Journal of Osteoporosis

New and Emerging Therapies for Osteoporosis

Guest Editors: E. Michael Lewiecki, Manuel Diaz Curiel, Joao Lindolfo Borges, Annie Kung, Maria Louisa Brandi, and Hans Peter Dimai

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Editorial

New and Emerging Therapies for Osteoporosis

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Osteoporosis is a common skeletal disease that increases the risk of fracture, with serious clinical and economic consequences. It can be diagnosed by dual-energy X-ray absorptiometry (DXA), even before a fracture has occurred, using the World Health Organization (WHO) criteria according to the patient's T-score. The WHO has also developed a fracture risk assessment tool (FRAX) to estimate the 10-year probability of major osteoporotic fracture (clinical spine, hip, proximal humerus, and distal forearm) and hip fracture, using clinical risk factors for fracture and femoral neck bone mineral density (BMD), if available. Cost-effective pharmacological agents that have been proven to reduce fracture risk in patients at high risk for fracture are now widely available. However, despite great progress in the management of osteoporosis, it remains a disease that is underrecognized and undertreated; if treatment is started, persistence is often poor, with only about 50% of patients who are prescribed medication for osteoporosis still taking it 1 year later. Even when treatment is taken correctly and for a sufficient length of time for the patient to benefit from reduction in fracture risk, there may nevertheless be limitations in effectiveness (note the lack of evidence for reduction in the risk of hip fractures or other nonvertebral fractures with some agents), limitations in the duration of therapy (e.g., no more than 24 months of lifetime teriparatide in the US), and concerns regarding long-term safety, such as atypical femur fractures and osteonecrosis of the jaw with bisphosphonates. For all of these reasons, the goal of reducing the global burden of osteoporotic fractures is not being fully achieved.

This special issue of the *Journal of Osteoporosis* describes new and emerging approaches to treatment that offer the potential to reduce the risk of fractures or manage their consequences better than what is currently observed in clinical practice. In recent years, our understanding of the pathophysiology of osteoporosis and the regulation of bone remodeling at the molecular level have undergone tremendous advances, leading to the investigation of drugs that target specific molecules in order to modulate the bone remodeling process. For example, the discovery that receptor activator of nuclear factor kappa B ligand (RANKL) is the principal regulator of osteoclastic bone resorption led to the development of denosumab, a fully human monoclonal antibody to RANKL. This potent antiresorptive agent, administered as a 60 mg subcutaneous injection every 6 months, recently received regulatory approval for the treatment of women with postmenopausal osteoporosis (PMO) at high risk for fracture. It has been shown to increase BMD, reduce bone turnover marker levels, and reduce the risk of vertebral fractures, hip fractures, and nonvertebral fractures in women with PMO.

Wnt signaling initiated by the binding of Wnt proteins to the low density lipoprotein-related protein (LRP5/6)-frizzled receptor complex has recently been recognized as an important upregulator of osteoblastic bone formation; sclerostin and Dickkopf-1 (DKK-1) are natural inhibitors of

Wnt signaling. In this issue, J. J. Mason and B. O. Williams describe a rare genetic disorder, sclerosteosis, resulting from a mutation of the *SOST* gene that encodes for sclerostin, and van Buchem disease, a related disorder caused by a mutation closely linked to *SOST* on chromosome 17q11.2. Patients with sclerosteosis and van Buchem disease have high bone mass due to downregulation of sclerostin, suggesting that a therapeutic agent that downregulates sclerostin in a controllable fashion might be a potent osteoanabolic treatment for patients with osteoporosis. Mason and Williams review many of the studies that have enhanced our understanding of the regulators of Wnt signaling and lead to the investigation of compounds with potential therapeutic applications through their effects on sclerostin or DKK1. A fascinating new finding, yet to be fully elucidated, is that serotonin produced by enterochromaffin cells in the duodenum also downregulates Wnt signaling, raising the possibility that modulation of serotonin production or activity might also be an effective treatment for patients with osteoporosis. In a related paper by S. Silverman in this issue, the preclinical and clinical studies of sclerostin inhibition are presented.

The drugs used to treat osteoporosis are generally considered to be in 1 of 2 categories—antiresorptive (e.g, bisphosphonates) or osteoanabolic (e.g., teriparatide). Interestingly, some drugs may "uncouple," at least in part, the closely related processes of bone resorption and formation. Strontium ranelate may be such a drug. Another, perhaps, is odanacatib, an investigational agent that inhibits cathepsin K, a protease produced by osteoclasts that is largely responsible for the degradation of the bone collagen matrix. J. L. Perez-Castrillon et al. review what is now known about the role of cathepsin K in health and disease, followed by data from phase 1 and phase 2 clinical trials with odanacatib. This drug is currently under investigation in a large phase 3 clinical trial to evaluate antifracture efficacy in women with PMO. Other papers in this issue cover new developments concerning skeletal heath in areas as diverse as bisphosphonate nanoparticles, melatonin, and thalassemia.

The papers in the issue were selected from many excellent submissions. We give our thanks to all authors of these submissions and to the numerous reviewers who kindly donated their time and expertise in helping select and revise those published here.

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Review Article

SOST and DKK: Antagonists of LRP Family Signaling as Targets for Treating Bone Disease

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The study of rare human genetic disorders has often led to some of the most significant advances in biomedical research. One such example was the body of work that resulted in the identification of the Low Density Lipoprotein-Related Protein (LRP5) as a key regulator of bone mass. Point mutations were identified that encoded forms of LRP5 associated with very high bone mass (HBM). HBM patients live to a normal age and do not appear to have increased susceptibility to carcinogenesis or other disease. Thus, devising methods to mimic the molecular consequences of this mutation to treat bone diseases associated with low bone mass is a promising avenue to pursue. Two groups of agents related to putative LRP5/6 functions are under development. One group, the focus of this paper, is based on antagonizing the functions of putative inhibitors of Wnt signaling, Dickkopf-1 (DKK1), and Sclerostin (SOST). Another group of reagents under development is based on the observation that LRP5 may function to control bone mass by regulating the secretion of serotonin from the enterrochromaffin cells of the duodenum.

1. Introduction

During the last decade, several groups working on the genetics of rare human skeletal disorders observed that mutations in what were thought to be core or regulatory components of the Wnt/β-catenin pathway lead to dramatic phenotypic effects. These mutations were either in the gene encoding the low density lipoprotein receptor-related protein-5 (*LRP5*) or in a gene (*SOST*) encoding a protein (Sclerostin) that potentially binds and regulates the function of LRP5 and its family members LRP4 and LRP6. This work has established LRP5 as a major target for drug development to treat osteoporosis and other bone diseases. This paper will discuss the development of two groups of agents designed to activate LRP5 (and the related LRP6) signaling pathway to increase bone mass. We will first review the core components of the Wnt signaling pathway to put the development of these agents into a cellular context.

These are by no means the only agents related to the identification of LRP5 that are in clinical development. One of the more interesting areas of research has centered around the observation by Yadav and colleagues that loss of LRP5 leads to low bone mass due to dysregulation of serotonin synthesis from the enterochromaffin cells of the duodenum [1]. Normally, LRP5 inhibits the expression of TPH1, the rate-limiting enzyme for serotonin production in enterochromaffin cells. In the absence of LRP5 in both humans and mice, serum serotonin levels were reported to rise and act on the HTR1B receptor in osteoblasts to inhibit their proliferation [1, 2]. Patients and mice carrying alleles of LRP5 associated with high bone mass are reported to have decreased levels of serum serotonin [1, 3]. This has led to the exciting possibility that pharmacological modulation of serum serotonin levels could be an effective treatment for low bone mass, a possibility supported by a recent report. More detailed discussions of this potential treatment can be found in several recent reviews [4, 5].

While not discounting the potential importance of serotonin-based therapies, this paper primarily focuses on the development of agents that potentially target LRP5, and the related proteins LRP6 and LRP4, in the bone itself.

2. Overview of Wnt Signaling

Mammals contain 19 genes encoding Wnt ligands. Wnts are cysteine-rich, glycosylated, and lipid-modified proteins that are highly associated with the extracellular matrix, particularly heparin sulfate glycoproteins [6]. Wnts can activate several signaling cascades, including one that results in the stabilization of β-catenin in the cytoplasm followed by its nuclear localization [7]. Wnts initiate signaling by binding to a member of the Frizzled family of seven transmembrane receptors and either LRP5 or LRP6 [8–10] (Figure 1), leading to downregulation of glycogen synthase kinase-3 (GSK-3) activity. In the absence of Wnt ligands, GSK-3 phosphorylates β-catenin on residues near it amino terminal end, marking it for ubiquitin-dependent proteolysis [11]. Inactivation of GSK-3 increases β-catenin levels in the cytosol. Recent work has also uncovered a parallel set of signals that are initiated by activation of the Wnt receptor complex that lead to the phosphorylation of β-catenin on serine residues (in regions C terminal to and independent from the GSK3 sites). Phosphorylation of these residues is required for efficient nuclear translocation [12, 13]. The combined effect of increasing levels of cytosolic β-catenin and facilitating its translocation to the nucleus allows β-catenin to form complexes with members of the Tcf/Lef class of DNA binding proteins [14]. These complexes modulate transcriptional activity of target promoters [14].

This core pathway is regulated by a large number of extracellular and intracellular proteins. Extracellular proteins that interact with this pathway include members of the Dickkopf family and Sclerostin and secreted frizzled related proteins (sFRPs) which regulate signaling at the level of the Wnt/Frizzled/Lrp interaction [15–21] (Figure 1). In addition, many proteins (including GBP/FRAT, axin, βTrCP, and APC, the product of the *adenomatous polyposis coli* tumor suppressor gene) control the pathway by regulating components of the intracellular signaling pathway [9, 14].

The effects of regulating GSK3 activity by Wnt signaling can also directly activate the mammalian target of rapamycin (mTOR) pathway by decreasing GSK-3-mediated activation of the TSC2/TSC1 complex [22]. This observation extends our understanding of the role of Wnt signaling in cellular regulation and identifies mTOR as an important downstream affector of Wnt signaling and, by extension, a potential downstream target of Lrp5 and/or Lrp6 during osteoblast differentiation. Activation of the mTOR pathway by Wnt ligands is independent of β-catenin, highlighting a signaling cascade that could explain different phenotypes seen when the pathway is inactivated at the level of Lrp5 and/or Lrp6 compared to inactivation of β-catenin. In addition to the canonical pathway [14], other signaling cascades initiated by Wnts include pathways that signal through the Rho GTPases and calcium-dependent pathways [23]. For more detailed descriptions of the Wnt signaling pathway, several excellent recent reviews on the subject are available [6–8, 24–27].

3. Overview of LRP Family Members

Although partially redundant, Lrp4, Lrp5, and Lrp6 display clearly distinct functions. For example, Lrp6-deficient mice die at birth [28], whereas Lrp5- and Lrp4-deficient mice are viable [29], suggesting unique functions of these receptors that cannot be compensated for by the others. While Lrp5-deficient mice develop a normal skeletal structure [30], Lrp6-deficient mice exhibit long bone formation defects. These defects are reminiscent of those observed in Wnt-7a and Wnt-1 mutant mice, indicating a possible link between Wnt1 or Wnt7a and Lrp6 that may not exist between these Wnts and Lrp5 [28]. Another possibility is that the role of Lrp6 may also involve down regulation of the Wnt5a noncanonical signaling pathways.

It was recently shown that Lrp6 physically interacts with Wnt5a, but that this does not lead to phosphorylation of Lrp6 or activation of the Wnt/β-catenin pathway. Overexpression of Lrp6 blocks activation of the Wnt5a-target, Rac, and this effect is dependent on intact Lrp6 extracellular domains. Surprisingly, some Lrp6$-/-$ birth defects were rescued by deletion of Wnt5a, indicating that the phenotypes resulted from noncanonical Wnt gain-of-function [31]. Finally, the Wnt5a loss-of-function birth defect is consistent with $Ca2^{+}$ modulation having an antagonistic interaction with Wnt/β-catenin signaling [32].

Similar to Lrp6, Lrp4 (a.k.a. Megf7) plays a role in limb development [33]. Lrp4-deficient mice have less severe phenotypes than those lacking Lrp6, but more severe than Lrp5-deficient mice. Phenotypes in Lrp4-deficient mice include a fully penetrant form of polysyndactyly and a mild and incompletely penetrant form of craniofacial abnormalities [34].

Detailed analysis of the functions of these receptors in several additional tissues using several mouse models are in progress. These include the mammary gland, where the functions of both Lrp5 and Lrp6 are linked to mammary progenitor cell regulation and where the proteins appear to function in at least a partially redundant fashion and affect the levels of Wnt/β-catenin signaling within the mammary gland [35–37]. In addition, changes in both LRP5 and LRP6 have been linked to alterations in glucose homeostasis and lipid metabolism [38–43], although it is not certain whether these latter functions are dependent on the Wnt/β-catenin signaling pathway.

Recent studies have also highlighted an important unanswered question related to the function of LRP family receptors in osteoblasts. Conditional deletion of β-catenin, or activation of the pathway by either inducible expression of a oncogenic version of β-catenin or via deletion of the Apc gene, leads to dramatic effects on osteoclastogenesis due to altered regulation of osteoprotegerin expression [44, 45]. In contrast, neither humans nor mice lacking LRP5 display any apparent alteration in osteoclast differentiation or function. There are several potential explanations for this. One is that

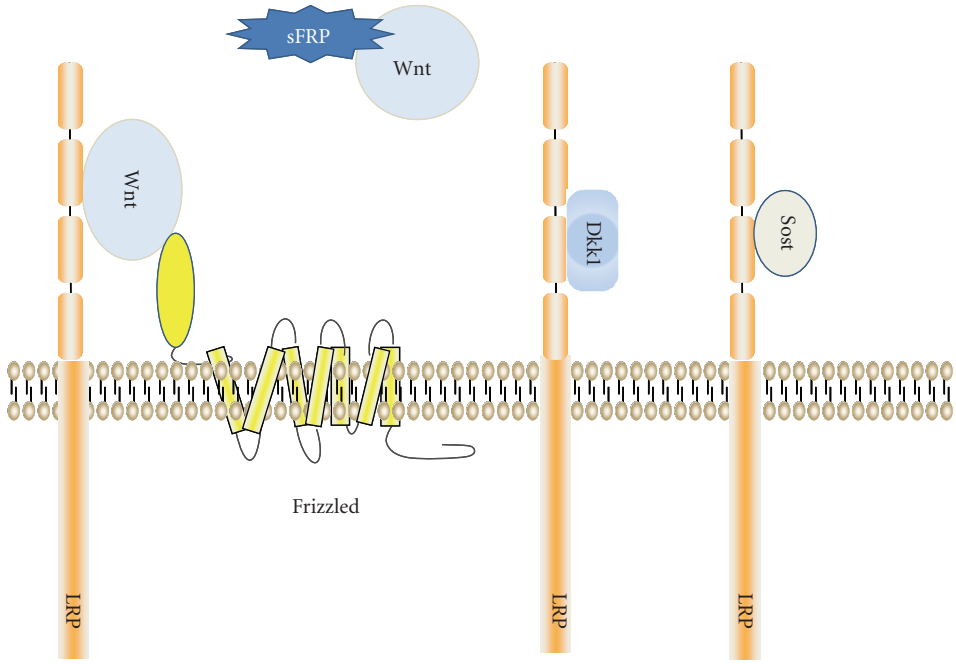

Figure 1: The current model for the induction of stabilization of β-catenin by Wnt ligands holds that Wnt proteins bind a complex that includes a member of the frizzled family of seven-transmembrane-spanning receptors and either LRP6 or LRP5. Several proteins have been identified that can block this process and are associated with downregulation of Wnt signaling. These include proteins which bind to the LRP component to prevent association of Wnt ligands with the LRPs (Dkk1 and Sost). In addition, secreted frizzled related proteins (sFRPs) also can block signaling by binding directly to Wnt ligands and potentially interfering with their ability to engage the receptor complex.

the functions of LRP5 within the duodenum may play a predominant role [1]. It is also possible that LRP6 and LRP5 play redundant roles in regulating this process [46]. These possibilities are being actively examined by several laboratories.

4. Linkage of LRP5 Mutations to Conditions with Altered Bone Mass

During the early part of this past decade, several reports linked changes in bone mass to alterations in *LRP5*. The first report found that patients with osteoporosis pseudoglioma syndrome (OPPG), an autosomal recessive disorder in which afflicted individuals develop severe, early-onset osteoporosis [47], are homozygous for inactivating mutations in *LRP5* [48]. These individuals have a very high susceptibility to multiple fractures and have severe deficits in vision due to persistence of the hyaloid vasculature often associated with retinal detachment [47]. Shortly after loss of LRP5 function was linked to OPPG, two groups independently reported that families with extremely high bone mass (HBM) carried a specific point mutation (G171V) in *LRP5* [49, 50]. The LRP5-G171V protein can no longer be bound by several proteins (such as Dkk1, Sost, and MESD) that may normally regulate its activity. Subsequently, work in mouse models by several laboratories provided further confirmation for a role of *Lrp5* in regulating bone mass [30, 46, 51–56].

LRP5 is a member of a multigene family and several other members of this family have shown to be involved in bone development and disease. For example, mutations in *LRP6*, which shares greater than 70% identity with LRP5, have been linked to changes in bone mass in both humans and mice [28, 30, 53, 56, 57]. In addition, it has recently been shown that *LRP4*, which is expressed in bone and cultured osteoblasts, binds Dkk1 and sclerostin in vitro and that *Lrp4*-deficient mice revealed shortened total femur length, reduced cortical femoral perimeter, reduced total femur bone mineral content (BMC), and bone mineral density (BMD) [58]. Thus, Lrp4 is also an osteoblast-expressed Dkk1- and sclerostin-receptor with a physiological role in the regulation of bone growth and turnover.

