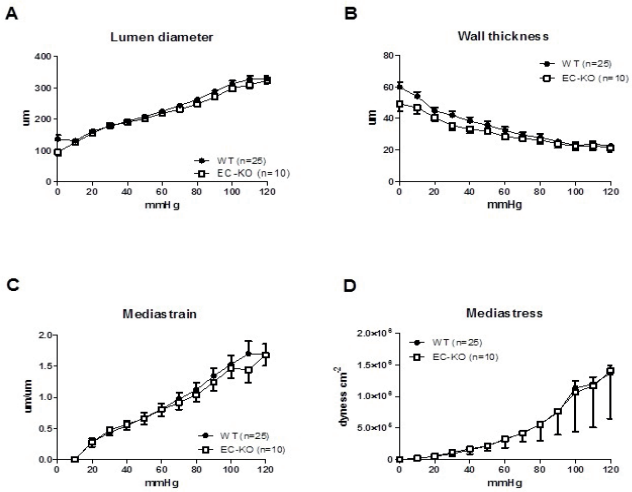
Values are Mean (SD).



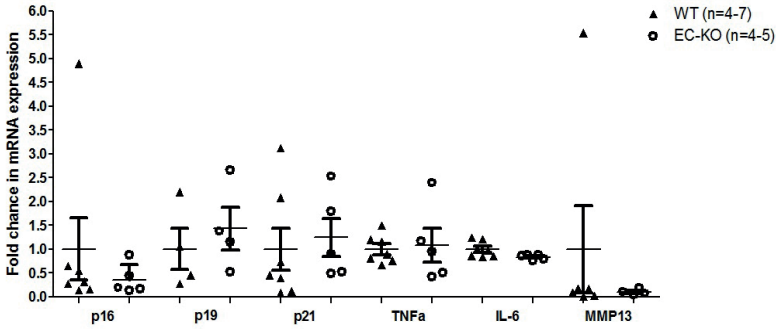
Supplementary Figure S1. General health and pathology findings. Kaplan-Meier survival analyses of EC-KO (n= 11) vs WT (n=12).



Supplementary Figure S2. Constrictor responses at 3 and 5 months in aorta and iliac arteries. Contractile responses (in milli Newtons) to KCl 100 mM in aorta and iliac arteries (A, E), and to angiotensin II (B, F), endothelin-1 (C, G) and phenylephrine (D, H) in iliac arteries at 3 and 5 months. * = p<0.05 (GLM-RM). Arteries from 9-10 EC-KO and 11-33 WT were studied.



Supplementary Figure S3. Mechanical properties of the carotid artery measured in a vascular perfusion set-up at 5 months. Lumen diameter (A), wall thickness (B), mediastrain (C) and mediastress (D) measured at increasing intraluminal pressures. Arteries from 10 EC-KO and 25 WT were studied.



Supplementary Figure S4. mRNA expression of SASP components measured in aorta. aortic mRNA levels of p16, p19, p21, TNFα, IL-6 and MMP13 in 5 months-old WT vs EC-KO mice. Samples from 4-5 EC-KO and 4-7 WT were included.

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CHAPTER 2.3

Dissecting the association of autophagy-related genes with cardiovascular diseases and intermediate vascular traits: a population-based approach

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Abstract

Autophagy is involved in cellular homeostasis and maintenance and may play a role in cardiometabolic health. We aimed to elucidate the role of autophagy in cardiometabolic traits by investigating genetic variants and DNA methylation in autophagy-related genes in relation to cardiovascular diseases and related traits. To address this research question, we implemented a multidirectional approach using several molecular epidemiology tools, including genetic association analysis with genome wide association studies data and exome sequencing data and differential DNA methylation analysis. We investigated the 21 autophagy-related genes in relation to coronary artery disease and a number of cardiometabolic traits (blood lipids, blood pressure, glycemic traits, type 2 diabetes). We used data from the largest genome wide association studies as well as DNA methylation and exome sequencing data from the Rotterdam Study. Single-nucleotide polymorphism rs110389913 in *AMBRA1* (p-value=$4.9\times10^{-18)}$ was associated with blood proinsulin levels, whereas rs6587988 in *ATG4C* and rs10439163 in *ATG4D* with lipid traits (*ATG4C*: p-value=2.5×10^{-15} for total cholesterol and p-value=3.1×10^{-18} for triglycerides, *ATG4D*: p-value=9.9×10^{-12} for LDL and p-value=1.3×10^{-10} for total cholesterol). Moreover, rs7635838 in *ATG7* was associated with HDL (p-value=1.9×10^{-9}). rs2447607 located in *ATG7* showed association with systolic blood pressure and pulse pressure. rs2424994 in *MAP1LC3A* was associated with coronary artery disease (p-value=5.8×10^{-6}). Furthermore, we identified association of an exonic variant located in *ATG3* with diastolic blood pressure (p-value=6.75×10^{-6}). Using DNA methylation data, two CpGs located in *ULK1* (p-values=4.5×10^{-7} and 1×10^{-6}) and two located in *ATG4B* (2×10^{-13} and 1.48×10^{-7}) were significantly associated with both systolic and diastolic blood pressure. In addition one CpG in *ATG4D* was associated with HDL (p-value=3.21×10^{-5}). Our findings provide support for the role of autophagy in glucose and lipid metabolism, as well as blood pressure regulation.

Introduction

Autophagy is a highly conserved catabolic process involved in the degradation of long-lived proteins and dysfunctional organelles (1). Under conditions of oxidative stress, hypoxia and nutrient deprivation, autophagy is activated as a key mechanism of cell survival by degradation and recycling of damaged organelles and protein aggregates (2). A disturbance in removal of these non-functional cellular components is a general impairment in housekeeping of the cell, and could lead to important phenotypic changes at cell and tissue levels (3). Several studies, mainly based on animal models, have provided evidence about the role of autophagy in the progression of ageing and in particular in atherosclerosis, inflammation, and cardiovascular disease (2, 4-8). A number of autophagy-related mechanisms have been studied for cell survival and in some instances the role of genetic variants involved in molecular mechanisms of autophagy were investigated in relation to cardiometabolic health in animal models (9). Systemic knockout of autophagy-related genes (ATG) in mice has shown the role of dysfunctional autophagy in hyperglycemia (10), hypoinsulinemia and increased basal Ca2+ concentrations in vascular smooth muscle cells (11, 12). However, this association has not been studied at population level. Genetic and epigenetic variations in ATG could affect the autophagy process in human cells, modify certain metabolic traits and eventually cause susceptibility to cardiometabolic disorders (13, 14). In this study, by using Genome-Wide Association Studies (GWAS) data and data from the Rotterdam Study, a population-based prospective cohort study, we aimed to examine the association of genetic and epigenetic variation in ATG with intermediate vascular traits and cardiovascular outcomes.

Materials and Methods

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Identification of autophagy-related genes

To select the set of autophagy-related genes to be studied, a literature search was performed and was updated by August 2018 in PubMed using the MeSH terms: Autophagy, genome-wide association studies, candidate gene studies, and knockout experimental models. We identified a total of 30 genes related to autophagy and associated pathways. We further included the genes in the pathway analysis done by QIAGEN's Ingenuity Pathway Analysis Software (IPA, http://www.ingenuity.com, Fig 1) to determine genes enriched for autophagy. Core Analysis implemented by IPA was used to interpret the role of these genes in the context of biological processes, pathways and molecular networks. IPA uses right-tailed Fisher exact test to identify enriched canonical pathways and diseases associated to these genes. A p-value < 0.05 was considered significant. A set of 21 genes showed significant enrichment for autophagy (p-value=1.33×10^{-40}) (Table 1). We studied genetic variants within the genes and those located within 10 MB upstream and downstream of the gene.

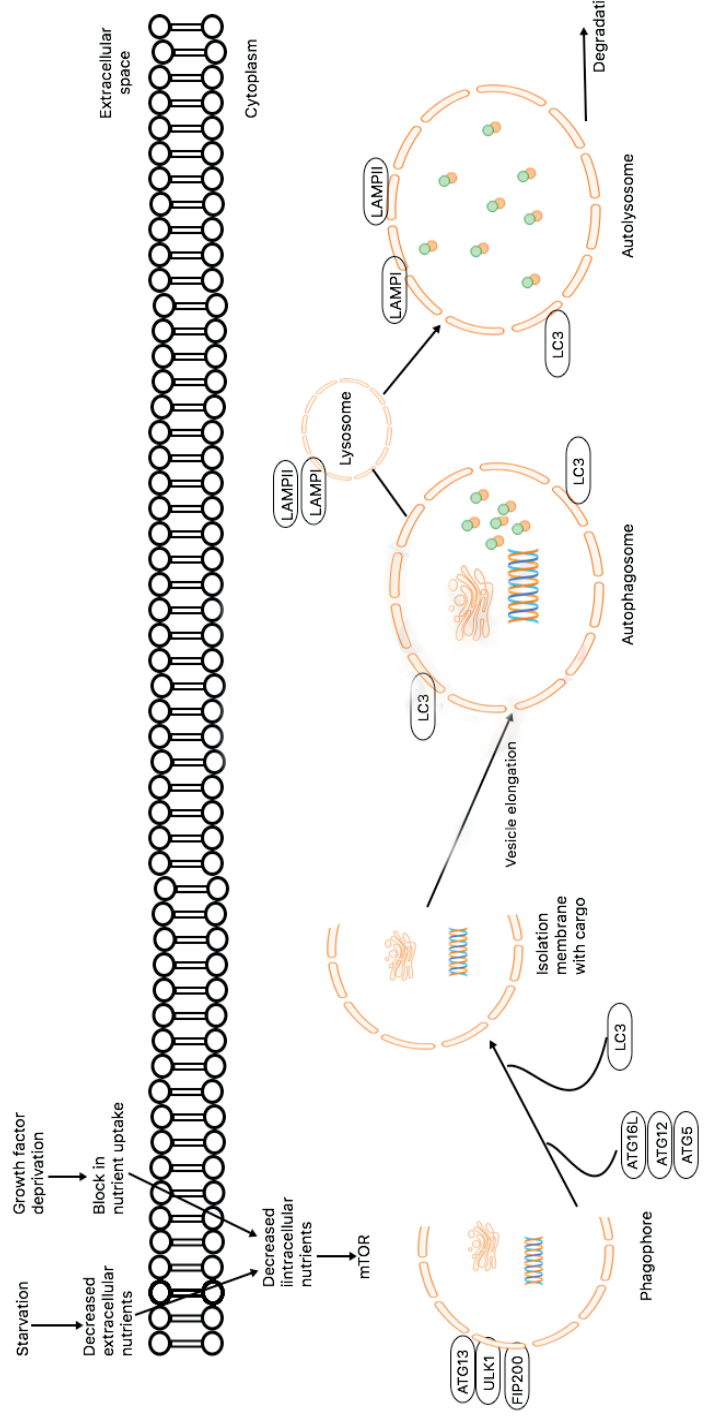

Figure 1. Molecular pathway of autophagy. Autophagy activation is controlled by several factors, including amino acid and nutrient deprivation, which stimulates mTOR-related pathways. Upon mTOR inhibition by starvation or rapamycin, ATG13/ULK1/FIP200 form a stable complex, essential for autophagy activity. Highly coordinated autophagy-related proteins are recruited to the phagosphore to participate in the autophagosome formation. ATG7 is one of the crucial regulators of this process. Lipidation of LC3-I known as LC3-II is attached to the autophagosome membrane and is widely used to monitor autophagy induction. When autophagosome formation is completed, the fusion between autophagosome and lysosome is mediated by lysosomal membrane protein LAMP-2. ATG12 conjugation to ATG5 is a crucial step in autophagosome formation. ATG7 regulates ubiquitin-like reaction that mediates this mechanism and subsequent processes in the autophagy machinery.

We excluded SNPs with a reported minor allele frequency (MAF) less than 1% in NCBI.

Table 1. Autophagy-related genes and the number of SNPs selected for this study

Gene	Pathway	Chromosome	Independent SNPs
AMBRA1	Autophagic vacuole formation	11	34
ATG1	Molecular activation of autophagy	12	18
ATG3	Protein transport. Protein ubiquitination	3	27
ATG4B	Autophagic vacuole formation	2	8
ATG4C	Autophagic vacuole formation	1	43
ATG4D	Autophagic vacuole formation	19	19
ATG5	Autophagic vacuole formation	6	49
ATG7	Protein transport. Protein ubiquitination	3	98
ATG9A	Autophagic vacuole formation	2	11
ATG9B	Autophagic vacuole formation	7	16
ATG10	Protein transport	5	101
ATG12	Autophagic vacuole formation	5	12
ATG16L1	Autophagic vacuole formation	2	43
ATG16L2	Protein transport	11	8
BECLIN-1	Regulation of autophagy	17	2
DRAM1	Autophago-lysosome formation	12	43
GABARAP	Autophagic vacuole formation	17	7
GABARAPL1	Autophagic vacuole formation	12	23
GABARAPL2	Autophagic vacuole formation	16	11
MAP1LC3A	Autophagic vacuole formation	20	16
MAP1LC3B	Autophagic vacuole formation	16	15

Genetic association

For each gene, we studied the association of the genetic variants intermediate vascular traits and cardiovascular outcomes. We extracted the summary statistics for the association of the selected SNPs with traits and diseases from the most recent GWAS meta-analysis of 12 traits: fasting glucose, fasting insulin, fasting proinsulin (15-18), type 2 diabetes (19), systolic blood pressure (SBP), diastolic blood pressure (DBP) (20), total cholesterol (TC), triglycerides (TG), HDL cholesterol (high density lipoprotein), LDL cholesterol (low density lipoprotein) (21) and coronary artery disease (CAD) (22). Details of the consortia included are provided in supplementary note and number of subjects included in each study is shown in Table 2.

Exonic variants

We sudied the association of exonic variants and DNA methylation status in ATG with lipid, glycemic and intermediate vascular traits using data from the Rotterdam Study. The Rotterdam Study is an ongoing prospective, population-based cohort study started in 1990 including subjects from Ommoord district in the city of Rotterdam (The Netherlands). The objectives and details of the study are described elsewhere (24). The Rotterdam Study

comprises a total of 14,926 subjects aged 45 years and over who were recruited in three sub cohorts in 1989-1993, 2000-2001, and 2006 (24). Genomic DNA was extracted from peripheral blood mononuclear cells. Whole-Exome sequencing (WES) was performed at the Rotterdam Genomics Core in Erasmus Medical Centre. WES was conducted on 2,998 paired-end sequenced samples using the Illumina hiSeq2000 (2×100bp reads). Indels and single nucleotide variants were filtered out and evaluated using GAKs Variant Evaluation; variants with a call rate <0.97, duplicate samples, duplicate variants and >5% of missing genotypes were considered as exclusion criteria. A total of 2628 samples passed through all technical quality control and GATKs Haplotype Caller was used to call SNPs and indels simultaneously. Annovar software tool was used to functionally annotate each genetic variant. Each variant was coded as 0, 1 and 2 representing two reference alleles, one reference allele and one mutated allele and two mutated alleles, respectively. Based on the functional annotation, we identified a total of 13 loss of function (LoF) variants defined as changes in DNA sequence predicted to completely disrupt the formation and/or function of a protein.

DNA methylation data collection and normalization

DNA was extracted from whole peripheral blood (stored in EDTA tubes) by standardized salting out methods. The Infinium HumanMethylation450 BeadChip array was employed to determine the gene methylation status using DNA from whole blood samples of 468 individuals from third visit of the second sub-cohort, 731 individuals from first visit of the third sub-cohort, and 251 individuals from second visit of the third sub-cohort (no-overlap). The array covers approximately 485,577 methylation sites (in 99% of Ref Seq genes approximately) with an average of 17.2 CpG sites per gene region. In short, samples (500 ng of DNA per sample) were first bisulfite treated using the Zymo EZ-96 DNA-methylation kit (Zymo Research, Irvine, CA, USA). Next, they were hybridized to the arrays according to the manufacturer's protocol. The methylation percentage of a CpG site was reported as a beta-value ranging between 0 (no methylation) and 1 (full methylation). The quality control of DNA methylation data was conducted for both sample and probe based on a p-value threshold for gene detection of 0.01. Data normalization was done based on color bias correction of methylated and unmethylated signals, per plate and separate color background adjustment. Outliers beyond the 3rd quartile were excluded and the final dataset comprised 463,456 CpG sites.

Clinical traits and outcomes

Lipid measurements were carried out using venous blood samples obtained from all participants of the Rotterdam Study. Total cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). Low density lipoprotein cholesterol levels were estimated indirectly from measurements of TC, HDL, and TG by means of the Friedewald equation (25). The corresponding interassay coefficients of variation was <2.1%.

During each visit, blood pressure was measured twice in the right arm using a random-zero sphygmomanometer, in sitting position, after a resting period of 5 minutes. After 2006 Omron M6 Comfort and Omron M7 devices were used. The measurements were taken in duplicate. The average of the 2 measurements was used in the analyses. A qualified physician at the research center collected data on indication for use of BP-lowering medication during the interview.

Glucose and insulin were measured from venous blood samples. Fasting insulin and glucose were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). The interassay coefficients of variations are <8% and <1.4% for insulin and glucose respectively. Type 2 diabetes was defined according to recent WHO guidelines, as a fasting blood glucose ≥ 7.0

mmol/L, a non-fasting blood glucose ≥ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication (26). Information regarding the use of diabetes medication was collected from both structured home interviews and linkage to pharmacy records (27).

Statistical Analysis

SNP pruning and Genome-wide association studies look up

We found 5398 SNPs in the 21 identified autophagy genes. Linkage disequilibrium (LD)-based SNP pruning implemented in PLINK software was applied using a genetic correlation threshold of 0.5 to calculate the number of independent SNP per gene (28). Using LD pruning, we identified 604 independent SNPs in 21 genes directly involved in autophagy pathway. We applied Bonferroni correction for 604 SNPs and 4 traits. The study-wide significance threshold was set at 2.07×10^{-5} (0.05/604 SNPs×4 traits).

Exome sequencing

We used Seqmeta, an R package to perform region-based tests of rare DNA variants, to conduct multiple regression models in order to determine association between LoF variants and intermediate vascular traits including age and sex as a covariates. The package allows to obtain the scores and MAF for each gene with the function prepScores and to calculate both gene-based and single variant-based associations based on the prepScores output (29, 30). The significance level was based on Bonferroni correction to adjust for multiple comparisons according to the number of variants and traits being tested. Here, we set a p-value threshold as 9.62×10^{-4} (0.05/(4 traits×13 LoF)).

Epigenome-wide association studies

We conducted epigenome wide association studies (EWAs) on blood pressure, glucose, insulin, HDL, LDL, triglycerides and total cholesterol. Beta-values to quantify the DNA methylation levels were used in a linear mixed-effect regression model with the outcomes of interest as a dependent variable. We fit the primary regression model adjusting by age and sex, cell counts, technical covariates and smoking. Further adjustments including, hypertensive or lipid lowering treatment, prevalent diabetes mellitus were employed for blood pressure and lipids levels respectively. We combined the results of the three sub-cohorts by conducting a fixed effect meta-analysis using the inverse variance method implemented in METAL (31). We established the target set of ATG-CpGs considering the CpG islands found in upstream, downstream and promoter regions of each gene. CpG sites per gene were obtained using the UCSC genome browser (http://genome.ucsc.edu/). Statistically significant association was set at 5.63×10^{-5} based on Bonferroni correction for 296 CpG sites and three trait groups (glycemic traits, lipids and blood pressure). Next to the identification of epigenetic marks in ATG genes, we extended our search of methylation quantitative trait loci (meQTLs) by the identification of both *cis* and *trans* associated SNPs using a large meQTLs dataset from mQTLdb resource (http://www.mqtldb.org/) (32).

Results

Genetic association

The results of genetic association of the SNPs and cardiometabolic traits are presented in Supplementary Table 1 (Table S1). The lead significant variants are presented at Table 3. Six SNPs at three genes, *AMBRA1* (Lead SNP: rs11038913), *ATG13* (rs8914), and *ATG16L1* (rs4944804) passed the significant threshold for association with pro-insulin serum levels.

Table 2. Description of GWAS meta-analysis on cardiometabolic disorders

Trait	Consortium	Sample size
Coronary artery disease	UK Biobank/CardiogramplusC4D (23)	122,733 cases / 424,528 controls
Fasting glucose	MAGIC (15)	133,01
Fasting insulin	MAGIC (16)	108,557
Proinsulin	MAGIC (17)	10,701
Type 2 diabetes	DIAGRAM (19)	26,676 cases / 132,532 controls
Blood pressure	UK biobank (20)	140
Total cholesterol	ENGAGE (21)	100,184
Triglycerides	ENGAGE (21)	96,598
HDL cholesterol	ENGAGE (21)	99,9
LDL cholesterol	ENGAGE (21)	95,454

MAGIC: Meta-analysis of Glucose and Insulin-related traits Consortium; DIAGRAM: Diabetes Genetics Replication and Meta-analysis; CARDIOGRAMplusC4D: Coronary Artery Disease Genome wide replication and Meta-analysis plus Coronary Artery Disease; HDL: High density lipoprotein; LDL: Low density lipoprotein

Total cholesterol level was significantly associated with 34 SNPs at *ATG4D* (rs10439163) and one SNP at *ATG4C* (rs6587988). HDL-cholesterol was associated with 41 variants at *ATG7* (rs7635838). One SNP at *ATG4D* (rs10439163) was associated with LDL-cholesterol and the one at *ATG4C* (rs6587988) was further associated with triglyceride levels. Moreover, 56 SNPs and 38 SNPs located in *ATG7* were associated with SBP and pulse pressure, respectively. Two SNPs (rs2424994 and rs6088521) located at *MAP1LC3A* showed an association with CAD.

In addition to this significant association, we also observed borderline significant association between SNPs at *ATG7* with SBP, *ATG4B* with fasting proinsulin, and *MAP1LC3A* with HDL-cholesterol. No associations were found between ATG and type 2 diabetes.

Loss of function variants

We used data from 2,628 participants of the first sub-cohort of the Rotterdam Study. Baseline characteristics of the individuals are presented in Supplementary Table S2 (Table S2). We identified 13 LoF stop-gain or splicing mutations in *ATG4C, ULK1, GABARAPL2, GABARAP, ATG4D, ATG3, ATG10* and *ATG5* (Table 4). We used single variant-based analysis implemented in SeqMeta to assess the association of LoF mutations with intermediate vascular traits. We identified one exonic variant in *ATG3* associated with DBP and fasting glucose (p-value for SBP: 0.054). The C/A variant found in this gene is considered a nonsense mutation and is related with stop-gain function of the protein. We also found a nominally significant association between an exonic variant in *ULK1* and triglycerides (p-value=0.0012). The results of association between these variants and studied traits and diseases are mentioned in Supplementary Table 3 (Table S3).

Table 3. SNPs in autophagy genes with the most significant association with cardiometabolic traits and diseases

SNP	Gene	Trait/Disease	P-value	MAF
rs7635838	*ATG7*	HDL-cholesterol	1.9×10^{-9}	0.49
rs2447607		Systolic blood pressure	3.2×10^{-8}	0.40
		Pulse pressure	3.5×10^{-7}	0.40
rs10439163	*ATG4D*	LDL-cholesterol	9.9×10^{-12}	0.43
		Total cholesterol	1.4×10^{-10}	0.43
rs7255312		Coronary artery disease	6.1×10^{-6}	0.10
rs6587988	*ATG4C*	Triglycerides	3.1×10^{-18}	0.24
		Total cholesterol	2.5×10^{-15}	0.24
rs11038913	*AMBRA1*	Fasting proinsulin	4.9×10^{-18}	0.08
rs8914	*ATG13*		7.1×10^{-18}	0.03
rs4944804	*ATG16L1*		8.9×10^{-18}	0.13
rs6088521	*MAP1LC3A*	Coronary artery disease	4.1×10^{-6}	0.50

MAF: minor allele frequency; HDL: High-density lipoprotein; LDL: low-density lipoprotein. Significance threshold: 2.07×10^{-5}

DNA methylation at autophagy genes

In total, 1450 individuals with a mean±SD age of 60.6 ± 5.3 years were included in the analysis. The association of the studied CpGs and all traits and diseases are presented in Supplementary Table 4 (Table S4). In a fully adjusted model, we found differential methylation at ATG4B and ULK1 were associated with blood pressure (Table 5). Hypermethylation in these CpGs were associated with lower SBP/DBP. On the other hand, no significant associations between DNA methylation and glycemic and lipid traits were found in the study population. Using mQTLdb database, we identified five trans-meQTLs associated with cg02710553. The variants were located in an intergenic region close to MAP3K7. Furthermore, 23 trans-meQTLs associated cg06006530 were identified close to CDH18 gene. meQTLs of cg02710553 were found to be associated with CAD at nominal significance level (p-value=0.02).

Discussion

In this study we used genetic and epi-genetic factors to assess the role of autophagy genes in cardiometabolic traits and disorders at population level. To this end, we utilized a number of genetic variants, LoF variants and DNA methylation in autophagy related genes. All approaches indicated associations between autophagy genes and certain cardiometabolic traits, mainly blood pressure, lipid levels and proinsulin levels but not coronary artery disease.

Our findings on the association between genetic variants in *AMBRA1, ATG13 and ATG16L1* and proinsulin levels are in agreement with previous evidence reporting autophagy deficiency as an important determinant in the pathogenesis of insulin resistance and diabetes (33). *AMBRA1* participates in the activation of beclin-1-regulated autophagy and favors the autophagosome core complex with the participation of other ATG proteins such as *ATG13* and *ATG16L1* (34). The upstream autophagy-signaling network controlled by *AMBRA1* is crucial for the metabolic response to a vast number of stress stimuli, ranging from starvation to hypoxia or DNA

Table 4. LOF variants associated with intermediate vascular traits

Variant	Gene	Trait	P-value
3:112277264	*ATG3*	Diastolic blood pressure	6.8×10^{-6}
		Fasting glucose	0.043
12:132404136	*ULK1*	Triglyceride	1.3×10^{-3}
12:132404136	*ULK1*	Total cholesterol	0.18
12:132404136	*ULK1*	HDL-cholesterol	0.08
12:132404136	*ULK1*	Fasting insulin	0.65
12:132404136	*ULK1*	LDL-cholesterol	0.19
12:132404136	*ULK1*	Systolic blood pressure	0.05

Significance threshold: 9.61×10^{-4}

Table 5. DNA methylation, blood pressure and HDL

CpG site	Gene	Trait	Effect	P-value*
cg08462942	*ATG4B*	Systolic blood pressure	-0.00026	2.0×10^{-13}
cg06006530			-0.0019	1.5×10^{-7}
cg02710553	*ULK1*	Systolic blood pressure	-0.0018	4.5×10^{-7}
		Diastolic blood pressure	-0.0018	1.0×10^{-6}
cg10819350	*ATG4D*	HDL-cholesterol	-0.0158	3.2×10^{-5}

*Adjusted for age, sex, cell counts, batch effects, anti-hypertensive and lipid-lowering medication. HDL: high density lipoprotein, significance threshold: 5.63×10^{-5}

damage. Dysfunctional autophagy has been suggested as a key process related to an impaired proinsulin/insulin homeostasis observed in pancreatic beta cells from experimental models (35). Conditional knockout of *ATG7* in high-fat-fed C57BL/6 mice has resulted in declined insulin secretion, impaired glucose tolerance and degeneration of pancreatic islets (36). In autophagy-deficient cells, the insulin secretion is restrained enabling the proinsulin accumulation in secretory granules and increased secretion in response to stimuli (37). At population level, in line with our findings, an exome array analysis conducted in 8229 individuals showed association between genetic variants in *AMBRA1* and *ATG13* with fasting proinsulin concentrations (38). The role of impaired autophagy in proinsulin degradation has highlighted the importance of the autophagy pathway on the design of novel therapeutic strategies, aimed to manipulate proinsulin clearance as means to increase the insulin secretion in diabetic population.

Our findings on common genetic variants in ATG and blood lipids support the hypothesis that autophagy may play a role in dyslipidemia. The role of impaired autophagy and lipid metabolism has been established from the identification of significant increased levels of hepatic triglycerides and cholesterol content in hepatocyte-specific knockout mice of *ATG5* (39). Moreover *ATG7* knockout mice models have displayed severe morphological abnormalities in the structure of white adipocytes, as well as an aggravated insulin resistance with increased lipid content and inflammatory changes (40, 41). In summary, upregulation of autophagy leads to a decrease of triglycerides and cholesterol in plasma, reduced lipid store as well as LDL oxidation and free fatty acid B-oxidation and an increase of folding and traffic

proteins (42). Further investigations are needed to clarify this association and potential pathways linking autophagy with blood lipids levels. Recent findings of a critical role for macroautophagy in the metabolism and storage of cellular lipids have now suggested that alterations in autophagy may mediate human disorders marked by excessive cellular lipid stores.

In contrast, mice with endothelial specific deletion of *ATG7* have shown normal blood pressure and normal vessel architecture compared to wild types (43). We further identified a suggestive association between LoF variants annotated in *ATG3* and glucose levels. It has been shown that glycogen autophagy in newborns serves as a mechanism of glucose homeostasis (44). In adult animals, the administration of glycogen autophagy-inhibiting insulin triggers a reduced rate of breakdown of liver glycogen by autophagy (45). Impaired mechanisms related to autophagosomal glucose production and the influence of gluconeogenesis may lead to a dissociation of gluconeogenic glucose production from blood glucose levels (46). On the other hand, we found no association between changes in DNA methylation patterns and glycemic traits. This might be explained by the fact that methylation is a tissue-specific process. Therefore, SNPs could be operating independently from methylation patterns.

We studied DNA methylation at autophagy-related genes and found differential patterns at *ULK1* and *ATG4B* associated with blood pressure levels. Increased blood pressure is determined by a complex machinery regulated, among others, by the renin-angiotensin system (RAS) (47). The interaction between autophagy and RAS has been previously examined. Porrello et al, provided the first evidence for an interplay between these two mechanisms in cardiomyocytes. This study reported that rat cardiomyocytes developed and augmented (via the angiotensin II receptor type 1) or inhibited (via the angiotensin II receptor type 2) autophagic response on stimulation by angiotensin II (48). The association between autophagy and blood pressure found in our study could be explained by its interaction with RAS and its role in hypertension. Given the fact that autophagy and RAS are both involved in many pathophysiological processes, further investigation is warranted to better understand the molecular mechanism behind this interaction and its role in pathological conditions. In addition, we found an association between DNA methylation in *ATG4D* and HDL levels. *ATG4D* is known to participate in the delipidation of GABARAP-L1, whereas the silencing of *ATG4D* abrogates GABARAP-L1 autophagosome formation (49). Experimental evidence of the role of this gene on lipid transport/metabolism is currently lacking.

Epi-genetic associations are subject to confounding and reverse causation. One approach to overcome these biases in molecular epidemiology is to use genetic instruments. In this study, we found that meQTL at cg02710553 was associated with CAD. This might indicate that the association of cg2710553 and CAD is causal.

We used both genomics and epi-genomics data to examine the role of autophagy related genes in cardiometabolic traits and diseases. Although we have several significant association, we did not find a consistent association with a certain gene or a certain trait across all approaches. It should be noted that genomic and epi-genomic approaches are not always pointing to the same gene. Moreover, it is not a surprise if a different gene in a certain pathway has more or less importance for a certain trait. The high complexity of the autophagy mechanism may be a major contributor of the heterogeneity of the findings in our results (50).

Our study has several strengths. First, we used data from largest GWAS, which has provided the best statistical power that could be achieved. Second, we used both genetic and epi-genetic approaches towards the research question. Third, we used exome sequence data, which in contrast to GWAS, is not based on a studying a proxy variant. Our study also had several limitations. First, the sample size for exome sequence analysis was much smaller than

the GWAS, thus the statistical power was significantly lower. Loss of function variants are mostly found in low frequency, indicating that they are enriched for mildly deleterious polymorphisms suppressed by negative natural selection (51). Therefore, smaller populations might also provide enough statistical power to detect their effect, however, this could not be ruled out that lack of association in our analysis might be a results of small sample size.

Second, DNA methylation pattern is tissue/cell line specific. As it is common in epidemiologic studies we used DNA methylation in whole blood, a cell type mixture, which might not be relevant for some of the traits. In our study DNA methylation was captured from the assessment of leukocytes. Although leukocytes are not relevant tissue for some of the traits that we have assessed in our analysis, they are the main tissue available in large scale in epidemiologic studies and evidence has demonstrated that methylation patterns might correlate between blood and the relevant tissues, suggesting that the use of blood tissue could yet be informative (52-55). However, such findings should be validated in subsequent studies.

In conclusion, this study is the first to examine the role of autophagy-related genes in intermediate vascular traits using a population-based approach. We have characterized the role of common and rare genetic variants as well as epigenetic variations of autophagy-related genes on several traits. Despite the heterogeneity of our findings across approaches employed and traits evaluated, we found many associations between autophagy genes and cardiometabolic traits and diseases. The integral approach covered by this study could contribute to further analysis evaluating the role of autophagy in other human diseases and traits, as well as the design of experimental studies targeting other autophagy-related genes and/or associated pathways.

Supplementary Information is available in the online version of the paper (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0214137).

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CHAPTER 2.4

Genetic and clinical determinants of abdominal aortic diameter: Genome-wide association studies, exome array data and Mendelian randomization study

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Abstract

Progressive dilation of the infrarenal aortic diameter is a consequence of the ageing process and is considered the main determinant of Abdominal Aortic Aneurysm (AAA). We aimed to investigate the genetic. We conducted a meta-analysis of genome-wide association studies in ten cohorts (n=13,542) imputed to the 1000 Genomes Project reference panel including 12,815 subjects in the discovery phase and 727 subjects (Partners Biobank cohort) as replication. Maximum anterior-posterior diameter of the infrarenal aorta was used as AAD. We also included exome array data (n=14,480) from seven epidemiologic studies. Single-variant and gene-based associations were done using SeqMeta package. A Mendelian randomization analysis was applied to investigate the causal effect of a number of clinical risk factors on AAD. In GWAS on AAD, rs74448815 in the intronic region of *LDLRAD4* reached genome-wide significance (beta=-0.02, SE=0.004, p-value= 2.10×10^{-8}). The association replicated in the PBIO1 cohort (p-value= 8.19×10^{-4}). In exome-array single-variant analysis (p-value threshold=9 $\times10^{-7}$), the lowest p-value was found for rs239259 located in *SLC22A20* (beta = 0.007, p-value =1.2×10^{-5}). In the gene-based analysis (p-value threshold=1.85×10^{-6}), *PCSK5* showed a positive association with AAD (p-value= 8.03×10^{-7}). Furthermore, in Mendelian randomization analyses, we found evidence for genetic association of pulse pressure (beta=-0.003, p-value=0.02), triglycerides (beta=-0.16, p-value=0.008) and height (beta= 0.03, p-value<0.0001), known risk factors for AAA, consistent with a causal association with AAD. Our findings point to new biology as well as highlighting gene regions in mechanisms that have previously been implicated in the genetics of other vascular diseases.

Introduction

Abdominal aortic diameter (AAD) is an index to measure the widening of abdominal aorta and therefore is a parameter for screening, surveillance and clinical management of abdominal aortic aneurysm (AAA), a focal dilatation of the abdominal aorta (1). An AAD equal or higher than 30 mm is used for diagnosis of AAA and an AAD ≥ 50-55 mm is an indication for surgical intervention, depending on the patient's risk factors (2, 3). Due to the risk of rupture, AAA is potentially lethal (4) and between 1990 and 2010 the global AAA death rate has increased from 2.49 per 100,000 to 2.78 per 100, 000 inhabitants (5). Progressive dilation of the aortic wall is positively associated with ageing, a higher collagen-to-elastin ratio, increased vascular stiffness and high pulse pressure (1).

Genetic and environmental factors are thought to affect the risk of AAD. Although a robust estimate of AAD heritability is lacking, AAA has been shown to be more than 70% heritable (6) and individuals with a first-degree relative with AAA have a 2-fold higher risk of developing an AAA (7). Initial genetic studies conducted in populations with AAA have identified genes related to abnormal aortic dilatation. These studies have encouraged the implementation of alternative approaches to further deepen investigation of the genetics underlying aortic dilation, including the use of aortic diameter as a continuous trait, leveraging additional power over discrete trait approaches for the limited sample sizes available (8). Here, we aimed to identify genetic factors that affect AAD. We applied GWAS to identify (common variants) associated with AAD using data from ten studies comprising 13,542 participants and exome array analysis to identify (rare variants), using data from seven studies on 14,480 participants. Further, we implemented Mendelian randomization (MR) analysis to investigate the causal effect of a number of risk factors on AAD.

Methods

Participating studies

The study was done primarily in the framework of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium, an international collaborative effort to facilitate GWAS meta-analysis and replication opportunities among multiple large cohorts (9). The discovery panel included European American participants from the Cardiovascular Health Study (CHS) (GWAS, n= 2,699; exome array, n=3,294), Cooperative Health Research in the Region Augsburg (KORA) (GWAS, n = 354; exome array, n=337), Framingham Heart Study (FHS) (GWAS and exome array, n = 3,262), Study of Health in Pomerania (SHIP-2 and SHIP-T) (GWAS, n = 1,010 [SHIP-2] and 827 [SHIP-Trend]; exome array, n= 2,848), The Multi-Ethnic Study of Atherosclerosis (MESA) (GWAS, n = 750; exome array, n=740), and the Rotterdam Study (RS) (GWAS, n = 3,913; exome array, n= 327) and BIOIMAGE (exome array, n=3,672). As a replication study, we used the Partners Biobank cohort (PBIO1) cohort (GWAS, n = 727). Details of the cohorts included are provided in supplementary information (supplementary Table S1a, S1b and Table S2).

Abdominal aortic phenotypes

Maximum aortic diameter was defined as the largest measurement of anterior-posterior diameter of the infrarenal aorta measured by MRI, ultrasound or CT-scan using previously described reading protocols. Due to positive skewness, we used natural log-transformed AAD measurements. A description of the method employed in AAD measurement by each study is provided in methods in the supplementary information.

Genotyping and imputation

The studies employed SNP arrays available from Illumina or Affymetrix. Using available imputation techniques, each cohort imputed approximately >37 million variants from 1000 Genomes reference panel (phase 1 version 3) and applied strict quality control checks. Further information on the genotyping and imputation methods is detailed in supplementary Table S3. Exonic and non-exonic variants were genotyped using the Illumina Infinium HumanExome BeadChip kit. The array covers > 240,000 markers, mostly coding variants discovered through exome sequencing in approximately 12 000 individuals and observed at least 3 times across at least 2 existing sequence datasets, including nonsynonymous, splicing, stop-altering variants, most of which are rare (http://genome.sph.umich.edu/wiki/Exome_Chip_Design). In order to remove suspect variants and to minimize population stratification, exome array data quality control was performed based on Best Practices and Joint Calling of the HumanExome BeadChip: The CHARGE Consortium (10). Further details on methods employed by each study are outlined in supplementary information.