While it is important to note that some HBM patients develop pain neurologic sequelae [59], the fact that these patients do not appear to have an obvious predisposition to cancer or other disease has led to several biotechnology and pharmaceutical companies investing large amounts of resources in developing agents in an attempt to mimic the effects of the LRP5 mutations associated with HBM [60]. Since the canonical Wnt pathway is ubiquitous in embryonic development and oncogenesis [7], targeting LRP4-6 directly may have unintended effects. For example, both *Lrp4* and *Lrp6*-deficient mice exhibit significant developmental deformities [28, 33]. However, by concentrating on agonists or antagonists specific to bone, we may significantly reduce those risks. Here, we focus on two groups of agents; those designed to inhibit the function of Sclerostin and those which block the activity of the DKK1 protein. Further, we briefly discuss sFRPs, whose affects on bone are only recently being reported.

5. Sclerosteosis and Van Buchem's Disease

Sclerosteosis is autosomal recessive disorder characterized by progressive skeletal overgrowth [61, 62]. Patients appear normal at birth, with the exception of some instances of syndactyly. Skeletal overgrowth, especially in the mandible and skull, commences early in life. This can cause compression of the 7th and 8th cranial nerves often resulting in facial palsy and conductive hearing loss.

In 2001, it was reported that a gene located on Chromosome 17q11.2 was mutated in sclerosteosis [63, 64]. This gene which encodes a secreted glycoprotein (Sclerostin or Sost) containing a cysteine knot-like domain with homology to the Cerebrus/DAN family of BMP antagonists [63, 64]. Subsequent work on a related disorder, Van Buchem's Disease, revealed that while there was no mutation in the coding region of the *SOST* gene, a homozygous 52 kB deletion in a region closely linked to the *SOST* gene was identified in these patients [65–67]. Patients with Van Buchem's display what is essentially a milder version of the symptoms observed in Sclerosteosis. Additional work suggests that this deletion results in downregulation of Sost expression [65–67].

Partly due to its homology to Cerberus and DAN family members, it was originally thought that loss of Sost lead to bone abnormalities primarily due to ectopic activation of BMP pathways [68, 69]. However, subsequent work demonstrated that it also bound Lrp5 and Lrp6 and could prevent their interaction with Wnts [17–19]. Thus, loss of Sost may lead to an inability to inactivate the Wnt signaling pathway. Consistent with the skeletal overgrowth seen in patients carrying the G171V mutation in LRP5, Sost is unable to interact with the mutated version of LRP5 [70, 71].

A key characteristic that makes Sost a particularly attractive target for the treatment of osteoporosis is that its expression is restricted to osteocytes [72]. Thus, unintended side effects caused by blocking activity of this protein in other tissues are less likely. Furthermore, genetically engineered mouse models designed to mimic the mutations seen in Sclerosteosis and van Buchem's patients accurately model the high bone mass changes seen in humans [73].

Based on these characteristics, several pharmaceutical companies have initiated programs to create biological agents that inhibit Sost activity. Amgen, Novartis, and Eli Lilly have all been reported to have developed monoclonal antibodies designed to inhibit SOST [74]. In addition, OsteoGeneX has reportedly developed a small molecule inhibitor of SOST that is in the preclinical development stage [74].

Evidence for the potential efficacy of such approaches has been found in at least two preclinical models. Amgen reported that an antibody that blocked SOST function increased bone formation, bone strength, and bone mass in a rat model of postmenopausal osteoporosis [75]. Furthermore, a similar antibody was reported to inhibit bone loss in a mouse model of chronic colitis [76].

6. Dickkopf 1

Dickkopf1 (DKK1) is the prototype of a 4 member gene family and was first identified in 1998 [77]. Dkk proteins contain two cysteine-rich domains. The more N-terminal domain is Dkk-family specific, while the second domain contains structural homology to the colipase fold [77]. At that time, it was reported to be a secreted protein that inhibited Wnt signal transduction, but did not bind directly to Wnt proteins.

After LRP5 and LRP6 were identified as putative coreceptors for Wnt ligands [28, 78, 79], several groups reported that Dkk1 inhibited Wnt/β-catenin signaling via binding directly to LRP5 and LRP6 and blocked the ability of Wnt ligands to interact with LRP5 and LRP6 [80–82]. In addition, some reports (but not all [71]) found that the version of the LRP5 protein found in HBM families (G171V) could not bind to or be inhibited by DKK1 [83, 84]. Subsequent work showed that three members of this family (DKK1, DKK2, and DKK4) were inhibitors of the Wnt/β-catenin pathway, while DKK3 was divergent in both function and structure [77].

Mouse models have provided further support for a key role for *Dkk1* in bone development. Germline deficiency for *Dkk1* results in embryonic lethality associated with absence of head structures anterior to the midbrain and abnormalities of digits in the limbs [85]. Studies of mice heterozygous for an inactivating mutation in *Dkk1* show high bone mass associated with a significant increase in the bone formation rate [86]. In addition, heterozygosity for a hypomorphic allele of *Dkk1* (doubleridge) also results in increased bone mass [87].

Based on these observations, several companies are pursuing therapeutic approaches for bone disease based on inactivating Dkk1 function. These include Nuvelo's development of a monoclonal antibody against Dkk1 and a small molecule inhibitor approach being pursued by Enzo Biochem [74]. To our knowledge, there has not been published evidence for efficacy studies in the area of osteoporosis. However, several studies have found anti-Dkk1 antibodies were effective in treating disease on preclinical modeling systems. For example, administration of such an antibody immediately following a fracture significantly enhanced bone repair [88]. There are also several examples of anti-Dkk1 antibodies modulating the severity of diseases such as multiple myeloma and osteoarthritis [89–91].

7. Secreted Frizzled Related Proteins

Secreted frizzled related proteins are similar to DKK1 and Sclerostin in that they also inhibit Wnt/β-catenin signaling. However, they do so through a different molecular mechanism. sFRPs inhibit canonical Wnt signaling by binding directly to the Wnt molecule itself [92, 93]. sFRPs share sequence similarity with the cysteine-rich domain (CRD) found in the extracellular region of frizzled. sFRPs bind the Wnt ligands through their CRD, thereby preventing their binding to Frizzled receptors [94]. Results related to the effects of sFRPs are fairly recent. To date, sFRP-1 has been shown to be involved in the anabolic affects of PTH; deletion of sFRP-1 resulted in increased trabecular bone mineral density in a mouse model [95, 96]. Furthermore, sFRP-2 has been shown to inhibit bone formation [97]. It should be noted, though, that down regulation of sFRP-1 predisposes

the mammary gland to tumorigenesis [98] while sFRP-2 is significantly downregulated in gastric cancer [99], and downregulation of both sFRP-1 and sFRP-2 contributes to cervical cancer progression [100]. Thus, approaches aimed at inactivating sFRP function [95, 101, 102] should be pursued with appropriate caution. Similar risks exist with DKK1 [103] and Sclerostin [104]. However, research to date has not shown any predisposition of treatments utilizing these molecules toward oncogenesis (see below).

8. Future Directions

Preclinical studies with agents designed to block the functions of Sost and DKK1 have shown promise in treating bone disease and will likely be soon entering human clinical trials for the treatment of osteoporosis. Ongoing work will undoubtedly identify other potential druggable targets within this pathway. For example, the finding that the Prorenin receptor acts as an rennin-independent adaptor between LRP6 (and potentially LRP5) and the vacuolar H^+–adenosine triphosphatase (V-ATPase) complex may provide new drug targets [105]. The subsequent acidification of this compartment is required for phosphorylation of the cytoplasmic tail of LRP6, which is necessary for activating the downstream signaling cascade [106–108]. One could envision approaches designed to enhance this event to increase Wnt signaling and bone mass.

In addition to the development of agents directly targeting components of this pathway, several current and potential treatments for low bone mass may interact with the Wnt signaling pathway. For example, the anabolic actions of Parathyroid hormone (the basis for Forteo/teriparatide [109]) have been proposed to directly and/or indirectly work through regulation of LRP6 and/or LRP5 signaling [110–112]. In addition, osteoprotegerin (OPG), a molecule produced by cells of the osteoblast lineage that inhibits activation of osteoclasts [113], is a direct transcriptional target for β-catenin [44, 45]. Denosumab, a monoclonal antibody in clinical trials developed by Amgen [114], is based on the function of OPG. However, while the regulation is clearly altered in mice carrying mutations which directly activate or inactivate β-catenin, it does not appear to be altered in mice or humans carrying inactivating mutations in *LRP5* [48, 51]. This demonstrates the potential complexity of regulation within these pathways, and emphasizes the critical need to increase our knowledge about the detailed regulation of Wnt signaling pathways within osteoblasts.

Activation of the Wnt signaling pathway is one of the most common events associated with human cancer [7, 115]. As previously noted, the potential for treatments aimed at activating the Wnt signaling pathway to increase bone mass must always been tempered by consideration of a potentially increased susceptibility to carcinogenesis or other deleterious consequences. However, several observations suggest that this may not be as large a concern in the context of treating bone disease as some originally feared. First, neither the LRP5 HBM patients nor those with Sclerosteosis are reported to have an increased rate of carcinogenesis. In addition, there have been no reports in the preclinical modeling studies of increased carcinogenesis in mice treated with agents that block Dkk1 or Sost. Finally, lithium chloride has been used for decades to treat psychiatric illnesses in humans without being associated with any apparent increase in cancer risk. Given that the main mechanism of action for lithium treatment is inhibition of GSK3 activity (associated with upregulation of β-catenin signaling) [116], this provides further confidence in the approaches discussed in this paper.

Some concerns should also exist regarding the effects of systemic upregulation of Wnt/β-catenin signaling on fracture healing. While it is presumable that efficacy of these drugs would reduce fragility fractures in osteoporotic patients, the likelihood of fracture due to moving vehicle accidents or other misfortune is not necessarily reduced. Therefore, the effects of potential drugs on healing cannot be ignored. Unfortunately, the role of Wnt/β-catenin signaling in fracture healing is only beginning to be understood and therefore could lead to difficulties. For example, nonsteroidal antiinflammatory drugs (NSAIDs) that inhibit inflammatory response through down regulation of Cox2 were expected to have little effect on bone healing since mice lacking Cox2 form normal skeletons. However, it was later shown that fracture healing failed in rats treated with COX-2–selective NSAIDs and consequently, it was concluded that COX-2 function is specifically essential for fracture healing but not embryonic skeletal development [117]. Early indications are that Wnt signaling is both upregulated and downregulated temporally throughout the healing process [118]. As such, regulation of canonical Wnt signaling during this process is presently unpredictable. Further investigation into the role of canonical Wnt signaling in fracture healing is required.

In summary, the discovery almost a decade ago that mutations in LRP5 were causally associated with alterations in bone mass has stimulated numerous lines of research that have identified a number of promising targets to treat osteoporosis. The next decade will undoubtedly see the further translation of these findings into clinical use.

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Review Article

Sclerostin

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The striking clinical benefits of intermittent parathyroid hormone in osteoporosis have begun a new era of skeletal anabolic agents. One potential new agent is monoclonal antibody to sclerostin, a potent inhibitor of osteoblastogenesis.

1. Introduction

The Wnt signaling pathway demonstrates a complex network of proteins well known for their roles in embryogenesis but also involving normal physiologic processes of bone formation in response to loading and unloading [1]. The Wnt pathway involves a large network of proteins that can regulate the production of Wnt signaling molecules [2]. Several proteins that inhibit Wnt signaling [2] have been described. One such protein is sclerostin which binds low-density lipoprotein receptor-related protein (LRP) and inhibits Wnt signaling. This paper discusses both preclinical and clinical data for sclerostin.

2. Sclerostin

Sclerostin which is a potent inhibitor of osteoblastogenesis is a glycoprotein secreted by osteocytes. Sclerostin after secretion by osteocytes travels through osteocyte canaliculi to the bone surface where it binds to coreceptors LRP5 and LRP6 thus preventing colocalization with frizzled protein and Wnt signaling, and thereby reducing osteoblastogenesis and bone formation [3].

Loss-of-function mutations in *SOST* are associated with an autosomal-recessive disorder, sclerosteosis, which causes progressive bone overgrowth [4]. A deletion downstream of the *SOST* gene, which results in reduced sclerostin expression, is associated with a milder form of the disease called van Buchem disease [5]. Furthermore, *SOST*-null mice have a high-bone-mass phenotype [6].

Sclerostin suppression is required for balanced remodeling in response to PTH [7]. Serum sclerostin levels are significantly higher in postmenopausal women than in premenopausal women with significant negative correlations between free estrogen levels and sclerostin as well as PTh and sclerostin [8].

The development of a monoclonal antibody to sclerostin that can be administered subcutaneously has allowed scientists to evaluate the effect of sclerostin blockade on bone metabolism and bone mass. Li et al. [9] treated estrogen-deficient osteopenic rats with biweekly subcutaneous treatment with 25 mg/kg of a monoclonal antibody to sclerostin for 5 weeks and restored trabecular bone mass to baseline levels. Surface-based histomorphometry determined that the increase in bone mass resulted from an increase in bone mass at all skeletal envelopes, including cancellous, cortical bone sites, and supervertebral sites. The increase in bone mass and the change in microarchitecture were associated with improved bone strength in both the appendicular and axial skeleton. This data shows that pharmacologic inhibition of sclerostin results in increased bone formation, bone mass, and bone strength in rodents. In a mouse model of colitis, short-term treatment with Scl Ab countered the effects of chronic inflammation on bone loss and resulted in increased bone strength and bone formation [10]. Similarly, in rodent models of fracture healing, Scl Ab treatment resulted in increased callus density and bone strength at fracture sites and accelerated bone repair [11]. This increased bone formation and bone mass by sclerostin antibody was not blunted in ovariectomized rats pretreated with alendronate [12]. Combination of both sclerostin antibody and zoledronic acid

resulted in additive effects on bone parameters and bone mass [13]. Treatment with sclerostin inhibitors is not gender specific; treatment increases bone formation in male mice [14].

Sclerostin inhibition in primates has recently been reported by Ominsky [15]. A humanized sclerostin neutralizing monoclonal antibody (Scl Ab) was administered to gonad-intact female cynomolgus monkeys. Two once monthly subcutaneous injections of Scl Ab were administered at three dose levels (3, 10, and 30 mg/kg) over two months. Scl Ab resulted in dose-dependent increases in bone formation on trabecular, periosteal, endocortical, and intracortical surfaces. Bone density measurement showed significant increases (11–29% compared to vehicle alone) at femoral neck and radial and tibial metaphysis. Additionally significant increases in trabecular thickness and bone strength were seen in the lumbar vertebrae at the highest dose strength. In another study by the same author, sclerostin antibody stimulated bone formation and improved strength of the fracture callus in a primate fibular osteotomy model [16]. Although these studies are short term, they suggest that sclerostin inhibition resulting in increased bone formation may be useful clinically in osteoporosis and fracture healing.

Despite the increases in anabolic activity, no increase in bone resorption as measured by serum CTX was found in the primate study, suggesting that coupling of resorption and formation did not occur consistent with prior results in sclerostin-knockout mice and in oophorectomized rats treated with sclerostin antibody. This may suggest that the mechanism of anabolic action differs from PTH where bone resorption markers are seen within one month of treatment. This may represent direct activation of bone formation (modeling) without activation of bone resorption (bone remodeling).

In humans, antisclerostin antibody results in dose-dependent increases in markers of bone formation in healthy postmenopausal women [17].

The bone-forming effects of the *SOST* antibody resemble in many ways those of high-dose intermittent PTH in rodents. Several studies have reported that sclerostin gene expression and protein levels are reduced in animals treated with daily injections of human parathyroid hormone (hPTH) (1–34). Preclinical studies with a sclerostin inhibitor appear to be somewhat different from those with hPTH (1–34). For example, all skeletal sites respond to anabolic daily PTH treatment; the trabecular bone is most responsive, followed by the endosteal surface and the periosteal surface. In contrast, inhibition of sclerostin also results in significant bone formation at the periosteal surface. Also, studies find the increases in bone formation induced by antisclerostin antibody, unlike PTH, not associated with increases in bone resorption in the aged rodent skeleton.

Reduced mechanical stimulation leads to disuse osteoporosis, as seen in bedridden patients and in astronauts. Lin et al. recently [18] reported that *SOST*-knockout mice were resistant to mechanical unloading bone loss. In contrast to wild-type mice, Wnt/β-catenin signaling was not altered by unloading in *SOST*-knockout mice. The data suggest a potential major role for sclerostin in mediating the bone response to unloading and propose it may be a promising target for preventing disuse osteoporosis [18].

At this time, monoclonal antibody to sclerostin is being considered for early phase 2 clinical trials in postmenopausal women with osteoporosis and in fracture healing. The long-term safety of sclerostin in humans has not been studied. Additional clinical study data is needed to determine if the rapid gain in bone mass is associated with bone of normal strength and architecture and if boney overgrowth occurs at areas such as the carpal tunnel resulting in carpal tunnel syndrome, or around the lumbar spine neural foramen resulting in lumbar radiculopathy or spinal stenosis [19].

In summary, treatments based on inhibition of sclerostin may be a powerful way to restore skeletal bone strength in our patients and may provide more efficacious protection from hip fracture than current therapies as well as potentially improve fracture healing.

3. Conclusions

We now have a diverse menu of osteoporosis therapies including both antiresorptive therapy and one anabolic therapy (teriparatide). Current research suggests that in the future we may have multiple different anabolic therapies such as sclerostin. The therapies may have orthopedic benefits in terms of fracture healing and fusions. The future of anabolic therapies looks bright.

Disclosure

The author is a consultant and speaker for Lilly and Amgen.

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Review Article

Odanacatib, a New Drug for the Treatment of Osteoporosis: Review of the Results in Postmenopausal Women

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Osteoclasts are specialized cells that initiate the process of bone resorption, which has two phases, dissolution of the mineral component and degradation of the organic matrix, in which cathepsin K plays a key role. Cathepsin K inhibitors, which block the activity of cathepsin on bone resorption lacunae, may be a new therapeutic option in osteoporosis. Odanacatib is a nonpeptidic biaryl inhibitor of cathepsin K. Two studies have evaluated the efficacy and safety of odanacatib, a phase I study to determine the dose and a phase II study of safety and efficacy. Due to the long half-life of odanacatib and the similar effects of different doses on bone remodeling markers, a weekly dosage was chosen for the phase II trail, with the best results being obtained with a dose of 50 mg. At 36 months, increases in bone mineral density similar to those produced by other powerful antiresorptive drugs (zoledronate and denosumab) were observed but there were differences in the behaviour of bone remodeling markers. Data on fractures from the phase III trial currently in development are required to confirm these possible advantages.

1. Introduction

Osteoporosis results from alterations in bone remodeling that cause an imbalance between bone formation and resorption, with a predominance of resorption resulting in a reduction in bone strength and the appearance of fractures. Bone remodeling is a physiological process whose function is the permanent renovation of the skeleton in order to ensure biomechanically correct bone function and the regulation of mineral homeostasis. It consists of an initial phase of bone resorption followed by a phase of formation, both of which are regulated by general (endocrine) and local (paracrine) factors. The main endocrine factors include calciotropic hormones (parathyroid hormone, and vitamin D) and sexual hormones, mainly estrogens and, to a lesser extent, androgens. Other hormones, including the thyroid hormones, growth hormone and leptin play a smaller role. Local factors include various cytokines and growth factors that regulate the process, with the inflammatory cytokines IL-1, IL-6, and TNF-α playing a key role [1]. The main regulator and final pathway of bone remodeling is the RANK/RANKL/OPG (Receptor Nuclear Activator Factor Kappa B/Receptor Nuclear Activator Factor Kappa B Ligand/Osteoprotegerin) system. During bone remodeling, bone marrow cells and osteoblasts produce RANKL, which binds with a transmembrane receptor of the osteoclast precursor, RANK, causing their differentiation and activation. Osteoprotegerin (OPG) is a glycoprotein that acts as a decoy receptor of RANKL, impeding the activation of osteoclastogenesis [2].

The most common form of osteoporosis is postmenopausal, which is initiated by a fall in estrogen levels

that provokes an imbalance in the TH1/TH2 ratio (type 1 Helper T cells/ type 2 Helper T cells), with a predominance of TH1 [3]. This is caused by an increase in local levels of IL-7 which provokes increased concentrations of inflammatory cytokines and RANKL and a reduction in TGF-β, which exerts a beneficial effect on bone, producing an increase in osteoblastic activity and a reduction in apoptosis [4].

Osteoclasts are specialized cells derived from the mononuclear phagocyte system that initiate the process of bone resorption in two ways; dissolution of the mineral component and degradation of the organic matrix. Bone resorption begins when osteoclasts bond firmly to the bone surface through actin-rich podosomes, which form extensions of the cytoplasm to the interior of the matrix, creating specific regions named resorption lacunae. An acid medium is produced in the interior of the resorption lacunae that provokes the destruction of the osseous mineral component, leaving the organic matrix exposed. Subsequently, the organic matrix is dissolved by two enzyme groups, matrix metalloproteinases and cathepsin K, which plays a key role in degradation of the matrix [5].

Osteoporosis treatment is currently based on two drug groups, antiresorptive and anabolic agents. Antiresorptive agents, whose function is to inhibit bone resorption and generate increased bone mineral density (BMD), were the first to be introduced. The gold standard is treatment with bisphosphonates, which accelerate the apoptosis of osteoclasts and have shown their efficacy in reducing vertebral and nonvertebral fractures. However, the chronic use of bisphosphonates may result in both osseous and nonosseous undesirable effects, and this has led to the search for alternatives [6], including denosumab, which blocks the RANK/RANKL/OPG pathway [7]. Other therapeutic targets have been drugs that block integrins, which play a key role in the bonding of osteoclasts to bone, and cathepsin K inhibitors, which block the activity of cathepsin on bone resorption lacunae [8]. This article reviews current evidence for the highly selective and specific cathepsin K inhibitor, odanacatib, including results from a phase II trial.

2. Cathepsin K

Cathepsins are lysosomal proteases that belong to the papain-like cysteine protease family. Eleven different types have been described (B, C, F, H, K, L, O, S, V, X, and W) with cathepsin K being the most important with respect to bone remodeling, since it is a protease with intense collagenase activity, especially with respect to acid pH, which is essential to dissolve calcic hydroxyapatite, the main mineral component of bone. It degrades the two main types of collagen, I and II and is predominantly expressed in osteoclasts [9]. Immunoreactivity has also been found in osteoblasts and osteocytes although its role in these cells is not known. It is coded by a gene located in chromosome 1q21. Transcription is initiated by different regulating elements, but IL1 and RANKL can stimulate expression of the gene in osteoclasts in a process modulated by NF-kB [10]. It is a protein of 329 amino acids that consists of an amino-terminal region of 15 amino acids, a propeptide of 99 amino acids and a catalytic unit of 215 amino acids [11, 12].

The role of cathepsin K in bone resorption was determined using evidence from an autosomal recessive osteochondrodysplasia named pycnodysostosis, a very rare disease characterized by high BMD, acroosteolysis of the distal phalanxes, short stature, and cranial deformities with late closing of the fontanelles. It is caused by a genetic alteration that produces mutations of the cathepsin K gene causing loss of function [13]. Studies in mice submitted to nonfunctional mutations of cathepsin have given rise to different models of osteopetrosis. Pennypacker et al. [14] found increases in bone volume and in the number and thickness of trabecules in the distal region of the femur in a group of homozygotic cathepsin-K-null mice.

Cathepsin K is a key enzyme in the process of bone resorption and its inhibition is a new therapeutic target for the treatment of osteoporosis. The antiresorptive treatment of choice is bisphosphonates, which reduce the risk of nonvertebral and vertebral fractures. However, bisphosphonates may have adverse consequences. They increase the total number of osteoclasts, although these are described as hypernucleated, detached proapoptotic and possibly dysfunctional [15]. In rhesus monkeys, administration of odanacatib produced changes in osteoclast morphology, with the accumulation of elongated intracytoplasmic granules, although the number and size of osteoclast nuclei was not affected, indicating normal fusion. In addition the number of osteoclasts increased [16]. In addition bisphosphonates are associated with osteonecrosis of the mandible, especially in patients with tumors or diaphyseal fractures of the femur, although these adverse effects are exceptional [17–19]. The search for new therapeutic alternatives, such as cathepsin inhibitors, is interesting. The ideal inhibitor should have a low molecular weight, exhibit a minimal peptidic character, be able to bond to cathepsin and have a high selectivity to inhibit cathepsin K without affecting other cathepsins. Various inhibitors have been developed including relacatib, balicatib, MIV-701/710, and odanacatib, the object of this paper.

3. Odanacatib

Odanacatib is a powerful, reversible nonpeptidic biaryl inhibitor of cathepsin K that inactivates the proteolytic activity of cathepsin k. It is synthesized by replacing the P2-P3 amide bond of an aminoacetronintrile dipeptide 1 with a phenyl ring. This results in a powerful, selective inhibitor with the capacity to inhibit cathepsin K in osteoclasts. The potency and selectivity is due to the presence of the 4-fluoroleucine side chain at the P2 position interacting within the S2 pocket [8]. Its selectivity is responsible for the lack of accumulation of undesirable collagen in cutaneous fibroblasts [20]. A lack of selectivity has led to the retirement of other inhibitors in phase 2 development due to the appearance of morphea-like skin lesions [21].

Two studies have been carried out to evaluate the efficacy and safety of odanacatib, a phase I study to determine the dose and a phase II study to evaluate the safety and efficacy.

4. Phase I Study

This was a randomized, placebo controlled, double-blind study in post-menopausal women, without menstruation during the previous three years or during the previous year and confirmation of an elevated follicle stimulating hormone level in the postmenopausal range. The study included two groups, one containing 49 women aged ≤75 years and another containing 30 women aged ≤70 years. The objective was to evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of odanacatib in order to select the best dose. The results were measured according to the response of bone remodeling markers including CTx (carboxyterminal telopeptide of type I collagen), 1-CTP (pyridinoline cross-linked carboxyterminal telopeptide of type 1 collagen), TRAP5b (tartrate-resistant acid phosphatase), urinary deoxypyridinoline *(uDPD)*, BSAP (bone-specific alkaline phosphatase), osteocalcin, and NTx/Cr (N-terminal telopeptide of type I collagen normalized to creatinine). CTx and NTx are generated by the catalytic action of cathepsin on collagen but DPD is not influenced by the effect of odanacatib.

The group of 49 women was used to evaluate the weekly dose. Doses of 5 mg, 25 mg, 50 mg, and 100 mg were used and 12 women were assigned to the placebo group. The group of 30 women was used to evaluate the daily dose. Doses of 0.5, 2.5, and 10 mg were used, with 6 women assigned to the placebo group. All doses were administered in fasting conditions.

Odanacatib had a long half-life of between 66 and 93 hours for all the regimes and doses used. The efficacy of weekly, and daily doses in modifying the markers was evaluated. The effect was dose-dependant although not dose proportional. Reductions in resorption markers were greatest for doses >50 mg weekly and doses ≥2.5 mg daily. Maximum suppression was achieved between day 3 and day 5 with the weekly dose and was maintained until the following dose. With the daily dose, equivalent suppression was also reached between day 3 and day 5 and remained stable whilst the drug was administered. These results suggest greater suppression with the daily dose but without significant differences with the weekly dose. Unlike other antiresorptive drugs, no effects on markers of formation, which remained at levels similar to placebo, were observed. This decoupling between markers of resorption and formation suggests a beneficial profile of odanacatib. No differences between odanacatib and placebo were observed in the number of adverse effects [22].

Due to the long half-life of odanacatib and the similar effects on bone remodeling markers between doses, the weekly dosage was chosen for the phase II trial.

5. Phase II Trial

This was a double-blind, randomized, placebo-controlled trial of 12 months duration with an anticipated extension period of 24 months. It included 399 post-menopausal women (no menstruation during the previous five years or bilateral oophorectomy) aged between 45 and 85 years, with a T-score <−2 but not less than −3.5 at any site. Patients were divided into five groups according to the dose: placebo, 3 mg/weekly, 10 mg/weekly, 25 mg/weekly and 50 mg/weekly. All patients received vitamin D3 (5600 U weekly) and calcium (500 mg/day in the form of calcium carbonate). The primary objective was changes in bone mass in the lumbar spine, and secondary objectives were changes in BMD in other sites, changes in bone remodeling, and adverse treatment effects.

Of 399 women randomized, 331 (83%) completed 12 months of treatment, and 320 participated in the extension study, which was completed by 270 patients (70%) at 24 months. No differences were found between women who completed or abandoned the study.

The results showed a dose-dependant increase in BMD in all sites. The greatest increase was obtained with the highest dose. Weekly administration of 50 mg of odanacatib increased bone mass by 5.7% in the lumbar spine, 4.1% in the total hip, 4.7% in the femoral neck, 5.2% in the trochanter and 2.9% in the distal third of the radius at 24 months.

Resorption markers (uNTX/Cr, sCTx, and uDPD) fell in a dose-dependant manner from the beginning of treatment and remained reduced during the first six months, after which they increased and the differences with placebo disappeared. Only the 50 mg dose showed statistically significant reductions in comparison with placebo. Bone formation markers showed no differences with placebo except for the 50 mg group, in which bone serum alkaline phosphatase (BSAP) and type I procollagen N-terminal propeptide (PINP) decreased initially but then gradually increased, with significant differences with placebo being observed for both at month 12 and month 24. Adverse effects were similar in both groups without significant differences. Bone biopsies were carried out in 28 patients and showed no adverse histologic effects [23]. The histologic effects of odanacatib were evaluated in a study carried out in ovariectomized monkeys in which the bone histomorphometry of the femoral neck was analyzed. In addition to an increase in BMD, different behaviour between cortical and trabecular bone was observed. In the trabecular bone, the behaviour was similar to established anticatabolic agents, inhibiting bone remodeling whereas in cortical bone increased bone formation was observed due to stimulation of periosteal apposition [24].

The results of the extension of the phase II study to 36 months have recently been reported. This included 189 women who were randomized to odanacatib 50 mg and placebo weekly. The study was completed by 169 women (89%). In the odanacatib group, BMD continued to increase (lumbar spine 7.5%, total hip 5.5%, femoral neck 5.5% and trochanter 7.4%). The urine NTX resorption marker was 50% lower compared with placebo, whereas there were no differences in the BSAP formation marker. At three years, formation markers were not only not reduced but in fact increased by 18% over baseline values. Table 1 shows the evolution of the markers. Patients in the placebo group lost bone mass although this remained above initial values and normal values of markers were re-established. This suggests that odanacatib continues to have an effect at three years and that the effect is rapidly reversible [25].

Table 1: Effect of 50 mg of odanacatib on formation markers and resorption at 12, 24, and 36 months.

	12 months	24 months	36 months
BSAP	−18%	−15%	+18%
NTx/Cr	−60.2%	−51.8%	−50%
CTx	−60%	−45%	−24%

NTx/Cr: N-terminal telopeptide of type I collagen normalized to creatinine.
BSAP: bone-specific alkaline phosphatase.
CTx: carboxyterminal telopeptide of type I collagen.

The phase I study showed the pharmacodynamics of odanacatib, with a prolonged half-life that permits weekly administration. No significant differences was observed between the daily and weekly dose in the suppression of bone resorption markers. There were no differences in adverse effects between placebo and odanacatib. Taking these data into account, the phase II trial used the weekly dosage, achieving the best effects with a dose of 50 mg. At 36 months, increases in BMD similar to those of most powerful antiresorptive agents (zoledronate and denosumab) [6, 26] were observed, but with differences in the behaviour of bone remodeling markers. Decoupling between markers of formation and resorption were observed in tandem with increases in the therapeutic window. There was a smaller reduction in markers of resorption in comparison with other powerful antiresorptive agents but, in turn, the reduction in levels of formation markers was much smaller.There are no data on fractures, a key element in demonstrating the efficacy of a drug against osteoporosis. To clarify this point, a study (ClinicalTrials.gov registration number: NCT00529373) is ongoing, with results expected in 2012. This is a clinical, randomized, double-blind, trial with 16,000 patients. The target population is postmenopausal osteoporotic women aged ≥65 years not previously treated for osteoporosis. Patients with metabolic bone diseases other than osteoporosis or with previous hip fracture will not be included. Odanacatib at a dose of 50 mg/weekly will be used and placebo will include calcium and vitamin D. The primary objective of the study is the reduction in osteoporotic fractures (vertebral, nonvertebral, and hip).

In conclusion, odanacatib is a cathepsin K inhibitor whose mechanism of action differs from that of other antiresorptive agents. It does not reduce the number of osteoclasts and does not alter their function, thereby offering theoretical advantages over bisphosphonates. The results of the phase III trial currently in development are required to confirm these possible advantages.

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Review Article

Vertebroplasty and Kyphoplasty Can Restore Normal Spine Mechanics following Osteoporotic Vertebral Fracture

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Osteoporotic vertebral fractures often lead to pain and disability. They can be successfully treated, and possibly prevented, by injecting cement into the vertebral body, a procedure known as vertebroplasty. Kyphoplasty is similar, except that an inflatable balloon is used to restore vertebral body height before cement is injected. These techniques are growing rapidly in popularity, and a great deal of recent research, reviewed in this paper, has examined their ability to restore normal mechanical function to fractured vertebrae. Fracture reduces the height and stiffness of a vertebral body, causing the spine to assume a kyphotic deformity, and transferring load bearing to the neural arch. Vertebroplasty and kyphoplasty are equally able to restore vertebral stiffness, and restore load sharing towards normal values, although kyphoplasty is better at restoring vertebral body height. Future research should optimise these techniques to individual patients in order to maximise their beneficial effects, while minimising the problems of cement leakage and adjacent level fracture.

1. Introduction

Vertebral fracture is the most common type of osteoporotic fracture and imposes a significant burden on society. In the year 2000, an estimated 1.4 million osteoporotic vertebral fractures were recorded in the world [1]. Such fractures can cause disabling pain and kyphotic deformity [2] leading to impaired physical function and reduced quality of life [3, 4]. For a significant number of patients the pain becomes chronic, even after several months of conservative treatment such as bed rest and analgesics. In recent years, a novel treatment named "vertebroplasty" has been used increasingly to treat painful osteoporotic vertebral fracture [5, 6]. It is a minimally invasive technique that involves injection of bone cement into the fractured vertebral body to stabilize the fracture and alleviate pain. A modification of the technique, called "kyphoplasty", involves inflating a balloon inside the fractured vertebral body in order to reduce the fracture and create a cavity for the subsequent injection of cement [7]. Kyphoplasty may reduce the incidence of cement leakage during injection [8, 9], and may also help to restore vertebral body height [10–15]. Numerous clinical studies have demonstrated that vertebroplasty is effective in relieving pain following vertebral fracture [16–19]. Furthermore, a recent systematic review of vertebral augmentation for treating vertebral compression fractures suggests that physical disability, general health, and pain relief show greater early improvements in patients treated with vertebroplasty or kyphoplasty compared to those undergoing medical management [20]. However, two recent randomized controlled clinical trials found that the pain relief effect of vertebroplasty is no better than local anaesthetic [21, 22]. These controversies suggest that the mechanical and clinical effectiveness of vertebroplasty needs further investigation [23].