Statistical analyses within studies

Each study implemented GWAS and exome array data analyses based on a predefined analysis plan. We conducted linear regression models to evaluate the association of log-transformed AAD with genetic variations. For each variant, each study fits additive genetic models regressing trait on genotype dosage (0–2 copies of the variant allele). In the primary model, each regression was adjusted by sex, age and principal components. Further adjustments including study site and/or familial structure were performed if necessary. We determined the association of each SNP with natural logarithm transformed AAD as the regression slope, its standard error and its corresponding p-value. Furthermore, we choose nine SNPs previously identified for AAA in GWAS and sought replication in our study (8, 11-14). To conduct study-specific analyses on exome array data, all the studies used the prepScores function, implemented in the R package "SeqMeta" (15). Briefly, the prepScores function calculates scores and minor allele frequencies (MAF) for each variant interrogated within a gene. Log-transformed AAD was regressed against sex, gender and principal component analysis. Subsequently, the calculated prepScores by each cohort were combined in a single variant meta-analysis and gene-based meta-analysis.

Genome wide association study quality control and meta-analysis

Each cohort conducted a GWAS on AAD in adult subjects with genetic information available. Subsequently, GWAS summary statistics were uploaded to a central repository for QC and meta-analysis. We conducted data quality control at study file level of GWAS outputs using EasyQC (16) to identify file naming errors, erroneous SNP genotype data and false association caused by incorrect analysis models. We filtered out variants with a poor imputation quality (SNPTEST INFO value or MACH RSQR <0.4, Probabel <0.3) prior quality control. We further calculated genomic inflation for each study to determine population stratification or any other inconsistencies that might have inflated the test statistics. After QC, we meta-analyzed a range of 8.8-11.1 million of variants per study. The meta-analysis of linear regression estimates and standard errors using an inverse-variance weighting approach was conducted using METAL (17). We reported SNPs that were present in at least five studies and with an average MAF of at least 1%. Variants with a meta-analyzed p-value $\leq 5 \times 10^{-8}$ were considered significant.

Meta-analysis of rare exonic variants from exome array

Study-specific summary statistics, such as estimated regression coefficient for each

variant and its standard error (prepScores) were meta-analyzed on single variant level, to perform score tests for single SNP associations, and in a gene-based test [sequence kernel association test (SKAT)] using seqMeta (15). Single variant results were filtered for a pooled MAF > 0.005 and those included in at least 50% of the cohorts were reported. Furthermore, variants with MAF ≤5% were included in the gene-level test. Additional arguments implemented in SKAT meta-analysis allow to specify the method of p-values calculation. We used the default method "saddlepoint", which appears to have higher relative accuracy (15, 18) . We defined a statistical significance threshold of single variant and gene-based exome array meta-analysis based on Bonferroni correction for multiple testing, ~55,434 variants (p-value< 9 $\times 10^{-7}$) and ~16,378 genes (p-value< 3 $\times 10^{-6}$), respectively. Genetic variants in exome array were annotated using SNPInfo file 12 version 1.0.

Identification of expression quantitative trait loci (eQTL)

Furthermore, we examined the effect of associated genetic variants on the expression of genes in cis and trans position. To characterize their effects, we first sought SNP associated with gene expression from the Genotype-Tissue Expression Project (GTEx portal, Analysis Release V7) a platform with available expression data on potential target organs (heart tissue, kidney tissue, brain tissue, aortic endothelial cells and blood vessels) as well as blood cell types (CD4+ macrophages, monocytes) (19) . The gene expression values are shown in TPM (transcripts per million), calculated from a gene model with isoforms collapsed to a single gene. Box plots are shown as median and 25th and 75th percentiles, outliers are displayed as dots if they are above or below 1.5 times the interquartile range (19). In addition, the platform also allows the assessment of isoform expression generated using RSEM, software package for estimating gene and isoform expression levels from RNA-Seq data.

Mendelian Randomization analysis

We implemented a two-sample Mendelian randomization analysis to evaluate causal effects of a number of risk factors on abdominal aortic diameter. Risk factors were chosen based on previous literature reporting them to be associated with AAA. We identified genetic instruments for systolic blood pressure (SBP) (20, 21), diastolic blood pressure (DBP) (20, 21), pulse pressure (PP) (20, 21), smoking (22, 23), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides (TG) (24, 25) , height (26), and body mass index (BMI) (27, 28) using the most up-to-date GWAS on these traits.. We used the inverse variance weighted (IVW) method to combine the effect of multiple instruments. We further used two sensitivity analyses, the weighted median and MR-Egger, to investigate potential effect of pleiotropic variants on the estimates. For lipid traits, we used a multivariable MR method (29). In this approach, a single regression model with outcome variable (β for AAD) was fitted for each of the predictor variables (β for LDL, β for HDL, and β for TG). The model was implemented as a multilinear regression of SNP association estimates weighted by the inverse variances of the estimated associations of SNPs with the outcome. All MR methods for multiple genetic instruments were conducted using "MendelianRandomization", a statistical package running under R (https://cran.r-project.org/web/packages/MendelianRandomization/index.html) (30). We used MR-PRESSO (Mendelian randomization pleiotropy residual sum and outlier) to identify horizontal pleiotropic outliers in multi-instrument summary-level MR testing (https://github.com/rondolab/MR-PRESSO) (31). Further details are outlined in Supplementary information.

Results

Study sample characteristics

Baseline characteristics for each cohort included in this meta-analysis of genome-wide association studies are summarized in Supplementary Table S1a and S1b. The discovery panel of genome-wide analysis for common variants included 12,815 participants from nine European cohorts. Mean age ranged from 45.1 to 74.9 years and the percentage of males ranged from 27.0 to 57.9. Replication of the GWAS findings was conducted in a sample of 727 individuals from PBIO1 cohort. The baseline characteristics of the samples included in the meta-analysis of exome array data are shown in Supplementary Table S2. We analyzed exome array data from 14,480 subjects from seven European cohorts whose mean age ranged from 49.4 to 75.3 years old and the percentage of male population ranged from 42.3 to 58.5.

Meta-analysis of genome-wide association studies

Fixed-effect meta-analysis of the summary statistics from each set identified one locus associated with AAD. Figure 1 presents the Manhattan plot of GWAS on AAD. Genome-wide significant SNPs are presented in Table 1. Quantile-quantile plots showed no genomic inflation (α=1.04) (Figure 2). The strongest association was found for rs74448815, located in the intronic region (chr18:13,593,315) of *LDLRAD4* (Low Density Lipoprotein Receptor Class A Domain Containing 4) (Figure 3a) where the C allele (MAF=0.04) was associated with a smaller abdominal aortic diameter (beta= -0.026, p-value= 2.10×10^{-8}). This SNP was replicated in PBIO1 cohort (p-value=8.19×10^{-4}). In addition, the combined meta-analysis showed that the top hit remained genome-wide significant (p-value= 9.2×10^{-10}) (Table 1).

Furthermore, we found suggestive associations (5×10^{-8} < p-value < 5×10^{-7}) for 12 highly correlated SNPs (genetic correlation> 0.80) located in *PLCE1* (Phospholipase C Epsilon 1) (chr10: 95,892,659-95,900,004) (Figure 3b) gene and 6 SNPs located in the intergenic region of *ADAMTS15* and *C11orf44* genes (Table 1). In a whole exome sequencing (WES) study *ADAMTS15* was found to be linked to intracranial aneurysm in a Japanese family (32). *PLCE1* has been identified as a locus associated with hypertension (33), and has been identified as to be associated with stroke in the dbGAP Gene-Trait Associations dataset (http://amp.pharm.mssm.edu/Harmonizome/gene_set/Stroke/dbGAP+Gene-Trait+Associations). However, these variants were not successfully replicated. in our analysis. Although this suggests that for the general population the contribution of both genes to aortic aneurysm is negligible, we cannot exclude any indirect effect through risk factors that affect AAD. We further examined the association of AAA-risk loci, previously reported by GWAS and candidate-gene based studies (8, 11-13, 34-36), with AAD (Table 2). *CDKN2BAS1/ANRIL* showed the strongest association with AAD (rs10757274; beta= 0.0058, p-value=1.71×10^{-4}) (35) and *ERG* was associated with AAD at nominal significance level (rs2836411; beta=0.0034, p-value=0.036) (8) (Table 2).

Association of the identified SNPs with gene expression

We found that *LDLRAD4* gene is highly expressed in several tissues, mainly brain as well as aorta, coronary artery and tibial artery (Supplementary Figure S1a). The expression levels of a set of ten isoforms of *LDLRAD4* are displayed in Supplementary Figure S1b. The isoform ENST00000359446.5, also known as hsa-miR-769-3p, reported the highest expression in arterial tissue (TPM=6.81) in comparison with other isoforms of the gene. Nevertheless, rs74448815 is an intronic SNP without a predicted function and without any proxy SNPs in coding or regulatory regions. Therefore, the effect of this SNP on miRNA regulation is yet to be explored. Likewise, we did not find expression quantitative trait loci associated with this top SNP at both cis/trans positions.

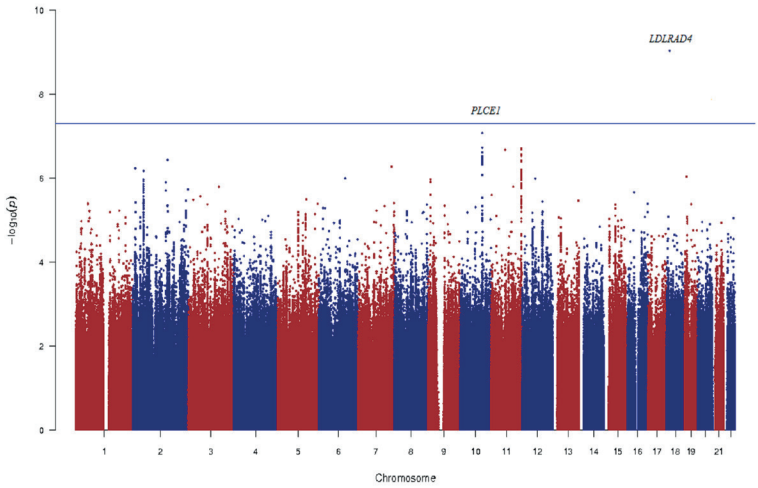

Figure1. Manhattan plot of meta-analysis of genome-wide association studies on abdominal aortic diameter. Manhattan plot showing the –log-10-transformed p-value of SNPs in the GWAS of abdominal aortic diameter. The blue line indicates the GWAS- significance level (p-value < 5×10^{-8}).

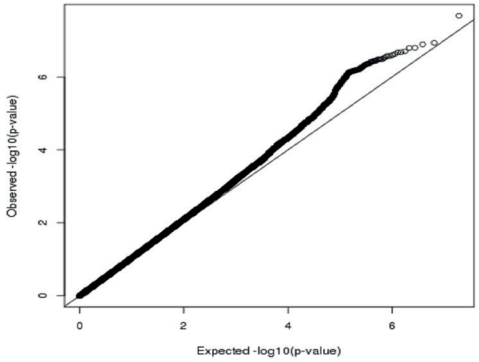

Figure 2. Quantile-quantile plot of genome-wide association studies on abdominal aortic diameter. Vertical and horizontal lines represent expected p-values under a null distribution and observed p-values, respectively.

Meta-analysis of exome array data

The meta-analysis of rare exonic variants associated with AAD was conducted in 14,480 ticipants from seven European cohorts (Table S2). Meta-analysis on single variant level of exome array variants with MAF >0.005 (number of variants=55,461) showed no significant associations with AAD. The lowest p-value was observed for rs239259 located in *SLC22A20* (Solute Carrier Family 22 Member 20), chromosomic region 11q13.1, where the T allele has a small effect on aortic diameter (beta=0.007, p-value=1.20×10^{-5}). Furthermore, gene-based meta-analysis revealed a positive association for *PCSK5* (Proprotein Convertase Subtilisin/Kexin Type 5) gene (p-value= 8.03×10^{-7})), composed by 18 variants. Four variants in the *LDLRAD4* gene were identified for gene-based analysis, however, no association with AAD was observed (p-value=0.21).

Table 1. associated and suggestive associated loci with abdominal aortic diameter in GWAS

Locus	Chr	SNP	maf	A1/ A2	β	p-value combined	p-value in discovery cohort	p-value in replication cohort
LDLRAD4	18	rs74448815	0.04	A/C	-0.02	9.15×10^{-10}	2.1×10^{-8}	8.19×10^{-4}
PLCE1	10	rs10882397	0.46	A/C	0.008	8.37×10^{-8}	2.13×10^{-7}	0.14
	10	rs10882398	0.44	A/T	0.008	1.86×10^{-7}	5×10^{-7}	0.12
	10	rs10736085	0.46	A/T	0.008	2.46×10^{-7}	5.9×10^{-7}	0.15
	10	rs10786152	0.47	A/G	-0.008	3.41×10^{-7}	8×10^{-7}	0.16
	10	rs2901761	0.43	A/G	0.008	4.37×10^{-7}	1.1×10^{-6}	0.14
	10	rs9663362	0.47	C/G	0.008	2.96×10^{-7}	7×10^{-7}	0.15
	10	rs932764	0.44	A/G	-0.008	4.36×10^{-7}	1.1×10^{-6}	0.14
	10	rs11187793	0.43	A/G	0.008	4.71×10^{-7}	1.2×10^{-6}	0.15
	10	rs731141	0.47	A/G	-0.008	4.58×10^{-7}	1×10^{-6}	0.17
	10	rs1806920	0.47	A/G	-0.008	3.86×10^{-7}	8.8×10^{-7}	0.17
	10	rs2797983	0.47	C/G	-0.008	4.04×10^{-7}	1×10^{-6}	0.13
	10	rs7070115	0.44	A/G	-0.008	3.31×10^{-7}	8.5×10^{-7}	0.13
ADAMTS15 - C11orf44	11	rs1689231	0.38	C/G	-0.008	3.81×10^{-7}	2.7×10^{-7}	0.95
	11	rs1630336	0.38	T/C	0.008	2.95×10^{-7}	2.1×10^{-7}	0.95
	11	rs2514390	0.39	A/G	0.009	2.77×10^{-7}	1.6×10^{-7}	0.91
	11	rs6590483	0.39	A/G	-0.008	1.96×10^{-7}	1.2×10^{-7}	0.92
	11	rs1921580	0.39	A/G	-0.008	2.09×10^{-7}	1.3×10^{-7}	0.94
	11	rs4937542	0.39	A/G	-0.008	4.03×10^{-7}	2.5×10^{-7}	0.94

A1: Coded allele; A2: non-coded allele; Chr: Chromosome, maf: minor allele frequency

Associations with cardiovascular traits

We further investigated the potential association between one genome-wide significant SNP and five suggestive SNPs found in our meta-analysis, as well as the variant with the lowest p-value reported from single variant analysis, with coronary artery disease (CAD) from the largest CAD GWAS to-date (37). We found association between our top hit, rs74448815, with a p-value= 0.005 reported for CAD (Table 3). In addition, one of the suggestive SNPs, rs932764 (*PLCE1*), was associated with both systolic and diastolic blood pressure (38) (Table 3).

Mendelian Randomization analysis

We further examined the potential causal association between AAA risk factors and abdominal aortic diameter, including SBP, DBP, PP, smoking, lipid traits (LDL, HDL and TG),height and BMI. We examined 104 SNPs reported for SBP (Supplementary Table S4), 139 SNPs associated with DBP (Supplementary Table S5), 109 SNPs reported for pulse pressure (Supplementary Table S6), 124 variants associated with smoking (Supplementary Table S7), 66 SNPs for LDL (Supplementary Table S8), 39 SNPs for HDL (Supplementary Table S9), 35 SNPs for triglycerides (Supplementary Table S10), 134 SNPs associated with

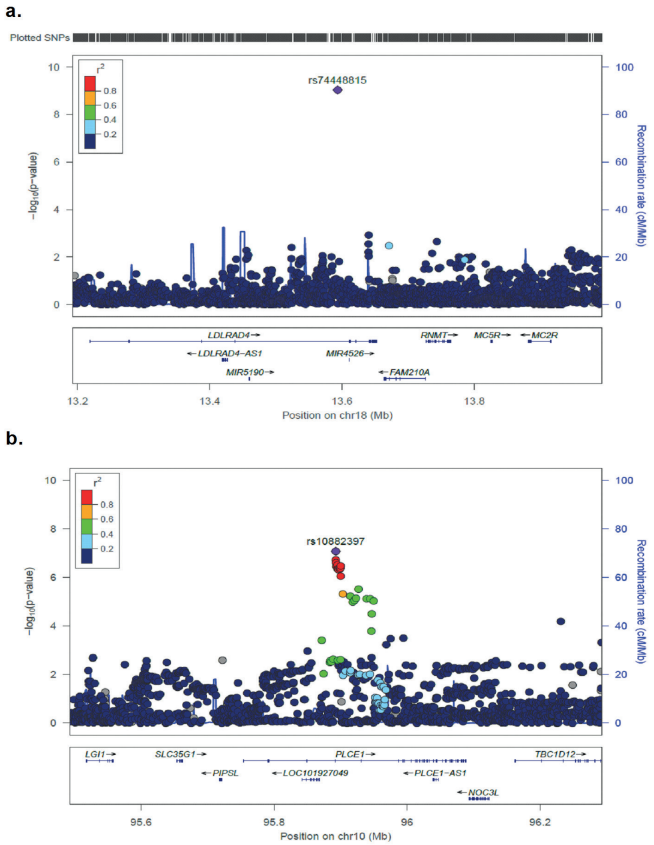

Figure 3. Regional plots showing the association of *LDLRAD4* and *PLCE1* genes with abdominal aortic diameter. P-values of genotyped SNPs (circle) and imputed SNPs (square) are plotted (as –log10 P value) against their physical location on chromosome 18p11.21 (a) and 10q23.33 (b). SNP's color indicates LD with rs74448815 (a), rs10882397 (b) according to a scale from r2 = 0 to 1 based on pair-wise r2 values from 1000 Genomes.

height (Supplementary Table S11) and 73 SNPs found for BMI (Supplementary Table S12).

Results from the conventional and sensitivity MR analyses are shown in Table 4. Penalized IVW estimates suggested that a 1-SD genetically-elevated height is associated with AAD (IVW estimate=0.03, p-value<0.0001) (Supplementary Figure S1). Furthermore, we found evidence to support that 1-SD genetically-increased pulse pressure demonstrated a significant association with infrarenal aortic diameter (IVW estimate=-0.003, p-value=0.02) (Supplementary Figure S2). For lipid traits, we conducted a conventional 2-sample MR analysis to assess the causal role of LDL-cholesterol on AAD. IVW estimates were not significant for any of the each lipid fractions (LDL: IVW=-0.005, p-value=0.2; HDL: IVW=-0.002, p-value=0.9; TG: IVW=-0.02, p-value=0.2). Nevertheless, MR-Egger estimate showed that 1-SD genetically-increase in triglycerides was significantly associated with smaller AAD (beta=-0.16, p-value=0.008, Supplementary Figure S3). We did not find evidence for causal association of systolic blood pressure (IVW estimate=0.0001, p-value for IVW=0.8, Supplementary Figure S2), diastolic blood pressure (IVW estimate=0.001, p-value for IVW=0.5, Supplementary Figure S3), smoking (IVW estimate=0.02, p-value for IVW=0.3, Supplementary Figure S5) and body mass index (IVW estimate=-0.005, p-value for IVW=0.1, Supplementary Figure S10) with AAD (Table 4).

Table 2. Association of the previously identified risk loci for AAA with abdominal aortic diameter

SNP	Nearest gene	A1/A2	β*	p-value**
GWAS				
rs602633	*PSRC1-CELSR2-SORT1*	T/G	0.002	0.32
rs4129267	*IL6R*	T/C	-0.001	0.47
rs10757274	*CDKN2BAS1/ANRIL*	A/G	-0.006	1.71×10^{-4}
rs10985349	*DAB2IP*	T/C	-0.001	0.58
rs6511720	*LDLR*	T/G	0.001	0.74
rs1795061	*SMYD2*	T/C	0.002	0.35
rs9316871	*LINC00540*	A/G	0.002	0.35
rs3827066	*PCIF1-ZNF335-MMP9*	T/C	0.002	0.44
rs2836411	*ERG*	T/C	0.003	0.036
Candidate-gene studies				
rs599839	*SORT1*	A/G	-0.001	0.46
rs7529229	*IL6R*	T/C	0.001	0.48
rs6743376	*IL1RN*	A/C	0.0001	0.93
rs1542176		T/C	-0.0006	0.68
rs10455872	*LPA*	A/G	-0.002	0.52
rs3798220		T/C	0.007	0.26
rs5186	*AGTR1*	A/C	-0.002	0.17
rs1036095	*TGFBR2*	C/G	0.001	0.53
rs764522		C/G	-0.003	0.08

A1: Coded allele; A2: non-coded allele; Chr: Chromosome, p-value threshold= 0.003

* Beta coefficients from combined AAD meta-analysis

** p-value from combined AAD meta-analysis

Discussion

The present study is the largest genome-wide study including both common and rare variants with AAD to date, utilizing GWAS data of 13,542 European ancestry participants from ten studies and exome array data from seven studies with 14,480 subjects. We identified two novel loci at *LDLRAD4* and *PCSK5* associated with AAD. At least one out of the 18 risk loci that were previously reported for AAA was also nominally associated with AAD. Furthermore, we showed that height and pulse pressure, known clinical risk factors for AAA, may be causally associated with AAD.

Our GWAS identified *LDLRAD4* to be associated with AAD. Prior studies have demonstrated an association of genetic variants in *LDLRAD4* with schizophrenia and bipolar disorder (39, 40), and a low frequency variant (rs8096897) in this gene showed evidence of association with systolic blood pressure (41). *LDLRAD4*, also known as *C18orf1*, is involved in the down regulation of transforming growth factor-β (*TGF-β*) by interacting with downstream effectors SMAD2 and SMAD3 via its SIM domain (42). *TGF-β* plays

Table 3. Association of the the identified AAD-SNPs with vascular traits

SNP	Nearest gene	p-value	Trait
rs74448815	*LDLRAD4*	0.005	CAD
rs932764	*PLCE1*	6.88×10^{-17}	SBP
		6.28×10^{-10}	DBP

CAD: coronary Artery Disease; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure

a crucial role during embryogenesis and its downregulation in adult life contributes to the development of vascular disorders, including abdominal aortic aneurysm (43). Heterozygous mutations in the genes encoding TGF-β receptors 1 and 2 (*TGFBR1* and TGF-*BR2*, respectively) cause Loeys-Dietz syndrome, an autosomal dominant aortic aneurysm syndrome, which predisposes patients to aggressive vascular disease with widespread systemic involvement (44). *SMAD3* mutations play an important role in familial aortic diseases, characterized by aneurysms(45). Thus, *LDLRAD4* variants might affect AAD through the TGF-β – SMAD3 signaling axis in the general population.

The genetic basis of aortic dilation was initially explored through candidate gene approaches. Candidate genes were selected based on their biological relevance and their potential role in the pathogenesis of AAA. So far, genetic variants in eleven genes including *SORT1, IL6R, LPA, AGTR1, TGFBR2, ACE, MMP3, MMP13, MTHFD1, MTRR* and *LRP5* mainly implicated in lipid metabolism, inflammation, blood pressure homeostasis, *TGF-β* signaling, degradation of extracellular matrix and methionine metabolism, have been subject of evaluation in candidate gene studies (34).

Furthermore, GWAS have so far identified genes in six chromosomal regions for AAA: *DAB2IP, LDLR, PSRC1-CELSR2-SORT1, CDKN2BAS1/ANRIL, IL6R, SMYD2, LINC00540, PCIF1-ZNF335-MMP9* and *ERG* (11-13, 35, 36). We thus explored the potential role of these AAA-risk loci with aortic diameter. Only one of these risk loci, namely *CDKN2BAS1/ANRIL* was associated with abdominal aortic diameter in our combined meta-analysis. *ERG* gene was found to be associated at nominal significance. Therefore, the underlying mechanisms for AAD and AAA may at least to a large extent be different. The lack of genetic overlap between both traits may also be determined by the limited power of this study. Another explanation is that the disparities observed might indicate to what extend aging and cellular senescence are important to form aneurysms. Genes like *IL6R, MMP9, MMP13* and *CDNK2* are intimately related to cellular senescence; hence cellular senescence-related pathways may have a great impact on the diameter of the abdominal aorta and its enlargement. *CDKN2BAS1/ANRIL*, located in the 9p21 chromosomic region, has been reported in numerous studies as a genetic risk locus for CAD, intracranial aneurysms, and diverse cardiometabolic disorders (46).

From the gene-based meta-analysis of exome array data we identified *PCSK5* as a risk locus for AAD. *PCSK5* encodes a member of the subtilisin-like proprotein convertase family, which is involved in the trafficking of peptide precursors through regulated or constitutive branches of the secretory pathway (47). This is also an important finding given that conditional inactivation of endothelial *PCSK5* has shown decreased collagen deposition in the heart and in the vasculature in aged mice, and may be relevant to aortic dilation biology (48). *PCSK5* is thought to process prorenin, pro-membrane type-1 matrix metalloproteinase as well as lipoprotein metabolism-related pathways (49, 50). Furthermore, genetic variations at *PCSK5* locus have been associated with HDL-C levels possibly through the

Table 4. Mendelian randomization results for each risk factor

Systolic blood pressure			
Method	**Estimate**	**p-value**	**p-value heterogeneity (IVW)**
IVW	-0.001	0.2	0.04
MR-Egger	0.001	0.8	-
MR-Egger (intercept)	0	0.6	-
Weighted median	0.0001	0.9	-
Diastolic blood pressure			
IVW	-0.001	0.6	0.2
MR-Egger	-0.002	0.5	-
MR-Egger (intercept)	0	0.6	-
Weighted median	0.001	0.8	-
Pulse pressure			
IVW	-0.003	0.02	0.02
MR-Egger	-0.009	0.04	-
MR-Egger (intercept)	0	0.1	-
Weighted median	-0.003	0.06	-
Smoking			
IVW	0.02	0.3	0.03
MR-Egger	0.01	0.9	-
MR-Egger (intercept)	0	0.9	-
Weighted median	-0.006	0.8	-
LDL			
IVW*	-0.005	0.2	0.04
MR-Egger	-0.004	0.5	-
MR-Egger (intercept)	0.0001	0.8	-
Weighted median	-0.006	0.3	-
HDL			
IVW*	-0.002	0.9	0.002
MR-Egger	-0.02	8	-
MR-Egger (intercept)	0	0.8	-
Weighted median	0.009	0.7	-
Triglycerides			
IVW*	-0.02	0.2	0.2
MR-Egger	-0.16	0.008	-
MR-Egger (intercept)	0.003	0.01	-
Weighted median	-0.03	0.1	-
Height			

Table 4. Mendelian randomization results for each risk factor (continued)

IVW	0.03	0.0001	0.05
MR-Egger	0.07	0.0001	-
MR-Egger (intercept)	-0.001	0.008	-
Weighted median	0.03	0.0001	-
BMI			
IVW	0.01	0.0001	0.06
MR-Egger	0.004	0.3	-
MR-Egger (intercept)	0.001	0.001	-
Weighted median	0.004	0.26	-

inactivation of endothelial lipase activity and atherosclerotic cardiovascular disease risk (50). *PCSK5* is also an important genetic predictor of tall stature as regulates the maturation of GDF15 (growth differentiation factor 15), a member of the TGF-β family, involved in body growth (51, 52). Although evidence is scarce, the role of this gene on the pathology of aortic diameter and aortic enlargement might be through collagen regulation and inflammatory pathways modulated by cholesterol metabolism and/or via activation of the renin-angiotensin system (53). In light of our findings, further research may be warranted.

Through the implementation of Mendelian randomization methods, we examined and determined if AAA-related risk factors may be causally associated with the variation of infrarenal aortic diameter observed in the studied population. MR uses genetic variants as proxies for the risk factor and the outcome of interest and can offer an opportunity to shed light on the causality of risk factors-outcome associations (54). Unlike the associations of a risk factor with aortic enlargement reported from observational data, genetic associations are not influenced by reverse causation because the genotype is unmodified by the development of the disease. Moreover, the randomized assortment of parental alleles at conception tends to balance confounding factors among groups of differing genotypes (55).

In this study we used multiple independent SNPs as instrumental variables, selected from studies which have reported a robust association with each risk factor evaluated. We found evidence that genetic variation in height, pulse pressure and triglycerides is associated with variation in abdominal aortic diameter, consistent with causal associations. Height, among the body size measurements, has shown the strongest association with aortic size. Evidence has demonstrated that height-based relative aortic measure predicts the risk of rupture, dissection, and death in patients with AAA (56). The association between height and AAA development and increasing risk of rupture might be explained by the presence of longer arteries in the AAA population (57). Nevertheless, the biological link between height and aortic enlargement remains unexplained.

Increased pulse pressure is associated with increased characteristic impedance (Zc) of the aorta and increased load on the heart, contributing to the risk of ischemia (58), diastolic dysfunction (59) and adverse clinical events (60, 61). A high pulse pressure, markedly after mid-life when Zc increases rapidly, may have an impact on the aortic diameter (62). Evidence has shown that higher pulse pressure in older people is associated with smaller aortic lumen area and greater aortic wall stiffness and thickness (63). A small aortic diameter related with a higher pulse pressure suggests a mismatch in hemodynamic load accommodation by the heart and aorta in aged population. In contrast, a causal association between AAA and pulse pressure has not been established.

Pleiotropic effects are a major challenge in the implementation of Mendelian randomization (54). Instrumental variables that affect the outcome via pathways not including the exposure could distort or bias MR results. To address this, we performed sensitivity analyses including MR-Egger. Our MR-Egger result suggests a potential causal effect of triglycerides on AAD. Prospective studies have shown positive relationships between triglycerides and AAA (64-66). Moreover, triglycerides is strongly related to risk of ruptured AAA (67). In line with our findings, a large meta-analysis MR study have reported significant associations between triglycerides and abdominal aortic aneurysm (68).

In contrast, we found that there was not significant evidence for other genet ically determined lipid fractions such as HDL-C and LDL-C having a causal effect on AAD. One explanation is that the genetic variants for LDL-C and HDL-C may explain a small proportion of the total variance in these lipid traits and this could affect their association with aortic diameter. Another explanation is that both lipids are indeed not causally related to AAD. Our findings support the clinical importance of the management of blood pressure/ pulse pressure and triglycerides for the prevention of AAA in high risk patients. Therefore, targeting risk factors related with aortic expansion at early stages could have important implications for the implementation of public health interventions aiming to reduce the prevalence of these risk factors and the morbidity and mortality caused by AAA.

Strengths and limitations

A key attribute of this study is the combination of a large, discovery sample with standardized AAD measurement and dense 1000 Genomes imputation, yielding a high-quality data set with ~9.8 million variants (69). Differences in the methods employed to measure AAD (ultrasound, MRI, CT-scan) may have introduced site-based effects into the study conferring error or bias in the measurements, which ultimately may have contributed to a lack of associated variants observed. We made use of log-transformed AAD to standardize the phenotype and minimize the impact of the variability present among the cohorts. We acknowledge several limitations in our work. The sample size for this GWAS has been the main limitation that hampered identifying further loci. However, we have searched extensively to include all studies with relevant data (37). Sexual dimorphism is relevant as it has been observed in the development of AAA (70). However, we did not perform sex-specific analyses given our limited sample size. Our study population was predominantly of European ancestry. Generalizing the results to other ethnic groups should be done with caution.

Conclusions

In summary, we identified one replicated locus and one suggestive locus associated with the diameter of infrarenal aortic aorta. In addition, we provided evidence that the main genetic determinants of pulse pressure and height also causally influence the diameter of the abdominal aorta. In contrast, we found that other genetically determined risk factors for vascular-related diseases had no effect on AAD. Our findings point to new biology as well as highlighting gene regions in mechanisms that have previously been implicated in the genetics of other vascular outcomes. Larger sample sizes together with functional studies, investigating the translational potential of these observations, are critical to characterize the molecular mechanisms regulated by the genes described in this study.

Table S1. Baseline characteristics table of the cohorts included in the genome-wide association meta-analysis

	Discovery cohorts									Replication cohort
Characteristics	**RSI**	**RSII**	**RSIII**	**KORA**	**FHS**	**CHS**	**SHIP2**	**SHIP trend**	**MESA**	**PBIO1**
N	700	1081	2132	354	3262	2699	1010	827	750	727
AAD, mean ± SD	19.21±5.24	19.76±3.67	17.60±3.09	15.1±2.2	18.22±2.81	18.43 ±4.64	19.1±2.3	18.5 ± 2.3	19.3±2.9	19±8
Ln_AAD, mean±SD)	2.92±0.22	2.96±0.16	2.84±0.15	2.7 ± 0.1	NA	2.89±0.21	NA	NA	NA	0.6 ± 0.22
AAA (%)	25 (3.57)	22(2.03)	17(0.79)	0	24(0.76)	85 (3.1)	2 (0.19)	0	0	10 (1.4%)
Male (%)	295(42.14)	365(34)	588(27)	205(57.9)	1686(51.7)	1040(38.5)	498 (49.3)	374 (45.2)	392 (52)	321 (44%)
Age (years), mean ± SD	61.24±4.5	72.01±4.9	56.54	56.6±9.1	60.18±9.01	74.87±5.04	45.06±12.78	50.72±13.34	66±9.6	39.5 ± 14.9
Total cholesterol (mmol/L), mean ± SD	5.84±0.97	5.81±0.92	5.64±2.08	5.66±0.93	5.07±0.91	5.23±0.95	5.49±1.08	5.51±1.03	10.35±1.99	4.61±1.16
HDL(mmol/L), mean ± SD	1.43±0.38	1.40±0.37	1.58±3.31	1.60±0.46	1.38±0.43	1.39±0.37	1.45 ± 0.39	1.47±0.36	2.90±0.87	1.51±0.57
Hypertension (%)	120(17.14)	166(15.35)	401(18.80)	122 (34.5)	1001(30.91)	1338 (49.6)	514(50.89)	322(38.93)	332(44)	441 (60.7%)
Diabetes (%)	48 (6.85)	86(7.95)	145(6.80)	46 (13)	186(5.76)	305 (11.6)	90(8.91)	27 (3.26)	70(9)	89 (12.2%)
Current smoker (%)	124(17.71)	105(9.71)	313(14.68)	70 (19.8)	393(12.13)	235 (8.9)	192(19)	169 (20.45)	88(12)	301 (41.4%) (EVER smokers)
Former smoker (%)	326(46.57)	587(54.30)	1075(50.42)	154(43.5)	NA	1170 (44.3)	429(42.47)	305 (36.88)	353(48)	NA
Never smoker (%)	227(32.42)	389(35.98)	744(34.89)	130(36.7)	NA	1238 (46.8)	386(38.21)	351 (42.44)	298(40)	399 (54.9%)
BMI (%)	26.43±3.49	26.85±3.51	27.17±4.04	28.1±4.9	27.77±5.29	26.35 ±4.34	27.65±4.39	27.18±4.29	27.8±5	32.5 ± 7.8
Height (cm), mean ± SD	168.90±32.82	168.58±23.73	171.48±9.44	171.5±9.9	NA	164.08 ±9.41	169.46±9.23	169.11±9.02	169.3±9.7	NA

AAD: Abdominal aortic diameter; AAA: Abdominal aortic aneurysm; HDL: High density lipoprotein; BMI: Body mass index

Table S2. Baseline characteristics table of the cohorts included in the exome array meta-analysis

Characteristics	RSI	KORA	FHS	CHS	SHIP	MESA	BIOIMAGE
N	327	337	3262	3294	2848	740	3672
AAD (mm), mean ± SD	19.55±5.35	15±2.1	18.22±2.81	18.71±4.90	18.8±2.3	19.3±2.9	18.3±3.6
AAA, (%)	13 (3.97)	0	24(0.76)	134 (4.1)	6 (0.20)	0	51(1.38)
Ln_AAD, mean±SD)	2.95±0.23	2.7 ± 0.1		2.90±0.22	NA	NA	0.59 ± 0.18
Male (%)	165 (50.45)	197(58.5)	1686(51.7)	1429 (43.4)	1416(49.71)	388(52)	1555(42.3)
Age (years), mean ± SD	68.34±5	56.4±9.3	60.18±9.01	75.24 ± 5.22	49.36±13.83	66±9.6	69.1±6.1
Total cholesterol (mmol/L), mean ± SD	5.80±0.95	5.65±0.95	5.07±0.91	5.21±1.01	5.50 ±1.08	10.36±1.99	11.33±2.09
HDL(mmol/L), mean ± SD	1.39±0.37	1.60±0.46	1.38±0.43	1.36±0.37	1.45±0.37	2.90±0.88	3.15±0.87
Hypertension (%)	61(18.65)	133(33.5)	1001(30.91)	1720 (52.2)	1322(46.41)	329(45)	929 (25.3)
Diabetes (%)	18(5.50)	41(12.2)	186(5.76)	434 (13.6)	246(8.63)	68(9)	291 (7.9)
Current smoker (%)	61(18.65)	68(20.2)	393(12.13)	286 (8.9)	612 (21.48)	86(12)	315 (8.6)
Former smoker (%)	155(47.40)	144(42.7)	NA	1500 (46.6)	1111 (39)	349(48)	1787 (48.7)
Never smoker (%)	216(66.05)	125(37.1)	NA	1436 (44.6)	1119(39.29)	294(40)	1570 (42.8)
BMI (%)	26.37±3.40	28(5)	27.77±5.29	26.41 (4.34)	27.60±4.35	27.8±4.9	28.2±5.1
Height (cm), mean ± SD	168.84±9.64	171.6±9.9	NA	164.61±9.55	170.10±9.26	169.3±9.6	166±9.8

AAD: Abdominal aortic diameter; AAA: Abdominal aortic aneurysm; HDL: High density lipoprotein; BMI: Body mass index

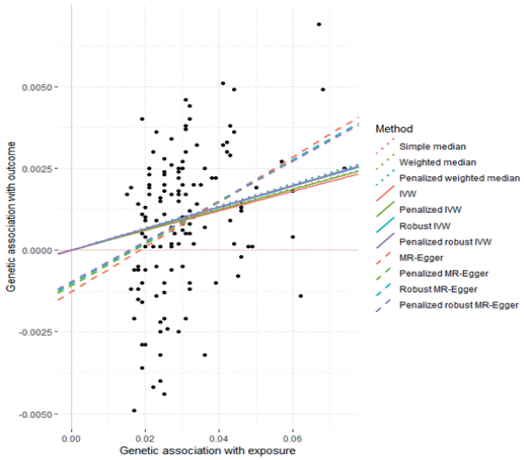

Figure S1. Mendelian Randomization plot height SNPs and AAD

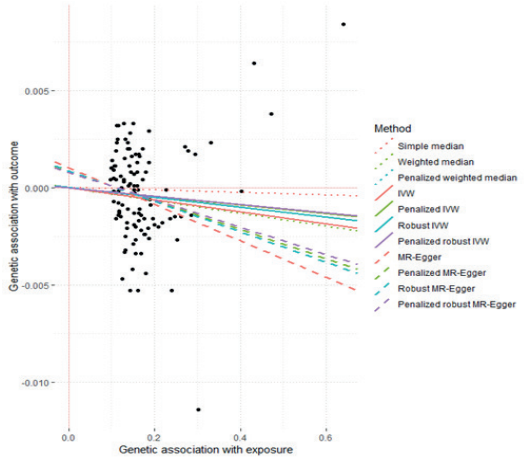

Figure S2. Mendelian Randomization plot pulse pressure SNPs and AAD

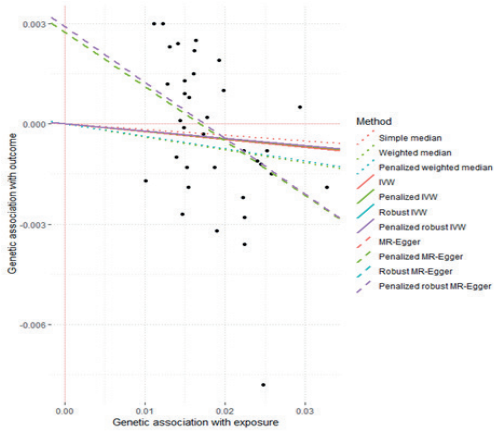

Figure S3. Mendelian Randomization plot triglycerides SNPs and AAD

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CHAPTER 2.5

Meta-analysis of epigenome-wide association studies of carotid intima media thickness

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Abstract

Common carotid intima-media thickness (cIMT) is an index of subclinical atherosclerosis, that is associated with ischemic stroke and coronary artery disease. We undertook a cross-sectional epigenome-wide association (EWA) study in 6,400 individuals of measures of cIMT. Mendelian randomization analysis was applied to investigate the potential role of DNA methylation in the causal link between cardiovascular risk actors and cIMT or coronary artery disease. The CpG site cg05575921 was significantly associated with cIMT (beta= -0.0264, p-value= 3.5×10^{-8}) in the discovery panel, and was replicated in replication panel (beta=-0.07, p-value= 0.005). This CpG is located at chr5:81649347 in the intron 3 of the aryl hydrocarbon receptor repressor gene (*AHRR*). Moreover, 34 differentially methylated regions (DMRs) were identified from which the region comprised by *ALOX12* showed the strongest association with cIMT (p-value=1.4×10^{-13}). Mendelian randomization did not support DNA methylation at cg05575921 to be causally implicated in the association between cardiovascular risk factors and cardiovascular diseases. In conclusion, DNA hypomethylation at *AHRR* is associated with cIMT.