In this paper, we will concentrate on the mechanical effects of vertebroplasty and kyphoplasty and how they might improve clinical outcome. Although the primary purpose of these procedures is to mechanically augment the fractured vertebral body in order to alleviate pain, the discussion of their mechanical effects should not be limited to their effects on this structure alone. As will be discussed later in this paper, osteoporotic fracture not only damages the fractured vertebral body, but also causes profound changes to the

mechanics of the whole spine. It is therefore necessary to take a wider perspective of the mechanical effects of vertebral augmentation.

This paper will present evidence from current studies to answer the following three questions: (1) What are the effects of osteoporotic vertebral fracture on spine mechanics? (2) To what extent can vertebroplasty and kyphoplasty restore these fracture-induced effects on spine mechanics? (3) What are the important modifiable factors that can influence the restoration effects of vertebral augmentation?

2. Osteoporotic Vertebral Fracture Disrupts Spine Mechanics

The main function of the human spine is to resist compressive load in order to maintain the upright posture, allow flexibility for body movements, and protect the spinal cord which lies within the bony vertebral canal [24]. Two main structures of the spine, that is, the vertebrae and intervertebral discs, help to accomplish these functions. The vertebral body has a high stiffness which enables it to resist axial loading, and the intervertebral discs allow for mobility while distributing compressive load to the adjacent vertebral bodies. In a young and uninjured spine more than 80% of the compressive load is transferred through the anterior column (vertebral bodies and discs), and the discs, which act like a water bed, distribute the resulting compressive stress evenly across the vertebral bodies in both flexed and erect postures [25].

As the spine ages, osteoporosis and disc degeneration can alter the load bearing properties of the spine. Osteoporosis leads to a loss of stiffness in the bone, and as a result vertebral bodies become more deformable and may show greater deformations than the discs under compressive loading [26]. Disc degeneration leads to a loss of fluid and of disc height. As a result, nucleus pressure falls and the disc loses its ability to distribute compressive stresses evenly on the adjacent vertebral bodies [27]. In flexed postures, stress concentrations develop in the anterior annulus whereas in erect postures, stress becomes concentrated in the posterior annulus and neural arch [27, 28]. These changes in spinal load sharing can lead to stress shielding of the anterior vertebral body in upright postures increasing the risk of osteoporotic vertebral fracture, which can lead to even more profound changes in the spine's mechanical function.

Osteoporotic vertebral fracture usually involves damage to the endplate, as well as to the trabecular and cortical bone [29]: this leads to a loss of stiffness and strength in the fractured vertebra. Damage is usually located in the anterior part of the vertebral body because this part has lower bone mineral density in elderly spines (Figure 1), and so is easily damaged during spinal flexion when load is concentrated on the anterior part of the disc and vertebral body [27, 29]. This reduces vertebral height anteriorly, leading to wedge shape vertebral deformity [29]. The time-dependent mechanical properties of the fractured vertebra also deteriorate. A recent study on cadaver motion segments found that creep deformation of damaged vertebra was markedly increased following fracture [30], suggesting that the damaged vertebral body may continue to lose height even if no further damage is sustained [30], leading to even more pronounced wedge deformity [31, 32].

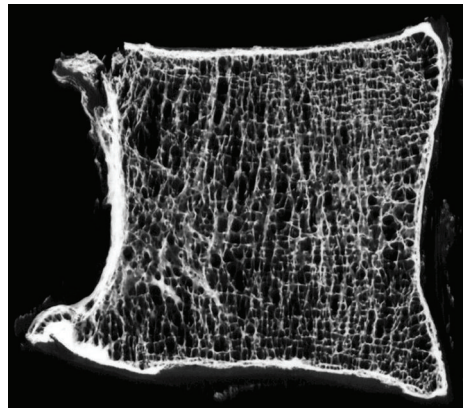

Figure 1: Microradiograph of a mid-sagittal-plane slice of an L2 vertebral body (male, aged 81 years), anterior on the left. Note the inferior trabecular architecture in the anterior region. (Reproduced with permission from Adams et al. [27]).

Vertebral fracture also causes mechanical changes to the surrounding structures. The damaged endplate and trabecular bone deform excessively under compressive load [33] allowing more space for the nucleus of the adjacent disc which is effectively a pressurised fluid [34]. This will induce a loss of intradiscal pressure [29]. A decompressed disc bulges radially and loses height, like a flat tyre [35] producing slack in the intervertebral ligaments, and reducing bending and compressive stiffness [36]. The decrease in nucleus pressure causes more compressive load to be resisted by the annulus. This increases concentrations of stress in the annulus, particularly the posterior annulus [37]. The compressive load resisted by the anterior vertebral body is correspondingly reduced [29, 38]. On the other hand, compressive load bearing by the neural arch is increased significantly because disc height loss brings adjacent vertebrae closer together, increasing contact stresses in the zygapophyseal joints, particularly in erect or extended postures [39].

These mechanical changes to adjacent structures following vertebral fracture may have serious consequences. The outer posterior annulus and zygapophyseal joints are innervated, so high stresses in these structures could contribute to the pain associated with osteoporotic vertebral fracture [40, 41]. In the long term, the transfer of compressive load from the anterior vertebral body to the neural arch will stress-shield the entire anterior column, reducing bone density in this region [28]. This could contribute to the risk of adjacent level fracture [27]. The altered disc mechanics such as loss of nuclear pressure and increased stress peaks in the annulus may also initiate or exacerbate disc degeneration [34].

Osteoporotic vertebral fracture also influences the mechanics of the whole spine. Increased vertebral wedging at the fractured level would act to increase flexion deformity so that greater extensor moments are required to counter gravitational forces on the trunk and maintain the upright

posture. As a result, the compressive forces acting down the spine will increase during standing [42, 43]. This increase in spinal loading may induce anterior wedging at adjacent and other levels with low anterior BMD leading to progressive spinal deformity and loss of sagittal balance [44, 45]. This effect may be exacerbated with time by the marked increase in creep deformation of damaged vertebra [30] which can result in a progressive increase in kyphosis [32].

The influence of osteoporotic vertebral fracture is two dimensional: it disrupts the mechanics of the whole spine in space, and this disruption is progressive over time. This poses a serious challenge for its treatment. In the following section, we will present evidence showing how vertebroplasty has the ability to restore spine mechanics in both of these dimensions.

3. Vertebroplasty Can Restore Normal Mechanics to an Injured Spine

3.1. Stiffness and Strength. Vertebroplasty increases the stiffness and strength of a fractured vertebral body towards prefracture levels [46, 47]. The compressive and bending stiffness of whole spinal "motion segments" (two vertebrae and the intervening disc and ligaments) is also partially restored by vertebroplasty [29]. These effects depend on the type and volume of injected cement, as discussed below.

3.2. Height and Wedge Angle. By increasing stiffness, vertebroplasty can effectively increase the height [48–50], and decrease slightly the wedge angle [49], of unloaded fractured vertebrae. Some *in vitro* biomechanical experiments have reported that, if enough cement is injected, then kyphosis angle can be restored to prefracture levels [51]. However, most experimental and clinical studies show that vertebroplasty does not entirely restore height and wedge angle [29, 49, 50, 52–55]. This may reflect the recent tendency to use small cement volumes in order to minimise the risk of leakage, resulting in an insufficient volume of cement being injected. Such a suggestion is supported by the findings of a cadaveric study which found that the restoration of local kyphosis angle was significantly correlated with cement volume [51].

Changes in vertebral body shape may be maintained during subsequent loading, although the evidence is equivocal. Augmented vertebral bodies have been reported to show improved fatigue properties compared with nonaugmented controls [56], and several *in vitro* studies have found no loss of restored vertebral height following cyclic loading [51, 57]. Clinical studies found that kyphosis was decreased immediately [54] and 6 months after vertebroplasty [58]. However, more recent clinical studies have noted that augmented vertebral bodies often lose height or recollapse during the follow-up period [59, 60], and in most cases, these changes were not due to trauma [60]. This raises serious concerns about the ability of vertebroplasty to fully and permanently restore height and shape to fractured vertebrae.

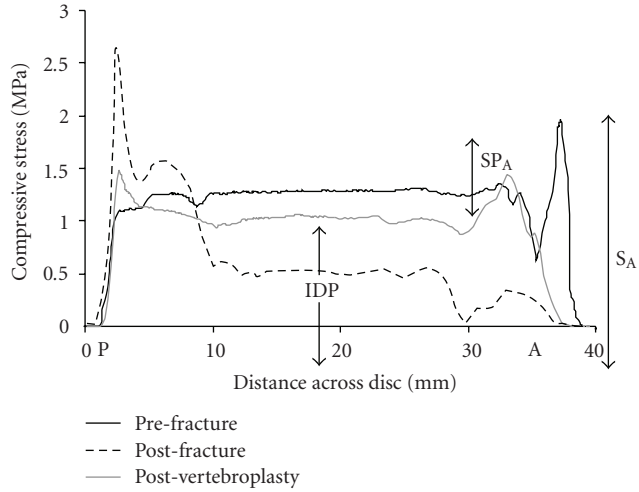

Figure 2: "Stress profiles" show the distribution of compressive stress within the intervertebral disc of a cadaver motion segment (Male 74, L1-2, A: anterior, P: posterior). In the nucleus of the disc, there is a hydrostatic pressure, the intradiscal pressure (IDP). Before fracture, stress is distributed evenly across the disc except for a small stress peak in the anterior annulus (SP$_A$). After fracture, IDP falls markedly and a large stress peak appears in the posterior annulus. Vertebroplasty restores IDP towards pre-fracture levels and also reduces the height of the stress peak in the posterior annulus. (Reproduced with permission from Luo et al. [29]).

3.3. Load-Sharing and Adjacent-Level Fracture. By augmenting the fractured vertebra, vertebroplasty can help restore normal mechanics to surrounding structures. Endplate deformation of fractured vertebrae under compressive load is reduced after vertebroplasty [61], restoring nucleus pressure in adjacent intervertebral discs, and reducing stress concentrations in the posterior annulus [29, 38]. Compressive load bearing by the anterior half of augmented and adjacent vertebral bodies is also increased, and neural arch load bearing correspondingly decreased [29, 38]. Fracture-induced changes are largely but not entirely reversed (Figure 2). By restoring normal load sharing, vertebroplasty has the potential to decrease the risk of recurrent and adjacent level fractures to an osteoporotic spine.

Despite these findings, there is persisting concern that vertebroplasty can increase the risk of fracture to adjacent vertebrae [62–64] by increasing the compressive stress acting on them [65–68]. Finite element studies suggest that vertebroplasty can increase endplate deformation in adjacent vertebrae by decreasing endplate bulging of the augmented vertebra and thereby increasing intradiscal pressure [65]. However, this is not supported by experimental studies which found that endplate deformation [69] and load transfer [70] do not increase following vertebroplasty.

4. Factors Influencing the Mechanical Efficacy of Vertebroplasty

The mechanical effects of vertebroplasty depend on the characteristics of the procedure (such as cement type,

volume, and distribution) and also on the characteristics of the augmented spine (including BMD, disc degeneration, and damage severity).

4.1. Properties of Bone Cement. Polymethylmethacrylate (PMMA) is currently the most widely used bone cement for vertebroplasty. However, it has several disadvantages, such as temperature rises during polymerization that can cause tissue damage [71], and lack of bioactivity [72]. Accordingly, new types of cement such as bioactive composite materials like Cortoss and calcium phosphate cement (CPC) have been developed. Although different cements have varying elastic modulus and compressive strength [47, 73], they are all able to increase stiffness and strength of fractured vertebrae. However, this ability depends on the volume injected [47].

A finite element study has suggested that stiffer cement can increase stress on the endplates immediately above and below it, leading to increased pressure in adjacent discs, and consequently greater stress on the endplate of adjacent vertebrae [74]. However, this was not confirmed in an experimental study on cadaver motion segments that compared Cortoss and PMMA [29]. Although Cortoss has an elastic modulus twice as high as PMMA [73], no differences were found between the two cements regarding the restoration of intradiscal pressure, spinal load sharing, and compressive and bending stiffness. This could be due to the fact that smaller volumes of Cortoss were used, and it suggests that the mechanical effects of vertebroplasty depend as much on cement volume and distribution as on cement modulus [74].

Less stiff bone cements, such as CPC, appear to have inferior fatigue properties as indicated by the appearance of small cracks after cyclic loading [56]. This could explain why clinical studies report that CPC-injected vertebral bodies are vulnerable to progressive collapse for 2 or more years after vertebroplasty [75]. Recently, efforts have been made to reduce the stiffness of PMMA cement for vertebral augmentation [76, 77] but the ability of softer cements to reduce the risk of adjacent-level fracture has yet to be demonstrated [78].

4.2. Volume and Distribution of Cement. Experiments on isolated cadaver vertebral bodies show that different volumes of cement are required to restore vertebral strength and stiffness. Strength can be restored to prefracture levels by using as little as 2 ml of PMMA cement [48], but full restoration of vertebral body stiffness requires injection volumes of approximately 4 ml in thoracic vertebrae and 6 to 8 ml in thoracolumbar vertebrae [48, 79, 80]. Restoration of strength and stiffness depends also on percentage volumetric fill [46, 81–83]: 16% [82] to 24% [83] percentage cement fill can fully restore vertebral strength to pre-fracture levels, but 24% [83] to 30% [82] fill is required to restore vertebral stiffness.

The restoration of mechanics to adjacent structures is also influenced by cement volume. One experiment on cadaver motion segments found that only a small amount

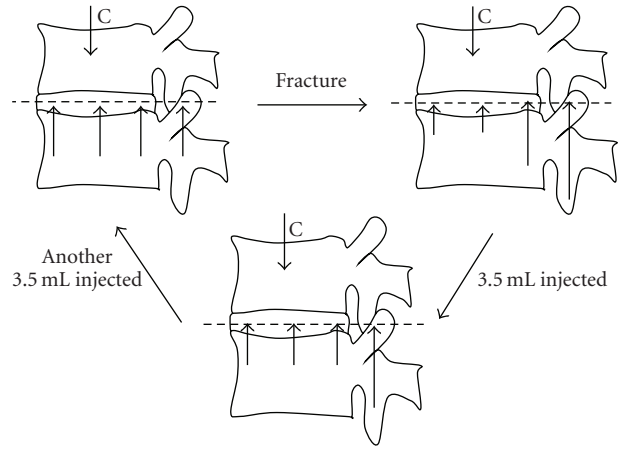

Figure 3: Diagram summarising the changes in load bearing by vertebrae following fracture and vertebroplasty. The length of the upward pointing arrows represents load bearing by different regions of the vertebra. Before fracture (A), the compressive load is borne mostly by the anterior column (disc and vertebral bodies) and stress is distributed evenly across the disc and adjacent vertebral bodies. After fracture (B), stress falls in central and anterior regions of the disc and increases in the posterior annulus and neural arch. Injecting 3.5 ml of cement into the fractured vertebral body (C) causes stress to be distributed more evenly across the disc, but loading on the neural arch remains elevated. Injecting a further 3.5 ml of cement restores neural arch load bearing towards pre-fracture levels (A). Based on data from Luo et al. [84].

(3.5 ml) of PMMA is needed to restore normal stress distribution to the fractured and adjacent vertebral bodies, but more cement (7 ml) is required to restore motion segment stiffness and load sharing between the vertebral bodies and neural arches [84], as shown in Figure 3. Restoration of vertebral body shape and kyphosis angle also increases with the cement volume injected [51]. These experimental findings appear to suggest that more cement is beneficial in restoring the spine's mechanical properties. However, a large cement volume may not be advisable clinically, because it increases the risk of cement leakage [85]. Overfilling of the fractured vertebra may also increase the risk of adjacent vertebral fracture [62, 65, 66].

This dilemma may be overcome by using larger volumes of more compliant cement, as discussed above. However, there is still a higher risk of cement leakage associated with greater cement volumes. Another solution is to place the bone cement in a more efficient way so that less cement is needed to achieve a better mechanical outcome. For example, placing the cement adjacent to the endplates so that a complete endplate-to-endplate fill pattern is achieved could maximise the increase in compressive stiffness and strength to a fractured vertebral body [74, 86]. Unfortunately, it may also induce excessive endplate deformation in the *adjacent* vertebra and cause adjacent-level fracture [65, 74]. It is therefore reasonable to suggest that a moderate amount of cement placed adjacent to the endplates could restore spine mechanics and minimise the risk of adjacent-level fracture. This suggestion is supported by two recent experimental

studies: one showed that the increase in nucleus pressure and the decrease in neural arch load bearing were correlated with cement fill in the region adjacent to the endplate [87]; the other showed that if cement is not in close contact with the endplates then it does not increase endplate deformation in adjacent vertebrae [69].

The efficiency of cement placement within the vertebral body can be controlled by cement viscosity during injection. Optimal cement viscosity can result in more evenly distributed cement and can significantly decrease the risk of cement leakage [88, 89]. A more evenly distributed cement pattern results in greater increases in vertebral body stiffness and induces smaller stress concentrations around the cement, which may decrease the risk of adjacent-level fracture [90].

4.3. Kyphoplasty versus Vertebroplasty. Kyphoplasty is a modification of the basic vertebroplasty technique. It involves forcibly inflating a balloon inside the fractured vertebral body in order to reduce the fracture and create a cavity for the subsequent injection of cement [91]. This modification is thought to have several benefits: it allows cement to be injected at lower pressure so that leakage is reduced [8, 9, 92], and it leads to compaction of bone around the balloon, elevating the fractured endplate and restoring vertebral body height [10–13, 15, 91, 93–95], which may be beneficial for restoring spine mechanics to patients with osteoporotic fracture [42].

In vitro biomechanical studies have shown that kyphoplasty can achieve a better restoration of vertebral height [57, 96, 97] and wedge angle [50] in fractured vertebrae. However, the short-term mechanical effects of kyphoplasty are similar to those of vertebroplasty, with both procedures restoring motion segment stiffness [50, 98], intradiscal pressure [50, 99], and spinal load sharing [50] by a similar amount. A recent randomized clinical trial comparing kyphoplasty and vertebroplasty found that the two procedures produced similar pain-relieving effects, although kyphoplasty was superior in restoring vertebral height and shape [100]. Nevertheless, an in vitro study found that, whilst kyphoplasty achieved a better initial vertebral height restoration than vertebroplasty, the restored height was lost during subsequent cyclic loading [57]. This highlights the importance of following-up changes over time, both *in vivo* and *in vitro*.

4.4. Characteristics of the Treated Spine. Cadaver experiments have shown that vertebrae with lower BMD tend to sustain more severe fractures and lose more height [29]. These same specimens show greater changes in mechanical function following fracture [29] and, encouragingly, benefit most from vertebral augmentation [29, 101, 102]. Evidently, vertebroplasty is particularly effective for restoring spine mechanics in patients with osteoporosis.

5. Summary and Future Directions

Osteoporotic vertebral fracture can induce profound disruption to normal spine mechanics which can have both short-term and long-term consequences. By augmenting the fractured vertebra, vertebroplasty largely restores normal mechanics to a fractured osteoporotic spine.

Further research is required to optimise vertebral augmentation procedures. Cadaveric experiments have been successful in identifying the mechanical consequences of fracture, for the affected and adjacent vertebrae, and demonstrating how they can be reversed. However, many variable and interacting factors can influence mechanical outcome, and clinical outcome will depend on even more variables, including the tissue origins of pain. It is becoming evident that mathematical modelling based on patient-specific anatomy and BMD will be required to provide optimal solutions for individual patients.

In addition, a wider view of vertebral deformity needs to be adopted. Approximately half of patients with osteoporotic vertebral fractures recall no traumatic onset [103], and many deformed vertebrae do not appear to be obviously fractured on radiographs. This suggests that vertebral deformity in many patients involves gradual processes such as "creep", which is continuing deformation under constant load [30, 104]. Cadaveric studies have recently demonstrated that creep can cause anterior wedge deformities in old human vertebrae bones [104], and that creep is accelerated greatly following vertebral microdamage [30]. Vertebroplasty may prove as successful in modifying these time-dependent processes as in reversing the effects of fracture.

Finally, more research is required to explain why vertebral deformity is so variably associated with pain, and why pain relief following vertebroplasty is so unpredictable.

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Review Article

Revisiting Estrogen: Efficacy and Safety for Postmenopausal Bone Health

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The rapid decline in endogenous estrogen production that occurs during menopause is associated with significant bone loss and increased risk for fragility fracture. While hormone therapy (HT) is an effective means to re-establish endogenous estrogen levels and reduce the risk of future fracture, its use can be accompanied by undesirable side effects such as stroke and breast cancer. In this paper, we revisit the issue of whether HT can be both safe and effective for the prevention of postmenopausal bone loss by examining standard and alternative doses and formulations of HT. The aim of this paper is to continue the dialogue regarding the benefits and controversies of HT with the goal of encouraging the dissemination of-up-to date evidence that may influence how HT is viewed and prescribed.

1. Introduction

Osteoporosis is a skeletal disorder characterized by low bone mass and low bone quality which leads to compromised bone strength and an increased risk of fracture [1]. The prevalence of osteoporosis in the United States and Canada is high, with 10 million Americans and 2 million Canadians affected with this disease. Several more millions, although not osteoporotic, suffer from low bone mass and will experience a fragility fracture in their lifetime [1, 2]. Along with its significant consequences to morbidity and mortality, osteoporosis has an annual cost of 20 billion dollars to the American and Canadian health care systems combined and, it is expected that this economic burden will rise due to rapidly aging populations [2, 3].

The decline in endogenous estrogen production that occurs during menopause has important implications for skeletal health because estrogen plays a major role in the development and maintenance of the human skeleton throughout the lifespan. In postmenopausal women, reduced levels of endogenous bioavailable estrogen are associated with lower bone mineral density (BMD) and higher risk for fragility fractures [4–7]. The repletion of endogenous estrogen through the use of hormone therapy (HT) effectively prevents postmenopausal bone loss and reduces the risk of fragility fractures [8–12].

While the benefit of HT, particularly conjugated equine estrogens (CEEs), on the prevention and treatment of postmenopausal osteoporosis and related fracture has been demonstrated, its effects on the health of other estrogen-sensitive tissues such as the uterus and breast must be carefully considered. In many circumstances, estrogen induces proliferation of the uterine and breast tissues [13–15]. In addition, postmenopausal women are at a higher risk for coronary heart disease (CHD) and breast cancer than their premenopausal counterparts, and the effects of HT may modify these risks. Moreover, estrogen has been shown to modulate vascular function; however, its effect on cardiovascular health is rather controversial. Evidence from observational trials [16, 17] suggests that HT is associated with a lower risk of CHD; however, subsequent data from two large randomized clinical trials (RCTs), the Heart and Estrogen/Progestin Replacement Study (HERS), and the Women's Health Initiative (WHI) trial did not confirm these benefits [9, 10, 18]. The HERS reported no benefit [18] from the use of HT, and the WHI study reported a significantly greater number of cardiovascular events in

women taking HT compared to women taking placebo [9, 10]. Despite important limitations including high drop-out rates in the WHI, major consequences of the findings from WHI were a significant drop in HT prescription and use worldwide, with a concomitant increase in the prescription and use of alternative therapies (e.g., bisphosphonates) for the treatment of postmenopausal bone loss, for which their long-term consequences remain unknown [19, 20]. Many women were also left with little option for the relief of symptoms associated with the onset of menopause.

Study findings including secondary analysis of the WHI have shown that HT is safe in younger postmenopausal women for the treatment of vasomotor symptoms and for the prevention of osteoporosis [21]. In fact, consensus statements from the North American and International Menopause Societies also support the notion that HT use may be safer in younger postmenopausal women (aged 50-59 years, or fewer than 10 years after the onset of menopause) [22–24]. In addition, RCTs are underway to confirm the safety of HT in younger postmenopausal women [25]. Nevertheless, long-term studies are needed to determine whether lower doses of HT with alternative modes of delivery are associated with fewer side effects compared to the standard HT formulations.

In this paper, we revisit the usefulness of estrogen for the prevention and treatment of postmenopausal bone loss by reviewing the literature on its effectiveness at attenuating bone loss and risk of fracture and its side effects, in the context of alternative formulations and lower doses than traditionally prescribed. Through continuous dialogue and emerging evidence, the question of whether HT *should* be recommended to postmenopausal women is shifting to the question of *which type* of HT can be prescribed for the treatment of menopausal-related symptoms including bone loss, particularly in younger postmenopausal women.

2. Effects of Standard HT on Skeletal Health during Postmenopause

The most popular HT formulation prescribed to postmenopausal women is the CEE derived from urine of pregnant mares [26–28]. Medroxyprogesterone acetate (MPA) is conventionally added to CEE for women with intact uteri but, for hysterectomized women, CEE alone is used for the treatment of postmenopausal-related conditions including loss of bone mass. CEE, as indicated by its name, is itself made up of several conjugated forms of estrogen, mainly estrone sulfate and equilin sulfate. The most prescribed dose of CEE is 0.625 mg/d because this is the dose that alleviates postmenopausal-related symptoms in the majority of women that endure ovarian failure [29]. Other forms of estrogen in HT include esterified estrogen, ethinyl estradiol, 17β-estradiol, estradiol acetate, and estropipate. Norethindrone, levonorgestrel, and progesterone are the commonly used forms of progestogens in HT.

Unquestionably, HT during postmenopause improves BMD and decreases fracture risk in the vertebrae as well as in nonvertebral sites including the hip (Table 1) [8–10, 12, 20, 31]. Observational studies have reported decreases of up to 71% in fracture risk with HT use [12, 20]. In a subgroup (n = 138,737) of postmenopausal women from the Million Women Study, current use of HT was associated with a 38% decrease in risk for total fractures, regardless of the type, dose, or delivery method of HT [20]. Similar observations were reported in other prospective cohorts including the Study of Osteoporotic Fractures trial, whereby current use of HT was associated with 34% and 38% decreases in risk for all nonspinal and hip fractures, respectively [31]. Moreover, current users that started HT within 5 years of menopause had a 50% decrease in risk for all nonspinal fractures and a 71% decrease in risk of hip fractures [31].

Similar to observational studies, RCTs have confirmed the protection of HT against fracture risk, albeit at more modest levels (~30%) (Table 1) [9, 10, 30]. The largest RCT reporting the effects of HT on fracture risk is the WHI. This trial was designed primarily to determine whether daily use of 0.625 mg CEE plus 2.5 mg MPA (for nonhysterectomized women) or 0.625 mg CEE alone (for hysterectomized women) reduces CHD in healthy postmenopausal women [9, 10]. Both the combination (CEE + MPA) and alone (CEE) arms were stopped early, after a mean of 5.2 and 6.8 years, respectively, because the number of observed side effects was deemed unacceptable. Nevertheless, CEE + MPA use was associated with a 34% decrease in risk for hip fractures and a 26% decrease in risk for total fractures [9]. Use of CEE alone was associated with a 39% decrease in risk for hip fractures and a 30% decrease in risk for total fractures (Table 1) [10].

While the positive effects of HT on bone health in postmenopausal women have been widely shown in both observational and clinical trials, the side effects popularized by its use in the WHI trial have resulted in dramatic decreases in its prescription and use worldwide [19, 26, 32]. Few women have been left with the option for HT use for simultaneously alleviating vasomotor symptoms and preserving bone tissue; however, the decision to discontinue HT may have been somewhat misguided.

3. Side Effects Associated with the Use of Standard HT

The potential for estrogen to modulate the health of many tissues in the body is based largely on the fact that estrogen receptors are located in both reproductive and nonreproductive tissues [33]. Thus, in addition to its ability to reduce vasomotor symptoms, vaginal atrophy and prevent postmenopausal bone loss, HT has the ability to modulate the health of many other tissues including the breast, liver, kidneys and cardiovascular tissues. Since postmenopausal women are at an increased risk for CHD and breast cancer, it is prudent to determine the safety profile of HT in these tissues.

Described as an estrogen-deficiency disease, menopause was previously portrayed as a catalyst for the development of other diseases such as CHD, due to the dramatic decline in endogenous estrogen production [28, 34]. Evidence from

Table 1: Selected studies on the effect of hormone therapy on bone metabolism in postmenopausal women

Study Name	Study Type	Sample Size, Mean Age in Years (Range), and Years since Menopause	Type of Therapy	Dose	Treatment Duration	Results for BMD, BMC, or Fracture Risk
WHI Estrogen plus Progesterone Trial [9]	RCT	16608, 63 (50–79), >6 months (>12 months for 50–54 years old)	CEE + MPA or placebo	CEE: 0.625 mg/d MPA: 2.5 mg/d (continuous)	Stopped after a mean of 5.2 years due to increases in breast cancer, stroke, CHD	HR, 0.66 [0.45–0.98] for incidence of hip fractures. HR, 0.76 [0.69–0.85] for incidence of total fractures.
WHI Estrogen alone Trial [10]	RCT	10739, 64 (50–79), previously hysterectomized	CEE or placebo	CEE: 0.635 mg/d	Stopped after a mean of 6.8 years due to increases in stroke	HR, 0.61 [0.41–0.91] for incidence of hip fractures. HR, 0.70 [0.63–0.79]for incidence of total fractures.
WISDOM Trial [30]	RCT	4385, 63 (50–69), >12 months or previously hysterectomized	CEE, CEE + MPA, or placebo	CEE: 0.635 mg/d MPA: 2.5 mg/d (continuous) or 5.0 mg/d (sequential)	Stopped after a mean of 11.9 months due to adverse findings inWHI [8]	HR, 0.69 [0.46-1.03] for incidence of osteoporotic fractures.
PEPI Trial [8]	RCT	875, 56 (45–64), 1 to <10 years (either naturally or surgically)	CEE, CEE + MPA, CEE + MP, or placebo	CEE: 0.625 mg/d MPA: 2.5 mg/d (continuous) or 10 mg/d (sequential) MP: 200 mg/d (sequential)	3 years	3.5–5.0% increase in LV BMD; 1.7% increase in hip BMD with these doses.
Danish Nurse Cohort Study [12]	Prospective	14015, 60 (≥50), Years since menopause not provided	All types (e.g., CEE, E_2) and delivery methods (ex: oral, transdermal)	All Doses (ex: CEE, >0.625 mg/d or ≤0.625 mg/d; E_2, >1 mg or ≤1 mg oral; E_2, >50 μg or ≤50 μg transdermal); Various doses of MPA and NA as continuous or sequential regimes	Mean years of follow-up: not reported (Range: 0–6 years)	HR, 0.69 [0.50–0.94] for incidence of hip fractures with ever users.
Million Women Study [20]	Prospective	138737, Mean age in years not provided (50–69), Years since menopause not provided	All types (ex: CEE, E_2) and delivery methods (ex: oral, transdermal)	All Doses (ex: CEE, >0.625 mg/d or ≤0.625 mg/d; E_2, >1 mg or ≤1 mg oral; E_2, >50 μg or ≤50 μg transdermal); Various doses of MPA and NA as continuous or sequential regimes	Mean years of follow-up: 2.8 (Range: 1.9–3.9 years)	RR, 0.62 [0.58–0.66] for incidence of fracture with current users. RR, 1.07 [0.99–1.15] for incidence of fracture with past users.
SOF Trial [31]	Prospective	9704, 70–72 (>65), 2 years before onset of menopause to >10 years	All types of oral preparations	All doses of oral preparations	Mean years of follow-up: 2.5 (Range: 0.02–6.5 years)	RR, 0.60 [0.36–1.02] for incidence of hip fractures with current users. RR, 0.66 [0.54–0.80] for incidence of all nonspinal fractures with current users. RR, 0.29 [0.09–0.92] for incidence of hip fractures with current users who started HT within 5 years of menopause. RR, 0.50 [0.36–0.70] for incidence of all nonspinal fractures with current users who started HT within 5 years of menopause.

RCT: Randomized Control Trial, CEE: Conjugated Equine Estrogen, MPA: Medroxyprogesterone Acetate, MP: Micronized Progesterone, NA: Norethisterone Acetate, E_2: 17β-estradiol, BMD: Bone Mineral Density, LV: Lumbar Vertebra, HR: Hazard Ratio, and RR: Relative Risk.

observational trials indicated HT as a postmenopausal therapy with many health benefits, and that it was marketed to prolong youth and libido, resulting in its popularity to quickly expand worldwide [28]. To validate the notion that HT supports health during menopause and prevents the development of side-effects associated with other diseases, two large trials, the HERS and WHI, were carried out on a combined 30110 postmenopausal women in multicentre, randomized, double-blind, placebo-controlled designs [9, 10, 18]. The HERS trial was the first large RCT to evaluate whether daily use of 0.625 mg CEE plus 2.5 mg MPA alters the risk of CHD events in 2763 postmenopausal women with established CHD [18]. After an average follow-up of 4.1 years, HT did not confer protection against cardiovascular events (nonfatal myocardial infarction or CHD death), and its use was associated with higher venous thromboembolic events compared to placebo. Interestingly, HT use was associated with higher risk for CHD events during the first year of treatment, however, in the fourth and fifth years of the trial, its use was associated with lower risk for CHD events compared to placebo. Thus, the HERS trial demonstrated that the use of HT does not provide an overall benefit to cardiovascular health in women that have never used HT but it may provide benefit to cardiovascular health in current users that continue HT [18]. Subsequently, the WHI, which was the largest RCT to examine the effect of HT on the prevention of CHD, demonstrated that HT is associated with increased risk of CHD, stroke, venous thromboembolism, and breast cancer in the combination group and stroke in the estrogen alone group. The WHI findings resulted in early termination of the trial and subsequently widespread discontinuation of HT among postmenopausal women worldwide [9, 10, 26, 35].

The reasons for inconsistent findings among previous observational trials and the HERS and WHI trials may be due to differences in subject characteristics and inherent designs. For example, observational studies that have investigated the safety of HT have included younger postmenopausal subjects; however, the HERS and WHI trials consisted of mainly older postmenopausal women. In addition, observational studies can be subjected to unknown confounders that influence the outcome variable and are susceptible to a number of biases including sampling bias and admission rate bias, whereas RCTs can experience inadequate randomization, unblinding, and loss of sampling units— all of which can influence the outcome variable [36]. Nevertheless, since RCTs by design are superior compared to observational studies, the WHI study would inevitably have the greatest impact on HT recommendations for the postmenopausal population.

4. Limitations of the WHI

Important limitations of the WHI have become the focus of subsequent dialogue and have stimulated experts to question whether the WHI findings can be generalized to all postmenopausal women receiving various forms of HT. These limitations, which will be briefly discussed, include (i) a high loss of subjects, (ii) an older and healthy postmenopausal population included as subjects, and (iii) the investigation of one formulation, dose, and delivery method of HT.