Introduction

Carotid intima-media thickness (cIMT) is defined as a progressive thickening of the arterial wall that is characterized by the presence of large arterial wall deposits. Ultrasound of the carotid artery is widely used as a non-invasive procedure to detect the presence of atherosclerotic plaques and as a marker of subclinical vascular disease which is strongly associated with aging (1). A cIMT value above 75th percentile threshold for a person's age, sex and race in asymptomatic individuals, the risk of myocardial infarction, stroke and death from coronary heart disease is significantly increased as compared to the average of the population (2). Furthermore, the addition of cIMT to the Framingham Risk Score has been shown to improve the 10-year risk prediction of myocardial infarction or stroke independent of age, sex and cardiovascular risk factors (3). Therefore, cIMT could add considerable utility to the study of the onset and progression of atherosclerosis (1).

Epigenetic modifications including covalent changes of DNA methylation and chromatin alterations, are known to induce changes in the regulation of gene expression, and are heritable during cell division (4). DNA methylation is considered as the most stable epigenetic mark and the most informative at explaining gene expression patterns and cell differentiation. DNA methylation varies with age, sex and environmental factors including diet and smoking (5). In recent years, there has been a growing interest in identifying whether DNA methylation variations contribute to the onset and progression of complex human diseases; accumulating evidence suggests that this is the case at least for some traits and disorders (6-10).

Technological advances and the implementation of epigenome-wide association studies (EWAS), have facilitated the systematic assessment of DNA methylation signatures, leading to the identification of novel mechanisms related to human diseases (11-13). Epigenomic dysregulation characterized by EWAS has been assessed mainly in leukocytes since this is the most accessible tissue in epidemiologic studies. Although the sampling of the cell type mediating the disease allows to infer more valid conclusions, it has been proven that the use of leukocytes, a more accessible surrogate cell type, yields useful information (7). Differences in leucocyte DNA methylation patterns in healthy persons vs. those at risk could either reflect the cumulative effects of CVD risk factors or indicate the changes the leucocytes undergo in the course of developing CVD. The latter might be mimicking or reflect similar processes in vascular cells. Using a multi-center approach (>6000 individuals of European ancestry), we are the first to assess the association between DNA methylation markers and cIMT by using data from eight cohorts participating in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. The analysis of epigenetic markers and their association with cIMT in this population could provide insight into mechanisms related to arterial thickness and atherosclerotic disease. In addition to assessing individual CpGs, we analyze and characterize differentially methylated regions (DMR) of the genome at which adjacent CpG sites show differential methylation levels across multiple samples. Information from multiple nearby methylation sites may aid biological inference as well as increase the power to detect associations with human traits (14).

Methods

Study population

This study was conducted using data from eight cohorts within the CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology) consortium, an international collaborative effort to facilitate collaborative efforts in omics era, providing opportunities for meta-analysis and replication among multiple studies (15). The discovery panel comprised of 6407 subjects from

Framingham Heart Study (FHS) (n=1977), Cooperative Health Research in the Region Augsburg (KORA) (n=1511), Rotterdam Study (RS) rounds RSIII-1 (n=731) and RSII-3 (n=468), MRC National Survey of Health and Development (NSHD) (n=600), The Lothian Birth Cohorts (LBC) (n=288), Study of Health in Pomerania (SHIP) (n=246), Young Finns Study (YFS) (n=191) and Cardiovascular Health Study (CHS) (n=191). For the replication panel, we used data from RSIII-2 (n=251). Details of the cohorts included are provided in Supplementary Appendix 1a.

Measurement of cIMT

Each study evaluated the carotid arteries using B-mode high-resolution ultrasound by trained operators. cIMT was calculated by averaging the maximum cIMT of the far walls of the right and left common carotid artery in the mid portion of the visible segment of the arteries in the neck. A longitudinal image was used, optimizing the image frame to be perpendicular to the arterial wall. We used natural log transformation to deal with the skewness of the cIMT measurements. A description of the method employed in cIMT measurement by each study is provided in Supplementary Appendix 1.a.

DNA methylation analysis

Genome-wide DNA methylation profiling was conducted using Illumina Infinium BeadChips arrays. FHS, KORA, RS, LBC, CHS and YFS used HumanMethylation450 BeadChip 450k array; which covers approximately 485,577 methylation sites. SHIP and NSHD employed Infinium MethylationEPIC BeadChip, which interrogates approximately 850,000 CpG sites across the genome. Beta values, defined as the ratio of intensities between methylated and unmethylated CpG alleles were used to represent DNA methylation levels. Further details regarding data pre-processing and quality control methods employed by each study are outlined in Supplementary Table S3.

Epigenome wide association analysis

Each study used linear mixed-effects models to identify associations between DNA methylation signatures and cIMT. Two regression models were conducted. DNA methylation levels at each CpG site were regressed against natural-log transformed cIMT with adjustment for age, sex, white blood cell proportions and smoking history as fixed effects, and microarray and position on the microarray as random effects. In the fully adjusted model, body mass index (BMI), HDL cholesterol/triglycerides ratio, systolic blood pressure, antihypertensive drug use, lipid lowering drug use and prevalent diabetes mellitus were added to the primary regression model. Further details are provided in Supplementary Table S3. All statistical analyses were conducted using R.

Epigenome wide association study quality control and meta-analysis

Each cohort conducted the EWAS of cIMT in adult subjects with DNA methylation profiles available. Subsequently, EWAS summary statistics were uploaded to a central repository. Prior to their inclusion into the meta-analysis, all probes on sex chromosomes, non-CpG probes, CPH probes, and cross-reactive probes were removed as suggested by Chen et al (16). Genomic correction was applied to any cohort-specific EWAS result with a lambda of more than 1. The total number of probes included in the meta-analysis was 473,755. We performed an inverse variance weighted meta-analysis EWA studies results using METAL (17). Bonferroni-corrected significance threshold (assuming 473,755 independent tests): 0.05/nprobes_max= 0.05/473,755 $\approx 1.05\times10^{-7}$ was considered to adjust for multiple comparisons and assumed as epigenome-wide significant.

Differentially methylated regions

The identification of DMR was conducted using Comb-p, a python library that combines and calculates the autocorrelation among adjacent p-values found in genomic regions, in order to determine statistical significance at region level (13). DMR analyses were conducted in the discovery panel and we seek for replication in RSIII2. Detailed description of DMR identification can be found in Supplementary Appendix 1.b.

Methylation risk score

A methylation risk score (MRS) was developed for each participant of the Rotterdam Study based on DNA methylation patterns of CpGs identified from differentially methylated regions. The risk score was estimated as:

*MRS= Bm1*CpG1+ Bm2*CpG2+....Bmk*CpGk*

Where Bm is the meta-analyzed effect of each CpG site on cIMT and k is the kth CpG. MRS was calculated for each DMR (MRS.DMR) combined. We conducted a linear regression model using log-transformed cIMT as dependent variable and MRS for each genomic region (MRS.DMR) as independent variables adjusted for sex, age, RS cohort and smoking history. In the fully adjusted model, cell counts and batch effects were added to the fitted linear-mixed model using lme4 package (16). Detailed information of the construction of MRS is outlined in Supplementary Appendix 1.c.

Analysis of Expression quantitative methylation

We examined the association between the DNA methylation levels of associated CpGs and gene expression found in 731 subjects from the third sub-cohort of the Rotterdam Study (RSIII-1). Significant associations were determined based on the Bonferroni-corrected p-value threshold (assuming 21,238 independent tests) = 2.3×10^{-6} (0.05/21,238 expression probes). Additionally, we performed a look-up of cis-eQTMs (expression-quantitative trait methylation) for the identified CpGs using the biobank BIOS-BBMRI data resource (17) (http://bbmri.researchlumc.nl/atlas/). Further details regarding gene expression quantification in Rotterdam Study are detailed in Supplementary Appendix 1.d.

Mendelian randomization analysis

We implemented a Mendelian Randomization (MR) study (21) (Supplementary Figure S1) to investigate the causal relation between the identified CpG sites, cardiovascular risk factors and the risk of vascular outcomes. First, we investigated whether the identified CpGs are causally affected by cardiovascular risk factors. We selected a panel of SNPs associated with each trait at a genome-wide level of significance ($P < 5\times10^{-8}$) and minor allele frequency >0.01 as genetic instruments using published GWAS. Only studies of individuals of European ancestry were considered. We selected 167 genetic instruments for systolic blood pressure (SBP) (22, 23) (Supplementary Table S4), 170 SNPs associated with diastolic blood pressure (DBP) (22, 23) (Supplementary Table S5), 235 SNPs reported for pulse pressure (PP) (22, 23) (Supplementary Table S6), 123 variants associated with smoking index (24, 25) (Supplementary Table S7), 101 SNPs for LDL (26, 27) (Supplementary Table S8) and 40 SNPs associated with glucose levels (28) (Supplementary Table S9). We included 362 genetic variants previously found to be associated with BMI, in a recent meta-analysis by the GIANT Consortium (29) (Supplementary Table S10). Second, we examined the causal effect of DNA methylation on cIMT and coronary artery disease (CAD). We chose instrumen-

tal variables for DNA methylation levels based on methylation quantitative trait loci (meQTL) data from the biobank BIOS-BBMRI data resource (20) (http://bbmri.researchlumc.nl/atlas/) and the meQTLs reported by Gao et al (30) (Supplementary Table S11). Beta estimates of meQTLs were obtained from GWAS on CpG sites performed in ~1,400 individuals from RS-II and RS-III. Detailed information on the estimation of meQTLs is provided in Supplementary Appendix 1.e. We used "MendelianRandomization", a statistical package running under R (31) (https://cran.r-project.org/web/packages/MendelianRandomization/index.html). Detailed description of MR methods is outlined in Supplementary Appendix 1.f.

Results

Study sample characteristics

Baseline characteristics of the discovery cohorts (n=6157) and replication cohort (n=251) in this meta-analysis are presented in supplementary Tables S1 and S2. Participants came from ten cohorts with sample sizes ranging from 191 to 1977. Nearly half of participants were female in all studies, ranging from 47.6% to 62%. Mean±SD age ranged from 40.3 ±3.3 to 76.1±5.1 years. The cohorts included participants of European ancestry.

Meta-analysis of epigenome-wide association studies

Figure 1 shows the Manhattan plot for the discovery meta-analysis results of the fully adjusted model. There was one CpGs associated with cIMT at the epigenome-wide-significance threshold (p-value=1.05×10^{-7}). The CpG site cg05575921 showed the lowest p-value= 3.5×10^{-8} (beta= -0.0264, SE= 0.0048), and was replicated in RSIII2 with a p-value= 0.005 (beta= -0.07) (Table 1). This CpG is located at chr5:81649347 in the intron 3 of the aryl hydrocarbon receptor repressor (*AHRR*) gene (Figure 2, (32)).Further adjustments for additional potential confounders including BMI, HDL cholesterol/triglycerides ratio, systolic blood pressure, antihypertensive and lipid lowering drug use, prevalent diabetes mellitus and smoking did not substantially change the effect estimates and p-values in both the discovery panel (p-value=2.2×10^{-8}, beta=-0.03) and replication cohort (p-value=0.005, beta=-0.08) (Table 1).

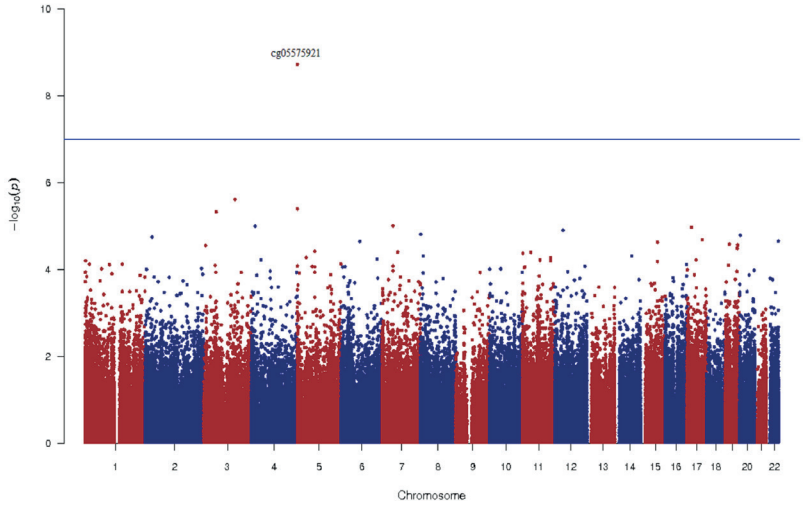

Figure 1. Manhattan plot Epigenome wide association between genome-wide DNA methylation and carotid intima media thickness

Table 1. Associated CpG sites with CIMT

			Discovery panel		Replication study		Combined analysis	
CpG site	Position	Gene	Beta	p-value	Beta	p-value	Beta	p-value
cg05575921*	Chr5: 373378	AHRR	-0.02	3.5×10^{-8}	-0.07	0.005	-0.03	3.17×10^{-9}
cg05575921**	Chr5: 373378	AHRR	-0.03	2.2×10^{8}	-0.08	0.004	-0.03	1.9×10^{-9}

*First model: BETA ~ Ln(cIMT) + age + sex + tech cov + cell counts + smoking status (+ study specific)
**Second model: BETA ~ Ln(cIMT) + age + sex + tech cov + cell counts + smoking status (+ study specific) + BMI + HDLC/TC ratio + SBP + antihypertensive + lipid lowering + pDM.

Gene expression levels

We further investigated the role of the methylation status in cg05575921 on gene expression levels measured in subjects from RSIII-1. We found 18 gene expression markers significantly associated with cg05575921 in trans eQTM database (Supplementa ry Table S12). The strongest association was found for the transcripts of the *LRRN3* (Leucine Rich Repeat Neuronal 3) gene. Hypermethylation status of cg05575921 was negatively correlated with ILMN_1773650 (p-value=4.1×10^{-22})and ILMN_2048591 (p-value= 4.2×10^{-22}), both located in *LRRN3* (Leucine Rich Repeat Neuronal 3).

In the look-up for cis-eQTM in the BIOS-BBMRI dataset, cg05575921 was associated with expression levels of *EXOC3* (exocyst complex component 3) (p-value=1.2×10^{-6}). We further explored whether the gene expression levels, associated with cg05575921, associate with cIMT. Using data from RSIII1, none of the gene expression levels were significantly associated with cIMT (α=0.05/18 expression markers = 0.002).

Assessment of differentially methylated regions

In the discovery panel, we identified 34 DMRs associated with cIMT composed of 247 CpGs. The strongest association (p-value=1.4×10^{-13}) was observed in a cluster of 12 CpG sites annotated to the promoter region of *ALOX12* (in the upstream genomic region of chr17: 6899085- 6899759) (Table 2, Figure 3). Most of the CpG sites clustered in these DMRs were annotated to the promoter region or transcription site of the gene. Nevertheless, none of these DMRs were partially nor fully replicated in RSIII2.

Methylation risk score

We conducted a methylation risk score to determine the variance of cIMT explained by 247 CpG sites located in the differentially methylated regions previously described. We found that 26% of the cIMT variance was explained by the methylation risk score of DMRs combined (MRS.DMR) (p-value=0.005). MRS accounting for correlated CpGs and analyzing independent CpGs alone (MRS.DMR.indCpGs) explained 28% of cIMT variation (p-value= 1.12×10^{-6}).

Mendelian Randomization

We applied Mendelian Randomization analysis to investigate the causal effect of cardiovascular risk factors on DNA methylation at cg05575921 as well as the causal effect of this CpG on the risk of cardiovascular diseases. First, we studied the causal effect of CVD risk factors on DNA methylation at cg05575921. Results from the conventional and sensitivity MR analyses are shown in Table 3 and Table 4. MR estimates did not support a causal effect for LDL (IVW. beta=0.0001, p-value=0.9; Supplementary Figure S2), SBP (IVW.beta=-0.001, p-value =0.7;

Table 2. Differentially methylated regions (DMRs) associated with CIMT

Gene	Locus	DMR position	CpGs	Regulatory feature	p-value
ALOX12	17p13.1	6899085-6899759	12	TSS1500; TSS200; 1st exon	1.4×10^{-13}
-	Chr 11	67383377-67384041	8	-	1.2×10^{-10}
-	Chr 5	71852464-71853216	4	-	2.6×10^{-10}
VARS	6p21.33	31762353- 31762902	15	Body	3.3×10^{-7}
-	Chr 16	53407421- 53407809	6	Body	1.1×10^{-6}
MYO1G	7p13	45002287- 45002920	4	Body; 3'UTR	3.6×10^{-6}
-	Chr 12	86229804- 86230368	4	TSS1500; TSS200; 1st exon;5'UTR	6.5×10^{-6}
MRVI1	11p15.4	10715175- 10715596	8	TSS1500; TSS200; 1st exon;5'UTR	9.9×10^{-6}
B4GALNT4	11p15.5	368351- 368848	19	TSS1500	1.9×10^{-5}
MAP3K6	1p36.11	27687074- 27687696	4	Body	1.1×10^{-4}
LTK	15q15.1	41805868- 41806235	5	TSS200; 1st exon;5'UTR	1.4×10^{-4}
ZC3H12D	6q25.1	149805995-149806733	10	TSS1500; TSS200; 1st exon; 5'UTR	1.7×10^{-4}
-	Chr 6	29648161-29648757	20	-	2.4×10^{-4}
-	Chr 17	3416252 -3416526	4	-	5.1×10^{-4}
SLC52A3	20p13	749148- 749621	7	TSS1500; TSS200; 1st exon;5'UTR	7×10^{-4}
AHRR	5p15.33	368447- 369089	4	Body	7.1×10^{-4}
TCL1A	14q32.1	96180319-96181045	11	TSS1500; TSS200; 1st exon; 5'UTR, body	8.7×10^{-4}
C10orf11	10q22.2-q22.3	77542302- 77542586	9	TSS1500; TSS200; 1st exon; 5'UTR	1.3×10^{-3}
KIF19	17q25.1	72350354-72350711	4	Body	1.5×10^{-3}
-	Chr 19	2858854- 2859154	4	-	1.9×10^{-3}
FOXK2	17q25.3	80545020-80545545	9	Body	1.9×10^{-3}
GNMT	6p21.1	42927940- 42928346	15	TSS1500; TSS200	2.2×10^{-3}
FCHO1	19p13.11	17877419-17877734	5	Body	3.4×10^{-3}
-	Chr 8	1443908-1444086	3	-	4.1×10^{-3}
NFIX	19p13.13	13135514-13135809	4	Body	6.4×10^{-3}
CMYA5	5q14.1	78985425-78985593	9	TSS1500; TSS200; Body	7.3×10^{-3}
MTHFSD	16q24.1	86588932- 86589513	10	TSS1500; TSS200; Body	8.2×10^{-3}
-	Chr 10	13481846-13481945	3	-	8.8×10^{-3}
FAM19A3	1p13.2	113265410-113265754	6	Body	1.2×10^{-2}
ZNF518B	4p16.1	10459929-10460238	3	TSS1500	1.4×10^{-2}
-	Chr 12	116919971-116920305	3	-	2.7×10^{-2}
-	Chr 16	30832346-30832795	4	-	3.3×10^{-2}

Table 2. Differentially methylated regions (DMRs) associated with CIMT (continued)

Gene	Locus	DMR position	CpGs	Regulatory feature	p-value
PTPRG	3p14.2	61547130-61547525	7	TSS1500; TSS200; 1st exon;5'UTR, body	3.5×10^{-2}
TRAF3	14q32.32	103367489-103367859	4	Body	4.1×10^{-2}

Supplementary Figure S3), DBP (IVW.beta=0.002, p-value =0.4; Supplementary Figure S4), pulse pressure (IVW.beta=0.003, p-value=0.2; Supplementary Figure S5); smoking (IVW. beta=0.04, p-value=0.09; Supplementary Figure S6); glucose (IVW.beta=-0.005, p-value=0.9; Supplementary Figure S7) and BMI (IVW.beta=-0.01, p-value=0.4; Supplementary Figure S8). Second, we investigated the causal effect of DNA methylation at cg05575921 on cIMT and CAD. We identified 11 genetic instruments for the CpG site (p-values $<5\times10^{-8}$, R2>0.8). The combination of independent SNPs showed that genetically determined methylation at cg05575921 did not reveal a causal association with cIMT (IVW.beta=-0.13, p-value =0.2; Supplementary Figure S9) and CAD (IVW.beta=-0.078, p-value =0.8; Supplementary Figure S10).

Discussion

This study is the first epigenome wide association study on cIMT, an index of atherosclerosis. We report differential DNA methylation at one CpG site and 34 DMRs with cIMT. The association found for the CpG cg05575921 was independent of potential confounders including BMI, lipid traits and blood pressure. The CpG sites found in DMRs combined in a methylation risk score, explained up to 26% of the variance observed in cIMT in Caucasian population. In addition, we found that DNA methylation at cg05575921 is not implicated in the causal association pathway between several risk factors and cardiovascular diseases.

Our top hit is located in the intronic region of the *AHRR* gene. This gene encodes aryl-hydrocarbon receptor repressor, a protein that participates in the aryl hydrocarbon receptor (AhR) signaling cascade, which mediates dioxin toxicity, and is involved in regulation of cell growth and differentiation. It functions as a feedback modulator by repressing AhR-dependent gene expression (33). Several studies have shown that the activation of the AhR pathway promotes atherosclerosis. Wu et al, showed that the treatment of macrophages with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) leads to AhR–dependent activation of inflammatory mediators and atherosclerotic plaque formation (34). In addition, Vogel et al demonstrated that TCDD promotes the differentiation of U937 macrophages to atherogenic foam cells, verified by lipid accumulation and extensive formation of blebs on the cell surface, which are characteristics of foam cells (35).

We also observed an inverse association between DNA methylation of cg05575921 and levels of 18 mRNA expression probes, in which the majority are transcripts of inflammation genes. These findings suggest that increased methylation at *AHRR* gene decreases expression of relevant genes that are critical in the regulation of the inflammatory mechanisms taking place in the vascular wall. In our study, two expression probes of the *LRRN3* gene (ILMN_1773650 and ILMN_2353732) showed the strongest association with methylation of cg05575921. The *LRRN3* has been shown to be differentially expressed between regions of plaque rich in smooth muscle cells and macrophages (36). Furthermore, *LRRN3* has been incorporated in predictive models in whole blood to evaluate self-reported smoking status (current and recently quit smokers vs. former and never smokers) (37). Based on

Table 3. Mendelian randomization analysis on the effect of cardiovascular risk factors on cg05575921

Method	Estimate	p-value	p-value heterogeneity (IVW)
LDL-cholesterol --> cg05575921			
IVW	0.0001	0.9	0.4
MR-Egger	0.008	0.6	-
MR-Egger (intercept)	-0.001	0.5	-
Weighted median	-0.019	0.2	-
Systolic blood pressure --> cg05575921			
IVW	0.001	0.7	0.6
MR-Egger	0.01	0.05	-
MR-Egger (intercept)	-0.002	0.05	-
Weighted median	0.0001	0.9	-
Diastolic blood pressure --> cg05575921			
IVW	0.002	0.4	0.2
MR-Egger	0.002	0.8	-
MR-Egger (intercept)	0	0.9	-
Weighted median	-0.002	0.5	-
Pulse pressure --> cg05575921			
IVW	0.003	0.2	0.6
MR-Egger	0.002	0.7	-
MR-Egger (intercept)	0	0.9	-
Weighted median	0.001	0.7	-
Smoking --> cg05575921			
IVW	0.04	0.09	0.5
MR-Egger	0.0001	0.9	-
MR-Egger (intercept)	0.001	0.6	-
Weighted median	0.04	0.2	-
Fasting glucose --> cg05575921			
IVW	-0.005	0.9	0.5
MR-Egger	-0.07	0.5	-
MR-Egger (intercept)	0.002	0.5	-
Weighted median	-0.02	0.6	-
BMI --> cg05575921			
IVW	-0.01	0.4	0.5
MR-Egger	-0.003	0.9	
MR-Egger (intercept)	0	0.8	
Weighted median	0.009	0.7	

BMI: Body mass index; CAD: coronary artery disease; cIMT: carotid intima media thickness; IVW: Inverse variance weighted; LDL: low-density lipoprotein

Table 4. Mendelian Randomization on the effect of cg05575921on cIMT and coronary artery disease

Method	Estimate	p-value	p-value heterogeneity (IVW)
cg05575921 --> cIMT			
IVW	-0.13	0.2	0.8
MR-Egger	-0.03	0.8	-
MR-Egger (intercept)	-0.001	0.5	-
Weighted median	-0.01	0.6	-
cg05575921 --> CAD			
IVW	-0.078	0.8	0.6
MR-Egger	-0.3	0.5	-
MR-Egger (intercept)	0.002	0.6	-
Weighted median	0.14	0.7	-

cIMT: carotid intima media thickness; IVW: Inverse variance weighted; CAD: coronary artery disease

our findings, the differentially expressed genes associated with cg05575921 could compromise downstream signals, resulting in the variability observed for cIMT in this population.

We also implemented bioinformatics tools to evaluate the presence of differentially methylated regions which are genomic regions with adjacent CpG islands that show differential methylation (14).The identification of DMRs is thought to provide a more comprehensive characterization of a genomic region based on the analysis of correlated CpGs (38). In this study, the assessment of DMRs allowed us to identify associations of target genomic regions with cIMT. Among the regions identified, the strongest association was observed for *ALOX12*. Furthermore, significant associations were observed for 33 additional DMRs located in genes involved in molecular mechanisms of cell signaling, vascular function and inflammation. *ALOX12* encodes a member of the lipoxygenase family of proteins. Lipoxygenases (LOXs) are dioxygenases that catalyze the formation of corresponding hydroperoxides from polyunsaturated fatty acids such as linoleic acid and arachidonic acid (39). Polymorphisms in *ALOX12* have shown to be genetically associated with subclinical atherosclerosis and with biomarkers of disease in families with type 2 diabetes (40). Mice models lacking *ALOX12* (P-12LO) exhibit a selective modulatory role for P-12LO in the ADP-induced pathway of platelet aggregation in mice, and increased mortality in an ADP-induced mouse model of thromboembolism (41). Lipoxygenases, especially ALOX12 may be considered as an interesting new genomic target for further investigations on traits related to vascular inflammation and impaired vascular function. Although these regions were not replicated on an independent sample, the genes identified have biologic relevance on the trait. The limited sample size of the replication cohort may have contributed to the lack of reproducibility of our findings.

The cross-sectional design of our study makes it difficult to determine whether the presence of cardiovascular risk factors are confounders or precursors in the reported methylation-cIMT associations. Causal inference in this setting is normally addressed by Mendelian

randomization methods, which rules out reverse causation, confounding and provides further understanding on the direction of risk factor-outcome association. We conducted MR analysis addressing the effect of cardiovascular risk factors on DNA methylation and the effect of DNA methylation on cardiovascular outcomes. This enabled us to get a better understanding of the potential role of epigenetic markers in mediating the environmental impact on complex disease (21). Our results found no causal effect of cardiovascular risk factors on methylation at *AHRR* and no causal link between DNA methylation and the risk for vascular disease. One explanation is that the genetic variants included in MR may explain a small proportion of the total variance in cardiovascular risk factors and DNA methylation status, and this could affect the statistical power to address any causal relations. Another explanation is that DNA methylation at cg05575921 may be implicated in the causal association between risk factors and cardiovascular diseases in relevant tissues but not in blood.

This study has several strengths and limitations that should be considered with the interpretation of the currently reported results. The meta-analysis results were obtained by combining DNA methylation results from European populations. The exclusion of 117 Afro-American (AAs) individuals from CHS cohort was based on potential differences of DNA methylation patterns observed between AAs and Caucasian ancestries. Indeed, the inclusion of data from non-Caucasian population led to different results in both single CpG meta-analysis and DMRs assessment. A sensitivity analysis to study the impact of ancestry in the results is a valuable approach, however the sample size was limited. Furthermore, DNA methylation was quantified from whole blood, a cell type mixture. We addressed this issue by incorporating white blood cell composition in our regression models. In addition, recent publications support that trait-specific differentially methylated sites identified in blood can show similar associations in the target tissue (23, 42, 43). This suggests that DNA methylation measured in blood can be used as a proxy of methylation in other tissues. Another limitation was the usage of 450K array by the majority of the studies included in this meta-analysis, which has limited coverage, allowing the interrogation of only ~2% of total human DNA methylation and limiting the discovery of other genetic regions. The strengths of our study include the investigation of a clinically relevant trait in the field of vascular biology and cardiovascular diseases; despite a potential lack of power for this trait in particular, our meta-analysis includes rigorous analytical methods and a large sample size compared to other epigenetic studies. In addition, we implemented a wide variety of resources in the characterization of our findings, including gene expression assessment, identification of the effect of genetic variants on DNA methylation levels, identification of differentially methylated regions and the implementation of a comprehensive Mendelian randomization approach.

In conclusion, we identified one CpG located in *AHRR* gene to be associated with intima-media thickness, a subclinical marker of atherosclerosis, from the largest epigenetic study conducted on this trait. DNA methylation at *ALOX12* and other 33 DMRs also contribute to the phenotype. Future experimental research, as well as an in-depth exploration of these genes, should be conducted to disentangle their role in pathophysiology related to arterial thickness.

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CHAPTER 3

Epigenetic modifications and cardiometabolic risk

3.1.

The role of DNA methylation and histone modifications in blood pressure. A systematic review

3.2.

Epigenetics and inflammatory markers: a systematic review of the current evidence

3.3.

The role of epigenetic modifications in cardiovascular disease: A systematic review

3.4.

Epigenetic link between statin therapy and type 2 diabetes

CHAPTER 3.1

The role of DNA methylation and histone modifications in blood pressure: a systematic review

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Abstract

Epigenetic mechanisms might play a role in the pathophysiology of hypertension, a major risk factor for cardiovascular disease and renal failure. We aimed to systematically review studies investigating the association between epigenetic marks (global, candidate gene or genome-wide methylation of DNA and histone modifications) and blood pressure or hypertension. Five bibliographic databases were searched until December 7th of 2019. Of 2,984 identified references, 26 articles based on 25 unique studies met our inclusion criteria, which involved a total of 28,382 participants. The five studies that assessed global DNA-methylation, generally found lower methylation levels with higher systolic blood pressure, diastolic blood pressure and/or presence of hypertension. Eighteen candidate gene studies reported, in total, 16 differentially methylated genes including renin-angiotensin-system related genes(*ACE* promoter, and *AGTR1*) and genes involved in sodium homeostasis and extracellular fluid volume maintenance system (*NET* promoter, *SCNN1A* and *ADD1*). Between the three identified EWAS, lower methylation levels of *SULF1*, *EHMT2*, and *SKOR2* were found in hypertensive patients as compared to normotensive subjects and lower methylation levels of *PHGDH*, *SLC7A11* and *TSPAN2* were associated with higher systolic and diastolic blood pressure. In summary, the most convincing evidence has been reported from candidate gene studies, which show reproducible epigenetic changes in the interconnected renin-angiotensin and inflammatory systems. Our study highlights gaps in literature on the role of histone modifications in blood pressure and the need to conduct high quality studies, in particular hypothesis-generating studies that may help to elucidate new molecular mechanisms.

Introduction

Hypertension, a long-term condition in which the blood pressure (BP) in the arteries is persistently elevated, and contributes to 4.5 % of the global burden of disease. The incidence of hypertension and associated outcomes have increased worldwide (1), playing a major role in the etiology of cerebrovascular and cardiovascular disease (CVD) (2) . The etiology of hypertension remains unclear, therefore, a better understanding of the risk factors is key to improve prevention strategies. Several environmental risk factors are contributing to hypertension (3-5). Genetic variants also determine BP and risk of hypertension which heritability has been estimated up to 30-50% (6). The most recent genome-wide association study on blood pressure phenotypes conducted in 321,262 participants found more than 241 loci, 44 of which were newly discovered. This study and previous genetic investigation of the biology of blood pressure regulation, have revealed new opportunities for future drug development and highlighted the shared genetic architecture between blood pressure and lifestyle exposures such as obesity, smoking, alcohol and high salt-intake (7-9). However, these variants explain only a minor fraction (<5%) of the inter-individual variation in the susceptibility for hypertension (10). Epigenetic modifications might contribute to the pathophysiology of hypertension (11). Epigenetics refers to, dynamic and potentially reversible, changes that alter gene activity and expression. DNA methylation and histone modifications are the most studied epigenetic mechanisms and have been involved in pathways related to dyslipidemia, type 2 diabetes, and cardiovascular disease, conditions that are much correlated with hypertension (11-13). To date, however, little work has been done to systematically assess the current evidence of the role epigenetic modifications on the risk of hypertension. We aimed to systematically review all the available evidence of the association epigenetics with hypertension. A critical appraisal of limitations and gaps in the field is also presented.

Methods

Literature search

This review was conducted and reported in accordance with the PRISMA (14) guideline (Appendix S1). We sought studies published before December 7th 2019 (date last searched) in five electronic databases: Embase.com, Medline (Ovid), Web-of-Science, Cochrane Central and Google Scholar. The search was done with the help of a medical information specialist. In databases where a thesaurus was available (Embase and Medline) articles were searched by thesaurus terms, title and/or abstract; in other databases only by title and/or abstract. The search combined terms related to the exposure (e.g. epigenetic, histone acetylation, methylation, demethylation, hypomethylation, hypermethylation, DNA methylation) and outcome (e.g. blood pressure, hypertension, and hypotension). We did not apply any language restriction, but we restricted the search to studies on humans. The full search strategies of all databases are provided in Appendix S2. The study identification also included manual search, based on the screening of the citations of the included studies.

Study selection and inclusion criteria

Studies were eligible for inclusion if they (1) were cross-sectional studies, case-control studies, or cohort studies; (2) were conducted among humans; (3) assessed epigenetic marks (global, site specific or genome-wide methylation of DNA or histone modifications); (4) collected data on blood pressure (systolic and diastolic blood pressure, hypertension, essential hypertension), and (5) reported the association of any of the above-mentioned epigenetic marks with blood pressure. We did not make restriction on the tissue examined for epigenetic marks. We excluded studies that examined epigenetic marks other than DNA methylation

and histone modifications, such as noncoding RNAs. We also excluded post-mortem studies. Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. We retrieved full texts of the selected references and two reviewers screened these full texts against the selection criteria. If no consensus was reached, a third independent reviewer solved discrepancies between the two reviewers.

Data extraction

A predesigned data collection form was prepared to extract the relevant information from the selected studies, including study design, characteristics of the study population, location of the study, sample size, and degree of adjustment. Furthermore, for each study, the tissue type and methods used to determine DNA methylation, specific CpGs sites, directions of the associations, and when possible the reported measures of associations (e.g., correlation analysis, beta-coefficients, odds ratio, relative risks, and confidence intervals) were reported. Assessing the risk of bias. Two reviewers independently rated the quality of studies based on the Newcastle-Ottawa Scale (NOS) (15), a semi-quantitative scale designed to evaluate the quality of case-control or cohort studies. We evaluated cross-sectional studies using an adapted version of the scale. Studies that received a score of nine stars were judged to have good quality and to be at low risk of bias; studies that scored seven or eight stars were considered medium risk of bias and those that scored less than seven were considered to be at high risk of bias.

Outcome assessment and statistical methods

For each study, we defined whether an association was reported, and when applicable, direction and effect sizes were reported. Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. However, due to differences in exposure and outcomes, and input parameters, it was not feasible to quantitatively pool the data.

Results

In total, we identified 2,984 unique references (Fig 1). Based on the title and abstract, we selected full texts of 55 articles for detailed evaluation. After full-text assessment, 26 of these articles based on 25 unique studies, met our eligibility criteria and were included in this review. The other 29 articles were excluded (Fig 1).

Characteristics of the included studies

Detailed characteristics of the 25 included studies are summarized in Table 1-3. Combined, the 25 studies included data from 28,382 individuals. Five studies assessed global DNA-methylation. From those, two studies (16, 17) also used candidate gene approach. Sixteen studies assessed the DNA methylation only in specific candidate genes, three studies used genome-wide approaches, and one study assessed histone modification in relation to BP. One study included South Asian and European population (18), and another one included individuals of European, African American, and Hispanic ancestry from different countries. Twelve studies included participants from China, three from Canada, two from USA, and the rest included participants from Brazil, Egypt, the Netherlands, Poland, Spain and Switzerland. The majority (n=22) of studies assessed epigenetic signatures in blood, two in visceral adipose tissue (VAT) and one in saliva. Eight studies were judged at medium risk of bias whereas the rest high risk of bias.

Outcome definition and assessment

The studies reported the outcomes in two different ways: measures of blood pressure (expressed as continuous variables) (n=7) or diagnosis status (presence or absence of essential hyper

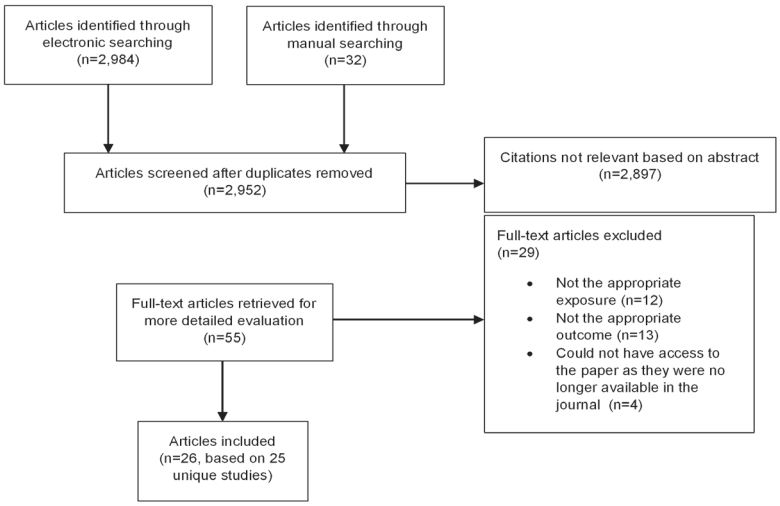

Figure 1. Flowchart of studies included in the systematic review

tension) (n=14). The remaining four studies reported both types of outcomes. Although studies that reported diagnosis status, used different cut-off to define the presence of essential hypertension, the majority (n=11) used the same criteria based on the European Society of Hypertension-European Society of Cardiology Guidelines of 2003 (19) (Table S3). Studies that assessed the blood pressure levels, usually measured it in a standardized way- after at least 10 minutes of rest, with multiple measures and waiting intervals between measurements of 10 minutes, either in different days or in different arms, in order to finally obtain an average measure (Table S3).