(i) The WHI suffered from a large loss of subjects. 42% of the subjects in the combination arm and 53.8% in the estrogen alone arm dropped out of the WHI resulting in reduced power of its design. The implication of a high drop-out rate may be an underestimation of observed effects, whether negative (ex: CHD and breast cancer) or positive (ex: fracture) [9]. However, being that losses in subjects are rarely a random occurrence [36], it is too simplistic to assume that the observed effects are merely an underestimation of what would have occurred if an appropriate number of subjects remained in the study throughout its total duration.

(ii) The WHI enrolled into its trial mostly older and healthy postmenopausal women. While the WHI reported an unfavorable risk-to-benefit ratio, this study was conducted in a predominantly older and asymptomatic menopausal population, many of which who were 13–15 years past the onset of menopause. However, HT is targeted primarily to younger and more recently postmenopausal women for the treatment of menopausal symptoms including hot flushes, vaginal dryness, and decreased libido. Thus, there is a clear disconnect between the conventional target group of HT and the subjects from the WHI in terms of age and health status, and the question of whether similar findings would be observed in a younger postmenopausal population still remains.

Observational studies point toward the notion that time since menopause may be an important factor on risk of CHD and other side effects of HT [24, 37]. Posthoc subgroup analysis by Rossouw et al. [21] of younger postmenopausal women (aged 50–59) in the WHI trials detected a tendency for HT to reduce the risk of CHD and total mortality. Further analysis of the WHI study by Manson et al. [38] determined that CEE alone resulted in lower coronary-artery calcification compared to placebo in the younger postmenopausal women aged 50–59 years. Nonetheless, these subsequent analyses were not adequately powered, especially in women aged 50–54 or closer to the onset of menopause (<5 years) [21]. Thus, difficulties remain in drawing clear conclusions from the secondary analyses of the WHI. While data from observational trials have shown that HT is protective against CHD, mortality, and other diseases in younger postmenopausal women, RCTs are underway to determine whether these previous findings can indeed be confirmed [25]. In the meantime, in March 2008, the International Menopause Society held a global summit to address the actual versus perceived risks of HT use in younger postmenopausal women [24]. Through the scrutiny of vast empirical evidence, this summit resulted in a consensus that there may be a "window of opportunity" whereby HT is safe in women that are closer to the onset of menopause (<10 years). Nevertheless, current opinion regarding the use of HT for the treatment of vasomotor symptoms and for the prevention of osteoporosis during the postmenopause is that the lowest effective dose should be used for the shortest duration possible.

(iii) Only two formulations of HT were investigated in the WHI, CEE + MPA (0.625 mg/d + 2.5 mg/d) for postmenopausal women with an intact uterus or CEE alone (0.625 mg/d) for postmenopausal women who had undergone hysterectomy. Both these formulations were provided as an oral tablet. Whether alternative formulations of HT, such as low-dose transdermal HT, result in fewer side effects than those observed with the HT used in the HERS or WHI trials is yet to be determined. However, compelling evidence points towards an advantage for lower doses of HT through the transdermal delivery method compared to oral standard dose HT.

5. Effects of Lower Doses and Transdermal HT on Skeletal Health during Postmenopause

The most commonly prescribed HT for postmenopausal women is oral CEE (0.625 mg/d) alone or in combination with MPA (2.5 mg/d). In pursuit of the lowest effective dose for the attenuation of menopausal-related conditions including bone loss, several studies chose to examine doses that are three-quarters, half, and a quarter of the standard dose. In addition, alternative modes of delivery, such as the transdermal patch, are being studied because evidence indicates that they are associated with fewer side effects compared to the oral standard dose formulations. For the purpose of this paper, 0.3 mg and 0.45 mg CEE, along with 25 μg transdermal 17β-estradiol are considered low-dose HT while 14 μg transdermal 17β-estradiol is considered ultra-low-dose HT.

Results from a number of trials have shown that lower doses of HT are effective in improving BMD in the hip and lumbar spine (Table 2) [39–47]. In a substudy (n = 822) of the Women's Health, Osteoporosis, Progestin, Estrogen (HOPE) trial, standard and lower doses of orally administered CEE alone or in combination with MPA resulted in significant improvements from baseline (1.33–3.46%) in spine and hip BMD, as well as biochemical markers of bone turnover in healthy postmenopausal women within 4 years since the onset of menopause [40]. These improvements contrasted from the placebo group, which experienced significant losses in spine BMD and total body bone mineral content. While CEE alone at the standard dose resulted in higher gains in spine BMD (2.43%) compared to CEE alone at the 0.3 mg/d dose (1.33%), the observed gains in BMD did not differ from the CEE alone at the 0.45 mg/d dose (2.09%). In addition, no differences in gains in hip BMD were observed between any of the treatment groups. Benefits in spine BMD of up to 3.5% have also been reported with the use of low-dose HT (0.3 mg/d CEE + 2.5 mg/d MPA) in older postmenopausal women with low bone mass [44].

The search for the lowest effective dose of HT has also been investigated using the transdermal delivery method [42, 47]. Using healthy postmenopausal women, both standard- and low-dose transdermal 17β-estradiol with 100 mg/d micronized progesterone resulted in similar increases in femur neck BMD (0.73% and 0.92%, respectively) after 18 months of treatment while the control group experienced a significant decrease in BMD (−2.23%) [42]. Another study observed improvements of 1.65% and 4.08% in spine BMD in healthy hysterectomized postmenopausal women that were treated for 2 years with unopposed standard- and low-dose transdermal 17β-estradiol, respectively [47]. Testing the effectiveness of unopposed ultra-low-dose transdermal 17β-estradiol, the Ultra-Low-dose Transdermal estRogen Assessment (ULTRA) observed a 2.6% increase in BMD at the lumbar spine in nonhysterectomized postmenopausal women after 2 years of treatment [39]. A non-hysterectomized sample was used since endometrial safety was also examined. Improvements in hip BMD and favorable changes in biochemical markers of bone turnover (osteocalcin and bone-specific alkaline phosphatase) were also observed from ultra-low-dose [39].

While no large head-to-head studies have been conducted between lower doses of HT and other pharmacologic agents used for the prevention or treatment of postmenopausal bone loss, ultra-low dose transdermal HT has also been shown to exert similar protection against bone loss in the spine as the selective estrogen receptor modulator, raloxifene [45]. In a 2-year RCT conducted in 500 osteopenic postmenopausal women, 77.3% of those treated with ultra-low-dose HT and 80.5% of those treated with raloxifene did not experience bone loss at the lumbar spine. Slight increases in total hip BMD were observed in both the ultra-low-dose HT and raloxifene groups, and raloxifene resulted in significantly greater improvements in hip BMD compared to ultra-low-dose HT. In addition, 63.8% of the ultra-low-dose HT and 81.3% of the raloxifene groups did not experience a loss of BMD at the hip. No difference in fracture incidence was observed between both groups but trials that are longer in duration are needed to adequately determine whether lower doses and alternative methods of delivery of HT modulate fracture risk [45].

6. Side Effects Associated with the Use of Lower Doses and Transdermal HT

Studies have shown that the dose and formulation of HT can be significant factors that determine the safety profile of HT. In the transdermal delivery system, 17β-estradiol is absorbed subcutaneously and is readily absorbed into tissues as it circulates systemically before reaching the liver. Because transdermal HT avoids first-pass liver metabolism, there is lower production of coagulation factors compared to oral formulations, which may result in a lower risk for cardiovascular events [48]. Thus, low-dose transdermal HT has the potential to provide significant benefit to skeletal health but with fewer side effects than the conventionally prescribed standard dose HT [49].

Evaluation of adverse events was included in the HOPE trial whereby a dose response in endometrial hyperplasia and vaginal bleeding was observed in women taking CEE alone, with the greatest number of reported adverse effects being in the 0.625 mg/d dose. A dose response in breast pain was observed in women taking CEE + MPA, with the greatest number of reported adverse effects being in the 0.625 mg/d

Table 2: Selected studies on the effect of lower doses of hormone therapy on bone metabolism in postmenopausal women.

Study Name	Study Type	Sample Size, Mean Age in Years (Range), and Years since Menopause	Type of Therapy	Dose	Treatment Duration	Results for BMD, BMC, or Fracture Risk
ULTRA Trial [39]	RCT	417, 67 (60–80), ≥5 years	E_2 patch or placebo patch*	E_2: 0.014 mg/day	2 years	2.1% greater LV BMD versus placebo.
HOPE Trial [40]	RCT	822, 51.6 (40–65), 1 to ≤4 years	CEE, CEE + MPA, or placebo*	CEE: 0.625, 0.45, or 0.3 mg/d MPA: 2.5 or 1.5 mg/d	2 years	1.33–3.46% increase in LV BMD; Approximately 1.5–3% increase in hip BMD; 1.03–1.74% increase in total BMC, with these doses.
Gambacciani et al, 2008 [41]	Open Trial	Sample size not provided, 57 (range not provided), ≥1 year	E_2 + NA (oral) or no treatment*	1 mg E_2 + 0.5 mg NA for 28 d or 0.5 mg E_2 + 0.25 mg NA per day	2 years	2–5% increase in LV BMD; 1.8–2.8% increase in femur neck BMD, with these doses.
Garcia-Pérez et al, 2006 [42]	Transversal Study	136, 53 for 0.05 mg/d and placebo groups to 56 for 0.025 mg/d group (range not provided), ≥1 year	E_2 patch + micronized progesterone, or placebo*	E_2: 0.05 or 0.025 mg/d progesterone: 100 mg/d	18 months	0.73–0.92% increase in femur neck BMD; −0.35–0.87% change in LV BMD, with these doses.
Gambacciani et al, 2001 [43]	Open Trial	38,54 (45–56), ≥1 year	CEE + MPA*	CEE: 0.3 mg/d MPA: 2.5 mg/d	2 years	2.7% increase in LV BMD.

CEE: Conjugated Equine Estrogen, MPA: Medroxyprogesterone Acetate, NA: Norethisterone Acetate, E_2: 17β-estradiol, BMD: Bone Mineral Density, BMC: Bone Mineral Content, and LV: Lumbar Vertebrae. *All subjects received additional supplementation with calcium alone or with vitamin D.

dose [40]. The ULTRA trial, which examined the effects of unopposed ultra-low-dose transdermal 17β-estradiol on BMD and biochemical markers of bone turnover in non-hysterectomized postmenopausal women, did not observe higher incidences of endometrial hyperplasia compared to the placebo group [39]. In addition, further examination of the ULTRA trial by Grady et al. [50] demonstrated that nonopposed ultra-low-dose transdermal 17β-estradiol does not significantly change breast density in postmenopausal women. Nielsen et al. [51] observed that similar to raloxifene, no significant change in breast density in osteopenic postmenopausal women with 2-year treatment with ultra-low-dose transdermal 17β-estradiol. Whether no significant change in breast density from lower doses of ET ultimately results in reduced incidence of breast cancer compared to standard ET therapies is not yet known. Indeed, there is a necessity for long-term trials to assess whether lower doses of HT using alternative modes of delivery affect disease risk profiles.

7. Conclusion

The benefit of HT for the prevention and treatment of postmenopausal bone loss and for relief of vasomotor symptoms is well acknowledged. Thus, HT is an attractive option for the treatment of postmenopausal-related symptoms. However, due to its well-known ability to modulate physiology and subsequent disease risk in other tissues, the safety of HT must always be considered before it is prescribed. While standard-dose HT through the oral delivery method is effective against both postmenopausal bone loss and vasomotor symptoms, discrepancies in its safety have been noted between observational trials and RCTs. While review of the studies to date reveals discrepancies among studies and limitations of trials using standard doses of HT, emerging data suggests that HT can be safe for younger postmenopausal women. Moreover, lower doses of HT and alternative modes of delivery such as the transdermal patch may be a safe alternative to standard HT in protecting against postmenopausal bone loss while relieving vasomotor symptoms. The dialogue regarding HT should continue as findings from new studies using lower doses of HT and alternative models of delivery are published.

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Review Article

Progesterone and Bone: Actions Promoting Bone Health in Women

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Estradiol (E_2) and progesterone (P_4) collaborate within bone remodelling on resorption (E_2) and formation (P_4). We integrate evidence that P_4 may prevent and, with antiresorptives, treat women's osteoporosis. P_4 stimulates osteoblast differentiation *in vitro*. Menarche (E_2) and onset of ovulation (P_4) both contribute to peak BMD. Meta-analysis of 5 studies confirms that regularly cycling premenopausal women lose bone mineral density (BMD) related to subclinical ovulatory disturbances (SODs). Cyclic progestin prevents bone loss in healthy premenopausal women with amenorrhea or SOD. BMD loss is more rapid in perimenopause than postmenopause—decreased bone formation due to P_4 deficiency contributes. In 4 placebo-controlled RCTs, BMD loss is not prevented by P_4 in postmenopausal women with increased bone turnover. However, 5 studies of E_2-MPA co-therapy show greater BMD increases versus E_2 alone. P_4 fracture data are lacking. P_4 prevents bone loss in pre- and possibly perimenopausal women; progesterone co-therapy with antiresorptives may increase bone formation and BMD.

1. Introduction

Osteoporosis has been considered primarily because of estrogen deficiency at menopause since Fuller Albright [1]. Most scientists view estradiol as women's sole bone-active gonadal steroid. In reality, estradiol and progesterone work together in every tissue in women's normal physiology [2]. Estrogen plays positive roles in bone biology and osteoporosis prevention and treatment primarily through decreasing bone resorption [3–5]. There is also compelling evidence that powerful bone-destructive cytokines such as IL-1, IL-6, and TNFα are released and increase rapidly with dropping estradiol levels, as occurs with surgical menopause [6]. Estradiol achieves its positive bone effects largely through two key actions: facilitation of vitamin D-related intestinal calcium absorption [4, 7] and suppression of bone resorption through the osteoprotegerin/RANK/RANKL system [7]. It is also clinically obvious that premenopausal women with amenorrhea have lower estradiol levels and lower bone mineral density (BMD) and/or lose bone rapidly [8].

Not until recently did randomized, placebo-controlled trial data from the WHI studies show that treatment with conjugated equine estrogen (CEE) plus medroxyprogesterone (MPA) or with CEE alone (in women with hysterectomy) prevented osteoporotic fractures in asymptomatic postmenopausal women ages 50–79 [5, 9]. Estradiol's role in human bone health is unmistakable. However, progesterone is usually a present, but an unrecognized partner in bone. With amenorrhea and surgical or natural menopause, not only are estradiol levels low or dropping, progesterone levels are also low. While, in these conditions, estrogen and progesterone deficiency are nearly indistinguishable, progesterone deficiency precedes low estradiol levels in perimenopause [10], for example, and with ovulatory disturbances, occurs silently in regular cycles with normal estrogen levels [11].

The purpose of this paper and meta-analyses is to study recent clinical evidence that endogenous progesterone plays a role in bone health. So far, three *in vitro* publications document progesterone's ability to increase

osteoblast numbers [12–14] as well as its effects to promote osteoblast maturation and differentiation [13]. Progesterone appears to play a differing but also physiological role in partnership with estrogen in achieving optimal peak bone mass. Medroxyprogesterone increases premenopausal spine BMD as physiological-dose cyclic therapy in a randomized controlled trial (RCT) for healthy women experiencing hypothalamic amenorrhea, oligomenorrhea, anovulation, or short luteal phase cycles [15].

Progesterone may also have a *therapeutic* role in postmenopausal osteoporosis if paired with an antiresorptive therapy. Thus this paper highlights the accumulating human evidence for a role of progesterone for increasing bone formation in estrogen-replete women with regular menstrual cycles.

From a teleological point of view, a higher trabecular bone mass in women is needed in preparation for building of the fetal skeleton during pregnancy. Interestingly, the third trimester of pregnancy, during which 80% of the fetal skeleton is mineralized, coincides with the maximum rate of progesterone production in human physiology. Under normal circumstances, enough trabecular bone has been accumulated and maintained in women's skeletons to serve as a reservoir for the calcium needs of both mother and fetus during the months of pregnancy, and for the infant during potential months of breast-feeding. The fact that bone morphogenic proteins play a crucial role in both ovulation and bone metabolism points towards a functional link between bone and reproductive systems aimed at preparing for the increased demands of pregnancy.

Knowledge of progesterone's actions in the context of the latest genetic, receptor, and bone ligand systems is in its infancy—relationships may well exist between progesterone and the immune system through osteoblast and hematopoietic stem cell interactions in bone marrow [16], through progesterone's known brain anti-inflammatory and antiapoptotic actions [17], and through potential relationships with emerging bone-related molecules such as sclerostin, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF), to name a few. These molecular biology issues, however, are beyond the scope of this primarily clinical and therapeutic review.

Over the last 20 years [14], a number of controlled trials and prospective studies suggest that progesterone may have a role in treatment of pre- or perimenopausal women with regular, estrogen-sufficient menstrual cycles who, however, are also experiencing ovulatory disturbances (anovulation, or short luteal phase length cycles). The most prevalent of abnormal cycles are *subclinical* ovulatory disturbances (SOD) that are unremarkable because they occur within regular, asymptomatic menstrual cycles [11, 18]. They have an increased incidence in normal weight women with subclinical cognitive dietary restraint [19], women working shifts and in stressful environments. However, currently there are no published data about effects of progesterone on human bone architecture and bone quantitative histomorphometry in either the cortical or cancellous bone compartments, or about the potential of molecularly identical progesterone to decrease fracture risk.