Global DNA methylation and blood pressure

Five studies examined the association between global DNA methylation and BP (Table 1). Four of them used blood samples to assess DNA methylation and only one was conducted in VAT (20). Three of the five studies assessed global DNA methylation in the repeat sequences and transposable elements in the genome. A large portion of methylation sites within the genome is found in these sequences, and is shown to correlate with total genomic methylation content (21). Of these three, one study (reported in two articles) (16, 22) assessed both long-interspersed nuclear element (LINE-1) and ALU transposable repeated elements, one study assessed solely LINE-1 methylation (20) and one solely ALU methylation (17). The two remaining studies assessed global DNA methylation as a percentage of total cytosine (methylcytosine/cytosine ratio) (23) or the level of 5-methylcytosine (5mC) (24). Two studies assessed BP as outcome, one study assessed hypertension and two additional studies (reported in three articles) (16, 22, 23) assessed both BP and hypertension.

The studies that assessed LINE-1 methylation showed association between lower methylation level and higher diastolic blood pressure (DBP) and hypertension (16, 20, 22). From the two studies that assessed methylation of ALU transposable repeated elements, one showed results consistent with the previous two studies, lower ALU methylation with higher DBP (17), whereas the other study reported both systolic blood pressure (SBP) and DBP to be positively associated with the degree of methylation of the gene for ALU (16). Of the studies that measured methylcytosine, one reported higher levels of 5mC in healthy controls compared to patients with hypertension (24), whereas the

other one reported no association between methylcytosine/cytosine ratio and BP(23).

Gene-specific DNA methylation and blood pressure

Eighteen studies examined methylation sites in specific candidate genes (Table 2).The rational and criteria for the selection of the candidate genes varied across studies. Some of the studies (18, 25, 26) investigated genes (*ADRB3, ABCG1, GALNT2 and HMGCR*) that were previously identified in genome- or epigenome- wide association studies on hypertension or cardiovascular disease. Other investigations studied pro-inflammatory genes (*TRl2, iNOS, IFNγ, F3, GCR, ICAM-1, TLR4, NFKB1, PPARγ and IL-6*) (16, 17, 27-29), or renin-angiotensin-system (RAS) genes (*ACE* promoter, and *AGTR1*) (30-33). Some others chose genes involved in the physiology of hypertension, e.g. related to the sympathetic nervous system, sodium homeostasis, extracellular fluid volume maintenance or proliferation of vascular smooth muscle cells (*NET* promoter, *SCNN1A, ADD1* and *MFN2*) (34-38).

Of the eighteen studies, one measured DNA methylation in VAT(20) and one in saliva (32), whereas the other studies used blood samples. Four of the studies did not report any level of adjustment or control for confounders, while the others controlled for age and additional confounders such as sex, body mass index, lipid levels, and smoking. Five studies assessed BP as outcome and twelve assessed hypertension. One additional study assessed both BP levels and hypertension as outcome (18).

Among the studies that assessed BP levels, three of them found hypomethylation of the genes (*TLR4,ACE* promoter and *NFKB1*) at higher levels of SBP (17,28,30) and one found hypermethylation of the gene (*ADRB3*) at higher levels of SBP (25). There was also no consensus for DBP (Table S4). Overall, among the other 13 studies whose outcome was hypertension, 12 studies found hypertension to be associated with hypomethylation of the candidate genes (*ADD1, ADD1* promoter, *GCK, AGTR1, IL-6, NET* promoter, *IFNγ* promoter and *MFN2*). *ADD1* and *AGTR1* were assessed by two studies each, finding congruent results that showed hypomethylation in patients with hypertension (Table S4). Only one study found higher levels of methylation of the gene among hypertensive patients (39).

Epigenome-wide analysis and blood pressure

Three studies investigated genomic DNA methylation in a hypothesis-free approach (Table 3). One of them adjusted for age and the other two, additionally, adjusted for sex, body mass index, and ethnicity, among others. The studies assessed DNA from blood and used replication cohorts to validate their findings. Wang et al., found seven out of the 10 differentially methylated top genes, to be hypomethylated in American hypertensive patients (40). The top two CpG sites (one located in SULF1 and one in PRCP) could not be replicated in two independent cohorts. The study of Boström et al. was performed among patients that underwent gastric surgery. They found differentially methylated genes correlated with change in SBP before and after the surgery. The association of the top CpGs with essential hypertension was evaluated (41). The replication cohort showed two CpGs (one in EHMT2 and one in SKOR2) to be significantly hypomethylated in cases compared to controls. Finally, Richard et al. conducted a study using data from CHARGE consortium. After replication, 13 CpG sites were associated with BP. All replicated CpG sites demonstrated associations of decreased DNA methylation with increases in BP. The top CpG sites for both SBP and DBP were located at PHGDH locus and SLC7A11 locus (42).

Table 1. Global DNA methylation and blood pressure

Author, year, quality*	Study Design	Outcome	%male/Age/ Sample size/ Country	Methylation sites/ method	Tissue type	Adjustment level	Main findings
LINE-1 methylation							
Baccarelli et al., 2010, 6/9 (23)	CS and PS	Hypertension	100/55-92/ n=712/USA	Bisulfite PCR-Pyrosequencing	WB	Age	Inverse association. LINE-1 methylation was inversely associated with an existing diagnosis of hypertension at baseline (age-adjusted OR=0.6 [0.3 to 1.0] for subjects in the lowest vs. highest quartile-based category of LINE-1 methylation).
Turcot et al., 2012, 7/9 (21)	CS	Blood pressure	18.28/35.1±7.73/ n=186/ Canada	Bisulfite PCR-Pyrosequencing	VAT	Age, sex, smoking, waist circumference	Inverse association. LINE-1 methylation was negatively associated with diastolic blood pressure (β =-0.65; P = 0.03) after adjustments for the effects of age, sex, waist circumference and smoking.
Alexeeff et al., 2013, 7/9 (17)	CS and PS	Blood pressure	100/74.1±6.7**/ n=789/ USA	Bisulfite PCR-Pyrosequencing	WB	Age, BMI, smoking, pack-years of smoking, DM, alcohol consumption, race, IHD or stroke, number of neutrophils in white blood count, season, and day of week.	Inverse association. LINE-1 methylation was inversely associated with DBP (β=-0.7, 95% CI -1.2, -0.2), yet the association with SBP was weaker, with the 95% CI including zero.
ALU							
Alexeeff et al., 2013, 7/9 (17)	CS and PS	Blood pressure	100/74.1±6.7**/ n=789/ USA	Bisulfite PCR-Pyrosequencing	WB	Age, BMI, smoking, pack-years of smoking, DM, alcohol consumption, race, IHD or stroke, number of neutrophils in white blood count, season, and day of week.	Positive association. ALU methylation was positively associated with Both SBP and DBP. An increase in inter-quartile range (IQR) in the methylation was associated with an increase of 0.97mmHg in DBP (95% CI 0.32–1.57) and with an increase of 1.51mmHg in SBP (95% CI 0.36-2.61)

Table 1. Global DNA methylation and blood pressure (continued)

Author, year, quality*	Study Design	Outcome	%male/Age/ Sample size/. Country	Methylation sites/ method	Tissue type	Adjustment level	Main findings
ALU							
Bellavia et al., 2013, 4/9 (18)	CS	Blood pressure	53.3/27.7±8.6/ n=15/Canada	TLR4, IL-12, IL-6, iNOS/ Bisulfite PCR-Pyrosequencing	WB	-	Inverse association. Decreased Alu methylation was associated with significantly increased DBP (β=0.41, p=0.04) and non-significantly increased SBP (β =0.40, p=0.15).
5mC							
Smolarek et al., 2010, 5/9 (25)	CC	Essential hypertension	63.33/ 36.74± 10.59/ n=90/ Poland	TLC analysis of the DNA nucleotide composition	Blood	Age, sex, BMI, duration of disease, smoking, concentration of cholesterol, ALT, AST, glucose, and others (not specified).	Inverse association. The mean level of 5mC was 1.80±0.69 in the healthy subjects, 1.14±0.48 in the whole group of patients with essential hypertension, 1.29±0.50 in the patients with stage 1, and 0.99±0.42 with stage 2 hypertension.
mCyt/tCyt ratio							
Luttmer et al., 2013, 7/9 (24)	CS	Blood pressure, hypertension	49.5/68.7±7.2 /n=738/	Liquid chromatography–tandem mass spectrometry.	PBL	Age, sex, use of antihypertensive medication.	No association. Mean systolic and diastolic blood pressure were not associated to MC/C ratio, nor was the presence of hypertension, with or without adjustment for antihypertensive treatment.

CS: cross-sectional; PS: prospective; WB: whole blood; VAT: visceral adipose tissue; BMI: body mass index; DM: diabetes mellitus; IHD: ischemic heart disease; DBP: diastolic blood pressure; SBP: systolic blood pressure; CC: case-control; TLC: thin-layer chromatography; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PBL: peripheral blood leukocytes. *Quality assessment based on the Newcastle-Ottawa Scale. Highest score: 9/9.**Mean age from the original cohort from which the patients were taken.

Table 2. Gene-specific methylation and blood pressure

Author, year, quality*	Study design	Out-come	Tissue type	%male /Age/ Sam-ple size/Country	Methylation sites/ method	Adjustment level	Main findings
Bellavia et al., 2013, 4/9 (18)	CS	Blood pressure	WB	53.3/27.7±8.6/ n=15/Canada	TLR4/Bisulfite PCR-Pyrosequenc-ing	-	Inverse association. Decreased TLR4 methylation was associated with significant increases of both diastolic (β=0.84, p=0.02) and systolic blood pressure (β=1.45, p=0.01).
Alexeeff et al., 2013, 7/9 (17)	CS and PS	Blood pressure	WB	100/74.1±6.7**/ n=789/USA	TRL2, iNOS, IFNγ, F3, GCR, ICAM-1/ Bisulfite PCR-Py-rosequencing	Age, BMI, smoking, pack-years of smoking, DM, alcohol consumption, race, IHD, number of neutrophils in with blood count, season, day of week.	They found a positive association between DBP and methylation of TLR2 and iNOS, and a negative association between DBP and methylation of IFNγ. No clear associations were observed between SBP/DBP and meth-ylation level of ICAM-1, GCR or F3.
Zhang et al., 2013, 6/9 (37)	CC	Essential hyper-tension (EH)	PB	50.1/50.2±5.3/ n=61/China	ADD1/Bisulfite PCR-Pyrosequenc-ing	Adjusted for age, sex, smoking, and drinking.	Inverse association. ADD1 CpG2-5 methyla-tion levels were significantly associated with essential hypertension (cases versus controls (%): 27.54±7.48 versus 31.44±5.30, adjusted p= 0.026).
Guay et al., 2014, 4/9 (26)	CS	Blood pressure	VAT	100/-/n=30/Canada	ADRB3/Bisulfite PCR-Pyrosequenc-ing	-	Positive correlations. Partial Pearson´s correlations (r) between mean ADRB3 DNA methylation in visceral adipose tissue and SBP and DBP: r=0.43, p=0.05 and r=00.45, p=0.04 respectively.
Peng et al., 2014, 8/9 (27)	CS	Hyper-tension	PB	64/59.39±9.14/ n=139/China	ABCG1, GALNT2, HMGCR/ Bisulfite PCR-Pyrosequenc-ing	Age, sex, smoking, lipid level, history of hypertension and history of diabetes.	Treating gene methylation as a dichotomous variable (methylated or unmethylated), none statistically significant difference was found between patients with or without hyperten-sion.
Rangel et al., 2014, 7/9 (31)	CS	Blood pressure	PBL	52/8.99±0.22/ n=115/Brazil	ACE promoter/ Bisulfite PCR-Py-rosequencing	Age, sex, birth weight, prematurity and family history of CVD.	Inverse association. Hypomethylation of the ACE promoter was associated with changes in SBP as well as ACE activity, even after adjustment for confounders. Pearson´s correlation coefficient: -0.206, p=0.031.

Table 2. Gene-specific methylation and blood pressure (continued)

Author, year, quality*	Study design	Outcome	Tissue type	%male /Age/ Sample size/Country	Methylation sites/ method	Adjustment level	Main findings
Fan et al., 2015, 6/9 (34)	CC	Essential hypertension	PB	M and W***/ 59.28±7.41/ n=94/ China	GCK, 4CpGs/ Bisulfite PCR-Pyrosequencing	Age-matched	Significantly lower CpG 1-3 methylation (cases vs. controls, 49.13±5.72 vs. 53.49±7.53%; adjusted p=0.006) and significantly higher CpG4 methylation (cases vs. controls, 46.34±6.48 vs. 34.74±12.73%; adjusted p=0.002) were observed in patients with hypertension.
Kato et al., 2015, 6/9 (19)	CS	SBP, DBP, and hypertension	PB	74.2/54.6± 9.99/ n=6,757/South Asian and European population.	28 CpG/Bisulfite PCR-Pyrosequencing	-	Based on their GWAS analysis on five blood pressure phenotype, 35 sentinel SNPs were identified. Then, they investigated the relationship of them with local DNA methylation and found that 28 of the 35 SNPs were associated with local methylation markers. Then, using Mendelian randomization, they showed that the observed effects of SNPs on blood pressure were correlated with the effects predicted through association with methylation (r=0.52, p=0.005).
Fan et al., 2015, 7/9 (32)	CC	Essential hypertension (EH)	PB	40/ 56.52±8.47/ n=192/China	AGTR1 promoter, 5 CpGs/Bisulfite PCR-Pyrosequencing	Age, gender, smoking, drinking, BMI, triglycerides, HDL, uric acid and homocysteine.	Inverse association. A significantly lower CpG1 methylation level was identified in EH cases than in controls (cases vs. controls: 6.74 ± 4.32% vs. 9.66 ± 5.45%, p = 0.007), and no significant association was observed in the remaining analyses. Receiver operating characteristic curves showed that CpG1 methylation was a significant predictor of EH.
Mao et al., 2016, 7/9 (36)	CC	Essential hypertension (EH)	PB	35/ 57.83±7.74/ n=180/China	SCNN1A/Bisulfite PCR-Pyrosequencing	Age, sex, gender, BMI, TC, TG, glucose, ALT, smoking and drinking.	Positive association. Incident cases had a higher SCNN1A methylation level than the non-EH controls (16.15±4.51 versus 13.66±4.08, p=0.041), and prevalent cases (16.15±4.51 versus 13.77±3.90, p=0.002). Logistic regression analysis results showed that SCNN1A hypermethylation was the risk factor of EH in incident cases compared with non-EH (OR=1.157, p=0.01), and in incident cases compared with prevalent cases (OR=1.149, p=0.013).
Bayoumy et al., 2017, 5/9 (38)	CC	Essential hypertension (EH)	WB	48/52.6±5.02/ n=250/Egypt	ADD1 promoter/ Bisulfite PCR-Pyrosequencing	-	Inverse association. Lower methylation of AAD1 CpG2-5 was associated with an increased risk of EH (29.21±6.81) compared to healthy group (34.63±7.5).
Lin et al., 2017, 6/9 (33)	CC	Essential hypertension	Saliva	51.4/40.76± 16.92*/ n=326/China	AGTR1/Bisulfite PCR-Pyrosequencing	Age, sex, education level, marital status, physical activity, diet regularity, smoking and drinking status, and sleep duration and quality.	Inverse association. Low methylation levels were associated with risk for hypertension in comparison to the control group. hypertensive group compared with the control group.

Table 2. Gene-specific methylation and blood pressure (continued)

Author, year, quality*	Study design	Outcome	Tissue type	%male /Age/ Sample size/ Country	Methylation sites/ method	Adjustment level	Main findings
Mao et al., 2017, 6/9 (28)	CC	Essential hypertension (EH)	PB	40/ 56.5± 8.5/ n=192/China	IL-6/Bisulphite pyrosequencing	Age- and gender-matched	Inverse association. CpG2 and CpG3 had lower methylation in EH group compared with controls (58.43 ± 7.53 versus 62.34 ± 9.65, P = 0.004 and 51.52 ± 6.18 versus 57.45 ± 8.29, P<0.001, respectively). Logistic regression analysis found CpG3 hypomethylation was a risk factor of EH (odds ratio = 1.111, adjusted p=0.004). Receiver operating characteristic curve analysis showed that CpG2 (area under the curve: 0.638, P = 0.001) and CpG3 (area under the curve: 0.704, P<0.001) had a diagnostic value to predict the risk of EH.
Meng et al., 2017, 6/9 (35)	CS	Hypertension	PBL	85.4/ 45.1±7.43/ n=162/China	NET promoter/ Pyrophosphate sequencing	Age and BMI	Positive association. The average and specific methylation levels were higher in non-hypertensive subjects except for CpG2.
Bao et al., 2018, 6/9 (30)	CC	Essential hypertension (EH)	PB	39.6/ 56.5±8.43/ n=192/China	IFNγ promoter, 6 CpGs/ pyrosequencing	Age, sex, smoking, drinking, uric acid, HDL and BMI	CpG2 was significantly hypomethylated among cases compared controls (p=0.032) and it was found to be an effective marker of EH based on the area under the curve.
Jin et al., 2018, 7/9 (39)	CC	Essential hypertension	WB or serum	59.2/ 50.6±2.54/ n=76/China	Mfn2/ Bisulphite DNA sequencing	Age- and sex-matched	The DNA methylation level of Mfn2 was significantly lower in hypertensive patients than in controls.
Macías-González et al., 2018, 6/9 (29)	PS	Blood pressure	PBMC	34.6/ 44.68±9.27/ n=60/Spain	PPARγ, SLC19A1, IL-6, NFKB1/ pyrosequencing	Age, sex, bariatric procedure, weight loss (%)	There was no statistically significant difference between the DNA methylation patterns of the PPARγ, SLC19A1 and IL-6 genes before and at 6 months after bariatric surgery. The promoter methylation levels of the NFKB1 gene were increased after surgery. This change of methylation level was associated with changes in both SBP and DBP (r=-0.513, p=0.003 and r=-0.544, p=0.002, respectively).
Xu et al., 2018, 6/9 (40)	CC	Essential hypertension	Serum	53.3/ 65.9±9.2 / n=461/China	MTHFD1 promoter/ methylation-specific PCR	Age, gender, total homocysteine, uric acid, TG, BMI, glucose, waist circumference, hip circumference, SBP, DBP, drinking, smoking.	The MTHFD1 promoter methylation was higher in hypertensive patients than healthy controls (median PMR were 8.97% and 5.69%, respectively, p < 0.001). Multivariable analysis showed MTHFD1 promoter hypermethylation increase the risk of essential hypertension. (OR= 1.336; 95%CI, 1.235–1.446; p < 0.001). The area under the curve of MTHFD1 promoter methylation was 0.739 in total patients with essential hypertension.

CS: cross-sectional; WB: whole blood; PS: prospective; BMI: body mass index; DM: diabetes mellitus; IHD: ischemic heart disease; DBP: diastolic blood pressure; SBP: systolic blood pressure; CC: case-control; PB: peripheral blood; VAT: visceral adipose tissue; PBL: peripheral blood leukocytes; CVD: cardiovascular disease; M: men; W: women; HDL: high-density-lipoprotein; TC: total cholesterol; TG: triglycerides; ALT: alanine aminotransferase; PBMC: peripheral blood mononuclear cells; PMR: percentage of methylated reference.
*Quality assessment based on the Newcastle-Ottawa Scale. Highest score: 9/9. **Mean age from the original cohor from from which the patients were taken. ***Percentage of men not described.

Histone modifications and blood pressure

There was only one study (43) that examined the association between histone modifications and BP. The authors assessed histone 3 acetylation and methylation levels in whole blood of Beijing workers and found higher levels of both acetylation and methylation associated with lower SBP and DBP.

Discussion

The present work is the first to systematically assess the current evidence of the association between epigenetic modifications and BP. We observed an association between a generalized hypomethylation status and high levels of DBP and SBP. Our findings suggest that epigenetic variations, mainly DNA methylation, may play an important role in the regulation of molecular mechanisms of BP. Accordingly, we showed that the genes reported in these findings are important regulators of inflammatory mechanisms (*NFKB1, IFNγ, MFN2, SULF1*), and RAS activity (*PRCP, ACE, AGTR1* genes). However, no overlap was found between the findings from EWAs and the studies that used candidate-gene approach. Conclusive evidence in alterations of histones in BP is still lacking.

Global DNA methylation

Global DNA methylation in DNA repetitive elements, such as ALU and LINE-1 are the most widely used in population-based studies (44). There are 1.4 million ALU repetitive elements and half a million LINE-1 elements interspersed throughout the human genome, which represents up to 50% of global genomic methylation (45).

Consistent trend of demethylation was observed with both LINE-1 and ALU. The studies that used LINE-1 concluded a significant association between decreased methylation levels and high SBP and DBP (16, 20, 22). Hypomethylation at ALU elements was related with higher BP (16). These findings are in line with other studies showing that hypomethylation at LINE-1 inversely correlate with coronary artery disease and stroke (11). In contrast, global DNA hypermethylation at LINE-1 appears to be associated with vascular inflammatory response to endothelial injury and an increased mortality from chronic kidney disease (46). Despite their widespread usage, little is known about the biological function of DNA repetitive elements. LINE-1 are transcribed into mRNA and translated into protein that acts as a reverse transcriptase (47). Moreover, low levels of methylation of these retrotransposable elements have been associated with genomic instability and were found to be highly expressed under conditions related with cellular senescence (46, 48). In contrast, ALU elements contribute to genetic diversity and disease through insertional mutagenesis, gene expression, polyadenylation and splicing (49). ALU elements are also highly correlated with age (50). Methylation changes in response to endogenous and exogenous factors can vary between ALU and LINE-1elements (51), supporting the assumption that LINE-1 and ALU methylation might represent distinct measures of methylation in different regions of the genome (52).

Epigenome-wide association studies

The implementation of EWAS, which are the large scale, systematic, epigenomic equivalent

Table 3. Epigenome-wide and histone modification in relation to blood pressure

Author, year, quality*	Study design	Out-come	Tissue type	%male/Age/ Sample size/ Country	Methyla-tion sites/ method	Adjust-ment level	Main findings
Epigenome-Wide Association Study							
Wang et al., 2013, 6/9 (41)	CC	Essential Hyperten-sion (EH)	PB	100/ 14-23/ n=16/ USA	Illumina Hu-manMeth-ylation 27K BeadChip	Age	7 out of the 10 most significant CpG sites were hypomethylated in cases. The two most significant CpGs (one CpG site in SULF1 gene and one in PRCP gene) were replicated in 96 patients. CpG in SULF1 remained signifi-cant even after adjustment for age (p=0.038). Validation of the CpG sites in the SULF1 gene was further conducted in a second replication sample of 70 patients and it was not found to be significantly different methylated among cases vs controls.
Boström et al., 2016, 6/9 (42)	CS and PS	Blood pressure and essential hyperten-sion (EH)	WB	49.8/46.9±11.9/ n=11/Switzer-land	Illumina Hu-manMethyl-ation 450K BeadChip	Age, sex, BMI and ethnicity.	In case of 24 CpG sites changes in methylation significantly correlated with the percentile change in SBP six months after RYGB surgery. Those CpG were further investigated for an association with EH in the verification cohort (n=539, aged 19 to 101 years), finding two CpG (one in EHMT2 and one in SKOR2) significantly hypomethylated in EH.
Richard et al., 2017, 8/9 (43)	CS	Blood pressure (BP)	WB and CD4+ T cells**	M and W***/ mean age be-tween 46.3 and 76.0/n=17,010/ Consortia	Illumina Hu-manMethyl-ation 450K BeadChip	Age, sex, blood cell counts, BMI, smoking, ances-try and technical covariates.	In the discovery stage, they conducted genome-wide associations of DNA methylation with SBP and DBP in nine cohort studies (n=9,828). Multi-ethnic meta-analyses identified methylation at 31 CpG sites associated with BP after Bonferroni correction. They replicated those 31 CpG in multieth-nic meta-analyses of six additional cohorts (n=7,182). Methylation at 13 of the 31 discovery CpG sites (corresponding to 8 genes) was associated with BP at p< 0.0016 in the replication meta-analysis (0.05/31). All of the 13 CpG demonstrated associations of decreased DNA methylation with increases in BP. The top CpG sites for both SBP and DBP were located at PHGDH locus and SLC7A11 locus. The investigators found a mediation of a causal relationship of cg23999170 with BP through expression of TSPAN2.

Table 3. Epigenome-wide and histone modification in relation to blood pressure (continued)

Author, year, quality*	Study design	Outcome	Tissue type	%male/ Age/ Sample size/ Country	Methylation sites/ method	Adjustment level	Main findings
Histone modification							
Kresovich et al., 2017, 6/9 (44)	CS	Blood pressure	WB	67/18-46/ n=240/ China	Histone 3 lysine 9 acetylation (H3K9ac), histone 3 lysine 9 tri-methylation (H3K9me3), histone 3 lysine 27 tri-methylation (H3K27me3), and histone 3 lysine 36 tri-methylation (H3K36me3), lysine 9 tri-methylation (H3K9me3), histone 3 lysine 27 tri-methylation, (H3K27me3), and histone 3 lysine 36 tri-methylation, (H3K36me3)	Age, sex, occupational group, BMI, work hours per week, day of the week, smoking habits, number of cigarettes smoked during examination time, alcohol drinking status, temperature, and 8-day ambient PM10.	Inverse association. In all participants, a one fold increase in H3K9ac was associated with 2.52mmHg lower mean SBP (95%CI: -4.22, -0.81, p<0.01) and 1.54 mmHg lower mean MAP (95%CI: -2.95, -0.14, p=0.03). A one-fold increase in H3K9me3 was associated with 2.04mmHg lower mean SBP (95%CI: -3.32, -0.77, p<0.01), 1.68 mmHg lower mean DBP (95%CI: -2.84, -0.52, p=0.01), and 1.75 mmHg lower mean MAP (95%CI:-2.86, - 0.64, p<0.01). Finally, we observed a one-fold increase in H3K27me3 was associated with 2.2 8mmHg lower SPB (95%CI:-4.42, -0.13, p=0.04).

CC: case-control; PB: peripheral blood; CS: cross-sectional; PS: prospective; WB: whole blood; BMI: body mass index; RYGB: Roux-en-Y gastric bypass surgery; M: men; W: women; SBP: systolic blood pressure; DBP: diastolic blood pressure.

*Quality assessment based on the Newcastle-Ottawa Scale. Highest score: 9/9.

**Of the 14 cohorts, 13 used whole blood samples to measure DNA methylation. One cohort (GOLDN) used CD4+ T cells.

***Percentage of men not described.

of GWAS, alongside with the development of microarray technologies, has allowed the interrogation of DNA methylation sites at single-nucleotide resolution (53). Findings from EWAS have shed light on the role of DNA methylation on gene expression regulation as well as the evaluation of phenotypic variation that is attributable to inter-individual epigenomic variation (53).

In the current review, three studies examined the association between differentially methylated regions and BP following a EWAS approach (40-42). The studies reported significantly hypomethylated CpGs in association with increase in BP. The hypomethylated CpG sites are located in the genes *SULF1* (Sulfatase 1), *PRCP* (Prolylcarboxypeptidase), *EHMT2* (Histone H3-K9 Methyltransferase 3), *SKOR2* (SKI Family Transcriptional Corepressor 2), *PHGDH* (Phosphoglycerate Dehydrogenase) and *SLC7A11* (Solute Carrier Family 7 Member 11). *SULF1* is a protein coding gene which catalyzes the hydrolysis of the 6-O-sulfate group attached to glucosamine residues in heparin sulfate proteoglycans (54). The pathways controlled by this protein are closely related with inflammation through the production of interleukin-6 (55). *PRCP* gene encodes a member of the peptidase S28 involved in the degradation of angiotensin II, one of the main regulators of BP and electrolyte balance (56). *EHMT2* encodes a methyltransferase that methylates lysine residues of histone H3 which is also associated with cellular responses to starvation, negative regulation of transcription from RNA polymerase II promoter and regulation of DNA replication (57, 58). *SKOR2* gene is an homolog to the SKI family of transcriptional corepressors (59) and has been mainly identified as a potential tumor suppressor in neck squamous cell carcinomas (60). *PHGDH* encodes phosphoglycerate dehydrogenase, a key enzyme for de-novo sphingolipid synthesis, membrane lipids involved in lipid metabolism(61). *SLC7A11* encodes a sodium-independent cysteine/glutamate antiporter resulting in protection from oxidative stress and ferroptotic cell death (62). Further research is needed to determine the functional relevance of *EHMT2, SKOR2, PHGDH* and *SLC7A11* genes in the pathogenesis of hypertension.

Gene-specific DNA methylation

The assessment of DNA methylation in candidate genetic regions provides further insight into the importance of relevant genes and pathways in the etiology of BP (63). Our review expands current knowledge of blood pressure-related pathways by supporting the role of (epi) genetic dysregulation of a specific set of genes in the development of abnormal BP levels. Several pieces of evidence included in this review are consistent regarding the role of hypomethylation in *ADD1* (Adducin1), *AGTR1* (angiotensin II receptor type 1) and *ACE* (angiotensin I-converting enzyme) in the pathogenesis of hypertension. *ADD1* is a protein coding gene, part of a family of cytoskeletal proteins (64), known to increase renal sodium reabsorption and involved in the pathophysiology of hypertension in the Asian population (65). The epigenetic variability found in genes, *AGTR1* and *ACE*, involved in the renin angiotensin system crucial mechanism in the etiology of hypertension, encourages the design of better approaches at both population and experimental level to get more insight into these mechanisms.

Genetic factors of blood pressure regulation are still not very well elucidated. Essential hypertension remains to be a conundrum diagnosis. Evidence suggests a key role for 11β-hydroxysteroid dehydrogenase (11βHSD) on the pathogenesis of EH (66). Patients with EH show a decreased production of the enzyme, related with a prolonged half-life of cortisol and an increased ratio of urinary cortisol to cortisone metabolites. Genetic variants in the coding gene, *HSD11B2*, contribute to the enhanced blood pressure response to salt in humans (67). However, the percentage of people with essential hypertension is very low and efforts have been focused in investigating overall blood pressure regulation and the influence of environmental factors on this phenotype.

In the context of gene-specific methylation is important to consider that most of the research is not streamlined and is too dispersed. In this way, all the attempts to find further contributors might not be successful. Therefore, the implementation of a better organization and research guidelines in the field should be strengthened.

The evaluation of genes whose expression is associated with blood pressure may shed light on novel mechanisms associated with blood pressure regulation as well as unravel how transcripts mediate genetic and environmental effects on blood pressure variability (68). Huan et al. evaluated the global expression signatures of blood pressure and hypertension in 7,017 individuals who were not receiving antihypertensive drug treatment. They identified 34 differentially expressed genes, involved in inflammatory response and apoptosis pathways, which some of them explain 5%–9% of inter-individual variance in blood pressure (68).

DNA methylation may differ by race or ethnicity, challenging replication across individuals of varying descent in epigenetic studies (69). Previous epigenome wide association studies of several cardiometabolic risk factors for example, C-reactive protein, have been able to provide trans-ethnic replication of the differentially methylated genes (70). Current evidence supports the notion that despite differing baseline epigenetic profiles, different ethnicities may have consistent epigenetic association. Although most of the candidate gene studies included in this review are coming from Chinese population, we were not able to evidence any overlap with investigations from other ethnicities. Future studies investigating associations of differentially methylated genes with blood pressure outcomes are needed to not only discover new genes but also replicate these epigenetic markers in trans-ethnic population.

Age and gender-specific effects on epigenetic variations

DNA methylation gradually changes with aging while gender-specific methylation patterns have been observed over the lifespan (71). Several studies reported higher global DNA methylation levels in males (72), whereas studies on gender-associated differences in DNA methylation at specific loci have yielded contrasting results (73). Interestingly, among twenty studies only three articles (with overlapping participants) stratified the analyses by gender (27, 31, 36). In Chinese Han population, DNA methylation of *ADD1* gene was significantly higher in females as compared to males, yet, *ADD1* promotor methylation was a risk factor in both, males (CpG2-5) and females (CpG1) (36). Similarly, *AGTR1* CpG1 methylation was a significant predictor of hypertension in both genders (31). Finally, at CpG1 and CpG2 sites of *IL-6* promoter males were hypomethylated as compared to females, yet, only hypomethylation of CpG3 site was significantly associated with hypertension risk in both genders (27). Gender stratification in epigenetics is lacking, as seen in the current review as well, thus we are not able to make any predictions regarding the role of gender-specific methylation patterns in hypertension risk. Four out of twenty studies included in our review looked at male population only (16, 22, 25, 40), therefore, the caution is needed when interpreting our findings as some of the conclusions of our review may not be generalizable to female population. Further research is needed to investigate the effects of gender on epigenetic regulatory processes in hypertension.

In the context of aging, chronological age is one of the main determinants for functional impairments in blood pressure regulation. Evidence also suggests that aging is associated with differential methylation (mainly hypermethylation) of some genomic loci (74). Multiple CpG sites have associated with aging and are currently used to estimate 'epigenetic age' (75). 'Epigenetic age', also called 'epigenetic clock', has been associated with mortality independently of chronological age and other risk factors, supporting the assumption that they capture some aspect of biological aging (76). Therefore, it has been established that chronological age may act as a proxy for biological aging-associated changes, including DNA methylation.

Nevertheless, there is no evidence of the potential impact of the 'epigenetic age' on blood pressure. Considering that DNA methylation patterns change overtime and are highly correlated with age, they may contribute to age-related traits such as blood pressure. Therefore, further research on the impact of 'biological age' on blood pressure variability is warranted.

Strengths and limitations

The strengths and limitations of the findings from this study merit careful consideration. The present analysis, involving data from nearly 28,382 individuals, is the first to systematically assess the evidence on the subject following an a priori designed protocol with clearly defined inclusion and exclusion criteria. However, as mentioned above, the majority of studies included are cross-sectional, making it difficult to determine whether epigenetic marks are a cause or a consequence of BP. In this scenario, the use of Mendelian randomization approaches, where genetic variants are used as instrumental variables for DNA methylation (77) is encouraged. Moreover, many epigenetic studies are often limited by the fact that only blood is studied rather than other more relevant tissues, since this is the most accessible tissue in epidemiologic studies. Although, the sampling of the cell type mediating the disease allows to infer more valid conclusions, the use of leukocytes, a more accessible surrogate cell type, is proved to yield sufficiently useful information (78). Although the use of standardized and validated protocols have allowed undertaking a comprehensive search of the literature, we cannot exclude the possibility of publication bias from underreporting negative findings.

Conclusions

The emerging evidence highlights the importance of epigenetic variation in the regulation and maintenance of BP pressure levels. The most convincing evidence has been reported from candidate gene studies, where mechanisms related to RAS activation and inflammation can be assumed to represent a substrate for epigenetic regulation. Further studies integrating the systematic analysis of epigenetic markers at genomic scale as well as the demonstration of the exact cellular and physiological role of target epigenetic modifications will be needed to elucidate alternative molecular pathways.

Supplementary Information is available in the online version of the paper (https://www.nature.com/articles/s41371-019-0218-7)

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CHAPTER 3.2

Epigenetics and inflammatory markers: a systematic review of the current evidence

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Abstract

Epigenetic mechanisms have been suggested to play a role in the genetic regulation of pathways related to inflammation. Therefore, we aimed to systematically review studies investigating the association between DNA methylation and histone modifications with circulatory inflammation markers in blood. Five bibliographic databases were screened until 21 November of 2017. We included studies conducted in humans that examined the association between epigenetic marks (DNA methylation and/or histone modifications) and a comprehensive list of inflammatory markers. Of the 3,759 identified references, 24 articles were included, involving, 17,399 individuals. There was suggestive evidence for global hypomethylation but better-quality studies in the future have to confirm this. Epigenome-wide association studies (EWAS) (n=7) reported most of the identified differentially methylated genes to be hypomethylated in inflammatory processes. Candidate genes studies reported 18 differentially methylated genes related to several circulatory inflammation markers. There was no overlap in the methylated sites investigated in candidate gene studies and EWAS, except TMEM49, which was found to be hypomethylated with higher inflammatory markers in both type of studies. The relation between histone modifications and inflammatory markers was assessed by one study only. This review supports an association between epigenetic marks and inflammation, suggesting hypomethylation of the genome. Important gaps in the quality of studies were reported such as inadequate sample size, lack of adjustment for relevant confounders and failure to replicate the findings. While most of the studies have been focused on C - reactive protein, further efforts should investigate other inflammatory markers.

Introduction

Inflammation is a critical response to pathogens and injuries in the human body. Specifically, chronic low-grade inflammation plays a key role in the pathogenesis of chronic conditions and diseases like obesity, diabetes mellitus, and cardiovascular disease (1-3). A better understanding of factors that contribute to the development of inflammation and its consequences on disease is essential to improve prevention strategies in inflammation-related disorders. Genome-wide association studies have identified several genetic variants associated with inflammatory markers such as C-reactive protein, the most widely studied marker (4, 5), but the explained variance is relatively small. In addition, non-genetic factors such as smoking and dietary behaviours have been shown to exhibit a strong influence on the inflammatory response (6, 7). Emerging evidence suggests that epigenetic processes, reflecting changes in gene expression that occur without sequence mutations, may offer opportunities to understand the pathophysiology of inflammation processes. The role of epigenetic determinants is increasingly being recognized as a link between (external) environmental factors and disease risk. Moreover, epigenetic modifications are also involved in differentiation of the immune cells, a key component of the inflammatory process. Epigenetics is defined as a group of chemical modifications of the DNA sequence, which could be affected by external factors such as BMI, smoking, inflammation and can be transmitted from one generation of cells to the other (8). The molecular basis of epigenetic mechanisms are complex and comprise DNA methylation, modifications of histones and gene regulation by non-coding RNAs (9). Unlike genetic variation, epigenetic modifications are dynamic and potentially reversible and, therefore, could be modified by lifestyle and other therapeutic approaches. Until now, a comprehensive and systematic appraisal of the current literature on the role of epigenetic modifications in inflammation measured by levels of inflammatory markers is missing. Therefore, we aimed to identify and synthetize all available evidence conducted in humans and quantify the association of two of the major epigenetic modifications, DNA methylation and histone modifications, with circulation inflammatory markers in blood.

Material and Methods

Literature search

This review was conducted and reported using a predefined protocol and in accordance with the PRISMA (10) and MOOSE (11) guidelines (Supplement Material S1 and S2). We sought studies published before 21 November of 2017 (date last searched) in five electronic databases: Embase.com, Medline (Ovid), Web-of-Science, Cochrane Central and Google Scholar. We did the search with the help of an experienced medical information specialist. In databases where a thesaurus was available (Embase and Medline), articles were searched by thesaurus terms, title and/or abstract; in other databases only by title and/or abstract. The search combined terms related to the exposure (e.g. epigenetic, methylation, demethylation, hypomethylation, hypermethylation, DNA methylation) and outcome (e.g. inflammation, C-reactive protein, cytokine). We did not apply any language restriction, but we restricted the search to studies on humans alive. The full search strategies of all databases are provided in Supplement Material S3. The study identification also included manual search, based on the screening of the citations of the relevant studies. Information about study selection and inclusion criteria, data extraction process, and risk of bias assessment is described in Supplement Material S4.

Results

In total, after deduplication, we identified 3,759 potentially relevant citations. Based on

the title and abstracts, full texts of 80 articles were selected for detailed evaluation. After full-text assessment, 24 of these unique studies met our eligibility criteria, and were included in this review. The other 56 articles were excluded for reasons shown in Figure 1.

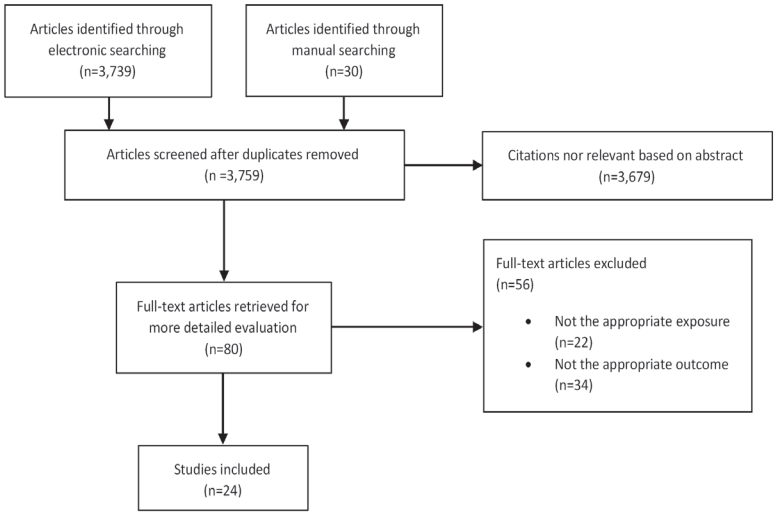

Figure 1. Flowchart of studies included in the systematic review

Characteristics of the included studies

Detailed characteristics of the included studies are summarized in Tables 1-3. All included studies were of cross-sectional design, except one study of prospective design (12). Overall, 17,399 individuals were participating in these studies. Nine studies included participants from the USA, three studies from China, three studies from Canada and the rest included participants from Brazil, Colombia, India, Ireland, Germany, Greece, Mexico, Spain, and Sweden. One of the studies (13) included participants from different cohorts such as USA, UK, Italy, Germany, and Netherlands. The majority (n=23) of studies assessed epigenetic signatures in blood, whereas other assessed epigenetic marks in tumour specimens (glioblastomas). Of the 24 studies included, four studies assessed global DNA-methylation only, eleven studies assessed DNA methylation in specific candidate genes, seven studies used genome-wide approaches, while one additional study examined both DNA methylation in specific candidate genes and globally (14). Only one study assessed histone modification in relation to inflammation markers (15). The most studied marker was C - reactive protein (CRP), which was evaluated in 17 studies. Interleukins like IL-4, IL-6, IL-8, IL-9, IL-10, and IL-18 were evaluated in 11 studies. TNF-α was assessed in three studies, fibrinogen in two and other markers such as VCAM, ICAM, VEFG, COX2, leptin, TNFR2, C-CAM1, alpha interferon and TGF-β were assessed in one single time. Nine studies were judged at medium risk of bias whereas the rest at high risk of bias.

Global DNA methylation and inflammation markers

Five studies examined the association between global DNA methylation and inflammatory markers in blood samples (Table 1). All of them used blood samples to assess DNA methylation. Four of these studies assessed methylation in long-interspersed nuclear element (LINE-1). A large portion of methylation sites within the genome are found in these repeat sequences and transposable elements, and correlate well with total genomic methylation content. From the four studies, two (14, 16) reported no association between global DNA methylation and CRP

levels, while the others showed lower methylation to be related with higher CRP levels (17, 18). One study (16), in addition to CRP levels, also evaluated the association of global DNA methylation at LINE-1 with VCAM-1 and ICAM-1, and reported an inverse association with VCAM-1 but no association with ICAM-1. One study quantified global DNA methylation by measuring the amount of methylated cytokines in the sample (5 mc) relative to global cytidine (5mC + dC) in a positive control and found no association between global DNA methylation and IL-6 serum levels.

Gene specific DNA methylation and inflammatory markers

There were twelve studies that examined methylation sites in, or near, candidate genes in relation to inflammatory markers (Table 2). One study measured DNA methylation in tumour specimens, whereas the other studies used blood samples to assess the DNA methylation. Of the twelve studies, eight did not report any level of adjustment or control for confounders, one of them (19) controlled for age and sex whereas the others (14, 20, 21) controlled for at least these two confounders. Of the twelve studies, three focused solely on CRP as outcome, one solely on interleukins, one solely in leptin and the others assessed a set of inflammatory markers including interleukins, TNF-α and fibrinogen.

In total, eight studies assessed CRP as inflammatory marker. Overall, these studies found higher levels of CRP to be associated with higher degree of methylation of *SOCS-1* (22), *LY86* (20) and *EEF2* (23) with lower degree of methylation of *AIM2* (24), *IL-6* (25) and *IL-6* promoter gene (19). One additional study (14) that examined methylation levels of *IL-6* promoter and CRP reported no association. In addition, no association was found between methylation status of *F2RL3* in peripheral blood cells and CRP levels.

Five studies evaluated the association of gene specific DNA methylation with *IL-6*. They found higher degree of methylation of *MGMT, RARβ, RASSF1A*, and *CDH13* in tumour specimens and of *SOCS-1* in peripheral blood with higher levels of IL-6, while others found less degree of methylation of *USP2, TMEM49, SMAD3, DTNB* and *IL-6* promoter with higher levels of IL-6. Other interleukins such as IL-8, IL-10 and IL-18 were only evaluated once. No significant correlation was found for IL-8, whereas for IL-10 and IL-18 inverse association was found with DNA methylation in *IL-10* promotor and *F2RL3*, respectively (Table 2).

Two studies evaluated leptin as outcome, showing contradictory results. One (23) reported inverse association between leptin levels and Leptin Receptor methylation, whereas the other reported no association between Leptin promoter and leptin levels (26). Two studies assessed the association of DNA methylation and TNFα levels. Higher levels of methylation of *EEF2* (23) and *SOCS-1* (22) were found with higher levels of TNFα. Also, six studies reported the association between methylation at different genes (*MGMT, RARβ, RASSF1A, CDH13, USP2, TMEM49, EEF2, COL18A1, IL4I1, LEPR, PLAGL1, IFRD1, MAPKAPK2, PPARGC1B, SMAD3, DTNB, LY86 and F2RL3*) with levels of several inflammatory markers other than CRP and interleukins (VEGF, VCAM1, C-CAM1, COX-2, sTNFR2 and fibrinogen) (Supplement Table 1).

Epigenome-wide analysis and inflammatory markers

Seven studies investigated differentially methylated regions in the genome in a hypothesis-free approach. Six of them adjusted at least for age and sex and four of them, additionally, for BMI, smoking or other confounders. All of the studies used blood samples to assess DNA methylation. Five studies assessed CRP, two studies evaluated *TNF* and interleukins such as *IL-1β, IL-6, IL-8 and IL-10* (Table 3). One study assessed 121 inflammatory biomarkers related with inflammation, cancer, and cardiovascular disease (27). Three out of seven studies used replication to validate their findings: two of them (13, 28) used external validations and one (29) internal validation. The identified genes were enriched by pathways

such as atherosclerosis, *IL-6, IL-9, IL-8*, growth hormone, and *JAK/STAT*, signalling pathways. Among the genes reported to be differentially methylated, *SOCS3* and *BCL3* were found to be significantly hypomethylated in two studies (13, 28). *BCL3* was no longer significant in the replication cohort, whereas *SOCS3* remained significant after replication.

Histone modifications and inflammatory markers

There was only one study that examined the association between histone modifications and inflammatory markers (15). The authors assessed levels of acetylated histone H4 in the peripheral blood mononuclear cells of Chronic Obstructive Pulmonary Disease (COPD) patients, and reported higher acetylation levels in patients with higher IL-8 levels and in patients with lower IL-4 levels.

Discussion

This is the first attempt to summarize current literature on the role of epigenetic marks in chronic inflammation. There is suggestive evidence for hypomethylation of overall genome in inflammatory processes, but better-quality studies have to confirm these results. Histone modification and inflammatory markers are scarcely investigated. Given the complexity and variability of proteins involved in the inflammation network, most of the studies focused on exploring CRP levels with few studies on *IL-6* and fewer investigations on *IL-8, IL-10, IL-18, VEGF, Cox-2, TNF-α, sTNFR2,* leptin and fibrinogen levels. The largest epigenome wide association study up to date found *AIM2* and *SOCS3* to be top genes related to CRP levels in whole blood.

Global DNA methylation

There were either no or an inverse association of inflammatory markers such as CRP, VCAM-1 and ICAM-1 in whole blood. Because we identified only a small number of studies, we cannot make any firm inferences on the overall hypomethylation of the genome due to inflammation. Moreover, populations were hardly comparable as two of the studies were conducted in children while the others in adults. As global DNA hypomethylation has become the hallmark of most human cancers, stroke and heart disease (30-33), the need to measure this epigenetic signature has become more essential. Global methylation would enable the ability to associate for example, LINE-1 or 5-mdC levels with correlative factors such as patient history or clinical outcome. The observed hypomethylation could lead to activation of dormant repeat elements and the subsequent aberrant expression of associated genes or may contribute to genomic instability and increased mutation rates. More intense efforts in studies investigating global DNA methylation through different methodologies such as Alu repeats and LUMA can hold future prospects for guiding risk stratification in individuals with high levels of inflammatory markers at an increased risk of chronic diseases.

EWAS vs candidate gene approaches

Ligthart et al. identified and validated 58 CpG sites located in 45 unique loci in whole blood in 12,974 individuals of European and African descent (13). The top signal near *AIM2* gene was found to be inversely associated with gene expression levels and with lower CRP levels. *AIM2* is a key regulator of human innate immune response implicated in defence mechanism against bacterial and viral pathogens (34, 35). Severalof these hits including cg18181703 (*SOCS3*), cg06126421 (*TUBB*), and cg05575921 (*AHRR*) were associated with future incidence of coronary heart disease and smoking (13), whereas two other CpGs were recently identified in an EWAS of type 2 diabetes (36). The gene *SOCS3*, suppressor of cytokine signalling 3, plays a pivotal role in the innate immune system as a regulator of cytokine signalling along the JAK/STAT pathway and was previously reported to be important in ath-

Table 1. Global DNA methylation and inflammatory markers

Author, Year	Study Design	Outcome	Population-Sex/Age/ Population/ Country	Tissue type	Adjustment	Association, reference
LINE-1 methylation						
Baccarelli et al., 2010(16)	CS	*VCAM-1*, *ICAM-1* and CRP	M /73.8 ± 6.7/ n=593/USA	WB	Age, BMI, smoking, pack years of smoking, IHD or stroke.	Inverse for *VCAM-1*, no association for *ICAM-1* and CRP.
Perng et al., 2012(17)	CS	CRP	M and F/ 8.8 ± 1.7/n=568/ Colombia	WBC	Sex, vitamin A, maternal BMI and household socioeconomic stratum.	Higher CRP was related to lower LINE-1 methylation.
Zhang et al., 2012(14)	CS	CRP	M and F/ 18-78/n=165/ USA	WBC		No association (β coefficient=-0.02, p=0.81).
Narayan & Dangi, 201. (18)	CS	CRP	M and F/7.9 ± 1.5/n=600/ India	WB	Sex, plasma Vitamin A, socioeconomic status	Global DNA methylation was inversely related to CRP concentrations and the association was stronger in male children.
5mdC						
Murphy et al., 2015(60)	CS	*IL-6* (protein and serum levels)	M and F/ mean=33.04/ n=47/Ireland	WB		No association. (r = -0.125, p=0.46).

CS: cross-sectional; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular adhesion molecule 1; CRP: C-Reactive protein; M: men; W: women; WB: whole blood; BMI: body mass index; WBC: white blood cells; IL: interleukin.

Table 2. Specific gene methylation and inflammatory markers

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjust-ment	Main findings	Clinical condition associated with the main find-ings*
Candidate gene approach								
Piperi et al., 2010(1)	CS	*IL-6, IL-8, VEGF, COX-2*	Tu-mour speci-mens	M and W/25-76/n=23/ Greece	*MGMT, RARβ, RASS-F1A, CDH13*/MS-PCR	-	*IL-6*: positive correlation with the four genes; *IL-8* and *COX-2*: no correlation for any gene; *VEGF* positive correlation with *MGMT* and *RARβ* no correlation with *RASSF1A* and *CDH13*.	IPA: Cancer, neu-rological disease, ophthalmic disease
Uddin et al 2010(2)	CS	*IL-6*, CRP	PBMC	M and W/ 45.3±16.7 6/ n=100/ USA	*IL-6*/Illumina Hum-anMethylation27K DNA Analysis Bead-Chip	-	Among patients with lifetime depression, there was a signifi-cant inverse correlation between methylation of *IL-6* and serum levels of *IL-6* and CRP (Pearson r=-0.54, p=0.001 and Pearson r=-0.48, p=0.006, respectively.	*IL-6*: Rheumatic diseases, inflamma-tory bowel disease, Kaposi sarcoma.
Fu et al., 2011(3)	CS	*IL-10* (mRNA)	PBMC	M and W/39 ± 10.8/ n=40/ China	*IL-10* promoter, 5 CpG sites/ Pyro Q-CpG system	-	Hypomethylation of -145C was correlated with higher *IL-10* mRNA expression (r=-0.746, P=0.001), The authors did not report the results for the other CpG sites.	*IL-10*: Susceptibility to HIV type 1, rheu-matic conditions, cutaneous leish-maniosis.

Table 2. Specific gene methylation and inflammatory markers (continued)

Author	Study design	Out-come	Tissue type	Population Sex/Age/ Population/ Country	Meth-ylation sites/ method	Adjust-ment	Main findings	Clinical condition associated with the main findings*
Zhang et al., 2012(4)	CS	CRP	PB	M and W/18-78/ n=165/ USA	*IL-6* promoter, 6 site/ bisulfite treatment	Age, sex, race, dietary folate intake, prudent diet pattern, western diet	No association was found, with a spearman correlation coefficient of 0.11 (p=0.18).	Not applicable
García-Cardona et al., 2014(5)	CS	Leptin	PB	M and W/ 10-16/ n=106/ Mexico	*LEP* promoter/ MS-PCR	-	No significant correlation was observed between the circulating levels of leptin and the methylation frequencies of the two selected CpG sites of the *LEP* promoter (at – 51 and – 31 nt).	Not applicable
Lai et al., 2014(6)	CS	*IL-6, TNF-α* and CRP	PB	M and W/ 36-80/ n=46/ China	*SOCS-1* gene, 11 CpG sites/ Bisulfite method	-	A positive trend between the levels of *SOCS-1* methylation and CRP levels was observed (R^2=0.1127, P=0.0278). Patients with serum *IL-6* above median showed a significantly higher *SOCS-1* methylation than the patients with serum *IL-6* below median (P<0.001). Similar results were observed for *TNF-α* (P<0.001).	*SOCS-1*: Cancer, hepatic system disease, ophthalmological disease.
Smith et al., 2014(7)	CS and pro-spective	*sTNFR2, IL-6*	PBMC	W/56.4 ± 9.4/ n=61/USA	*USP2, TMEM49, SMAD3, DTNB,* 8 CpG sites/ Human-Methyl-ation450 Bead Cheap	-	At baseline, lower methylation at each of the 8 CpG sites was significantly correlated with increased s*TNFR2* and *IL-6*.	IPA: Gastrointestinal diseases, hepatic system diseases, cancer (like gynaecological cancer), dermatological diseases.

Table 2. Specific gene methylation and inflammatory markers (continued)

Author	Study design	Out-come	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings	Clinical condition associated with the main findings*
Wang et al., 2014(8)	CS	Fibrino-gen and CRP	PB	M and W/16.2 ±1.2/ n=703/ USA	*LY86* gene, 6 CpG sites / Human-Methylation27 BeadChip and ThumanMethyla-tion450 BeadChip from Illumina	Age, sex, race, BMI and batch	They performed a principal component analysis to combine the six CpG sites into one score. The score of these CpG sites was sig-nificantly associated with fibrinogen (partial r=0.145, p<0.001) and CRP (partial r=0.114, p=0.005).	*LY86*: Pelvic inflammation, pulmonary inter-stitial emphy-sema
Wei et al., 2016(9)	CS	CRP	WBC	M and W/ / n=673/China	*IL-6* promoter/EZ DNA Methylation Kit	Age and sex	Plasma CRP levels were significantly asso-ciated with IL-6 promoter methylation (P = 0.025). One interquartile range increase in plasma CRP was associated with a decrease in IL-6 methylation by 0.78% (95% CI: -1.47% to -0.1%).	*IL-6:* Rheumatic diseases, inflam-matory bowel disease, Kaposi sarcoma.
Arpón et al., 2017(10)	CS	*TNF-α , VCAM-1, sICAM-1,* CRP, leptin	PB	M and W/63.8±2.74/ n=36/ Spain	*EEF2, COL18A1, IL4I1, LEPR, PLAGL1, IFRD1, MAPKAPK2 and PPARGC1B*/ Illumina Infinium HumanMeth-ylation450K BeadChip	-	Results showed correlations between LEPR methylation and concentration of LEP (r=-0.24, p=0.047). Also, between EEF2 methyla-tion and concentration of TNF-α (r=0.24, p= 0.0408) and CRP (r=0.24, p=0.0457).	IPA: Inflamma-tory response, cardiovascular disease, repro-ductive system disease.

Table 2. Specific gene methylation and inflammatory markers (continued)

Author	Study design	Outcome	Tissue type	Population Sex/ Age/Population/ Country	Methylation sites/ method	Adjustment	Main findings	Clinical condition associated with the main findings*
Jhun. et al., 2017(11)	CS	CRP, *IL-6*, *IL-18*, fibrinogen	PBL	M and W/66 7.5/ n=822/ USA	Cg0363183 in *F2RL3*/ Illumina Infinium HumanMethylation27 BeadChips and the Illumina BeadXpress reader.	Age, sex, four principal component, five cell proportions, plate and random intercepts for family.	DNA methylation level of Cg03636183 in F2RL3 was significantly associated with log (IL-18) levels (-0.11, 95% CI (-0.19, -0.04)).	Unknown
Miller et al., 2017(12)	CS	CRP	WBC	M and W/32.08±8.36/ n=286/ USA	*AIM2*,cg10636246/ Illumina Infinium HumanMethylation450K		Log CRP levels were negatively correlated with cg10636246 ($r = -0.264$, $p < 0.001$).	*AIM2*: Skin disease, melanoma.

*We used Ingenuity Pathway Analysis (IPA) for studies that found significant association between multiple inflammatory markers and/or methylation in multiple genes (7]) (1, 10). For the other studies, the connection between findings and disease was assessed through literature review and gene cards (https://www.genecards.org/).
CS: cross-sectional; *IL*: interleukin; *VEGF*: vascular endothelial growth factor; *Cox-2*: cyclooxygenase; M: men; W: women; MS-PCR: methylation-specific PCR; IPA: Ingenuity pathway analysis. PBMC: peripheral blood mononuclear cells; PB: peripheral blood; *TNF- α*: tumour necrosis factor-alpha; *sTNFR2*: soluble tumour necrosis factor receptor 2; BMI: body mass index; WBC: white blood cells; *VCAM-1*: vascular cell adhesion molecule 1; *sICAM-1*: soluble intercellular adhesion molecule 1; PBL: peripheral blood leucocytes.

Table 3. Genome-wide and histone acetylation approaches and inflammatory markers

Author	Study design	Out-come	Tissue type	Population Sex/ Age/Population/ Country	Methyla-tion sites/ method	Adjust-ment	Main findings
Epigenome-Wide Association Study							
Guénard et al., 2013(13)	CS	CRP	WB	M and W/12.25±5.77/ n=50/Canada	Infinium HumanMeth-ylation450K BeadChip	Age and sex	From 17 genes involved in the IL-8 signalling pathway, significant correlations between gene methylation and plasma CRP levels was found for 16 genes. Of these, 9 showed inverse correlation and 7 posi-tive. Out of those 16 genes, 13 remained significant after adjustments.
Sun et al., 2013(14)	CS	CPR	PBL	M and F/ 66.27±7.58 n=966/ USA	Infinum HumanMeth-ylation27K BeadCheap	Age, sex, BMI, smok-ing	207 out of 257 CRP-associated DNAm sites, showed an inverse cor-relation of greater methylation with lower level of CRP. Twenty-four out of the top 30 CpGs remained significant in both replication subsets with KLK10, LMO2 and TM4SF4 as top genes ($p=5.85x10^{-12}$, $p=1.69x10^{-11}$ and $p=2.05x10^{-10}$, respectively).
Ligthart et al., 2016(15)	CS	CRP	WB	M and W/mean age between 60 and 87/n=8863/ Consortia	Illumina Infinium HumanMeth-ylation 27K and 450K BeadChip.	Age, sex, white blood cell proportion, technical covariates, smoking, BMI.	Of the 218 CpG sites (125 CpGs positively associated and 93 nega-tively associated) significantly associated with CRP, 58 CpG sites, in 47 genes, were still significantly associated in the replication cohort (n=4111). The top CpG site were located in AIM, RPS6KA2 and PHOSPHO1 ($P = 2.53x10^{-27}$, $2.06x10^{-26}$ and $4.87x10^{-25}$, respectively).
Marzi et al., 2016(16)	CS	CRP	PB	M and W/60.9±8.89/ n=1741/ Germany	Illumina HumanMeth-ylation450K BeadChip	Age, sex, BMI, smok-ing, white blood cells composi-tion.	Four CpG sites located at AQP3, BCL3, SOCS3, and intergenic at chromosome 19p13.2 were significantly hypomethylated at high CRP concentrations. Those four sites were replicated in three subcohorts. CpG at AQP3 remained significant in two of the subcohorts and the one at SOCS3 remained significant in one of the subcohorts.

Table 3. Genome-wide and histone acetylation approaches and inflammatory markers (continued)

Author	Study design	Outcome	Tissue type	Population Sex/Age/ Population/ Country	Methylation sites/ method	Adjustment	Main findings
Ahsan et al., 2017(17)	CS	121 biomarkers related with inflammation, cancer, and cardiovascular disease.	PBL	M and W/ 14-97/ n=698/ Sweden	Illumina HumanMethylation450K BeadChip	Age, sex, batch and plate effects, year of sampling and cell fractions.	For 36% (44/121) of the studied biomarkers, the abundance level was associated with DNA methylation, but for 52% these biomarkers (23/44), the associations were explained by genetic variants. For a subset of biomarkers, the association with DNA methylation was confounded by environmental factors (e.g., smoking), but for the majority of the associations, no such relationship could be found.
Verschoor et al., 2017 ("The relation between...") (18)	CS	TNF, IL-6, IL-8, IL-10	WBL	M and W/48-78/n=14/ Canada	Illumina Infinium HumanMethylation 450 K BeadChip	Age and sex	Serum IL-10 levels exhibited the most substantial association to DNA methylation patterns, followed by TNF, IL-6 and IL-8.
Verschoor et al., 2017 ("DNA methylation...") (19)	CS	TNF, IL-6, IL-1β, IL-10 and CRP	PBMC	M and W/82-98/n=23/ Canada	Illumina Infinium HumanMethylation 450 K BeadChip		Authors performed linear regression between each factor assessed and the scores of top 10 principal components (PCs) of the DNA methylation dataset. Only IL-6 and IL-10 were found to be associated, both of which with PC7 (ln IL-6, p = 0.002; ln IL-10, p= 0.03). Ln CRP was positively associated with DNAmAge using Hannum's approach (β = 0.21, p = 0.007), which relates to approximately 5-years age acceleration per 1-unit change in ln CRP (β= 0.20, p =0.008).
Histone acetylation							
da Silva et al., 2017(20)	CS	IL-4, IL-6, IL-9, INF- γ and TGF-β	PBMC	M and W/ 68.5±6.49/ n=10/ Brazil	Global Histone H4 Acetylation Assay Kit		At 24th session, the basal values of global histone H4 acetylation levels were correlated with basal IL-4 and IL-8 levels (r =−0.65, p= 0.04 and r=0.85, p=0.01, respectively).

CS: cross-sectional; WB: whole blood; M: men; W: women; IL: interleukin; PBL: peripheral blood leucocytes; BMI: body mass index; PB: peripheral blood; TNF: tumour necrosis factor; WBC: white blood cells; PBMC: peripheral blood mononuclear cells; INF-γ: interferon-gamma TGF-β: transforming growth factor-beta.

erosclerosis processes (37). Moreover, another epigenome-wide association study in 1,741 individuals of European descent reported *SOCS3*, among others, as significantly associated with systemic CRP levels, not only in peripheral blood tissue, but also in human liver tissue (28).

Given the reported association of CRP levels and these cardiometabolic clinical outcomes, it seems that inflammation-related epigenetic features may explain part of the observed associations reported in epidemiology. However, the results should be interpreted with caution, as the association of CRP and DNA methylation were not adjusted for these factors. Most of the replicated CpG sites reported in the study of Lighart et al. were associated with different cardiometabolic phenotypes (body mass index, fasting glucose, fasting insulin, triglycerides, total cholesterol, HDL-cholesterol), highlighting the evidence of a pleiotropic network of epigenetics across various phenotypes. This information is promising as it holds new insights into shared epigenetic mechanisms and provide opportunities to link inflammation processes with clinical outcomes. Moreover, a general inverse association between hypomethylation and higher levels of CRP was observed by two large cohorts: KORA and GENOA study (28, 38). The latter reported a similar trend of hypomethylation among individuals of older age and suggested that these pattern of modifications of DNA methylation on CpG islands between aging and inflammatory markers may indicate shared molecular mechanisms underlying chronic diseases through epigenetic changes (38).

Differentially methylated genes associated with CRP levels and other inflammatory markers did not directly overlap with the genes identified from previously reported genome wide association studies influencing CRP levels and other biomarkers. The non-overlap between GWAS and EWAS identified genes shows that clinical phenotypes are being influenced by different molecular mechanism, all of them important to explain phenotypical variation. Most of the identified genes are involved in common inflammation pathways related to cancer, rheumatic diseases and gastrointestinal pathologies (22, 25). Nevertheless, candidate gene approaches have less stringent criteria to assign significance on the expense of a narrower focus on genes. This might explain the absence of reproducibility of results in the reviewed epigenome wide association studies, except for *TMEM49*, which was found to be inversely associated with *sTNFR2* and *IL-6* levels in the candidate gene approach study of Smith et al, and shared the same direction of association with CRP levels, in the EWAS study of Lighart et al.

Histone modification

This review demonstrated that evidence involving inflammation and histone modification mechanisms are inexistent. Histone modifications are another epigenetic mark that play a pivotal role in the epigenetic regulation of transcription and other functions in cells. In addition, histone modifications have been linked to other inflammatory-related disorders, such as dyslipidaemia, obesity, diabetes, cancer and cardiovascular disease (39-41). Future studies on histone modifications and inflammation markers might shed light into their functional role in chronic diseases and might provide novel target therapies for inflammatory conditions.

Bias, confounding, and tissue specificity

There is quite ample evidence showing differential DNA methylation differing by ethnicity (42). Therefore, it is recommended that studies investigating epigenetics of genes related to inflammation should replicate their findings in diverse populations. The largest to date epigenome wide association study investigating DNA methylation and CRP levels used as discovery set a large European population (n = 8,863) and sought trans-ethnic replication in African Americans (n = 4,111) (13). As in genetic studies, the importance of replication of the significant findings in epigenetic association studies is a paramount in order to prevent false-positive results (43, 44).

Unlike genetic association studies that are less prone to confounding, epigenetic signatures

throughout the genome, are highly labile due to temporal or spatial factors affecting DNA such as age, gender, demographics, lifestyle, comorbidities, and medication used. It has been shown that methylation investigations harbour new information in explaining the variation of complex traits such as inflammation characterized by a strong influence of environment (4, 13, 45). CRP, one of the most studied inflammatory markers, and others, are affected by both genetic and environmental factors. Therefore, controlling for confounders in epigenetic studies is crucial. In our review, the majority of our studies (62.5%) were classified as low quality largely explained by the lack of proper adjustment in the statistical models. Whereas epigenome wide studies controlled for life-style factors such as smoking, alcohol consumption and BMI, candidate-gene approach studies were heavily suffering from incomplete adjustments.

Most of the inflammatory markers and especially the ones of the acute phase are predominantly synthesized in liver cells, hepatocytes, and regulated via transcription factors such as *STAT3, C/EBP* family members and NF-kappa B by the pro-inflammatory cytokines IL-6 and IL-1beta (46, 47). Nevertheless, extra-hepatic expression to a lesser degree has been reported for adipose tissue and blood cells (47). DNA methylation profiles have been commonly studied in whole blood due to the easy access to the biological samples. Environmental exposure signatures such as smoking, alcohol and other condition involving the circulatory system and the immune response are well reflected in whole blood. This tissue is primarily composed of leukocytes, a key component of the human immune system and therefore, highly relevant to systematic inflammation. However, since peripheral blood constitutes a heterogeneous admixture of different cell populations, it is possible that the results reflect inflammation-related DNA methylation changes that influence a single cell type component of blood cells. Adjusting for measured or estimated blood cell proportions or future studies conducted in cell specific tissues would help to rule out presence of any residual confounding caused by white blood cell distribution.

Causality and study designs

In the last years, the GWAS have resulted in the identification of many genetic variants that are associated with clinical traits and diseases but together, these variants explain only a small fraction of the variability. It has been suggested that epigenetics might hold promise to uncover the rest of the missing heritability. Moreover, it has been commonly hypothesized whether epigenetic signatures might be a cause for disease, rather than consequence. With the current evidence, it is unclear if epigenetic variation is causal to these inflammatory markers. In a recent study of Ahsan et al, the authors investigated the genetic and epigenetic influence in a large set of disease related inflammatory markers (27). Combining results of GWAS/EWAS in around 1,069 individuals and employing a complex bidirectional model to asses causality between genetic variation-DNA methylation-inflammation markers, concluded that DNA methylation has a limited direct effect on inflammatory markers and it reflects the underlying pattern of genetic variants, environmental exposures or secondary effect of the pathogenesis of disease. In line with recent evidence, DNA methylation seems to be a consequence of clinical traits rather than a cause, for example, BMI (48).

All of the included studies in this systematic review were of cross-sectional design, except for one (42), meaning that both epigenetic signatures and outcomes were measured at the same time. This design challenges further inferences concerning causal directionality of associations, a typical vulnerability of epigenetic studies. In longitudinal cohort designs, repeated measurements for both inflammatory markers and dynamic methylation changes could improve our knowledge of directionality of events. While performing direct experiments with randomization of individuals into exposed and not exposed to a specific inflammatory marker would be of preference, these requires a large amount of resources. Therefore, statistical approaches like Mendelian Randomization, in which genetic variants are used as proxies for DNA methylation and the outcome of interest, offer new opportunity to

investigate the directionality of evidence from cross-sectional data (49). The identification directionality and molecular pathways underlying the relation between epigenetic signatures and inflammatory markers represent promising targets for future functional studies.

Epigenetic screening

In the last years, many advances in technologies related to measurements of epigenetic signatures have been developed to respond to the fast-growing pace of the field (50). These techniques allow to investigate DNA methylation either on candidate genes or on the whole-genome level. However, as the number of genes of interest increases along with the number of tissues of relevance, investigating the role of DNA methylation in different clinical traits could be very costly and time consuming. Progressing to more cost-effective solutions, high-throughput technologies have open new opportunities for epigenome wide investigations in large-scale screening such as in population-based cohort studies. Furthermore, gene-specific assays such as bisulfite conversion provide a quick and efficient result for epigenetic investigations requiring relatively low DNA input with minimum DNA loss (51, 52). Cloning, the gold standard method for gene-specific DNA methylation studies, followed by Sanger sequencing is another technological option (53). Although the time for the procedure itself has been significantly reduced, the sequencing step might introduce several sources of errors (50, 54). Another technique, pyrosequencing, represents a high throughput quantitative method used for bisulfite sequencing (55, 56). This technique, which can be used for both DNA methylation and genetic variation (single nucleotide polymorphism) analysis, takes less time than cloning providing accurate reads within each run. Yet, optimal DNA quality is important to avoid misreads of pyrosequencing (50). Mass spectrometry assay, on the other side, is a tool that can be used for the discovery and quantification of DNA methylation sites based on difference in fragments weights that have been cleaved depending on the methylation status (54). This technology is highly sensitive and has the ability to sequence reads up to 600bp, which is considerably longer than other methods. Quantitative Polymerase Chain Reaction (qPCR) arrays are another alternative of methylation quantification techniques operating on fluorophore-labelled probes that emit fluorescence when bound to a complementary DNA sequence. This method might not be ideal for regions with multiple CpG sites because many probes need to be created, resulting rather costly. However, if a region is characterized by a few CpGs, qPCR method might provide a simple and relatively inexpensive way to conclude a high-powered study (50).

Other chip techniques for epigenetic studies, in particular for histone modifications, include chromatin immunoprecipitation (ChIP), methylated DNA immunoprecipitation (MeDIP) platforms, as well as methyl-binding protein immunoprecipitation platforms. A major limitation to these techniques in epigenome-wide analysis is the quality of the antibody, which plays an important role in the proper enrichment of DNA. In general, the immunoprecipitation techniques require the availability of large sample volumes and only measure relative enrichment of epigenetic markers.

Concerning large-scale epigenetic analysis, the most widely used platforms, as shown from our review, are from Illumina. Illumina Methylation profiling is based on bisulfite converted DNA genotyping (57). For example, The Illumina Infinium HumanMethylation27 (27,000 CpG site) and Human-Methylation450 Bead (450,000 CpG sites) arrays provide genome-wide coverage, featuring methylation status at CpG islands, CpG shores, nonCpG sites, promoter regions, 5′ UTR, 3′ UTR, as well as gene bodies. More recent platforms such as Infinium MethylationEPIC BeadChip Kit, have increased the number of interrogated sites to more than 850,000 CpGs across the genome at single-nucleotide resolution for only of 250ng DNA as input quantity (58). Moreover, TruSeq Methyl Capture EPIC Library Prep Kit, is another option that combines whole-genome bisulfite sequencing with methylation arrays that can support

both screening and biomarker discovery studies targeting over 3.3 million CpGs (59). These technologies rapidly produce a large amount of data at relatively low costs and are mostly preferred in population studies. On the other hand, epigenome-wide sequencing is another technology that is holding high hopes for future discoveries in the field of epigenetics. Currently, its widespread use is hampered by the high costs and computation burden of the analysis.

Clinical implications

Understanding the epigenomic regulation of loci related to inflammatory markers might hold the possibility to discover attractive targets to control inflammatory processes and consequently, improve therapeutical interventions for chronic diseases that share in their aetiology, inflammatory-related pathophysiology. The identified epigenetic patterns may be used not only in functional studies to provide further insights into molecular mechanisms of inflammatory processes but also in biomarker studies using whole blood to improve the prediction of inflammation related clinical disorders or events.

Conclusions

Current evidence suggests a potential role of epigenetics on the level of inflammatory markers in blood. Studies reporting on the association of inflammation with global DNA methylation show a hypomethylation trend. However, this evidence is not conclusive. Further studies are recommended to explore this relation. Moreover, studies on the role of histone modifications in inflammation markers are scarce. While most of the studies have been focused on CRP, reporting replicated genes across cohorts such as SOCS3, further efforts should focus on other biomarkers of the inflammatory cascade such as interleukins. Most importantly, given the systemic nature of inflammation, validation of the methylation sites among different tissues is paramount. The identified and reported genes so far involve epigenetics of inflammation with cardiometabolic factors, but also cancer and rheumatic diseases highlighting the potential of these regions as translational targets in the future. Given that we observed a lack of high quality investigations included in this review, we recommend future studies to improve some of the most urging factors such as design of studies (choosing for example, repeated measurements of epigenetic marks or prospective designs of conducted studies that would allow to draw insights on one of the most important drawbacks of epigenetic data, assessing the directionality of effects), increase the sample size (to provide adequate power) and perform proper adjustment of analysis to account for the role of environment on both epigenetics and inflammation. Lastly, the identified genes need to be validated in functional (in vitro and in vivo) studies in order to draw

Supplementary Information is available in the online version of the paper (https://www.hindawi.com/journals/iji/2019/6273680/).

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CHAPTER 3.3

The role of epigenetic modifications in cardiovascular disease: A systematic review

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Abstract

Epigenetic modifications of the genome have been reported to play a role in processes underlying cardiovascular disease (CVD), including atherosclerosis, inflammation, hypertension and diabetes. Eleven databases were searched for studies investigating the association between epigenetic marks (either global, site-specific or genome-wide methylation of DNA and histone modifications) and CVD. Of the 3459 searched references, 31 studies met our inclusion criteria (26 cross-sectional studies and 5 prospective studies). Out of the included studies, 6 studies examined global DNA-methylation, 20 studies assessed DNA methylation in candidate genes, 3 studies applied genome-wide approaches, one study examined histone modifications and one study examined both DNA methylation and histone modifications in specific candidate genes in relation to CVD. Overall, 12,648 individuals were included, with total of 4037 CVD events. The global DNA methylation assessed at LINE-1 elements was inversely associated with CVD, independent of established cardiovascular risk factors. Conversely, higher degree of global DNA methylation measured at Alu repeats or by the LUMA method was associated with the presence of CVD. The studies reported epigenetic regulation of 27 metabolic genes (involved in fetal growth, glucose and lipid metabolism, inflammation, atherosclerosis and oxidative stress) in blood cells to be related with CVD. Current evidence supports an association between genomic DNA methylation and CVD. However, this review highlights important gaps in the existing evidences including lack of large-scale epigenetic investigations, needed to reliably identify genomic loci where DNA methylation is related to risk of CVD.

Keywords: epigenetic, DNA methylation, histone modifications, LINE-1, cardiovascular disease

Key messages

- The global DNA methylation assessed at LINE-1 elements was inversely associated with cardiovascular disease, independent of established cardiovascular risk factors.
- Higher degree of global DNA methylation measured at Alu repeats was associated with the presence of cardiovascular disease.
- The contradicting observations with different markers of global DNA methylation may raise a question on how functionally such a measure could be useful.
- Epigenetic regulation of 27 genes in blood cells were related with cardiovascular disease through mechanisms including inflammation, hyperlipidemia and oxidative stress.
- This study highlights a key role for epigenetic marks and particularly DNA methylation in cardiovascular disease.

Introduction

There is a worldwide epidemic of cardiovascular disease (CVD) causing one-third of all deaths worldwide and counting for trillions of dollars of health care expenditure (1, 2). This figure will surely increase in both developing and developed countries as risk factors for the disease, such as dyslipidemia, hypertension, obesity and diabetes continue to increase(2).