2. Materials and Methods

Studies on endogenous and/or physiologic progesterone concentrations and bone are very scarce: a PubMed search carried out in January, 2010 using the MeSH terms "endogenous progesterone and bone" yielded 51 results since 1975. Similarly, 83 papers since 1968 were found using the terms "physiological progesterone and bone." We excluded all citations concerning animals and/or nonhuman cell lines (most of which have been previously reviewed [14]), those relating to preterm infants, and publications on synthetic androgenic or estrogenic progestins, depot-MPA or other injectable progestins, or those in supraphysiological doses. This paper will focus on the physiological and pharmaceutical actions of progesterone and/or "physiological dose" medroxyprogesterone acetate (MPA) as the progesterone-derived therapy most commonly prescribed in the USA and Canada. It is important to note that, when using the word, "progesterone," we are always discussing the native human steroid.

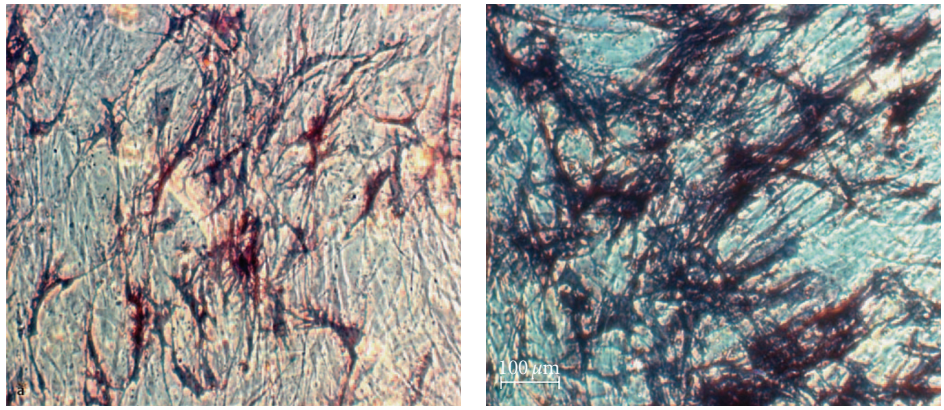
If reference to the actions of a progestin or progestogen is required, this paper will specifically state the compound involved. NETA—and other progestins that primarily are metabolized to estrogen or that act through androgen osteoblast receptors—is not covered because our focus is on physiological bone actions of the human steroid, progesterone.

Given our broad purpose, we are evaluating data from diverse sources; we are also, of necessity, comparing studies with differing methodologies and designs. Therefore, although numerical summaries are created where possible, we have not subjected these combined data to statistical analysis.

3. Results and Discussion

3.1. Progesterone and Bone Formation in Osteoblasts. Most studies of the action of progesterone on human osteoblasts *in vitro* have assessed effects over a maximum of only 72 hours' duration [12–14]. One recent study from Munich, however, used long-term cultures of human osteoblasts (HOBs) to characterize the influence of progesterone and estradiol on bone proliferation (using a hexosaminidase assay) and differentiation (using alkaline phosphatase [ALP] staining). This study quantified ALP production photometrically with extinction at 405 nm following incubation with p-Nitrophenyl-Phosphate (pNPP) and buffer [20] (Figure 1). These primary osteoblast cultures, derived from nonosteoporotic perimenopausal women undergoing hip replacement surgery, were exposed to 7 or 21 days of progesterone with and without estradiol pre- and cotreatment [20].

The effect of progesterone *in vitro o*n differentiation of osteoblasts was dose-dependent for progesterone [20], independent of estradiol, and reached its maximal stimulation at concentrations of 10^{-9} M progesterone (Figure 1). This progesterone level corresponds with luteal phase serum progesterone levels in ovulatory cycles. Seven days of exposure to physiologic levels of progesterone (6.4×10^{-7}–10^{-9} M) led to increased ALP concentrations of 70% ($P = .004$–.019),



(a) (b)

Figure 1: This photomicrograph (at 400 power magnification) shows human osteoblasts in culture after 28 days stained to show Alkaline Phosphatase production as dark blue. (a) Estradiol at a physiological concentration. (b) Estradiol alone for 7 days combined with Progesterone for 21 days. Note the lack of alkaline phosphatase staining in (a) exposed to estrogen alone, and the marked ALP staining indicating osteoblast differentiation/maturation induced by the addition of progesterone, (b) This figure is reprinted from [20] with permission from authors (Schmidmayr M and Seifert-Klauss V). Publisher permission provided.

while a *supraphysiological* progesterone concentration (6.4×10^{-6} M) caused a significant 50% reduction in ALP ($P = .028$). After 21 days of physiological progesterone exposure, the ALP production increased 2.7-fold ($P = .000$ to .004). At supraphysiological progesterone concentrations ALP staining decreased by 80% ($P = .03$). Thus there was a physiological osteoblast differentiation dose-response curve optimal at luteal phase levels with suppression at high progesterone doses. In contrast to expectation and the observations of others [13], this effect was independent of pre- or cotreatment with estradiol. Proliferation, however, was not significantly affected by progesterone in the absence of estradiol [20]. These results suggest an osteoanabolic function of progesterone, while showing for the first time that supraphysiological progesterone concentrations suppressed osteoblast differentiation. These data are the longest of any *in vitro* data on human osteoblasts and progesterone. They clearly show a progesterone osteoblast differentiation dose response and independence from estradiol.

3.2. Progesterone and Bone within Menstrual Cycles, Relation to Peak Bone Mass, and Premenopausal Bone Loss Prevention

3.2.1. Progesterone and Bone Remodeling in the Menstrual Cycle. During the bone remodeling cycle within a single bone multicellular unit (BMU), activation is followed by increased resorption, which in turn is followed by osteoid formation and osteoid mineralization [21]. Within a single BMU, formation takes approximately two to three weeks while formation and initial (incomplete) mineralization requires at least three months [21]. Perhaps in compensation, osteoblasts appear to be more abundant and also more plastic than osteoclasts and evolve to become both lining cells and osteocytes [22].

Although a number of studies have been performed of bone turnover markers across the menstrual cycle [14], most of them used less precise or specific markers and methods, inadequately differentiated ovulatory from anovulatory cycles (by hormonal measures), or recorded too few cycles to be helpful. Some studies with careful cycle bone marker documentation are now available [23, 24] and tend to show increased follicular phase urinary markers of bone resorption in addition to increased luteal phase markers of bone formation. Chiu et al. found the bone resorption marker deoxypyridinoline (D-Pyr) to be higher during the follicular phase than in the luteal phase and to correlate negatively both with E_2 values measured 6 and 8 days earlier and with progesterone levels measured 2–6 days earlier [23]. Unfortunately, the authors did not differentiate between ovulatory and anovulatory cycles, which judging from the wide span of progesterone values must have been mixed in their study. They concluded that "normal women experience monthly episodes of increased bone resorption from menarche to menopause" [23]. In another study, 10 Japanese women with normal ovulatory function showed significant decreases in CTX, free D-Pyr, and serum intact carboxyterminal telopeptide (ICTP) during the luteal phase and significantly higher serum PTH levels during the follicular than the luteal phase [25], perhaps because of coupling of resorption and formation. Caufriez et al. very recently proposed a potential role of endogenous progesterone in modulation of GH-secretion (along with prolactin and TRH) during the normal menstrual cycle [26]. In their study of 10 young Belgian women, 24-hour growth hormone secretion was associated with higher progesterone levels, and daytime GH secretion was increased in the luteal phase compared with the follicular phase [26]. Another European group had already found that PTH concentrations were highest on day three of the menstrual cycle, but had not monitored ovulation and found no relation to progesterone levels [27].

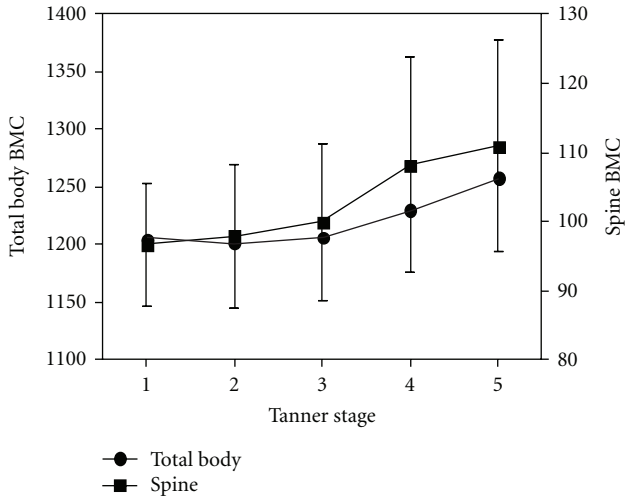


FIGURE 2: This diagram illustrates changes in Total Body (black circle) and Spine (black square) Bone Mineral Content (BMC) adjusted for body size in a population-based cohort of adolescents (mean 11.8 years old) by Tanner Stages on the *X*-axis. It is drawn from data in Table 3 [28]. Endocrine Society permission provided.

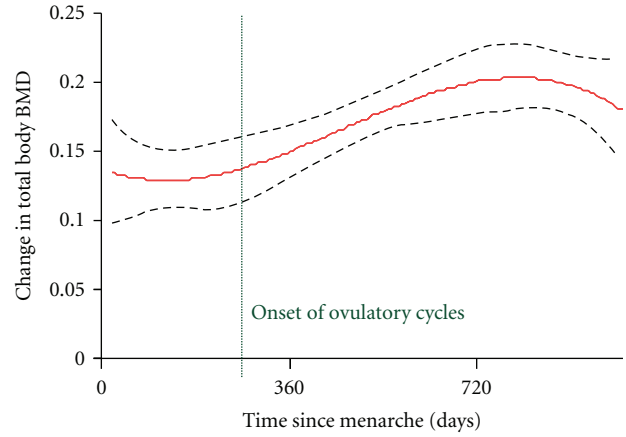


FIGURE 3: This graph shows the multivariable regression for the mean and the 95% confidence interval of the change in total body bone mineral density (BMD) over 3 years in relationship to time since menarche in 38 peripubertal girls studied prospectively [30]. The vertical line shows the earliest, in a subset of 13 girls who provided menstrual calendar data and salivary progesterone levels, that ovulation could be diagnosed [34]. Reprinted with permission of the authors. Society for Bone and Mineral Research permission provided.

Earlier, a Danish serial serum hormone study in eight healthy women aged 20–47 found osteoblastic activity to be higher during the well-documented luteal phase by measurements of osteocalcin (OC) and bone-specific alkaline phosphatase (BAP) [24]. In addition, this study also observed the highest level of IGF-1 (then called somatomedin C) during the luteal phase of the menstrual cycle [24], with which the recent growth hormone data agree [26]. Thus several studies confirm higher follicular phase bone resorption rates and higher luteal phase rates of bone formation.

3.2.2. Progesterone, Ovulation, and Peak Bone Mass. Young women gain body size (BMI), bone size, and BMD rapidly around the time of peak height velocity and menarche [29, 30]. Although not well characterized, it is known that levels of estradiol, testosterone, and growth hormone are increased during this period of bone growth and reproductive maturation. However, ovulatory cycles are rare at menarche and become more prevalent only with increasing time since the first period [31, 32]. There are sparse data about relationships between bone change and ovulation, documented either by Tanner breast stages (in which Tanner stages 4 and 5 indicate the presence of progesterone [33]) and/or by hormonal measures.

A recent population-based study of estradiol receptor polymorphisms and BMC data (corrected for bone area but not BMI or body size) compared BMC with breast Tanner Stage in a cross-sectional study of girls who averaged aged 11.8. These data showed that bone size-adjusted BMC is greater in Tanner Stage 5 than in Stage 1 (Figure 2) [28].

Such cross-sectional population-based data fit with prospective observations in the Teen Bone Study [30], a prospective, observational study in a convenience cohort of 38 girls aged 9–11 (mean initial age 10.6 ± [SD] 0.6 years) that documented total body and spine BMD and BMC at baseline and yearly. In addition six monthly measurements were made of weight, height, seated height, wrist width, BMI, and questionnaires about calcium intakes and exercise [30]. These young women were also examined every six months for pubertal maturation. The onset of menstruation, menstrual cycle calendar data, and weekly salivary progesterone levels were used to assess the prevalence of ovulation based on a threshold value of higher than 40 nmol/L [34, 35].

Menarche occurred for 33 young women during the course of the 3-year study [34]. Based on 93 menstrual cycles from 13 young women that averaged 36 days long (range 20–119) and their weekly salivary progesterone data (n = 163 samples), 27 (29%) cycles were ovulatory while 66 (71%) were anovulatory. Ovulation was documented no earlier than 10 ± 5 months postmenarche [34]. Figure 3 shows that total body BMD increased significantly by the number of days since menarche (day 0 in Figure 3); in particular BMD increased more following the onset of ovulation [34]. Gains in bone density were greater 10 ± 5 months after menarche following which time ovulation first developed (shown with the dotted vertical line) than before (r^2 = 0.40, $P < .0001$). It is known that pubertal bone gain strongly relates to body size and weight. Despite the observation that BMI increased with time following menarche, no significant relationship between changes in BMI and changes in spine BMD or BMC was found. However, changes in BMI did significantly relate to changes in total body BMC (r = .421, P = .001). In summary, these prospective teen bone and ovulation data, although limited, suggest that progesterone adds to the bone gains of menarche. Following the onset of ovulation (that is delayed on average for almost a year after menarche) bone gain is greater than early after menarche suggesting that

progesterone contributes to a high ideal peak bone mass in adolescent girls [36].

3.2.3. Menstrual Cycle Disturbances and Bone (Amenorrhea and Oligomenorrhea). Absence of menstruation following menarche is associated with low levels of both estradiol and progesterone, whether related to hypothalamic suppression (usually due to calorie insufficiency for the level of energy expenditure, emotional/social stressors, or physical illness) or to ovarian dysfunction (such as Turner's Syndrome or other causes of premature menopause). In general, longer cycle lengths are associated with lower estradiol levels and BMD values [37, 38]. In some young women, however, oligomenorrhea is related to anovulatory androgen excess (AAE, as in "polycystic ovary syndrome," PCOS) and associated with higher LH, testosterone, DHEAS, androstenedione, and estrogen levels, but absent or rare ovulation and absolute or relative progesterone deficiency [39]. Because these oligomenorrheic, hyperandrogenic young women likely have different changes in bone compared to regularly cyclic or to amenorrheic women, and their prospective bone changes are not well characterized, we will not review AAE/PCOS further here.

Epidemiological data suggest that primary amenorrhea is rare in the population (about 0.1%) [40]; secondary amenorrhea is also uncommon (<1.3%) in the premenopausal population, although it is more prevalent in teen-aged population-based samples [41, 42]. Both hypothalamic amenorrhea and oligomenorrhea are associated with significantly lower BMD values as well as lower FSH levels [43]. Furthermore, there is rapid bone loss after the onset of amenorrhea [44]. However, with longer durations of amenorrhea (more than three years), bone turnover and bone loss both appear to decrease [36] while absolute BMD values remain low. Thus, among premenopausal women, long cycles associated with hypothalamic oligomenorrhea and amenorrhea are both risk factors for bone loss and low BMD.

3.2.4. Ovulatory Disturbances (Anovulation/Short Luteal Phases) and Bone in Regular Cycles. Regular cycles with normal estradiol levels may vary in their progesterone characteristics. Such cycles may be normally ovulatory, anovulatory or have short luteal phase lengths that result in decreased total progesterone production [11]. Subclinical ovulatory disturbances (SODs, meaning regular cycles with either anovulation and short luteal phase lengths) may pose a risk for bone remodelling imbalance and bone loss despite regular, estrogen-sufficient menstrual cycles.

Currently five published observational cohort studies in a total of 458 women have prospectively examined menstrual cycles by ovulatory characteristics and change in BMD [11, 45–48] measured by dual energy X-ray absorptiometry measures (DXA) or spinal quantitative computed tomography (QCT). These studies span one to four years in healthy, largely Caucasian premenopausal women (ages 20–42) not using oral contraceptives (OCs) or other bone-active therapies. Documentation of luteal phase lengths used Quantitative Basal Temperature (QBT) methods (validated against the serum LH peak [49] and daily urinary progesterone excretion by pregnanediol (PdG), resp. [50]).

Ovulatory characteristics are variously described in the five studies. Table 1 shows the similarities and differences among these published studies—all are in primarily well-educated, white women who average >10 years since menarche and are mainly in their 30s (mean age 31.4) except for the younger women (mean age 22.1) in the Bedford study [48].

In these five studies assessing prospective bone change by the incidence of ovulatory disturbances, the total number of cycles with documentation of ovulation (Table 1) varied from a median of 10/year in the Prior 1990 study to 5/year in the Waugh investigation, to 6.8/year in the Bedford study, to 2.7/year in the Waller, and 1.5/year in the Prior 1996 data. In this latter four-year follow-up study, women collected menstrual cycle and QBT data for 3–6 months at the end of the fourth year and before repeat BMD measurement at the five-year anniversary of their initial QCT. Here, the median number of cycles of data collected by the 27 reported women was 6 (range 3–46) with a minimum of 3 cycles [45].

In those three studies that had documentation of ovulation in at least five cycles/year, which reached a total of almost 400 women, those with more prevalent normally ovulatory cycles had a +1.23% gain versus the −1.00% loss/year in those with ovulatory disturbances (Table 1) [11, 46, 48]. By contrast, studies with fewer cycles between bone density measurements [45] or few measurements not within the bone change window [47] were not able to show any such ovulation-bone change relationship [45, 47]. However, the luteal index (mean luteal phase length divided by mean cycle length) from year one of the Prior study [11] continued to relate positively ($r = 0.339$. $P = .043$) to the entire five-year bone change [45]. Also in the total body bone density reported by Waller, normally cycling women experienced a +0.02% change while those with ovulatory disturbances experienced a –1.7% loss ($P = .08$) [47]. Furthermore, the Bedford study showed that total hip BMD change, in addition to spinal BMD, was significantly related to ovulatory disturbances (−0.6% versus +0.9%, $P = .001$) [48]. In summary, it appears that five or more cycles of ovulation-documented data per year are needed to "see" any bone change related to progesterone production within regular menstrual cycles.