Current scientific knowledge does not completely explain the complex pathophysiology underlying CVD and therefore other pathways are constantly looked for. Epigenetic modifications of the genome might constitute an additional pathway leading to CVD (3). Epigenetics refers to various dynamic features that modify the genome's functionality under exogenous influence and also provide a molecular substrate that allows for the stable propagation of gene expression states from one generation of cells to the next (4). DNA methylation and histone modifications are the best understood of the epigenetic mechanisms thus far (4), and have been suggested to regulate gene expression and affect CVD risk factors including atherosclerosis, inflammation, hypertension and diabetes (5-7). Unlike mutations and other genetic abnormalities, epigenetic modifications are dynamic and could be modified by lifestyle and perhaps other therapeutic approaches (8, 9). Therefore, it has been suggested that these epigenetic mechanisms can be important regulatory key players not only in understanding CVD's pathophysiology but also in both its diagnosis and treatment(10). To date, however, little work has been done to systematically appraise the current evidence for the role of DNA methylation and histone modifications on the risk of CVD.

We aimed to conduct a systematic review and meta-analysis of all available evidence in humans to quantify the association of DNA methylation and histone modifications with cardiovascular outcomes.

Material And Methods

Literature Search

This review was conducted using a predefined protocol and in accordance with the PRISMA(11) and MOOSE(12) guidelines (eAppendix 1 and 2). Eleven bibliographic databases (Embase.com, Medline (Ovid), Web-of-Science, Scopus, PubMed, Cinahl (EBSCOhost), Cochrane Central, ProQuest, Lilacs, Scielo and Google Scholar) were searched until May 27th 2015 (date last searched) without any language restrictions, with the help of an experienced medical information specialist. The search strategy combined terms related to exposure (e.g., epigenetics, DNA methylation, histone, CpG) and outcomes (e.g., cardiovascular disease, coronary disease, heart disease, cerebrovascular disease, myocardial infarction, stroke, ischemia, carotid artery disease). In databases where a thesaurus was available (embase, medline and cinahl) articles were searched by thesaurus terms and in title and/or abstract, in other databases only by title and / or abstract. We restricted the search to studies on humans. The full search strategies of all databases are provided in eAppendix 3. After eliminating duplications, in total, we identified 3459 potentially relevant citations.

Study Selection and Inclusion Criteria

Studies to be included either described an association between epigenetic marks (global, site specific or genome-wide methylation of DNA or histone modifications) and cardiovascular outcomes defined as fatal or non-fatal coronary heart disease (CHD) and stroke. CHD events included myocardial infarction, coronary artery bypass graft, ischemic heart disease or sudden cardiac death if caused by myocardial infarction and CHD deaths. Stroke included both hemorrhagic and ischemic cerebrovascular events. Two independent re-

viewers, screened the retrieved titles and abstracts and selected eligible studies. In case of disagreement, decision was made through consensus or consultation with a third independent reviewer. Full texts were retrieved for studies that satisfied all selection criteria.

Data Extraction

A predesigned data collection form was prepared to extract the relevant information from the selected studies, including study design, study population, location, age range, duration of follow up (for longitudinal studies), and degree of adjustment. The degree of adjustment was defined as '+' when the measures of association were adjusted for age and non-established cardiovascular risk factors (e.g., education, income, ethnicity) and "++" when further adjustment was done for established vascular risk factors and potential mediators (eg, smoking status, body mass index, lipids, hypertension), tissue sample and method used to assess epigenetic marks, for type and numbers of cardiovascular outcomes and reported measures of associations (e.g., correlation analysis, odds ratio, relative risks).

Assessing the risk of bias

Bias within each individual study was evaluated using the validated Newcastle-Ottawa Scale, a semi-quantitative scale designed to evaluate the quality of nonrandomized studies(13). Study quality was judged on the selection criteria of participants, comparability of cases and controls, and exposure and outcome assessment. Studies that received a score of nine stars were judged to be at low risk of bias; studies that scored seven or eight stars were considered to be at medium risk; those that scored six or less were considered to be at high risk of bias.

Outcome Assessment and Statistical Methods

For each study, we defined whether an association was reported, and when applicable, direction effect sizes were reported. Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. If pooled, results were expressed as the pooled estimate and the corresponding 95% confidence intervals.

Results

In total, after deduplication, we identified 3459 potentially relevant citations (Figure 1). Based on the title and abstracts, full texts of 35 articles were selected for detailed evaluation. Of those, 31 articles met our eligibility criteria and were therefore included in the analysis (Table 1 and Table 2).

Summary of Included Studies

Overall, 12,648 individuals were included within the systematic review, with a total of 4037 CVD outcomes (3599 prevalent CVD outcomes and 439 incident CVD events) (Table 1 and Table 2). Of the 31 studies included, 6 studies assessed the global DNA-methylation (4 case control studies and 2 prospective studies), 20 studies assessed the DNA methylation in specific candidate genes (17 cross-sectional studies and 3 longitudinal studies), 3 studies (all case control studies) used genome-wide approaches, one study assessed histone modifications and one study (case-control) examined both DNA methylation and histone modifications in specific candidate genes in relation to CVD (Table 1, Table 2 and Supplementary Table S1). Seven studies included participants from China, 4 studies from India and the rest included participants from Canada, Germany, Italy, Spain, Scotland, Sweden, Romania, Iran and the USA (Table 1 and Table 2). All available studies were cross-sectional, case control or prospective cohorts in design and were judged as low or medium-quality studies, with only one study to be judged as a high quality (Table 1, Table 2 and Supplementary Table S1).

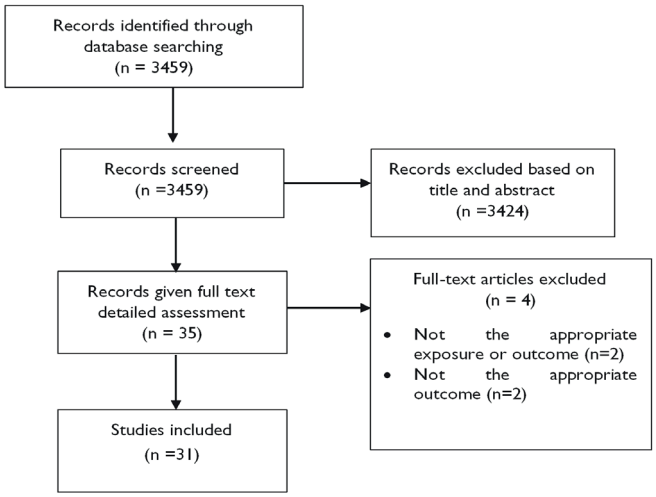

Figure 1. Flowchart of studies investigating epigenetic marks in relation to cardiovascular diseases

Global DNA Methylation and Cardiovascular Disease

Global methylation refers to the overall level of methylcytosine in the genome, expressed as percentage of total cytosine. A large portion of methylation sites within the genome are found in repeat sequences and transposable elements, such as Alu and long-interspersed nuclear element (LINE-1) and correlate with total genomic methylation content(14-16). Methylation of these repetitive elements is thus used as a surrogate for the overall methylation of the genome (17, 18). Other methods (e.g., Luminometric Methylation Assay, LUMA and the [3H]-methyl acceptance based method) to asses global genomic DNA methylation are primarily based on the digestion of genomic DNA by restriction enzymes HpaII and MspI(19). All six studies that examined global DNA methylation and risk of CVD used blood samples to assess DNA methylation (Table 3). Three studies used LINE-1 methylation(20-22), one study used Alu methylation(23) and two studies used the LUMA method and the [3H]-methyl acceptance based method to estimate the global genomic methylation(24, 25). Four studies were case control(20, 21, 23, 24), one study was prospective(25) and one study used both cross-sectional and longitudinal designs(22). Five studies (20-22, 24, 25) adjusted for established CVD risk factors whereas one study (23) did not (Table 1).

(i) LINE-1 Methylation and Cardiovascular Disease

One cross-sectional study showed that lower levels of LINE-1 methylation were associated with the presence of CHD in both men and women (comparing 1st quartile vs. 4th quartile: odds ratio (OR) = 2.3, 95% confidence interval (CI) = 1.6-3.5), and this association tended to be stronger among subjects with higher levels of homocysteine and among hypertensive subjects (20) Another study, using both a cross-sectional and longitudinal design demonstrated that lower degree of LINE-1 methylation was associated with both prevalent and incident ischemic heart disease and stroke (the longitudinal analysis, <median vs. ≥ median global DNA-methylation: hazard ratio (HR) = 2.9, 95%CI = 1.3-6.2) (22). Finally, a cross-sectional study reported an inverse association between LINE-1 methylation and ischemic stroke in men (per decrease of 1% in DNA-methylation level, OR = 1.2, 95%CI = 1.1-3.2) but not in women (21).

(ii) Alu Methylation and Cardiovascular Disease

One study of cross-sectional design examined Alu methylation in relation to myocardial infarction and reported a higher degree of methylation in cases compared to healthy controls(23).

(iii) DNA Methylation as assessed By LUMA or [3H]-methyl acceptance and Cardiovascular Disease

One cross-sectional study reported Global DNA methylation hypermethylation to be associated with the presence of coronary artery disease (CAD) within chronic kidney disease patients who underwent hemodialysis(24). Similarly, a prospective study showed that Global DNA hypermethylation was associated with increased risk of CVD-mortality (<median vs. ≥ median global DNA-methylation, HR= 13.9, 95%CI = 1.8-10.3) (25).

Gene Specific DNA Methylation and Cardiovascular Disease

DNA methylation, the addition of a methyl group to the 5 position of cytosine in a dinucleotide CpG site, is an important mechanism in gene expression regulation(26). Loss of DNA methylation promotes gene expression (27), however, the association of DNA methylation with gene expression depends on where within the gene sequence the methylation occurs. DNA methylation in the promoter region of the gene down-regulates its expression whereas higher methylation in the gene-body promotes the expression of the gene (28). This can be evaluated using candidate gene studies and genome-wide approaches:

(i) Candidate Gene Studies

There were 21 studies (18 cross-sectional studies and 3 prospective studies) that examined methylation sites in, or near, known candidate genes for CVD susceptibility in relation to CVD outcomes (Table 2). Most of the studies used a hypothesis-driven approach, whereas in others, the choice of genes was based on prior analysis of gene expression differences in the same subjects. The candidate gene methylation studies examined a range of genes involved in fetal growth(29), glucose(30) and lipid metabolism(31-35), inflammation(35, 36), vascular reactivity(37, 38), coagulation(39-41), atherosclerosis(35, 37, 42), obesity, oxidative stress(36, 43) and in the homocysteine and folate metabolic pathways(44, 45) (Table 4). Adjustment for established CVD risk factors were done in 13 studies (Table 2). DNA methylation assessment was only done in the promoter region of the gene in 18 studies. One study assessed DNA methylation in the body of gene (30) and one study assessed DNA methylation in both sites (44) (Table 4).

Overall, these studies showed that compared to subjects without CVD, subjects with an established CVD have higher methylation levels of Niemann-Pick disease type 1 (*NCP1*), ATP-binding cassette sub-family A (*ABCA1*), *PLA2G7, GALNT2, INS* and *GNASAS* in peripheral blood leucocyte, extracellular superoxide dismutase (*EC-SOD*), estrogen receptor alpha (*ERα*), tissue inhibitors of metalloproteinase (*TIMP-1*), methylenetetrahydrofolate reductase (*MTHFR*) in whole blood, F7 in peripheral blood mononuclear cells, glutathione-S-transferase P1 (*GSTP1*) in PB, FOXP3 in CD4+CD25+ T-cells and of genes involved in homocysteine metabolism and one-carbon metabolism pathway (*TCN2, CBS* 5'UTR and *AMT* in males and *PON1* and *CBS* 5'UTR in females) in peripheral blood, and lower methylation levels of *ABCG1* in peripheral blood leucocytes, adenovirus interacting protein 3 (*BNIP3*) in peripheral blood, and of *F2RL3*, tumor necrosis alpha (*TNF-α*), *LIPC, GCK, F2RL3* and *BCL2/E1B* in whole blood (Table 4). Furthermore, one study that analysed platelet mitochondrial DNA methylation levels of genes associated with ATP synthesis and of tRNA leucine gene 1 (*MT-TL1*) showed protein-encoding cytochrome c oxidase genes (*MT-CO1, MT-CO2* and *MT-CO3*) and *MT-TL1* to be hypermethylated in CVD cases compared to healthy controls(46).

Table 1. General characteristics of studies investigating the association between Global DNA methylation and cardiovascular disease

Lead Author, Publication Date	Study Design	Location	Age range (years)	Follow-up	Total Participants	Adjustment	Outcome	Quality
Cross-sectional studies								
Wei L et al. 2014(20)	CCS	China	Middle-aged	NA	1122	Age, sex, smoking status, BMI, total cholesterol, triglyceride, homocysteine, HTA and DM.	CHD	6
Lin TR et al. 2014(21)	CCS	China	40-80	NA	560	Age, sex, HTA, DM, hyperlipidemia and smoking history	Ischemic Stroke	8
Sharma, P et al. 2008(24)	CCS	India	27-75	NA	287	Age, BMI, DM, HTA, vegetarian status, smoking status.	CAD (N=137)	
Kim M et al. 2010(23)	CSS (SCHS)	China	45-74	NA	286	Age and gender	MI and/or Stroke (n=14)	6
Prospective studies								
Baccarelli A et al. 2010(22)	CSS and PS, NAS	USA	55-92	Median follow-up=63 months	712	Age, BMI, smoking history, alcohol drinking, hypertension, diastolic blood pressure, systolic blood pressure, DM, HDL, serum total cholesterol, statin use, plasma homocysteine, glomerular filtration rate, percent neutrophils and lymphocytes.	Incidence non-fatal IHD and stroke, IHD and stroke mortality	7
Stenvinkel P et al. 2007(25)	PS (CKD patients)	Sweden	54 ± 2[a]	36 ± 2 months[a]	56	Age, gender, diabetes mellitus, CVD, inflammation and mode of dialysis	CVD-mortality	8

[a] mean ± standard deviation

CCS, case control study, CSS, cross-sectional study; PS, prospective study; NAS, Normative Ageing Study; CKD, chronic kidney patients; CAD, coronary artery disease; MI, myocardial infarction; IHD, Ischemic heart disease; CVD, cardiovascular disease; HTA, hypertension; DM, diabetes mellitus; BMI, body mass index; NA, non-applicable; USA, United States; SCHS, Singapore Chinese Health Study; CKD, chronic kidney disease

Table 2. General characteristics of studies investigating specific gene methylation in relation to cardiovascular disease

Lead Author, Publication Date	Study Design	Location	Age range (years)	Follow-up	Total Participants	Adjustment	Outcome	Quality
Cross-sectional studies								
Afzali M et al 2013(31)	CCS	Iran	59.85 ± 12.13	NA	100	Age, sex, lipid lowering drugs, ethnicity, history of CVD, HTA and DM.	CVD (n=50)	4
Baccarelli, A et al. 2015(46)	CCS	USA	23-54	NA	27		CVD (n=10)	4
Fiorito G et al. 2014(44)	NCCS Epic Cohort	Italy	35-75	Mean: 12.52 ± 2.3 years	412	Age, season, center of sampling, vitamin B intake, BMI, waist to hip ratio, physical activity, smoking, socio-economic status, alcohol consumption, coffee intake, HTA, hyperlipidemia, DM and menopausal status in females.	MI (n=206)	6
Friso S et al. 2012(39)	CCS	Italy	59.5 ± 10.9	NA	253	Age, HTA, DM, smoking habit, BMI and total plasma homocysteine.	CAD (n=165)	6
Gomez-Uriz AM et al. 2014(36)	CCS	Spain	72.1 ± 11.8	NA	24	-	Stroke (n=12)	6
Guay SP et al. 2012(32)	CS	Canada	42.1 ± 1.71	NA	97	Age, sex, waist circumference and fasting triglycerides.	CAD (n=71)	5
Guay SP et al. 2014(33)	CCS	Canada	63.3 ± 1.1	NA	88	Age and medication	CAD (n=38)	6
Guay SP et al. 2014(34)	CS	Canada	44.4 ± 2.6	NA	44	Age, sex and lipid profile	CAD (n=22)	4
Huica I et al. 2011(37)	CCS	Romania	20-86	NA	62	-	CVD (n=37)	2
Jiang D et al.(35)	CCS	China	62.1 ± 5.4	NA	72	Age, smoking, DM and HTA.	CHD (n=36)	4
Lakshmi SVV et al. 2013(43)	CCS	India	20-80	NA	177	-	CAD (n=94)	5

Table 2. General characteristics of studies investigating specific gene methylation in relation to cardiovascular disease (contuned)

Lead Author, Publication Date	Study Design	Location	Age range (years)	Follow-up	Total Participants	Adjustment	Outcome	Quality
Lu CX et al. 2013(2)	CCS	China	48.8 ± 2.8	NA	124	-	ACS (n=89)	6
Peng P et al.(78)	CCS	China	59.4 ± 9.14	NA	139	Age, gender, smoking, blood lipids, HTA and DM	CHD (n=85)	7
Sharma, P et al. 2008(24)	CCS	India	27-75	NA	287	-	CAD (n=137)	7
Wei LK et al. 2015(45)	CCS	Malaysia	18-70	NA	407	Age, gender, waist to stature, DM, HTA, hypercholesterolemia, heart disease, smoking, coffee drinking, tea drinking and serum vitamin profiles.	Ischemic stroke (n=297)	7
Xu L et al. 2014(30)	CCS	China	59.24 ± 9.68	NA	72	Age, history of smoking, DM and HTA.	CHD (n=36)	6
Xu L et al. 2015(38)	CCS	China	59.24 ± 9.68	NA	1530	-	CHD (n=784)	6
Zhuang J et al. 2012(42)	CCS	China	64.4 ± 11.64	NA	205	Age, sex, BMI, smoking, HTA, DM, triglyceride, cholesterol, LDL-C, HDL-C, BUN, creatinine, statin, *ACEI/ARB*, rs10757274, *ANRIL* exon 1-5, *ANRIL* exon 4-5, *ANRIL* exon 17-18.	CAD (n=95)	7
Gomez-Uriz AM et al. 2015(47)	Genome-wide approaches	Spain	Discovery study: 69.6 ± 3.6 years. Replication Study: 66.2 ± 3.2	NA	Discovery study: n=24. Replication Study: n=115	-	Ischemic stroke (Discovery study: n=12.	6
Sharma P et al. 2014(48)	Genome-wide approaches	India	Mean age= Discovery study, 52 for case and 50.6 for controls Replication study, 49-57	NA	Discovery study: n=132. Replication Study: n=96	-	CAD (Discovery study: n=18. Replication Study: n=48)	7

Table 2. General characteristics of studies investigating specific gene methylation in relation to cardiovascular disease (contuned)

Lead Author, Publication Date	Study Design	Location	Age range (years)	Follow-up	Total Participants	Adjustment	Outcome	Quality
Guay SP et al. 2015(49)	Genome-wide approaches	Canada	Discovery study, 63.3 ± 1.7. Replication study, 49.7 ± 1.7	NA	Discovery study: n=12. Replication Study: n=161	Age, waist circumference, BMI, glucose levels and medication.	CAD (Discovery study: n=6. Replication Study: n=45)	5
Prospective Studies								
Breitling, LP et al. 2012(40)	KAROLA, a population-based prospective cohort with established CVD	Germany	30-70	8 years	1206	Age, sex, smoking, N-terminal pro-B-natriuretic, high sensitivity C-reactive protein, serum albumin and HTA.	CVD-mortality (n=64)	7
Talens RP et al. 2011(29)	PROSPER trial, a prospective study of Pravastatin in the Elderly at Risk	Scotland	70-83	Mean follow-up=3.2 years	248	Batch effects, country, age at baseline, sex and traditional risk factors (not specified)	MI (n=122)	8
Zhang Y et al.2014(41)	ESTHER study, a population-based prospective cohort	Germany	50-75	Median follow-up=10.1 years	3572	Ag, sex, batch effect, smoking status, methylation intensity, BMI, alcohol consumption , physical activity, systolic blood pressure, total cholesterol, HTA, CVD, DM and cancer	CVD-mortality (n=151)	9

BMI, body mass index; CAD, coronary artery disease; CCS, case control study; CHD, coronary heart disease; CS, cross-sectional study; CVD, cardiovascular disease; DM, diabetes mellitus; HTA, hypertension ; MI, myocardial infarction ; NA, non-applicable

Table 3. Global DNA methylation and cardiovascular disease

CVD outcome	Tissue type	Population	Association, reference	Comment
LINE-1 methylation				
CHD, n=344	PBL	M and F, n=1122	Inverse association(20)	LINE-1 methylation level was inversely associated with the risk of CHD (Relative to the subjects in the fourth quartile of LINE-1 methylation ORs for CHD were 0.9 (95%CI, 0.6-1.4), 1.9 (95%CI, 1.3-2.9), and 2.3 (95%CI, 1.6-3.5) for the subjects with methylation in the third, second and first quartile; P_{trend}<0.001). The association tended to be stronger among subjects with higher levels of homocysteine ($P_{interaction}$=0.04) and those with diagnosis of hypertension ($P_{interaction}$=0.01)
Ischemic Stroke, n=280)	PB	M and F, n=560	Inverse association in men; no association in women(21)	In men, a decrease of 1% methylation level in men was associated with an increased risk of stroke (OR=1.2; 95%CIs: 1.1-1.32).
Non-fatal IHD (prevalent, n=212, incident, n=36) . Non-fatal Stroke (prevalent, n=51; incident, n=8). IHD mortality (n=35). Stroke mortality (n=10). Combined IHD and Stroke	WB	M, n=712	Inverse association(22)	The associations were significant in both cross-sectional (combined incident non-fatal IHD and stroke: 1st quartile vs. 4th quartile: OR= 2.2 95%CI=1.2-3.9) and prospective analysis (combined incident non-fatal IHD and stroke: (<median vs. >median: HR= 4.1, 95%CI=1.9-8.7; combined incident fatal IHD and stroke: (<median vs. >median: OR= 2.9, 95%CI=1.3-6.2)
ALU				
MI and/or Stroke, n=14	PBL	M and F, n=286	Positive association(23)	Subject with a MI and/or stroke at baseline showed a significantly higher mean of combined methylation in ALU and SAT 2 repetitive elements (geometric mean=201 and 95%CI= 145-180, P=0.045). This association was prominent in men (P=0.02) but not in women (P=0.66).
HpaII/MspI ratio				
CAD, n=137	PBL[b]	M and F, n=287	Positive association(24)	Global DNA methylation was positively associated with CAD (P=0.02). The results differ by the levels of plasma homocysteine.
CVD-mortality, n=13	PBL	M and F, n=56	Positive association(25)	Global DNA hypermethylation (HpaII/MspI ratio <median) was associated with increases risk of CVD mortality (HR=13.9, 95%CIs: 1.8-109.3.)

[a] Significant inverse association with IHD and IHD and stroke combined but not with stroke.
[b] Levels of methylations estimated in terms of [3H] dCTP following MspI and HpaII.
CAD, coronary artery disease; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease; F, female; IHD, Ischemic heart disease; M, male; MI, myocardial infarction; n, number; OR, odds ratio; PB, peripheral blood; PBL, peripheral blood leukocytes

The most consistently reported epigenetic association was that of methylation at the *F2RL3* in whole blood with the risk of CVD mortality which was reported in 2 prospective studies, one that included CVD-free participants and the other that included participants with established CVD (40, 41). Both studies showed that hypomethylation at *F2RL3* was associated with increased risk of CVD-mortality (per 10% less methylation, Breitling et al. 2012: HR = 1.30, 95%CI, 1.04-1.63; Zhang Y et al. 2014: HR = 1.38, 95%CI, 1.14-1.66. Three studies showed sex-differences in the association between gene-specific DNA methylation and CVD (29, 35, 44). Collectively, these studies suggest that altered epigenetic regulation of a number of metabolic genes could be involved in cardiovascular disease ethiopathogenesis.

(ii) Genome-Wide Analysis for Cardiovascular Disease

Due to advent of genome-wide arrays for quantifying site-specific DNA methylation, several studies have investigated differentially methylated regions in the genome in a hypothesis-free approach. Three studies looked for CVD-associated differentially methylated sites in peripheral blood cells (47-49). All three studies used a replication study to validate their findings. Collectively, up to 1675 CpG dinucleotides were identified with potential DNA methylation related to risk of CVD. The identified genes were enriched for genes (known from genome-wide association studies) with epigenetic changes in biological pathways relevant to CVD-development, such as cellular homeostasis, proliferation of connective tissue cells, angiogenesis and cardiovascular system (Table 4). Also, new candidate genes emerged such as *COL14A1* (hypomethylation) and *MMP9* (hypermethylaion) which were reported to be associated with CAD (49).

Histone Modifications and Cardiovascular Disease

Two studies examined the association between histone modifications and CVD (Supplementary Table S1)(36, 50). One study showed that the levels of acetylated histone H3 in the peripheral blood mononuclear cells of acute ischemic stroke patients were lower than normal controls(50) whereas the other study reported no difference in binding pattern of H3K4me3 and H3K9ac in a region of *TNF-α* when comparing non-stroke with stroke patients (36).

Discussion

The present work is the first to systematically review the current evidence for the role of epigenetic marks in CVD. Our findings indicate that global DNA methylation might influence CVD risk and this could occur beyond the traditional cardiovascular risk factors. Furthermore, DNA methylation at 27 genes seem to be associated with the risk of CVD through mechanisms including inflammation, hyperlipidemia and oxidative stress.

The results of the present review support global DNA methylation measured in LINE-1 repeats to be inversely associated with the risk of CVD, independent of established cardiovascular risk factors. LINE-1 methylation was used in several studies as a marker of global DNA methylation. Given that LINE-1 is the most common repetitive sequence in the human genome and one third of DNA methylation in the genome occurs in these elements, the use of LINE-1 methylation as a marker of global DNA methylation seems justified(17, 51). Moreover, LINE-1 methylation correlates with other methods including genomic 5-methyl cytosine content and luminometric methylation assay (LUMA) (18, 52).

Table 4. Specific gene methylation and cardiovascular disease: gene and genome-wide approaches.

Author	CVD outcome	Tissue type	Population	Methylation sites/ method	Main Finding
Candidate gene approach					
Afzali M et al 2013(31)	CVD (n=50)	PBL	M and F, n=100	*NPC1* promoter/ Nested methylation specific polymerase	The frequency of semi-methylated *NPC1* promoter (methylated/ un-methylated) is higher in CVD patient than in controls (OR=6.521, 95%CIs, 2.211 -19.215). The prevalence of methylated allele was elevated in CVD patients than healthy subjects (OR=2.011, 95%CIs, 1.116 -3.594)
Baccarelli, A et al. 2015(46)	CVD (n=10)	Platelet	M and F, n=27	Mitochondrial genes (cytochrome c oxidase (*MT-CO1, MT-CO2, MT-CO3*), tRNA leucine (*MT-TL1*), ATP synthase (*MT-ATP6* and *MT-ATP8*) and NADH dehydrogenase (*MT-MD5*)/ bisulphite sequencing	Mean DNA methylation of *MT-CO1, MT-CO2, MT-CO2 MT-TL1* were higher in CVD cases than in non-CVD subjects (P<0.001). No differences were observed in DNA methylation at *MT-ATP6, MT-ATP8* or *MT-ND5* between CVD cases and non-CVD patients.
Breitling, LP et al. 2012(40)	CVD-mortality (n=64)	WB	M and F, n=1206	*F2RL3*/ 6 CpGs/Sequenom SpectroACQUIRE and MassARRAY EpiTyper.	No association was observed between *F2RL3* CpG_4 methylation levels and CVD-mortality comparing the 1st quartile with the forth (HR=2.32, 95%CIs: 0.97 -5.58). Per 10% less methylation: HR=1.30, 95%CIs: 1.04-1.63.
Fiorito G et al. 2014(44)	MI (n=206)	PB	M and F, n=412	33 genes involved in homocysteine metabolism and one-carbon metabolism pathway, 575 CpG sites/ HumanMethylation450 BeadChip	3 differentially methylated regions in males (*TCN2* promoter, *CBS* 5'UTR, *AMT* gene-body) and 2 in females (*PON1* gene-body, *CBS* 5'UTR) were identified, each of them characterized by an increased methylation in MI subjects. Four clusters of distinct methylation profile were identified, which were differently associated with the risk of MI (High risk vs. Low risk methylation profile groups: OR=3.49, P=1.87 x 10^{-4} and OR=3.94, p=0.0317 in males and females respectively).
Friso S et al. 2012(39)	CAD (n=165)	PBMC	M and F, n=253	*F7* gene promoter, 6 CpGs in SNPs/methyl specific PCR primers and bisulphite sequencing	CAD-free subject showed a higher F7 methylation index compared to CAD patients (32.12 ± 9.80, p=0.012). Among the 6 CpGs, the CpG2, 3 and 6 accounted for the largest difference in methylation.

Table 4. Specific gene methylation and cardiovascular disease: gene and genome-wide approaches (continued)

Author	CVD outcome	Tissue type	Population	Methylation sites/ method	Main Finding
Candidate gene approach					
Gomez-Uriz AM et al. 2014(36)	Stroke (n=12)	WB	M and F, n=12	*TNF-α* promoter, 19 CpGs/ Sequenom EpiTyper MassARRAY	Lower values of total *TNF-α* promoter methylation, using the median value as cut-off (median 0.918) was associated with higher odds of having stroke (OR=9.0, 95%CIs, 1.4 -57.1). The binding pattern of H3K4me3 and H3K9ac in a region of *TNF-α* was similar when comparing non-stroke and stroke conditions.
Guay SP et al. 2012(32)	CAD (n=71)	PBL	M and F, n=97	*ABCA1* gene promoter, 26 CpGs/ bisulfite-pyrosequencing	CAD subjects had higher *ABCA1* DNA methylation levels compared with those without CAD (34.3 ± 8.4 versus 4.2 ± 15.2, p=0.003)
Guay SP et al. 2014(33)	CAD (n=88)	PBL	M, n=88	*ABCA1* gene, 8 CpGs/ bisulfite-pyrosequencing	Subjects with a previous history of CAD showed higher mean *ABCA1* DNA methylation levels than subjects without CAD (38.7 ± 1.2 versus 36.0 ± 1.0, p=0.04). These results differ by age (older CAD-subjects had higher methylation levels than young CAD-subjects and non-CAD subjects).
Guay SP et al. 2014(33)	CAD (n=22)	WB	M and F, n=44	*ABCG*1-CpGC3, *LIPC*-CpGA2, *PLTP*-CpGC/ bisulfite-pyrosequencing	Subjects with a previous history of CAD showed lower mean *LIPC*-CpGA2DNA methylation levels than subjects without CAD (83.8 ± 1.9 versus 85.2 ± 1.9, p=0.02). No differences were observed in *ABCG1*-CpGC3 and PLTP-CpGC DNA methylation.
Huica I et al. 2011(37)	CVD (n=37)	WB	ND, n=62	Estrogen receptor alpha (*ERα*) and tissue inhibitors of metalloproteinases (*TIMP-1*) specific PCR primers and bisulphite sequencing	*ERα* and *TIMP1* presented a statistically significant frequency of hypermethylation in CVD cases compared to non-CVD cases (p<0.001 for each gene). Hypermethylation of *ERα:* OR=43.1, 95%CIs: 9.8-192.3. Hypermethylation of *TIMP1:* OR=15.3, 95%CIs: 3.8-61.3

Table 4. Specific gene methylation and cardiovascular disease: gene and genome-wide approaches (continued)

Author	CVD outcome	Tissue type	Population	Methylation sites/ method	Main Finding
Candidate gene approach					
Jiang D et al. 2013(35)	CHD (n=36)	PBL	M and F, n=72	*CF1* region of *PLA2G7* gene promoter, 4 CpGs/ pyrosequencing	There was a higher promoter DNA methylation of *PLA2G7* gene in the CHD cases than in non-CHD controls (6.41 ± 2.62 versus 4.98 ± 3.06, p=0.025). The stratified analysis by gender, showed that the significant association was found in females (p= 0.003) but not in males (p=0.096). Receiver operating characteristic curves showed that *LA2G7* methylation could predict the risk of CHD in females (area under the curve =0.912, p= 2.40E-5).
Lakshmi SVV et al. 2013(43)	CAD (n=94)	PB	ND, n=177	BCL2/E1B adenovisur interacting protein 3 (*BNIP3*), exctracellular superoxide dismutase (*EC-SOD*) and glutathione-S-transferase P1 (*GSTP1*), methyl specific PCR primers and bisulphite sequencing	Hypomethylation of *BNIP3* promoter in CAD cases compared to controls (41.95 ± 26.91% vs. 53.51 ± 42.78%, P=0.03). *EC-SOD* promoter hypermthelyation was observed in CAD compared to controls (62.23 ± 33.36 % vs. 32.35 ± 24.76%, P<0.0001). *GSTP1* promoter hypermthelyation was observed in CAD compared to controls, but not significant (45.63 ± 22.74 % vs. 41.67 ± 25.26%, P=0.28)
Lu CX et al. 2013(2)	ACS (n=89)	$CD4^+CD25^+$ T-cells	M and F, n=124	*FOXP3* gene, 2 CpGs/ pyrosequencing	Demethylation of DNA at *FOXP3* gene in ACS subjects was significantly lower than in non-ACS subjects (p<0.0001). Analysis of operating characteristic curve showed an area under the curve of 0.916 (p<0.001), supporting the notion that *FOXP3* demethylation can distinguish ACS subjects from non-ACS subjects.
Peng P et al.2014(78)	CHD (n=85)	PBL	M and F, n=139	*ABCG1, GALNT2* and *HMGCR* gene promoter/Bisulphite - specific PCR	Promoter hypomethylation of the *ABCG1* gene was associated with the risk of CHD (OR=19.966, 95%CI: 7.319-54.468). Methylation status of the *GALNT2* gene promoter was associated with the risk of CHD (OR=2.978, 95%CI: 1.335-6.649). There was no association between methylation status of the *HMGCR* gene promoter and the risk of CHD (OR=2.978, 95%CI: 1.335-6.649).
Sharma, P et al. 2008(24)	CAD (n=137)	PBL	M and F, n=287	*ApoE*, 25CpGs/ Bisulphite sequencing	No significant difference in methylation patterns between CAD and non-CAD subjects.

Table 4. Specific gene methylation and cardiovascular disease: gene and genome-wide approaches (continued)

Author	CVD outcome	Tissue type	Population	Methylation sites/ method	Main Finding
Candidate gene approach					
Talens RP et al. 2011(29)	MI (n=122)	PBL	M and F, n=248	*IL10, LEP, ABCA1, IGF2, INS* and *GNASA*, 49 CpG sites/ Sequenom EpiTyper MassARRAY	Overall, DNA methylation was modestly higher in MI cases at *GNASAS* compared with the control group (P=0.03). No differences in DNA methylation were observed at the other loci. Sex differences were observed for *INS* (p-interaction=0.014) and *GNASAS* (p-interaction=0.031). In women, DNA methylation at *INS* (p=0.002) and *GNASAS* (p=0.009) were higher in MI cases compared to control. In men, no differences in DNA methylation was observed. Overall, hypermethylation at one locus (*INS* or *GNASAS*) and at both loci (*INS* and *GNASAS*) was associated with OR=1.7 (95%CI: 2.7-27.9) and 2.8 (95%CI=1.4-5.9) respectively. In men, no associations was observed.
Wei LK et al. 2015(45)	Ischemic stroke (n=297)	WB	M and F, n=407	*MTHFR*, CpG A and B/ Bisulphite - specific PCR	CpG A methylation levels were associated with a higher risk for ischemic stroke (OR=4.73, 95%CI: 2.56 -8.75). CpG B methylation levels were not associated with ischemic stroke (OR=0.90, 95%CI: 0.56 -1.45).
Xu L et al. 2014(30)	CHD (n=36)	PB	M and F, n=72	*GCK* gene-body, 4 CpGs/ bisulfite-pyrosequencing	CHD cases had a significantly lower methylation level (49.77 ± 6.43%) compared with controls (54.47 ± 7.65%, P=0.018). Similar trends were observed in three CpGs (CpG2, 3 and 4; P for all <0.05).
Xu L et al. 2015(38)	CHD (n=784)	PB	M and F, n=1530	4 CpGs of the vascular-related genes (*VEGFA, CST3, AGTR1, ACE*)/ Sequenom EpiTyper MassARRAY	None of four CpG-SNPs in the vascular related genes was associated with the risk of CHD.
Zhang Y et al.2014(41)	CVD-mortality (n=151)	WB	M and F, n=3572	*F2RL3*, 4CpG (2 to 5)/ Sequenom EpiTyper MassARRAY	Lower methylation was associated with increased risk of CVD mortality (comparing the lowest vs. highest quartile of *F2RL3* CpG_4 methylation, (HR=2.94, 95%CIs: 2.45-4.68). Similar results were observed for other CpGs. Per 10% less methylation: HR=1.38, 95%CIs: 1.14-1.66.

Table 4. Specific gene methylation and cardiovascular disease: gene and genome-wide approaches (continued)

Author	CVD outcome	Tissue type	Population	Methylation sites/ method	Main Finding
Candidate gene approach					
Zhuang J et al. 2012(42)	CAD (n=95)	PBL	M and F, n=205	*BAX, BCL-2, TIMP3, p14^{ARF}, p15^{INK4b}* and *p16^{INK4a}*,seven CpGs at *p15^{INK4b}*/ MethyLight	*p15^{INK4b}* was associated with the presence of CAD (OR=2.55, 95%CIs, 1.26-5.01). No association was observed between *p16^{INK4a}* and CAD (OR=1.14, 95%CIs, 0.59 -2.36). CpGs +314 and +332 at *p15^{INK4b}* site, were significantly increased in CAD patients compared with controls. No difference was observed for CpGs +269, +272, +280, +303 and +321
Genome-wide approaches					
Gomez-Uriz AM et al. 2015(47)	Ischemic stroke Discovery study: n=12. Replication Study: n=60	PBL	M and F, Discovery study: n=24. Replication Study: n=115	27,578 CpG sites, 14,495 genes/ Illumina human methylation 27 Beads and MassARRAY EpiTyper	80 CpG sites differentially methylated in patients who suffered an ischemic stroke compared to them who did not ($p<0.05$). 59 CpG sites presented interaction between stroke and obesity. Among 21 CpG sites and 15 genes selected as candidates: CpG sites 19 and 20 of the gene Wilm's tumour 1 (WT1) showed a higher methylation levels in stroke patients compare to non-stroke subjects ($p<0.05$). These results were not replicated in the validation study. The promoter region of peptidase M20 domain containing 1 (*PM20D1*) gene was significantly hypermethylated in stroke patients ($p<0.05$). Differences in this region were significant at the CpG sites 1_2_10_11_12_13_14_16_17_18_22. These results were not replicated in the validation study. CpGs 8_9 of KQT-like subfamily, member 1 (*KCNQ1*) explained 31and 33% respectively of the variability for the case stroke in the validation study.
Sharma P et al. 2014(48)	CAD (Discovery study: n=18. Replication Study: n=48)	WB	M, Discovery study: n=132. Replication Study: n=96	Bisulphite sequencing by 454 platform	19 differentially methylated regions were significantly hypermethylated in CAD subjects compared to controls. In the validation study: Out of the 12 differentially methylated regions selected, 6 CpGs sties in 4 regions falling within three differentially methylated regions had significant higher methylation in CAD patients: of the 6 sites, 3 were in intronic region of *STRADA* gene (Homo sapiens *STE20*-related kinase adaptor alpha) flanking *CCDC47* and *LID2*, 2 were in the first exon of *C1QL4* gene flanking *TROAP* and *FLJI3236* while the other was in the intronic region of *HSP90B3P* gene flanking *CD27* and *TGFBR3*

Table 4. Specific gene methylation and cardiovascular disease: gene and genome-wide approaches (continued)

Author	CVD outcome	Tissue type	Population	Methylation sites/ method	Main Finding
Genome-wide approaches					
Guay SP et al. 2015(49)	CAD (Discovery study: n=6. Replication Study: n=45)	WB	M, Discovery study: n=12. Replication Study: n=161	27,578 CpG sites, Bisulphite sequencing by 454 platform	There were 1765 CpG dinucleotide with potential DNA methylation differences between CAD and non-CAD men (P<0.05). Among 1765 CpGs, 369 CpG dinucleotide were considered as most promising differentially methylated loci. The gene ontology analysis revealed significant enrichment of genes with epigenetic changes in biological pathways relevant to CVD development, such as cellular homeostasis, proliferation of connective tissue cells, angiogenesis and cardiovascular system. New candidate genes emerged: *COL14A1* (hypomethylation) and *MMP9* (hypermethylaion) were associated with CAD (however, the findings were not replicated in the validation study).

ABCA1, ATP-binding cassette sub-family A; ACS, acute coronary syndrome; CAD, coronary artery disease; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease ; *EC-SOD*, extracellular superoxide dismutase; *ERα*, estrogen receptor alpha; F, female; *GSTP1*, glutathione-S-transferase P1 ; HR, hazard ratio; M, male; MI, myocardial infarction; *MTHFR*, methylenetetrahydrofolate reductase; *MTHFR*, methylnetetrahydrofolate reductase; NCP1, Niemann-Pick disease type 1; Niemann-pick type C1; NPC1; PB, peripheral blood; PBL, peripheral blood leucocyte; PBMC, peripheral blood mononuclear cells; *TIMP-1*, tissue inhibitors of metalloproteinase ; WB, whole blood.

However, little is known about the biological function of LINE-1. The majority of LINE-1 copies are found to be inactive, however, multiple somatic cells express it which triggers senescence(53). LINE-1 hypomethylation in the peripheral blood cells has been associated with diabetes, obesity, lower levels of HDL-cholesterol, elevated levels of total cholesterol and inflammation and a higher risk of metabolic status worsening(20, 54-58). Also, higher plasma glucose levels and blood pressure, along with greater risk for metabolic syndrome (59) have been reported to inversely associate with LINE-1 methylation levels in other tissues, such as visceral fat. All these suggests that LINE-1 hypomethylation is associated with an unfavorable cardiovascular risk profile (58, 60). Furthermore, it has been previously shown that suppression of LINE-1 expression improves the outcomes after MI by ameliorating post-ischemic functional recovery and decreasing infarct size through Akt/PKB signaling (61).

In contrast to studies that used LINE-1 as an index of global DNA methylation, other studies reported a higher degree of global DNA-methylation to be associated with CVD. These studies assessed global DNA methylation in other repetitive elements such as Alu repeats, and/or used other methods to asses DNA methylation rather than bisulphate pyrosequencing. LINE-1 and Alu repeats represent distinct measures of dispersed DNA methylation, and might have different functions(62). The quantitative assessment of DNA methylation at ALU is about one-third to one-fourth of methylation at LINE-1, which may suggest that epigenetic changes at LINE-1 and ALU might measure different traits(62). Moreover, assay used and the source of DNA are important determinants in the interpretation of global DNA methylation patterns. For instance, global DNA methylation assessed by LUMA modestly correlates with LINE-1 methylation(63). Furthermore, DNA methylation occurs throughout the genome in a sequence-context–dependent fashion, and the extent to which regional sequence context might affect different measures of DNA methylation is unknown. Finally, similar opposing effects between LINE-1 and Alu methylation is observed with cardio-metabolic risk factors and other diseases, such as cancer and Alzheimer(58, 64-66).

The contradicting observations with different markers of global DNA methylation may raise a question on how functionally such a measure could be useful. It should be noted that methylation has a different effect depending on its position towards coding genes. Hypermethylation of the promoter CpG island is usually associated with gene transcriptional silencing and their hypomethylation of CpG islands is generally associated with increased gene expression(28). In contrast, hypermethylation at the gene-body is associated with increased gene expression(67). Therefore, further efforts are needed to dissect the molecular phenotype of these alterations, their link to disease processes and methodologies capable of distinguishing differences in methylation extent from inherent genomic variability of these elements.

Our study denotes 27 genes to be differentially methylated according to the presence of CVD. It is hypothesized that the epigenome regulates gene expression, cardiovascular risk factors and eventually risk of CVD (43). Of note, these gene/genes regions are known to affect biological processes related to CVD, such as homeostasis, endothelial dysfunction, inflammation, oxidative stress and lipid metabolism. Also, methylation of some of these genes has been associated with cardio-metabolic risk factors, e.g., epigenetic changes at the *ABCA1* gene promoter region contributes to the inter-individual variability in plasma HDL-cholesterol and methylation at *F7* and *TNF-α* promoter region has been associated with plasma factor VII concentrations and body weight respectively (32, 36, 39). It could be expected that the identified regions constitute only a small fraction of epigenome related to CVD. Further research is therefore needed to identify such regions and establish cause- and effect- relation between methylation of these loci and development of CVD and elucidate the underlying mechanisms, including eventual possibilities of developing epigenetic risk assessments for CVD as well as prevention strategies.

This review underscores a number of gaps in the literature concerning CVD and histone modifications, an important epigenetic mechanism that can be involved in CVD. Modifications in histone H3 in smooth muscle cells in atherosclerotic regions compared to normal arteries have been uncovered(68, 69). Also, recent studies have shown that histone modifications may have an impact in adipogenesis, energy homeostasis inflammation and diabetes (70-73).

The strengths and limitations of the findings from this study merit careful consideration. The present analysis, involving data from nearly 13,000 individuals, is the first systematic review on the subject that critically appraised the literature following an a priori designed protocol with clearly defined inclusion and exclusion criteria. However, on the majority of studies included are cross-sectional assessments, making it difficult to conclude whether specific epigenetic marks are a cause or consequence of CVD. Also, the included studies were of limited in sample size and while individual studies attempted to adjust for established cardiovascular risk factors, the levels of adjustment was inconsistent across the studies. Although every effort has been made to undertake a comprehensive search of the literature, we cannot exclude the possibility of publication bias from underreporting negative findings. Furthermore, a meaningful quantitative pooling of the existing data was unfeasible due to heterogeneity in the input parameters, assumptions and the study design.

The study of epigenetic markers is emerging as one of the most promising molecular strategies for risk stratification for complex disease, and when implemented will have a sizable public health impact. As epigenetic DNA modifications are potentially reversible and may be influenced by nutritional-environmental factors and through gene–environment interactions, future therapies targeting epigenome can be a novel preventive strategy and treatment for CVD. For example, some studies show that supplementation with methyl donors such as folate, choline and vitamin B12 may influence DNA methylation and may have beneficial effect on CVD risk, but results are still inconsistent(74-77). Also, epigenetics drugs have been shown to successfully reverse several epigenetics marks and disease symptoms and have been approved by FDA for use in cancer (9). However, epigenetics therapeutics should aim to modify various epigenetic elements in a complex and intricate cross-talk without disturbing further pathways. Thus, before undertaking any initiative, large-scale epigenetic investigations are needed to reinforce the current findings and extend our understanding on the role of epigenetics on CVD.

Supplementary Information is available in the online version of the paper (https://www.sciencedirect.com/science/article/pii/S0167527316304788?via%3Dihub).

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CHAPTER 3.4

Epigenetic link between statin therapy and type 2 diabetes

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(Submitted).

Key points

Question. Does epigenetics play a role in the diabetogenic effect of statin therapy?. **Findings**. This meta-analysis of epigenome-wide association studies and associations between DNA methylation, gene expression and glycemic traits involving 5 population-based studies (8270 adults), found differential methylation levels among statin users at key genes for cholesterol and insulin metabolism (DHCR24, ABCG1, SC4MOL). Increased methylation at the gene ABCG1 was associated with lower expression of ABCG1, increased insulin levels, insulin resistance and increased risk of type 2 diabetes. **Meaning.** DNA methylation at ABCG1 may constitute a potential mechanism through which statins increase type-2 diabetes risk.

Abstract

Importance. Evidence associates statins with increased risk of type-2 diabetes (T2D). The underlying mechanisms are unclear. Statins may impact DNA methylation, a regulator of gene expression, implicated in T2D pathophysiology. **Objective.** To investigate the role of epigenetics on the diabetogenic effect of statins. **Design, Setting and Participants.** Overall, 8270 individuals from five population-based cohort studies were involved. **Exposure.** Statin users were defined as on statin therapy at the time of blood draw (self-report, general practitioner or pharmacy records), otherwise participants were coded as non-current users. **Main outcomes and measurement.** DNA methylation in blood was assessed using Illumina 450K or EPIC array. Glycemic traits included plasma glucose, insulin levels, insulin resistance and incident T2D. **Results.** The epigenome-wide association study among current versur non-statin users in the discovery panel (n=6820; 52.1% male; mean [SD] age from 51.5 (13.8) years in the youngest cohort to 62.1 (6.5) years in the oldest) and replication analysis (n=1450; 44.1% male; mean [SD] age, 63.8 [5.4] years), revealed five methylation sites to be significantly associated with statin use: cg17901584 ($P=1.12x10^{-25}$; gene *DHCR24*) , cg10177197 ($P=3.94x10^{-08}$; *DHCR24*), cg06500161 ($P=2.67\ x10^{-23}$; *ABCG1*) , cg27243685 ($P=6.01x10^{-09}$; *ABCG1*), cg05119988 ($P=7.26x10^{-12}$; *SC4MOL*). Three sites were associated with glycemic traits. Hyper-methylation at cg06500161 was associated with downregulation of *ABCG1* expression, fasting glucose, insulin and insulin resistence, and also the mediation analysis showed that it may partially mediate the effect of statins on increasing insulin and insulin resistance. **Conclusions.** This study sheds light on potential mechanisms linking statin use with risk of T2D by providing evidence on DNA methylation at *ABCG1* partially mediating statin effects on insulin levels and insulin resistance independent of blood lipid levels. Future studies should explore whether such biomarkers could help in tailoring T2D prevention strategies among statin users.

Introduction

Statins effectively reduce the risk of cardiovascular disease.(1) However, clinical trials and observational studies show that statins lead to insulin resistance and type-2 diabetes (T2D).(2-4) The underlying mechanisms remain unclear. Statins are associated with epigenetic changes, including histone acetylation, microRNA regulation(5-7) and DNA methylation, particularly at genes related to lipid and insulin metabolism(8). DNA methylation is linked to T2D pathophysiology(9), thus it may be a potential mechanism contributing to the increased risk of T2D observed in statin therapy. Nevertheless, this hypothesis has not been investigated(7). Knowledge of these effects will provide insights into novel pathways for prevention and more targeted treatment.

We conducted an Epigenome-wide Association Study (EWAS) in blood investigating the association between current statin use and changes in DNA methylation at sites in the genome called CpGs. Further, we sought to replicate the findings, study the associations of the statin-related CpGs with gene-expression, glycemic traits and T2D. Finally, we examined the potential mediator role of methylation at the statin-related CpGs in the association of statins with glycemic traits and T2D risk.

Methods

Study design and population

Overall, 9286 participants from six populations-based studies were included. The EWAS discovery panel was comprised by 6820 Caucasian individuals from the population studies: Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung (ESTHER),(10) Kooperative Gesundheitsforschung in der Region Augsburg-F4 (KORA-F4),(11) Study of Health Pomerania-Trend (SHIP-Trend),(12) and by South-Asians living in England: London Life Sciences Prospective Population Study (LOLIPOP).(13) The replication panel included 1450 Caucasians from the Rotterdam Study (RS, subcohorts RS-III-1 and RS-BIOS).(14) Associations of the statin altered CpGs with glycemic traits (n=1165) and gene expression (n=631) were investigated in the RS. The study design is summarized in Figure 1. For study specific information see the eAppendix in the Supplement.

Statin use

Information on medication was obtained from pharmacy records in RS, self-declaration in KORA-F4, LOLIPOP and SHIP-Trend, and participant's general practitioner in ESTHER. Statin-use status was categorized in current users and non-current users. Current users were defined if the prescription of statins occurred at the time of blood drawing for assessment of DNA methylation, otherwise participants were coded as non-current users. The RS had information on former statin users, defined as participants who had previously used statins but were no longer current users on the blood draw date

DNA methylation data

DNA was extracted from whole peripheral blood (stored in EDTA tubes) by standardized salting out methods. Genome-wide DNA methylation in whole blood was measured in bisulfite-converted genomic DNA for all samples using the Illumina-Infinium Human Methylation 450K or the Illumina-Infinium Methylation EPIC BeadChip, according to standard manufacturer's protocols. The methylation proportion of a CpG was reported as a normalized value ranging from 0 to 1, where 1 represents 100% methylation. For cohort specific methods see eTable 1 and eMethods in the supplementary.

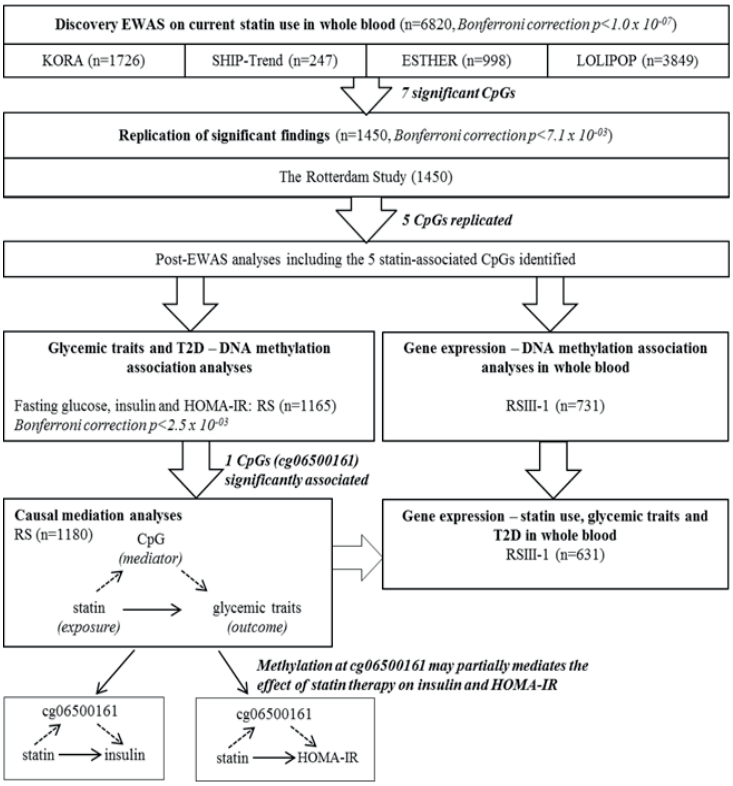

Figure 1. Study design. An epigenome-wide association study was conducted to identify CpGs related to current statin use, compared to non-current use. Four cohorts were used as a discovery panel and finding were replicated in an independent cohort. The replicated CpG sites were investigated in their association with glycemic traits. The significant associations from this analysis were tested in a causal mediation analysis. Association analyses of the identified CpGs, current statin use and glycemic traits with gene expression were performed.

Messenger RNA expression data

Messenger RNA data used comprised 21238 expression probes measured in 631 participants (RSIII-1), using the Illumina HumanHT-12 v4 Expression BeadChip, as described by the manufacturer's protocol. Details on samples processing quality control are found in eMethods.

Covariates, glycemic traits and T2D

Covariates were selected based on previous literature and included: age, sex, body mass index (BMI), smoking status (current, former and never smokers), history of coronary heart disease (CHD) and anti-hypertensive medication, systolic blood pressure (SBP), serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides and low-density lipoprotein cholesterol (LDL-C). Glycemic traits used in this study were: plasma glucose, insulin, homeostatic model assessment-insulin resistance (HOMA-IR) and T2D incidence. Variables with a right-skewed distribution were transformed to the natural logarithmic scale (glucose, insulin, HOMA-IR, HDL-C and triglycerides). Details on covariates assessment are found in eMethods.

Statistical analyses

Epigenome wide association study (EWAS)

The association between CpGs methylation and current versus non-current statin use was assessed in an EWAS in two phases, discovery and replication. Linear mixed-effect models were performed using the 'lme4' package in R v.3.4.2 (https://cran.r-project.org/web/packages/lme4/index.html). In the regression analysis, normalized DNA methylation β values at each CpG was used as the dependent variable and the current use of statis (yes=1, no=0) as the predictor of interest. Per individual CpG, participants with methylation levels higher than three times the interquartiles range (IQR) were excluded.

The main model was adjusted for batch effects (array number and position on array), leukocyte proportions or measured leukocytes, sex, age, smoking status, BMI, SBP, anti-hypertensive medication, prevalent CHD and T2D (assessed at the time of blood drawing for methylation). Leukocyte proportions (B-cells, CD4, T-cells, CD8, T-cells, granulocytes, monocytes and NK-cells) were estimated in the R 'minfi' package, as described by Houseman.(15, 16) Statistical significance for discovery was based on a Bonferroni-corrected threshold (1.0×10^{-7}). Heterogeneity (I2) of effect estimates was assessed to account for differences between cohorts. Results across the discovery cohorts were combined in a single meta-analysis by inverse variance weighted method in METAL v.2011-03-25.

The identified CpGs were replicated in an independent panel using identical EWAS model. Bonferroni-corrected P-value for replication was 0.05 divided by the number of discovery findings. Results of discovery and replication panel were combined in a meta-analysis as described above.

Associations of the statin-related CpG sites with glycemic traits and incident T2D

Linear mixed-effect models were performed for the association between the standardized methylation values at the replicated CpGs and fasting glucose, insulin and HOMA-IR (RS, n=1165), excluding prevalent T2D cases (in an effort to prevent reverse causation) and former statin uesrs (given the dynamic nature of DNA methylation). Results across cohorts were integrated using METAL v.2011-03-25.

The adjusting covariates were leukocyte proportions, batch effects, sex, age, smoking status, SBP, anti-hypertensive medication, CHD and statin use. Bonferroni-correction was applied (P-value <0.0025).

Causal mediation analysis

To investigate causal relationships, the significant associations in the previous stage were tested in a non-parametric causal mediation analysis crossectionally. The 'mediation' package in R(17) was used to calculate the proportion of the effect of statins on each trait that is mediated by methylation at the statin-related CpGs, as used elsewhere.(18) Data from the RS were used (n=1180), excluding the prevalent T2D cases and former statin users. The normalized CpG methylation value was considered as mediator while each CpG-associated trait as outcome. We considered sex, age and cohort as confounders. To investigate potential unmeasured confounding the sequential ignorability assumption (no confounder assumption) was tested in a sensitivity analysis using the correlated residuals method (19, 20) (eMethods).

Gene expression association analysis

To investigate a possible biological pathway, gene expression association analyses were performed. First, the associations between the identified CpGs and expression probles for cis and trans

genes were investigated using linear regression in a subset of the RS (n=731). The Bonferroni-corrected P-value was 0.05 divided by the number of expression probes times number of CpGs tested.

Next, the significant expression probes were tested in association with current statin use status (n=631) and the glycemic traits that were significant outcomes in the causal mediation analyses (n=616). Type 2 diabetes cases and former statin users were excluded from these analyses.

Dose effects

In 303 current statin users from SHIP-Trend and RS with available data on statin dose, we examined the association between the replicated CpGs and the defined daily dose (DDD, WHO ATC/DDD-classification, https://www.whocc.no/atc_ddd_index/), using the main EWAS model. Next, The association between gene expression and statin dose was stadied in the RS.

Confounding and Ethnicity

To investigate whether the associations of statin use with the replicated CpGs were independent of blood lipids, we additionally adjusted the EWAS for serum total cholesterol or for individual blood lipids instead (HDL-C, LDL-C and triglycerides) in both discovery and replication panels. In an effort for ruling out confounding by indication, we restricted the EWAS to subjects with LDL-C ≥70 mg/dL or ≥100 mg/dL, cut-offs used to advice statins use.(21, 22). Further, we re-run the EWAS excluding cases of prevalent T2D and pre-diabetes.

To account for the different ethnicities among the cohorts of the discovery panel, we re-ran the EWAS, taking only Caucasians (n=4349) for discovery and British-South-Asians (n=3849) for replication.

Results

Association between statin use and DNA methylation

The mean (SD) age in the discovery panel ranged from 51.5 (13.8) years in SHIP-Trend to 62.1 (6.5) in KORA-F4. Of the total discovery panel, 2969 (43.5%) participants were women. Other baseline characteristics are shown in eTable 2a-b.

In the discovery panel, seven CpGs passed the Bonferroni threshold for significance (P-value $<1.0\times10^{-7}$), being differentially methylated in current statins users compared to non-current users. Of them, current users had lower methylation at four CpGs, annotated to the genes *DHCR24* (cg17901584), *FAM50B* (cg03467813), *SC4MOL* (cg05119988) and *AHRR* (cg05575921), while three CpGs annotated to *ABCG1* (cg06500161, cg27243685) and *DHCR24* (cg10177197) showed higher methylation among current users (Table 1; eFigure 1). All associations were replicated in the independent panel, except for the two CpGs annotated to *AHRR* and *FAM50B*, which failed to reach statistical significance after Bonferroni correction for replication (P-value $<7.14\times10^{-3}$). The meta-analysis across the discovery and replication panels revealed one new CpGs at *DHCR24* (cg17475467). The sensitivity analysis using RS data with never statin users as reference group (former users excluded) revealed similar results with the same five CpGs being replicated (eTable3).

Associations of the identified statin-related CpGs with glycemic traits and T2D

After a Bonferroni correction (P-value=$2.5\text{x}10^{-3}$) and comprehensive assessment of potential confounders, increase in one standard deviation of methylation at cg06500161 (*ABCG1*) was associated with fasting glucose (P-value=$1.03\text{x}10^{-3}$), insulin (P-value =$4.63\text{x}10^{-8}$) and HOMA-IR (P-value=$1.05\text{x}10^{-8}$). Increase in one standard deviation of methylation at cg27243685 (*ABCG1*) was suggestively associated with augmented insulin (P-value=$4.62\text{x}10^{-3}$), but not with glucose or HOMA-IR. No significant findings were

observed for cg05119988 (*SC4MOL*), cg17901584 and cg10177197 (*DHCR24*) (Table 2).

Causal mediation analyses

When cg06500161 was tested as a potential mediatior in the association between current statin therapy and the associated glycemic traits (Figure 3), significant results were obtained for the models with outcome fasting insulin and HOMA-IR, under the sequential ignorability assumption. For both cases the results showed that: i) current statin use has a significant overall effect of 0.275 (0.190 - 0.360) on insulin, and of 0.291 (0.199 - 0.380) on HOMA-IR; ii) part of the effect goes directly or via other mediator different from cg06500161, with an average direct effect (ADE) of 0.233 (0.149 - 0.320) on insulin, and of 0.242 (0.151 - 0.330) on HOMA-IR; iii) there is an indirect effect of statins that is partially mediated by cg06500161 methylation, with an average causal mediator effect (ACME) of 0.043 (0.024 - 0.060) on insulin and of 0.049 (0.028 - 0.070) on HOMA-IR; iv) of the total effect of statins on insulin and HOMA-IR, 15.5% (0.086 - 0.260) and 16.8% (0.095 - 0.270) are mediated by methylation at cg06500161, respectively. No significant findings were observed for the mediation model with outcome glucose (Table 3). The sensitivity analysis showed that a residual correlation due to unmeasured confounding larger than 0.20 would be needed to violate the sequential ignorability assumption.

Gene expression association analyses

The 5 identified CpGs were tested in their association with transcriptome-wide gene expression measured in the RS. cg06500161 was inversely associated with two expression probes corresponding to *ABCG1* (ILMN_1794782 and ILMN_2329927), after Bonferroni correction (P-value $<4.71\times10^{-7}$). An inverse association was also observed for cg27243685 and one probe (ILMN_1794782). Both indicate a lower expression of *ABCG1* with increasing methylation of cg06500161 and cg27243685. Furthermore, methylation at cg17901584 was directly associated with *ABCG1* expression (ILMN_1794782) and suggestively associated with another probe at the same gene (ILMN_2329927). There were no significant findings for any of the other two CpGs after Bonferroni correction (eTable 4).

Additionally, exposure to *ABCG1* expression was associated with lower insulin and HOMA-IR (eTable 5), while exposure to current statin use was associated with lower *ABCG1* expression (eTable 6).

Dosage effect

Statin dose was significantly associated with the five identified CpGs after Bonferroni correction (P-value <0.01) (eTable 7). Moreover, exposure to increasing statin dose was nominally associated with lower levels of *ABCG1* expression at ILMN_1794782 and suggestively with ILMN_2329927 (eTable 8).

Sensitivity analyses

Residual confounding and confounding by indication: i) Additional adjustment of the EWAS for serum total-cholesterol or blood lipids HDL-C, LDL-C and triglycerides instead (eTable 9); ii) exclusion of prevalent cases of T2D and pre-T2D (eTable 10); or iii) restriction of the EWAS to participants with LDL-C ≥70 mg/dL or ≥100 mg/dL only (eTable 11) did not change the associations of the identified five CpGs.

Trans-ethnic replication: when a new EWAS was performed taking Caucasians only (n=4349) as discovery panel and the British-South Asians (n=3849) as replication, the five CpGs passed Bonferroni threshold (eTable 12).

Table 1. DNA methylation sites associated with current statin use (compared to non-current use)

					Discovery panel [a]			Replication panel [b]			Both panels combined		
CpG	**Chr**	**Position**	**location**	**Gene**	**Effect**	**SE**	**P-value**	**Effect**	**SE**	**P-value**	**Effect**	**SE**	**P-value**
cg17901584	1	55353706	TSS1500	*DHCR24*	-0.0219	0.0021	$1.12x10^{-25}$	-0.0143	0.0033	$1.47x10^{-05}$	-0.0197	0.0018	$6.03x10^{-29}$
cg06500161	21	43656587	gene body	*ABCG1*	0.0103	0.0010	$2.67x10^{-23}$	0.0115	0.0023	$3.76x10^{-07}$	0.0105	0.0009	$6.59x10^{-29}$
cg03467813	6	3851047	gene body	*FAM50B*	-0.0218	0.0023	$2.11x10^{-21}$	0.0008	0.0069	0.9073	-0.0196	0.0022	$2.77x10^{-19}$
cg05119988	4	166251189	5'UTR	*SC4MOL*	-0.0120	0.0017	$7.26x10^{-12}$	-0.0128	0.0032	$7.55x10^{-05}$	-0.0121	0.0015	$2.57x10^{-15}$
cg27243685	21	43642366	gene body	*ABCG1*	0.0044	0.0008	$6.01x10^{-09}$	0.0069	0.0015	$6.41x10^{-06}$	-0.0115	0.0021	$2.52x10^{-08}$
cg05575921	5	373378	gene body	*AHRR*	-0.0132	0.0022	$3.20x10^{-09}$	-0.0011	0.0056	0.8445	0.0049	0.0007	$5.15x10^{-13}$
cg10177197	1	55316481	gene body	*DHCR24*	0.0039	0.0007	$3.94x10^{-08}$	0.0054	0.0016	$7.10x10^{-04}$	0.0041	0.0006	$1.65x10^{-10}$
cg17475467	1	55316769	3'UTR	*DHCR24*	0.0033	0.0008	$1.68x10^{-05}$	0.0049	0.0013	$2.11x10^{-04}$	0.0037	0.0007	$2.41x10^{-08}$

Model adjusted for leukocyte proportions, batch effects, sex, age, smoking status, body mass index, systolic blood pressure, anti-hypertensive medication, presence of coronary heart diseases, prevalent type 2 diabetes.

Bonferroni correction for significance for discovery $P < 1.0x10^{-07}$ and $P < 7.14x10^{-03}$ for replication.

[a] Discovery panel (n=6820): Cohorts KORA-F4, SHIP-Trend, ESTHER, LOLIPOP.

[b] Replication panel (n=1450): the RS (sub-cohorts RSIII-1 and RS-Bios)

Abbreviations: Chr, chromosome; SE, standard error. Bold text indicates statistically significant associations.

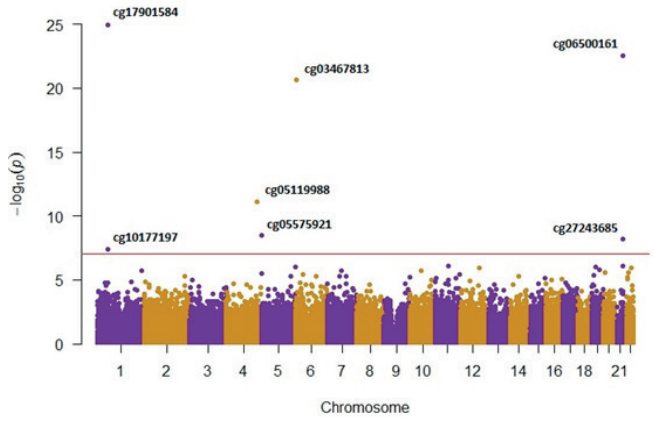

Figure 2. Manhattan Plot of the epigenome-wide associations between current statin use and DNA methylation (compared to non-current use)

Table 2. Association between DNA methylation of the replicated CpGs associated with statin use, and glycemic traits and type 2 diabetes

	log Glucose [a]			log Insulin [a]			log HOMA-IR [a]		
CpG	**Effect**	**SE**	**P value**	**Effect**	**SE**	**P value**	**Effect**	**SE**	**P value**
cg17901584	-0.006	0.004	$1.63x10^{-01}$	-0.023	0.019	$2.35x10^{-01}$	-0.029	0.021	$1.62x10^{-01}$
cg06500161	0.010	0.003	$1.03x10^{-03}$	0.076	0.014	$4.63x10^{-08}$	0.086	0.015	$1.05x10^{-08}$
cg05119988	-0.003	0.003	$3.59x10^{-01}$	-0.004	0.015	$7.84x10^{-01}$	-0.007	0.016	$6.65x10^{-01}$
cg27243685	-0.002	0.003	$5.04x10^{-01}$	0.041	0.014	$4.62x10^{-03}$	0.039	0.016	$1.39x10^{-02}$
cg10177197	-0.001	0.003	$8.01x10^{-01}$	-0.015	0.015	$3.08x10^{-01}$	-0.016	0.016	$3.18x10^{-01}$

Model adjusted for leukocyte proportions, batch effects, sex, age, smoking status, systolic blood pressure, anti-hypertensive medication, presence of coronary heart disease, statin use and body mass index.
Bold text indicates statistically significant associations after Bonferroni correction of $p < 2.5x10^{-03}$.
[a] Sample n=1165 (RS) complete cases, of which 119 were current statin users. Non-fasting samples (n=25), prevalent type 2 diabetes cases (n=181) and former statin users (n=74) were excluded from the analysis.
Abbreviations: Chr, chromosome; SE, standard error; T2D, type 2 diabetes; HOMA-IR, homeostatic model assessment insulin resistance

Discussion

The current study sheds light on potential mechanisms linking statin use and risk of T2D, by first, identifying and replicating associations between statin therapy and methylation at five CpGs (cg06500161, cg27243685, cg17901584, cg10177197 and cg05119988); and secondly, providing evidence on the parcial mediator role of *ABCG1* methylation in the effect of statins on increased levels of insulin and insulin resistance under the sequential ignorability assumption.

Our results on the association between statin use and differential methylation at cg17901584, cg06500161 and cg05119988 agree with a report from the Framingham Study.

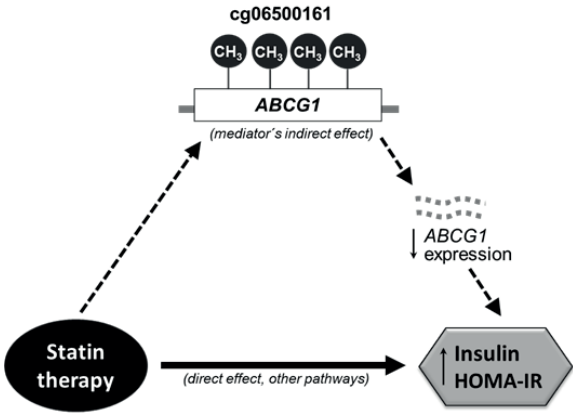

Figure 3. Scheme of the hypothesized mechanism linking statin therapy and risk of type 2 diabetes The solid black arrow represents the effect of statins on plasmatic insulin and HOMA-IR levels that goes directly or thro ugh a pathway different from the mediator analyzed in the current study (methylation at cg06500161). The dotted black arrows represent the suggested alternative pathway, where an indirect effect of statins on insulin and HOMA-IR is mediated by cg06500161 methylation. The black thin arrows indicate decreased or increased level of the parameter; in this figure they are representing decrease in gene expression of *ABCG1* and increase in serum levels of fasting insulin and HOMA-IR.

Table 3. Causal mediation analysis on the significant associations between the statin-related CpG (cg06500161) and glycemic traits

Outcomes	ACME estimate of mediator cg06500161 (95% CI)	ADE estimate (95% CI)	Total Effect (95% CI)	Proportion mediated by cg06500161 (95% CI)
log Glucose [a]	0.006 (0.003, 0.010)	0.009 (-0.007, 0.020)	0.015 (-6.79E-03, 0.030)	0.404 (-1.250, 2.600)
log Insulin [a]	0.043 (0.024, 0.060)	0.233 (0.149, 0.320)	0.275 (0.190, 0.360)	0.155 (0.086, 0.260)
log HOMA-IR [a]	0.049 (0.028, 0.070)	0.242 (0.151, 0.330)	0.291 (0.199, 0.380)	0.168 (0.095, 0.270)

Models adjusted for sex, age and cohort.
Bold text indicates statistically significant results.
[a] Sample n=1180 (RS) complete cases, of which 178 were current statin users. Non-fasting samples (n=25), prevalent type 2 diabetes cases (n=181) and former statin users (n=74) were excluded from the analysis.
Abbreviations: CI, confidence interval; ACME, average causal mediator effect; ADE, average direct effect; T2D, type 2 diabetes; HOMA-IR, homeostatic model assessment insulin resistance.

(8) They examined this association in a smaller sample (1545 participants versus 6820 of our discovery panel), and contrary to the current investigation, they did not proceed with replication nor examined their associations with gene expression, metabolic markers and risk of T2D. Moreover, our study adds to current knowledge that statin dosage might be implicated on the degree of methylation at the CpGs and *ABCG1* expression. The last finding goes in line with and experimental study where macrophages treated with vari-

ous types of statins showed lower levels of *ABCG1* expression as statin dose increased.(23)

Sites cg06500161 and cg27243685 are annotated to the *ABCG1* gene, cg17901584 and cg10177197 to the *DHCR24* gene, and cg05119988 to the *SC4MOL* gene. *ABCG1* (ATP-Binding Cassette Member-1 Subfamily-G) encodes a protein that mediates the transport of different of molecules, such as the cholesterol efflux to the high density lipoprotein, oxysterols and phospholipid transport in macrophages.(24-27) It is also involved in insulin secretion and sensitivity.(28) *ABCG1* expression has been found reduced in statin users compared to non-users,(29) and in T2D patients.(30, 31) The genes *DHCR24* (24-Dehydrocholesterol Reductase) and *SC4MOL* (Sterol-C4-methyl oxidase-like) code enzymes catalyzing different steps during the cholesterol biosynthesis. *DHCR24* mutations are related to desmosterolosis(32-34) and Alzheimer´s disease,(35) the last being linked to T2D and considered as type-III diabetes.(36) Deficiency of the *SC4MOL* protein produces congenital cataracts, microcephaly, growth delay, skin conditions and immune dysfunction.(37, 38) An observational study found that *DHCR24* and *SC4MOL* were up-regulated among statin users,(29) while *SC4MOL* up-regulation increased T2D risk.(30, 31)

Methylation at four of the identified statin-related CpGs (cg06500161, cg27243685, cg17901584 and cg05119988) has been previously associated with glycemic traits, T2D and blood lipids. Among them, cg06500161 (*ABCG1*) has been associated with increased levels of glucose, insulin, HbA1c, HDL-C and triglycerides.(39-44) Our causal mediation analyses provided evidence on cg06500161 methylation to partially mediate the association between statin use and higher fasting insulin and HOMA-IR. Although, given the crossectional nature of our analyses and the sensitivity analyses evidence, our results must be interpreted with caution. Since the mediator was measured at the same time as the outcome, reverse causation can not be completely ruled out. However, in an effort to overcome this issue, we excluded from the mediation analyses participants with T2D.

Based on our findings and the available evidence, we hypothesize that hypermethylation at cg06500161 may be a consequence of statins use, and may induce a decrease on *ABCG1* transcription in in blood. This down-regulartion could in turn compromise downstream signals, resulting in impaired insulin metabolism. In this line, imapired insulin sensitivity and secretion as a consecuence of statin treatment have been recently observed in a longitudinal study.(45) Furthermore, a functional study suggested a possible epigenetic regulation of *ABCG1* mediated by methylation-dependent transcription factor binding.(46) Nevertheless, future epidemiological and experimental studies are needed to prove this hypothesis and assess the effect of statins in specific tissues like pancreas, and to what extent these findings are generalizable to population with different ethnic background.

We highlight the large sample size with DNA methylation data available in our study, the use of a replication panel and the comprehensive assessment of confounding factors. The addition of complementary data like gene expression, the trans-ethnic replication and the use of a causal inference method as mediation analysis, also add value to our research. One limitation is the lack of data on DNA methylation and gene expression from specific tissues of interest for T2D and drug metabolism (pancreas, liver, adipose tissue) as both DNA methylation and gene expression may be tissue specific.(47, 48) Most cohorts had limited information on former statin users, thus it is likely that they were included in the reference group. However, in the replication panel we showed that the same results are valid for the group of current statin users only, although we had limited numbers to investigate such associations. Further, it was not possible to explore a potential effect of cessation time on DNA methylation, nor a potential effect of treatment duration. Studies included in our work used different methods to diagnose T2D, which could have introduced error measurement. Finally, some analyses

were performed using data from the replication panel only, which may introduce type-II error.

Conclusion

We identified and replicated five DNA methylation sites associated with current statin therapy, one of which (cg06500161) may have a causal role mediating the effect of statins on increasing insulin levels and insulin resistance. This could be one potential molecular mechanism explaining the link between statin therapy and early onset of T2D observed in clinical trials and observational studies. Nevertheless, the possibility of other biological pathways should not be discarded. Based on our study, it is not clear what might be the biochemical mechanism by which statins may induce changes in DNA methylation.

Our study may serve as a start for future investigations on new strategies for T2D prevention in patients undergoing statin therapy, like the use epigenetic biomarkers. Meanwhile, close monitoring of the risk factors for T2D in these patients is imperative to prevent further complications.

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CHAPTER 4

Summary and general discussion

Summary

The main objective of this work was to unravel novel pathways and associated risk factors underlying age-related changes in the vasculature. We implemented a multidirectional approach using several molecular epidemiology and experimental tools, including genetic association analysis, differential DNA methylation analysis and the assessment of cardiovascular function in a mouse model of accelerated aging. In this section, the main findings are summarized (Figure 1).

In **Chapter 2.1** we reviewed the evidence of the role of DNA damage in vascular aging and we described potential remedies against vascular aging induced by genomic instability. There is evidence that nuclear DNA lesions, shortened telomeres and mitochondrial DNA damage are involved in increased blood pressure, vasomotor disturbances and increased vascular stiffness. Cellular senescence, apoptosis, autophagy, stem cell exhaustion and altered proliferative capacity of vascular cells due to DNA damage could be important herein. Potential mediating signaling pathways involved include components of the survival response, notably antioxidants under regulation of Nrf2 (beneficial), increased inflammatory status (detrimental) and decreased IGF-1/GH signaling (detrimental), as well as the interplay between mTOR, AMPK and NFκB, SIRT-1, and PAI-1, p53- and p21- and p16-related signaling. Proposed remedies against genomic instability–related vascular aging include *PAI-1* inhibition, mTOR inhibition, DR, senolytics, *PDE1* and 5 inhibitors and stimulators of *Nrf2*. The role of gene mutation and of compromised transcription remains unknown.

In **Chapter 2.2** we evaluated cardiovascular function in a mouse model with loss of *ERCC1* DNA repair in vascular endothelial cells (EC-KO). EC-KO mice displayed a decrease in microvascular dilation of the skin, lung perfusion and aortic distensibility and coronary artery dilator function. We observed sudden death without noticeable preceding health problems at the age of 5 to 6 months in EC-KO mice. Nitric oxide mediated endothelium-dependent vasodilation was decreased in aorta and coronary artery. These features of age-related endothelial dysfunction can be largely attributed to loss of endothelium-derived nitric oxide. Interventions that potentiate the NO signaling pathway, such as pharmacotherapy with guanylyl cyclase stimulators or activators and specific phosphodiesterase inhibitors can potentially alleviated the disturbed vasomotor responses, albeit through improvement of cGMP signaling in VSMC.

In **Chapter 2.3,** we evaluated genetic variants and DNA methylation status in autophagy-related genes in relation to vascular outcomes and related traits. Autophagy was chosen because it represents an important cellular stress-coping mechanism, amongst others after DNA damage. Also, it is activated by caloric restriction, a well-known metabolic intervention with anti-aging and vascular protective effects. Single-nucleotide polymorphisms were associated with blood proinsulin levels, lipid traits, systolic blood pressure, pulse pressure and coronary artery disease. Loss of function variants showed association with diastolic blood pressure. Epigenetic variations were associated with systolic and diastolic blood pressure as well as HDL cholesterol levels. The integral approach covered by this study may contribute to the design of experimental studies targeting other autophagy-related genes and/or associated pathways.

In **Chapter 2.4** we conducted a meta-analysis of genome-wide and exome association studies to identify the genetic variants influencing abdominal aortic diameter (AAD), a main trait of vascular aging. We identified one replicated locus, *LDLRAD4*, that associated with AAD. In addition, we provided evidence that the main genetic determinants of pulse pressure, triglycerides and height also influence the diameter of the abdominal aorta. Our findings point to a potential novel part of the pathways underlying AAD, as this gene is one of the main regulators of *TGF-β* (transforming growth factor), which is important in the pathogenesis of aortic dilation. This study also highlights gene regions in mechanisms that have previously been implicated in the genetics of other vascular outcomes. The implementation of functional studies is critical to characterize the molecular mechanisms regulated by the genes described in this study.

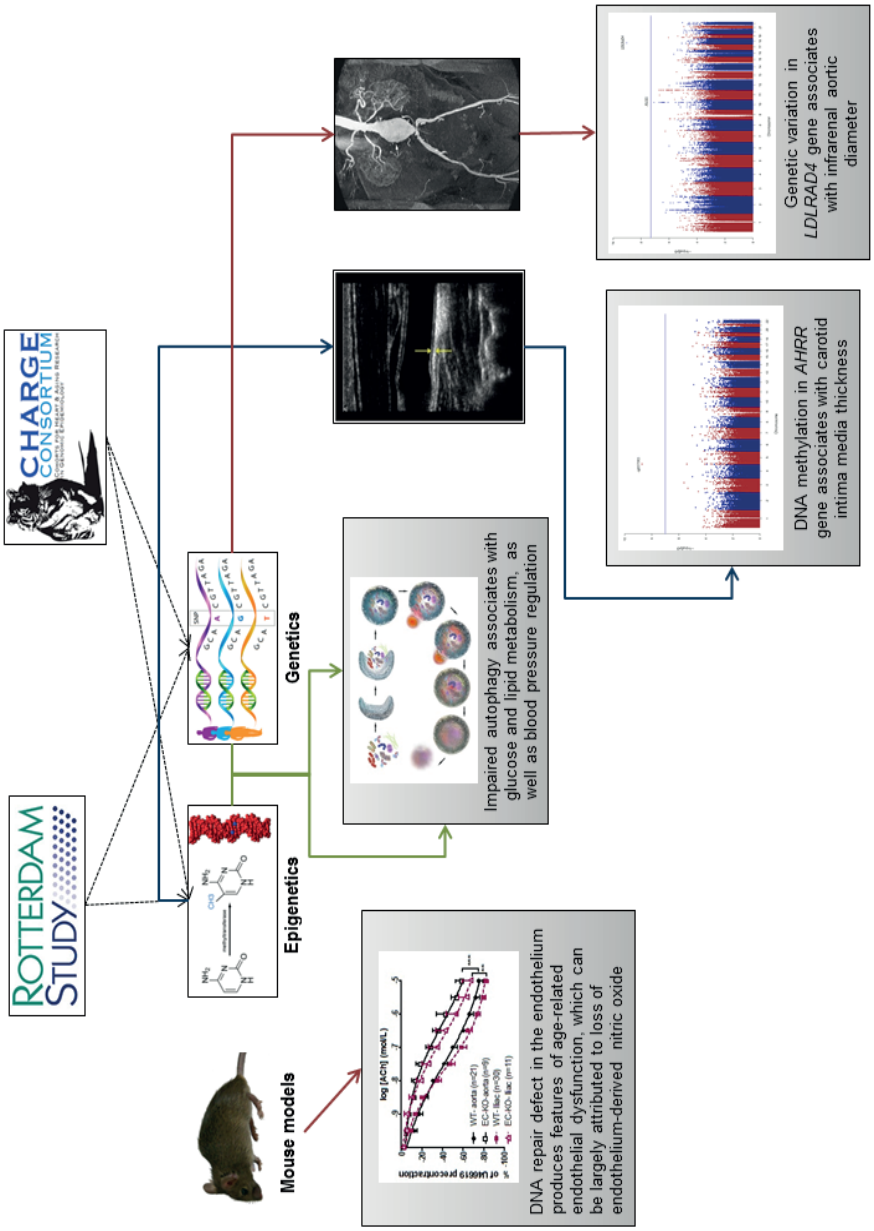

Figure 1. Summary of the findings described in this thesis

Apart from genetic variants, epigenetic regulation can play a role in disease. Epigenetic disease mechanisms are important in aging because they are thought to mediate, at least in part, the relationship between the genome and the environment (1). In **Chapter 2.5** we undertook a meta-analysis of epigenome-wide association studies on common carotid intima media thickness (cIMT), another main trait of vascular aging. We also characterized differentially methylated regions (DMRs) to aid biological inference on the trait. We identified one CpG, cg05575921, located in *AHRR* associated with cIMT and 34 DMRs important in inflammation and lipid metabolism pathways. Mendelian randomization approaches did not support DNA methylation at cg05575921 to be implicated in the causal association between cardiovascular risk factors and cardiovascular diseases. The in-depth exploration of the regulation of the genes described in this chapter warrants further study on the pathophysiology related to arterial thickness.

Chapters 3.1, 3.2 and **3.3** described a comprehensive and systematic review of the literature on the two most widely recognized mechanisms of epigenetic change, DNA methylation and histone modifications, and their role in blood pressure, inflammation and age-related cardiovascular disease. We observed an association between a generalized hypomethylation status and high levels of blood pressure, inflammatory markers and cardiovascular disease risk. The genes described in these chapters regulate key mechanisms related to renin-angiotensin system activation, inflammation, hyperlipidaemia and oxidative stress. These reviews highlighted important gaps in the quality of studies such as inadequate sample size, lack of adjustment for relevant confounders and failure to replicate the findings.

In **Chapter 3.4** we conducted a meta-analysis of EWA studies on epigenetic factors that determine the requirement of statin therapy, and described its link with type 2 diabetes (T2D) risk through epigenetic modifications. Statin therapy is the most applied therapy to prevent the development of atherosclerosis, one of the most life-threatening features of the aging vasculature. We found and replicated five CpGs annotated to genes *DHCR24, SC4MOL, ABCG1*, involved in lipid and carbohydrate metabolism, and molecular transport. Hyper-methylation at CpG site cg06500161(*ABCG1*) was associated with fasting glucose, insulin and insulin resistance. Methylation at *ABCG1* could partially mediate the effect of statins on increasing insulin and insulin resistance. Future studies should explore whether such biomarkers could help identifying statin users at risk of developing adverse events such as T2D. This might allow for more targeted treatment, and can guide the design of new drugs with a greater consideration of pharmacological safety.

General discussion and future perspectives

Genomic instability and vascular aging: research opportunities and potential interventions

The present results in cell-specific models also open novel opportunities for the study of differences in gene expression levels in association with the altered vascular features observed in EC-KO mice, which resembles normal aging, in particular of the endothelium. As mentioned in the summary, interventions that potentiate nitric oxide signaling pathway such as pharmacotherapy with soluble guanylyl cyclase (sGC) stimulators or activators and specific phosphodiesterase (PDE) inhibitors might alleviate the disturbed vasodilator responses. The EC-KO mouse appears to be an useful model to test the therapeutic efficacy of such drugs. Especially PDE1 and PDE5 inhibitor are attractive to test, since these PDE subtypes are commonly expressed in vascular tissue and known to be involved in vascular aging (2). Alternatively, or perhaps preferably, smooth muscle Ercc1 knockout mice (SMC-KO) could be used, because PDE1 and 5 are predominantly, if not exclusively, present in VSMC. Based on the observation that VSMC responses to NO are decreased in Ercc1d/- mice, which lack Ercc1 in all body cells, one would expect that, unlike EC-KO, SMC-KO would display reduced NO responses of VSMC as well. This could be easily tested in isolated aortic segments in organ bath chambers, either in experiments that use chronic (in vivo) or acute (in vitro) PDE inhibition to explore the involvement of PDE1 vs. 5. Such studies should also explore the effect of chronic PDE inhibitor treatment on molecular markers of (cellular) aging as to investigate if PDE1 and 5 are actually involved in the aging process.

There is however a great challenge to study effects of these drugs on vascular aging. Firstly, the expression levels of cell senescence-related genes were not different in comparison to wild type. Secondly, cell senescence markers alone are difficult to define, being limited to a few markers such as p16, p21, p27, p53, and a number of inflammatory markers, all of which may not specifically relate to cellular senescence. To overcome this problem I propose the implementation of a more comprehensive evaluation of the transcriptional landscape in various aging vascular beds, mainly aorta artery, and the microvasculature of lung and kidney, which have displayed a larger detrimental effect of the genotype and may have a greater clinical utility. PDE1 and 5 play important roles in the kidney and lung respectively, e.g. PDE5 inhibition is used as a therapy against pulmonary hypertension (3). Thus, the association of transcriptomic changes, e.g. of regulatory RNA's, to these enzymes can be integrally part of such evaluations. In this scenario, a whole transcriptome RNA-sequencing would be the preferred method, which will provide a high-resolution profile of differentially expressed transcripts, comprising canonical protein-coding transcripts, transcripts isoforms, and non-coding RNA transcripts. In addition, the assessment of the gene expression profile as an 'intermediate phenotype' of the physiological decline over time in this model, may allow a full depiction of the aging transcriptome in the vasculature. Therefore, the vascular tissue of which the transcriptome is evaluated should in parallel be tested for functional changes that are known to take place during aging, such as vasomotor disturbances, extracellular matrix changes, increase of stiffness, etc.

Although we did not observe senescence markers in EC-KO described in Chapter 2.2 nor in *Ercc1d/-* mice in a previous study (4), senescent endothelial cells and vascular smooth muscle cells are found in atherosclerosis and may be implicated in the progression of the atherosclerotic plaque (5), possibly through paracrine effects on surrounding healthy cells. Specific suppression of senescent cells in a background of vascular-specific genomic instability may be a promising approach to explore the importance of local senescent vascular cells in age-related vascular diseases. In this scenario, the implementation of "senolyt-

ic" therapies has been approached as an intervention to specifically target senescent cells, eliminate them, and thus diminish the contribution of SASP (senescence-associated secretory phenotype) and SMS (senescence-messaging secretome) (6), the malignant paracrine phenotype. Such therapies have shown beneficial effects improving the survival and recovery in aged mice following acute myocardial infarction (7). Nevertheless, the burden of cell senescence biomarkers and SASP needs to be further studied, for instance by studying the effects of senolytics to Ercc1d/- and EC-KO to study the effects on vascular aging. Also, the importance of cell senescence in vascular aging in humans needs to be explored. This might be accomplished only through long term intervention studies in patients at risk for CVD, and therefore this is a long and expensive process. Nevertheless, the 9p21 locus, which regulates important senescence pathways, displays the strongest association with risk for CVD of all loci found until now (8). Therefore, this is a promising field of research.

Cell death and survival mechanisms and vascular aging: an opportunity to link human and mouse studies

Autophagy

The evaluation of transcriptomic data and their specific association with vascular aging, made possible in EC-KO and SMC-KO mice, can become a giant puzzle since enormous amounts of transcripts can be involved. Our human studies can, however, give direction as to in what direction to look. One such direction is autophagy-related genes which are important for cell survival. Knockout mouse models have greatly contributed to understanding the physiological roles of autophagy in vivo. There are approximately 20 core autophagy-related genes involved in autophagosome formation in mammals. So far, 14 of them have been knocked out in mice (9) including *BECN1, PIK3C3/VPS34, ATG9A, RB1CC1, ATG13, ULK1/2, ATG3, ATG5, ATG7, ATG12, ATG16L1, ATG4B, ATG4C, MAP1LC3B*, and G*ABARAP*. In aging, genetic ablation of *ATG7* and *ATG5*, causes an accelerated appearance of vascular aging hallmarks in mice (10, 11). In chapter 2.3, we showed that a loss of function variant (genetic variant predicted to severely disrupt protein-coding genes) in *ATG3* confer risk to changes in blood pressure regulation. The complete ablation of *ATG3* in mouse is lethal leading to death within one day after birth (12) and, to the best of our knowledge, a specific *ATG3* gene knockout mouse model has not yet been developed. Therefore, I postulate to first assess the viability of using a conditional-tissue specific *ATG3* gene knockout mouse targeting both endothelial and vascular smooth muscle cell. If the model is feasible, blood pressure and overall cardiovascular function in adult mice can be evaluated, much in the way it was done in Chapter 2.2 for EC-KO. Potential compensating mechanisms in response to the ablation of *ATG3* are currently unknown. Another alternative, is to examine the regulation of candidate blood pressure-related pathways in *Atg3*-knockout cell cultures.

An additional research approach in vascular aging that warrants further investigation is the relation between autophagy and genomic instability. The interchange between autophagy and DNA repair is complex and it may occur in both directions. Autophagy is triggered by several types of DNA lesions and it can regulate mechanisms involved in DNA damage response (DDR), such as cell cycle checkpoints, cell death and DNA repair (13). Given our findings in chapter 2.2 in EC-KO, and previous findings in Ercc1d/- mice, it would be interesting to investigate the role of autophagy in these mouse models. Genetic ablation of important autophagy genes such as ATG3, 5 and 7 in such mice might be a tool to explore the connection between DNA damage and autophagy in vascular aging.

Cell death and survival

Apart from autophagy, other survival pathways, and also cell death mechanisms, are important both in the DNA damage response as well as in vascular aging. In this light I propose to study the changes in cell cycle, apoptosis and necrosis pathways in the vasculature of *Ercc1* mutant mice to explore the role of these survival pathways in DNA damage-induced vascular aging. In other tissues like the liver and kidney some studies confirm changes in the survival mechanisms in these models (14-16). In many vascular disease models and aged vascular tissue similar pathways are changed (17, 18). Important pathways include: the TOR kinase pathway, caspases, anti-apoptotic FLIPs (Flice Inhibitor Protein), the death-associated protein kinase (DAPK) family, p53 and mitoptosis (19). At least, genetic association studies demonstrated that genetic variations in some of these pathways associate with vascular diseases (20, 21).

Connecting autophagy with these mechanisms, pharmacological intervention in autophagy targets two main regulatory signaling machineries involved in dysregulated autophagy: AMP-dependent protein kinase (AMPK) and class I phosphatidylinositol-3-kinase(PI-3K)/Akt signaling, both resulting in over activated mechanistic target of rapamycin (mTOR) signaling (22). These drugs, especially rapamycin, have shown a beneficial effect in cardiovascular disease improving endothelium-dependent vasodilation and preventing or delaying atherosclerosis in old rodents (23, 24). Moreover, autophagy flux might be an attractive route for the treatment of age-related cardiovascular disease. However, this hypothesis in CVD research remains controversial due to the limited number of drugs that specifically inhibit autophagy-related proteins.

Metabolism

In Chapters 3 we have found various genes in the lipid and inflammatory and oxidative stress pathways to be associated with features of vascular aging that are expected to be found in metabolically challenged *Ercc1* mutant mouse models. Studies in the mouse models might provide further insight in the link of the identified genes to actual vascular dysfunction that is observed during aging. This might start with simple expression or gene methylation profiles and their association with read outs of the observed vascular dysfunction. Such data could feed future studies into the deeper mechanisms, and potential drug targets, of vascular aging. The variety of mechanisms that are candidate for further explorations is expected to be of considerable size and variety. The fruitful implication of each of these mechanisms in the development of health care is a task to be executed by large numbers of research groups and experts.

Integrated omics and phenotypic variation

The assessment of genetic and epigenetic variations has increasingly gained importance for unraveling molecular pathways underlying human traits and disorders. In the context of GWA and EWA studies, functional validation is key to obtain a mechanistic understanding of disease risk loci identified by these two approaches.

Population-based studies have shown that higher infrarenal aortic diameter during long-term longitudinal measurements is a validated tool for the identification of individuals with clinically significant AAA and is associated with significantly increased risk of future CVD events and total mortality (25, 26). From a molecular perspective, the evaluation of gene expression of relevant risk loci in several vascular beds may increase our knowledge of common pathologic pathways underlying vascular outcomes. In chapter 2.4, a genetic variation located in *LDLRAD4* was associated with infrarenal aortic diameter in a cross-sectional population sample. From in silico predictions, it appears that this gene is important in the down regulation of *TGF-β* which contributes to the development of vascular disorders, including abdominal aortic aneurysm (27). Since the cross-sectional measurement gives no

definite evidence for a relationship with AAA, the role of *LDLRAD4* on AAA can be further explored through the measurement of gene and protein expression in AAA tissue, obtained from human or mouse models of AAA. Preliminary data (not shown in this thesis) has shown that the expression of *LDLRAD4* in AAA tissue is not significantly changed in comparison to non-AAA tissue. This is however not conclusive evidence that *LDLRAD4* is not involved in AAA, nor in the development thereof. An additional approach would be to assess the expression of *LDLRAD4* at different stages of the lifespan of AAA mouse models to determine the importance of this gene during AAA development. Another addition would be the exploration of the functional link between *LDLRAD4* and *TGF-β* in cultured human vascular cells.

The findings from EWAs presented in this thesis propose some mechanisms that relate the identified associations with pathways affecting different disease in humans that merit further investigation. Functional validation in epigenetic studies face important challenges as epigenetic variations are subject to numerous environmental influences (i.e., hormones and smoking) and they are tissue specific. In chapter 2.5, we identified one CpG site, located in *AHRR* gene, to be associated with carotid intima media thickness. In this study, an approach to experimentally show the importance of *AHRR* in vascular thickness is to first examine the expression levels of this gene in the vasculature. The ablation of this gene in cultured endothelial and vascular smooth muscle cells may provide a mechanistic insight of up/down regulated genes and pathways dependent of *AHRR*.

Methodological considerations

Mouse models of accelerated aging

A potential limitation of mouse models of accelerated aging for cardiovascular research is the severe aging phenotype characterized by general frailty, small size and premature death (28). Cell type-specific models provide a solution to a certain extent. Nevertheless, Chapter 2.2 of this thesis proved that EC-KO had also a strongly reduced lifespan, with a median of 24.6 weeks. The cause of the death of this model remains unclear. We were able to only evaluated one animal in the final moments before death, which showed necrosis of the renal papilla and leakage of renal vasculature. We therefore suggested that acute tubular necrosis followed by severe electrolyte unbalance might have caused a fast onset of death, e.g. by induction of cardiac arrest. Ambulant measurement of blood pressure and heart rate, or electrocardiogram via implantable radio telemetry devices, is the preferred method to monitor arrhythmia frequencies and to detect if arrhythmias are the cause of death (29). However, such assessment was not possible within the period that this thesis was generated.

Systematic reviews

there are inherent flaws associated with systematic reviews such as location and selection of studies, heterogeneity, loss of information on important outcomes, inappropriate subgroup analyses, conflict with new experimental data, and duplication of publication (30). Moreover and although every effort was made to undertake a comprehensive search of the literature, we cannot exclude the possibility of publication bias from underreporting negative findings.

Genome wide association and epigenetic studies

Genome wide association studies provide an agnostic approach for investigating the genetic basis of complex disease. Technological progress and the plummet of costs of assays have made genotyping more 'affordable' leading to an increase of sample size and therefore statistical power. This will lead to the discovery of further genetic associations that have not yet been highlighted using other research and that may be biologically informative or collec-

tively useful in prediction (31). Moreover, as the genotyping coverage has increased around the world, the formation of international consortia have led to many analyses being solely based on the meta-analysis of genome-wide SNP data (chapter 2.4). In addition, the continuous development of imputation platforms has facilitated the harmonization of data generated by different array formats and has allowed SNP frequencies as low as 0.1% to be accurately imputed (32), extending the utility of GWAS to decipher the allelic structure of the susceptibility to complex disorders. Yet, GWAS merely identify loci that are directly associated with complex phenotypes and the identification of causal genes remains challenging.

Unlike genetic studies, epigenetic studies comprise a broader range of techniques and assays. The epigenetic study design is more prone to classical epidemiology caveats and therefore, clinical application seems distal, limiting their value. The key issues include selection of samples (tissue-specificity), sample size, replication and causality. Although the sampling of the cell type mediating the disease allows to infer more valid conclusions, the use of leukocytes, a more accessible surrogate cell type, is proved to yield sufficiently useful information (33). Furthermore, epigenetic alterations can be sensitive to temporal factors and micro or macro environmental determinants such as age, gender, demographics, ethnicity cellular composition, comorbidities, medication use, and lifestyle (smoking, alcohol consumption and BMI) (34-36), so it is essential to incorporate confounding factors into epigenetic analysis.

Causality estimation

Establishing the causal direction between biomarkers and disease remains one of the most challenging aims in epidemiology research, as in most studies the measurement of exposure and outcome are measured in the same time (37). Causal inference in epidemiological cross-sectional studies is normally addressed by mendelian randomization methods, which allow the estimation of causal effect from observational data in the presence of confounding factors and rules out reverse causation. In Chapters 2.4 and 2.5 of this thesis, an analytical framework was applied in an attempt to integrate genetic predictors of risk factors and DNA methylation levels to evaluate causal relationships and identify their contribution in causal pathways of vascular traits. we implemented a two-sample MR analysis using summary association results estimated in non-overlapping sets of individuals. These data sets can be obtained from large publically available GWAS, provided by consortia or estimated-directly from individual-level participant data (38). Despite the potential importance and promising benefits that MR methods may hold in the field of cardiovascular research, several limitations should be considered. These include, among others, population stratification, linkage disequilibrium, statistical power, pleiotropy and weak instrumental variables (39).

Concluding remarks

The main aim of population-based molecular epidemiology is to identify genes involved in the regulation of biological mechanisms underlying health and disease which may also lead to novel therapies. In this thesis, we made use of aging mouse models and molecular methods coupled with classical epidemiological approaches, resulting in the discovery of genes and a better understanding of the mechanisms behind variation in vascular disease and cardiometabolic traits. This integrative approach is likely to become increasingly popular in the forthcoming years as a result of major advances in molecular biology, technological progress and the plummet of costs of assays that belong to the 'omics' spectrum. The integration of high-throughput omics technologies will potentially unravel the 'hidden micro-universe' of molecular mechanisms through the usage of high-dimensional data modeling and the application of machine learning algorithms (40). To achieve this, the creation of large computational resources as well as computational capabilities are crucial. For the moment, the de-

velopment of multi-cohort projects, in consortia such as CHARGE, to increase sample size and replication opportunities have served as powerful incentives for scientific collaboration.

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CHAPTER 5

Samenvatting

Veroudering is één van de belangrijkste onafhankelijke risicofactoren voor hart- en vaatziekten. Door de stijgende leeftijd van de bevolking is deze groep ziekten gegroeid. Het verouderingsproces zorgt voor ongunstige functionele en structurele hermodelering van de bloedvaten, waaronder verstijving, plaquevorming en verkalking. In dit proefschrift is gezocht naar nieuwe mechanismen voor deze vaatveroudering. Hiervoor hebben we een multimodale benadering gebruikt die bestaat uit verscheidene moleculaire epidemiologische methodes, waaronder genetische associatie studies, DNA methylatie analyses, metaboloom analyses, and het verrichten van metingen van cardiovasculaire functie in een muismodellen van versnelde vaatveroudering.

In **Chapter 2.1** hebben we de rol van DNA schade in vaatveroudering beschreven op basis van literatuuronderzoek. We beschrijven er ook mogelijke therapiën. Er is groeiend bewijs dat genomische schade bijdraagt aan vaatveroudering, en dat het vaatverwijding en –constricties verstoort, bloeddruk verhoogt, vaatverstijving verhoogt, en aderverkaling verergert. Celveroudering, celdood middels apoptosis en autofagie zijn belangrijke mediatoren in dit proces. Mogelijke behandelingen zijn *PAI-I* inhibitie, mTOR remming, dieet restrictie, senolytica, *PDE1* en 5 inhibitors en *Nrf2* stimulatoren.

In **Chapter 2.2** hebben we cardiovasculaire functie in een muismodel waarin het DNA reparatie eiwit *ERCC1* specifieke in endotheelcellen (EC-KO) is uitgeschakeld onderzocht. Er werd een progressieve vermindering in vaatverwijdende functie gevonden in combinatie met verminderde perfusie van de long en een verhoogde vaatverstijving in vergelijking tot gezonde muizen. De verminderde vaatverwijding was louter te wijten aan verminderde nitriet oxide (NO) -gemedieerde responsen.

In **Chapter 2.3** hebben we de rol van autofagie in vaatveroudering en relevante metabole risicofactoren onderzocht middels epidemiologische methodieken. GWAS analyse liet zien dat genetische variaties (SNP's) in genen die coderen voor eiwitten betrokke bij autofagie gerelateerd zijn aan proinsuline niveau's, lipiden spectra, bloeddruk en coronair vaatlijden. Zeldzame genetische varianten laten verbanden zien met diastole bloeddruk. Epigenetische varianten laten verbanden zien met bloeddruk en HDL cholesterol.

In **Chapter 2.4** hebben we een meta-analyse en genetische associatie studie met exon varianten voor abdominale aorta diameter, een proxy voor aneurysmavorming, verricht. Hieruit blijkt dat het *LDLRAD4* gen geassocieerd is met verwijding van de intrarenale aorta. Ook bepalen belangrijke genetische factoren die betrokken zijn bij pulsdruk en lichaamslengte verbonden aan aorta diameter. Zodoende zijn er bekende mechanismen bevestigd en is er een nieuwe gevonden.

In **Chapter 2.5** is er een epigenetische associatiestudie verricht en gekeken naar wanddikte van de arteria carotis. Daaruit bleek dat het *AHRR* gen, die de toxiciteit van dioxine bepaalt, verband heeft met verdikking van de vaatwand.

In **Chapter 3.1** werd gekeken naar epigenetische eigenschappen en bloeddruk middels een systematisch literatuuronderzoek. In het algemeen laat hypomethylatie van het genomische DNA een verband zien met verhoogde bloeddruk. Onderzoek naar methylatie van individuele genen laten zien dat daarbij genen uit het renine angionsine systeem en de regulatie van ontstekingsprocessen een rol spelen.

In **Chapter 3.2** is verder gekeken naar epigenetische markers en histonmodificaties middels systematische literatuuronderzoek. Ook hier komen hypomethylatie en onstekingsfactoren naar voren, en bovendien een verband met epigenetische factoren die ook een rol spelen bij kanker en rheuma.

In **Chapter 3.3** wordt middels systematisch literatuuronderzoek getoond dat globale DNA methylatie een inverse relatie vertoond met cardiovasculaire ziekten. Hierbij zijn 27 genen betrokken die een functie hebben in onsteking, hyperlipidemie en oxidatieve stress.

In **Chapter 3.4** is er een meta-analyse verricht voor epigenetische studies om het verband met statine behandeling en type 2 diabetes te bestuderen. Daarbij vonden we dat

epigenetische veranderingen in de genen *DHCR24, SC4MOL, ABCG1*, die betrokken zijn bij lipide en koolhydraat huishouding, het gebruik van statines beïnvloeden. Methylatie van *ABCG1* lijkt het effect van statines op verhoogde insuline en insuline resistentie te beïnvloeden. Mogelijk kan dit van invloed zijn bij het bepalen van de behandeling.

CHAPTER 6

Appendices

About the author

List of publications

PhD Portfolio Summary

Words of Gratitude

About the author

Eliana Cristina Portilla Fernandez was born on December 5th 1988 in Cali, Valle del Cauca, Colombia. From 2005 to 2012, Eliana studied Biology at Universidad del Cauca. From 2010 she worked as a Research Assistant at the Human Genetics Research Group, Universidad del Cauca, in the framework of the project entitled "Atherosclerosis: Interactions among risk factors, genetic polymorphisms and Ancestry". The development of this project lead to Eliana and her team to determine the prevalence and risk factors associated with Peripheral arterial disease in Colombia. In the summer of 2014 she came to Erasmus MC to do the Master of Sciences on Health Sciences- Genetic Epidemiology at the Netherlands Institute for Health Science (NIHES) under the supervision of Prof. Oscar Franco. From 2015 to 2017 she studied a Doctor of Science in Genetic Epidemiology at NIHES. In 2017 she joined the departments of Epidemiology and Pharmacology-Internal Medicine to do her PhD under the supervision of Prof. Arfan Ikram, Prof. Abbas Deghan, Prof. Jan Danser and Dr. Anton Roks.

List of publications

Publications from this thesis

**denotes equal contribution*

Bautista-Niño PK, **Portilla-Fernandez E**, Vaughan DE, Danser AH, Roks AJ. DNA Damage: A Main Determinant of Vascular Aging. Int J Mol Sci. 2016; 17(5).

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Chapter 17- The role of epigenetic modifications in cardiometabolic diseases. Kim V.E. Braun, **Eliana Portilla**, Rajiv Chowdhury, Jana Nano, Jenna Troup, Trudy Voortman, Oscar H. Franco, Taulant Muka. Epigenetics of Aging and Longevity. Translational Epigenetics Vol 4. Translational Epigenetics. 2018, Pages 347-364.

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PhD Portfolio Summary

Name of PhD student:	Eliana Portilla Fernandez
Research School:	Netherlands Institute for Health Sciences
Erasmus MC Department:	Epidemiology and Internal Medicine
PhD Period:	September 2014-September 2019
Promotors:	Prof. Dr. A.H. Jan Danser and Prof. Dr. Arfan Ikram
Co-promotors:	Ass.Prof Anton J.M Roks and Dr. Abbas Dehghan

PhD training (Courses and Workshops)	**Year**	**ECTS**
1. Master of science in Health Sciences (NIHES)	2014/2015	**70**
Principles of Research in Medicine (ESP01)		0.7
Genome Wide Association Analysis (ESP29)		1.4
Conceptual Foundation of Epidemiologic Study Design (ESP38)		0.7
Principles of Genetic Epidemiology (ESP43)		0.7
Genomics in Molecular Medicine (ESP57)		1.4
Markers and Prediction Research (ESP62)		0.7
Advances in Genomic Research (ESP63)		0.4
The Practice of Epidemiologic Analysis		0.7
Study Design		4.3
Biostatistical Methods I: Basic Principles		5.7
Biostatistical Methods II: Classical Regression Models		4.3
Genetic-epidemiologic Research Methods		5.1
SNP's and Human Diseases		1.4
Linux for Scientists		0.6
Repeated Measurements in Clinical Studies		1.4
Missing Values in Clinical Research		0.7
Principles of Epidemiologic Data-analysis		0.7
Advances in Genome-Wide Association Studies		1.4
Family-based Genetic Analysis		1.4
An Introduction to the Analysis of next-generation sequencing data		1.4
Planning and Evaluation of Screening		1.4
English Language		1.4
Introduction to Medical Writing		1.1
Development Research Proposal		2.5

Oral Research Presentation		1.4
Research Period		29.6
2. Research Integrity	2017	**0.3**
3. Doctor of Science in Genetic Epidemiology (NIHES)	2015/2017	**70**
Introduction to Bayesian Methods in Clinical and Epidemiological Research (ESP68)		1.4
Research period Doctor of Science		62.3
Causal Inference (ESP48)		0.7
History of Epidemiologic Ideas		0.7
Causal Mediation Analysis (ESP69)		0.7
Topics in Meta-analysis (ESP15)		0.7
Women's Health		0.9
Courses for the Quantitative Researcher		0.0
Logistic Regression (ESP66)		1.4
Human Epigenomics (ESp75)		0.7
Cardiovascular Epidemiology		0.9
4. Additional courses		
Scripting for Life Science Researchers	2019	0.8
Epigenetic regulation in health and disease	2018	0.8
Python programming	2018	1.0
BBMRI-Omics	2017	0.8
Attended Conferences		
Science Days, Sint-Michielsgestel, Poster presentation	2019	1.2
Dutch Pharmacology Day, Utrecht, Poster Presentation	2018	1.2
Dutch German Meeting, Amsterdam, Poster Presentation	2018	1.2
Science Day, Antwerp, Poster presentation	2017	1.2
CHARGE Investigator Meeting, New York City, Poster presentation	2017	1.2
CHARGE Investigator Meeting, Charlottesville, Poster presentation	2016	1.2
Teaching		
Teaching assistant at Master Course: Methodological Topics in Epidemiological Research	2016	0.8
Teaching assistant in practical sessions of the "Pharmacological effects on the autonomic nervous system" course	2016	0.8
Attended Seminars		

Seminars of the Department of Epidemiology	2014-2019	0.2
2020 meetings	2017-2019	0.2
Cardiovascular Group Meetings	2014-2019	0.6
Pharmacology & Vascular Medicine Group Meetings	2015-2019	1.2

Words of Gratitude

"The desire for knowledge shapes a man" — Patrick Rothfuss, The Wise Man's Fear. Five years have passed since I left my home country and family behind to 'pursuit knowledge'. Since I first arrived to Rotterdam in 2014, initially to stay for one year only to complete my MSc, 'the desire for knowledge' supported each decision I made and shaped my mind and view of research and science overall. I learned a lot during this time. This process thought me more about myself than I would have ever imagined and I learned the value of patience, resilience and adaptability. Thus, I want to express my gratitude to everybody who contributed to this process, were always ready to help and were fun to be around.

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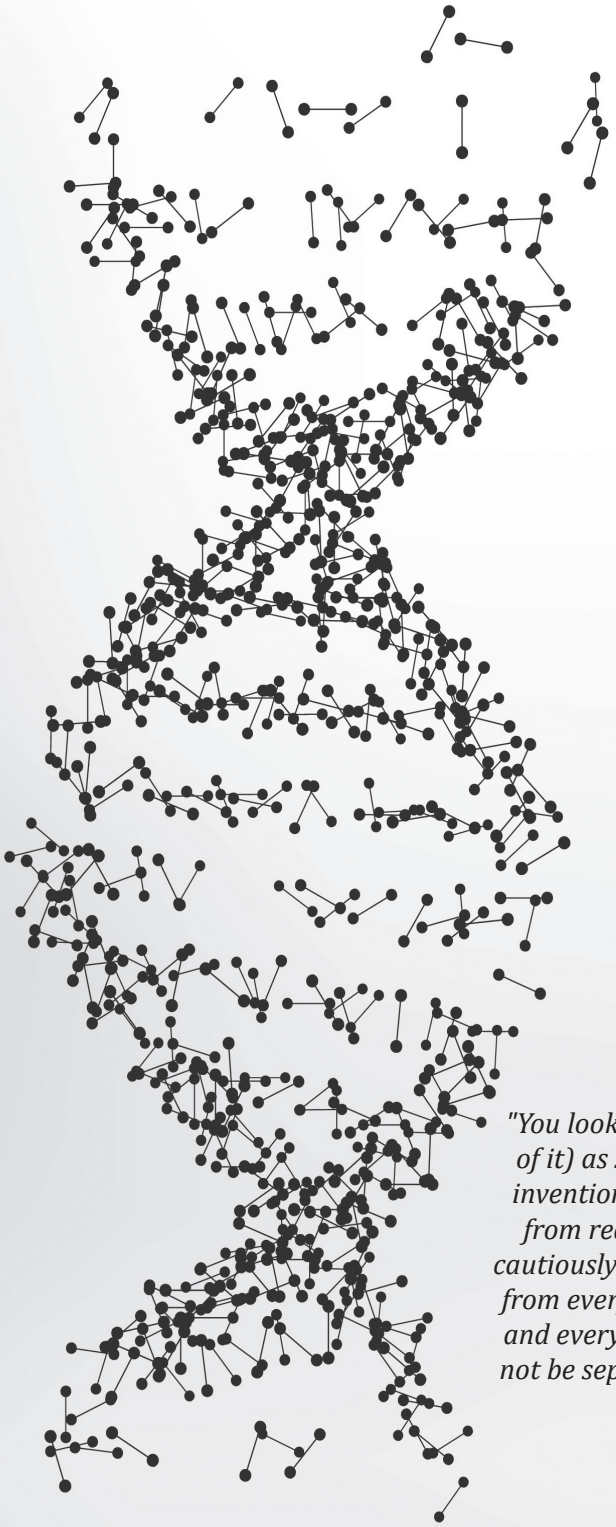

"You look at science (or at least talk of it) as some sort of demoralising invention of man, something apart from real life, and which must be cautiously guarded and kept separate from everyday existence. But science and everyday life cannot and should not be separated". **Rosalind Franklin (1920-1958)**

In this thesis, we aimed to untangle novel mechanisms underlying the aging of the vasculature. Among the multiple 'unknowns' in the field of cardiovascular physiopathology, we addressed the effect of local DNA damage in endothelial cells on vascular aging. We also studied the role of dysfunctional autophagy in cardiometabolic traits, which remains an open question in cardiovascular and cardiometabolic health research. Moreover, we characterized novel mechanisms of aortic diameter and arterial thickness through genetic and epigenetic studies. To accomplish this aim, we implemented a multidisciplinary approach, referred to as the "vascular aging study toolbox", which combines animal models and (big) data from human studies as a source of target mechanisms and a fundament for validation of the models.