Several cross-sectional studies have also addressed the issue of ovulation and BMD. The most influential of these has a nested case-control design within a population-based sample (The Michigan Bone Health Study) [54]. A randomly sampled cohort of premenopausal women ages 25–45 ($n = 582$) all had BMD measured by DXA. Those in the lowest 10th percentile of bone density (cases) and those in the 50th to 75th percentile of BMD (controls), who had regular cycles, were on no hormones ($n = 31$ cases and 34 controls) collected daily first morning urines for LH, FSH, and excretory products of estrogen (E1C) and progesterone (PdG) over two cycles or 84 days [54]. Cases (women with low BMD) were smaller and leaner than controls (BMI 23.6 versus 26.1), probably of lower socioeconomic status (based on significantly fewer years of education) and were less likely to use alcohol or to have taken oral contraceptives [28].

Table 1: Prospective studies of spinal Bone Mineral Density (BMD) change by ovulatory menstrual cycles compared with ovulatory disturbances (anovulation and short luteal phases within normal length cycles). BMD is by Quantitative Computed Tomography (*) or Dual Energy X-ray Absorptiometry (+). All data are shown as mean ± SD.

Manuscript	Number women	Duration (years)	Age ± SD (range)	Body Mass Index	# Cycles/year	Cycle length (days)	% Bone change/year-spine	
							Normal•	Ovulatory disturbances
Prior 1990 [15]	66	1	33.7 ± 7.1 (20–42)	22.0 (18–24.9)	10 (6 to 13 cycles)	28.2 ± 2.6	(*) +0.2	(*) –3.3
Prior 1996 [51]	27	4	35.9 ± 4.9	21.7 (18–24.9)	1.5 (3–46 cycles)	27.8 ± 2.4	(*) $n = 14 - 0.98°$	(*) $n = 13 - 0.94°$
Waller 1996 [52]	53	1.5	33.4 ± 4.3	NR^	2.7	NR^	(+) −0.05	(+) +0.55
Waugh 2007 [53]	189	2	32.4 ± 4.6 (21–40)	24.3 (range not given)	5	28.9 ±3.9	(+) +1.6	(+) −0.4
Bedford 2010 [48]	123	2	22.1 ± 3.3 (19–35)	21.8 ± 2.5	6.8 ± 7.0	30.8 ± 4.1	(+) +1.9	(+) +0.7
Totals (Mean)	458	2.1	31.4	22.5	6.6	28.9	+0.53	−0.68

°Based on a median split of % all cycles with ovulatory disturbances. "Normal" = 0%–33% of all cycles with ovulatory disturbances and "Ovulatory Disturbances" = 34%–100% of cycles with ovulatory disturbances.
•"Normal" means normal menstrual cycle length with ovulation and a normal luteal phase length
Numbers of cycles/year in which ovulation and ovulatory disturbances as well as cycle length were documented.
^NR means not recorded.

Results of this cross-sectional study showed lower PdG and E1C excretions across cycles in cases compared with controls. All three summary measures of PdG were lower in cases (peak, mean, and area under the curve with $P = .002–.006$). E1C was also lower (with $P = .01$ to .008). Although not statistically significant, anovulation rates were higher in cases (14.8%) than in controls (8.8%). The data suggest that lower BMD values are related to subtle disturbances in ovulation and perhaps estradiol levels within regular cycles.

The final two cross-sectional studies failed to confirm a relationship between ovulatory disturbances and bone change in premenopausal women [51, 53]. These studies typically have measured ovulatory function in only one cycle and/or did not document short luteal phase lengths that are the most prevalent subclinical disturbances of ovulation [51, 53]. It is likely that a cross-sectional study does not have the power to show a bone-ovulation relationship because of the great within-woman variability of subclinical ovulatory disturbances.

3.2.5. Progesterone Therapy for Premenopausal Bone Loss Prevention. If the above associations of ovulatory disturbances with less positive changes in bone hold true, women with subclinical ovulatory disturbances who are currently undiagnosed and overlooked as having bone risks might be experiencing bone loss over many asymptomatic premenopausal years.

Data on progesterone's osteoblast-differentiation effects suggest that luteal phase "progesterone replacement" may be an effective treatment for SOD. So far, no progesterone trial with bone endpoints has been undertaken, but there are two published trials of physiologic dose (not depot) cyclic medroxyprogesterone (MPA) of which we are aware, one a randomized controlled trial in healthy, normal weight, physically active women in their early 30 s [15] and one an open although apparently randomized trial in underweight teenagers with amenorrhea or oligomenorrhea [52]. The prospective trial of cyclic medroxyprogesterone acetate (MPA, 10 mg/day for 12 days a month) for underweight teenagers [52] is compared with the randomized, double-blind placebo-controlled two by two factorial design trial of cyclic MPA (10 mg/day for 10 days a month) and/or calcium supplementation (1000 mg/d) [15]. Women participating in this latter one-year trial differed from those in the teen study in being healthy, of normal weight, and physically active. In addition, they had a range of menstrual cycle and ovulatory disturbances including hypothalamic amenorrhea, oligomenorrhea, or ovulatory disturbances within regular cycles [15]. Bone change across one year was compared by randomization to cyclic MPA or to placebo [15].

Women with regular cycles were required to have two consecutive cycles with proof of ovulatory *disturbances* by QBT before enrolment. Participants were stratified by amenorrhea, oligomenorrhea, anovulation, and short luteal phase cycles into one of four groups—(1) cyclic MPA (10 mg for 10 days a month or cycle days 16–25) with active calcium (an additional 1000 mg/d); (2) cyclic MPA with placebo calcium; (3) placebo cyclic MPA with active calcium or (4) both MPA and calcium placebos [15]. The primary outcome, BMD of L1-4 in the spine, was measured at the beginning and the end of the year, as were body weight, height, and skin folds. Women also recorded 3-day diet diaries every three months and daily completed a Menstrual Cycle Diary [55] record daily, as well as recording their basal temperatures and exercise duration, type and mean exercise heart rates.

Results in the 61 women completing this cyclic MPA trial showed that bone change over one year was positive in those assigned to cyclic MPA with or without calcium supplementation and averaged +1.7 ± 0.5 (SEM) percent

(2×2 ANOVA $F = 19.43$, $P = .0001$). The effect of calcium supplementation was not quite significant ($F = 3.34$, $P = .073$); however it prevented some bone loss (mean change $= -0.7\%$, $P = .28$). Women assigned to both placebos lost bone at a significant rate (-2.0%, $P = .005$) despite being of normal weight, having regular exercise and adequate calcium intakes [15].

In a small open, apparently randomized (no clear RCT methodology provided) trial of underweight or anorexic teenagers with amenorrhea, who had inadequate calcium intakes (less than 1300 mg/d), the girls were assigned to cyclic MPA (10 mg for 12 days/month, $n = 5$), oral contraceptives (35 μg ethinyl estradiol, $n = 5$), or placebo ($n = 5$) [52]. Young women in the same study who had oligomenorrhea were assigned to cyclic MPA ($n = 5$) or placebo ($n = 4$). Amenorrheic women on oral contraceptives appeared to gain spine BMD while those on cyclic MPA and placebo lost BMD [52]. However, these results are flawed by the differences of endogenous estradiol between oligomenorrhea and amenorrhoea as well as by the undernutrition of enrolled young women, and the few participants.

3.3. Progesterone and Bone in Perimenopause

3.3.1. Bone Turnover Markers in Perimenopause. Although perimenopause is understood to be a time of dropping estrogen levels, its hormonal changes are much more complex than estrogen deficiency [10]. Hormonal perimenopausal changes involve altered control of gonadotrophins [56], disturbances of feedback of ovarian hormones at the pituitary and hypothalamic levels, at least partly through the inhibins [57], and erratic ovarian follicular growth despite decreasing numbers of follicles [58]. Ovulation disturbance is one of the consequences of these perimenopausal hormonal changes [59], which, in turn, may accentuate the changes.

In the normal menstrual cycle, progesterone exerts feedback on the hypothalamic GnRH pulse generator and slows the frequency of GnRH pulses [60, 61]. However the amplitude of the pulses is higher during the luteal phase compared with the follicular phase. Towards the end of the luteal phase, the decreasing progesterone concentrations cause the GnRH-pulse generator to accelerate again. During these few days of acceleration, GnRH-receptors in FSH-producing cells are particularly sensitised, so that, for a few days before, during and after flow, FSH-levels rise [62]. This mechanism is pronounced during perimenopause, when increasing numbers of anovulatory cycles go hand in hand with rising early follicular phase FSH levels.

This next section will first review several studies of cross-sectional BMD values and bone resorption markers related to pre-, peri-, and postmenopausal status (Table 2). Results of prospective changes in bone turnover markers in women who differed in reproductive status but were all over age 40 and changes in spinal BMD by QCT in untreated pre-, peri-, and early postmenopausal women at baseline, two, and six years [63] will be studied.

The majority of studies on perimenopausal bone change have been conducted using dual (energy) X-ray absorptiometry (DXA) of the hip and/or spine. DXA provides an areal, rather than true volumetric summary measurement of mineral content including both cortical and trabecular bone. Cancellous or trabecular bone, which is more responsive to hormonal changes, and measured volumetrically by QCT, provides a more sensitive assessment of change in BMD within the more metabolically active cancellous compartment and may result in earlier detection of bone loss.

Since perimenopause is characterized by unpredictable and unstable endocrinological changes, systematic comparison and classification are difficult. Efforts to establish standards for scientific comparisons and for clinical use have led to five phases of reproductive transition, based on endocrinological and clinical criteria defined in international boards such as the WHO Scientific Group [71] and Workshops (e.g., Staging of Reproductive Aging Workshop (STRAW) [72]), with the aim of achieving comparability of scientific results on perimenopause. To date international standardisation has not been achieved, and the newly defined criteria are still not used consistently. Therefore publications reporting that they studied "perimenopausal women" may include them either with premenopausal or postmenopausal groups, as by Kushida et al. or Melton et al. in Table 2 [66, 68], or mix them with others to form a group of middle-aged women such as ages 40–59 years [65] without applying any distinct hormonal or menstrual cycle criteria. Another way of dealing with the problem of definition has been to take change from premenopause to postmenopause over a time course of many years, which is only possible in studies such as those published by Löfman or Ravn [67, 69] (5-year follow-up, see Table 2). These studies, however, carry the risk of not capturing perimenopause itself over such long intervals.

Amongst the published cross-sectional data, only Ebeling et al. [64] and Sowers et al. [70] studied truly perimenopausal groups of women. Ebeling found elevated bone resorption rates and declining bone density in 118 perimenopausal women. Sowers, in the baseline data of the multiethnic participants ($n = 2336$ women aged 42–52) from SWAN, the Study of Women Across the Nation, also showed that increased bone turnover begins years before menopause [70].

A meta-analysis of within-centre studies documenting both perimenopausal and postmenopausal rates of spinal bone loss earlier showed significantly greater rates of loss in perimenopause (-1.8 versus -1.2%/year) [10]. That analysis reported preliminary Melbourne Midlife Women's Health study results prior to their publication [73]. Using DXA, this study showed that spine bone loss was increased during perimenopause. However, in 224 untreated pre-, peri, and postmenopausal participants ($n = 78$ early perimenopausal, $n = 12$ late perimenopausal), the greatest amount of loss occurred in the first three years following the final menstrual flow [73]. This may have been because of inclusion of the first year after the final menstrual flow in "postmenopause" rather than perimenopause [59], or because of the relative time lag of DXA for bone changes affecting mainly or exclusively the trabecular compartment. Accordingly, early cycle elevated FSH and low estradiol values, as are commonly found in postmenopause, correlated with this increased loss [73].

Another study by Slemenda et al. showed increased bone loss in 62 perimenopausal women from a total of 231

Table 2: Cross-sectional studies on perimenopausal bone metabolism and bone mineral density.

Author	Title	Design	Methods	Relevant findings	Conclusion
Ebeling et al. 1996 [64]	*Bone turnover markers and bone density across the menopausal transition*	281 women, 45–57 years. 3 groups: 60 premenopausal 118 perimenopausal 103 postmenopausal (of these, 36 with HRT)	DXA E2, FSH, LH, inhibin on day 4–8 of menstrual cycle if applicable Bone formation: OC, BAP, PICP Bone resorption: PYD, DPD, NTX	Postmenopausal group: BMD↓↓. Loss in BMD correlated with age in perimenopausal group. Perimenopausal group: LH, FSH doubled versus premenopause; E2, BAP did not differ between premenopausal and perimenopausal group. ↑ PYD, DPD, NTX, BAP, and OC in postmenopausal versus premenopausal group. perimenopausal group: Positive correlation of BMD with DPD. All women: correlation of BMD with NTX, BAP, OC, FSH	Perimenopause: increased bone resorption rate and decreased bone density. Other factors apart from E2 are involved in the development of postmenopausal osteoporosis
Khosla et al. 2005 [65]	*Relationship of volumetric bone density and structural parameters at different skeletal sites to sex steroid levels in women*	235 untreated women 3 groups: (i) premenopausal (20–39 years.) (ii) mixed (40–59 years.) (iii) postmenopausal (>60 years.)	QCT E2, Testosterone	Postmenopausal group: significant correlation of low bioavailable E2 and BMD (trabecular and cortical). 40–59 years: significant correlation between average rise in bioavailable E2 and loss in trabecular BMD.	Trabecular bone reacts faster to lowering E2. The threshold for estrogen deficiency in cortical bone in women appears to be lower than that in trabecular bone.
Kushida et al. 1995 [66]	*Comparison of markers for bone formation and resorption in premenopausal and postmenopausal subjects, and osteoporosis patients*	95 premenopausal women, 30–53 years. 66 postmenopausal women, 50–69 years 29 untreated women with osteoporosis, 55–91 years. No distinct perimenopausal group, but included in pre- and postmenopausal	No BMD measurement Bone formation: AP, OC, PICP, Bone resorption: PYD, DPD	Postmenopausal group: AP, OC, PICP, PYD, DPD significantly higher than in premenopausal group. In osteoporosis: PICP, PYD, DPD significantly higher than in postmenopausal group. Women ≥ 50 years.: PYD, DPD higher than in women 30–49 years.	Markers in postmenopause higher than in premenopause. In women with osteoporosis resorption markers are higher than formation markers.
Löfman et al. 2005 [67]	*Common biochemical markers of bone turnover predict future bone loss: a 5-year follow-up study*	Cross sectional study (+ longitudinal) 192 women, 21–79 years. 3 groups (i) premenopausal (ii) perimenopausal (i.e., premenopausal at baseline and postmenopausal after 5 years). (iii) postmenopausal	2x DXA Bone formation: BAP, OC, AP Bone remodelling: Hpr Ca	Baseline values of markers correlated negatively with baseline BMD. AP, OC, Hpr, Ca rise at the "beginning of menopause" 15 years after menopause: OC and Hpr are still elevated.	Bone markers and current BMD could give information about coming loss of BMD.

Table 2: Continued.

Author	Title	Design	Methods	Relevant findings	Conclusion
Melton et al. 1997 [68]	*Relationship of bone turnover to bone density and fractures*	351 women, 20–80 years. 2 groups: 138 premenopausal 213 postmenopausal (i) 47 with HRT (ii) 166 without HRT of these, 89 cases of osteoporosis No distinct perimenopausal group	DXA Bone formation: OC, BAP, PICP Bone resorption: PYD, DPD, NTX	Premenopausal group: OC, NTX negatively correlated with BMD. Postmenopausal group: increase of markers with age. Postmenopausal group: OC, BAP, NTX, PICP negatively correlated with BMD. Osteoporosis: markers↑, BMD↓.	Combination of markers with BMD measurement is sensible for prediction of individual fracture risk. NTX is best predictor of loss in BMD.
Ravn et al. 1996 [69]	*High bone turnover is associated with low bone mass in both pre- and postmenopausal women*	979 women, 30–75 years. 2 groups: 334 premenopausal 645 postmenopausal 5 year-longitudinal analysis. No distinct perimenopausal group, but included in pre- and postmenopausal	DXA Bone formation: OC, AP Bone remodelling: HydroxyProline Bone resorption: CTX	Premenopausal <50 years: markers stable. Women with highest markers had significantly lower BMD. OC and CTX correlated with BMD. Postmenopausal group: CTX, OC sign. higher than in premenopausal group. 5 years. after menopause: CTX, OC stable again.	Bone metabolism is accelerated in perimenopause and early postmenopause.
Sowers et al. 2003 [70]	*The association of endogenous hormone concentrations and bone mineral density measures in pre- and perimenopausal women of four ethnic groups: SWAN*	2336 women, multiethnic, 42–52 years. 2 groups: (i) premenopausal (ii) perimenopausal	DXA E2, FSH, T, DHEAS, SHBG (day 2-7 of menstrual cycle if applicable)	Perimenopausal group: FSH higher and BMD lower than premenopausal group. All women: negative correlation of FSH with BMD. No correlation of E2 and BMD.	Loss of BMD starts before menopause.

untreated women. Apart from low estrogen, bone loss in this study was also associated with lowered serum androgen levels [74]. The Michigan Bone Health Study cohort (513 women, aged 25–45 randomly sampled from the population) documented that DXA of the spine was three percent lower in perimenopausal than in premenopausal women and that annual bone loss was significantly elevated in perimenopausal women, when compared with premenopausal participants [75]. These observations were confirmed by the even larger SWAN study ($n = 2311$) [76]. In the first 4 years of this study with annual bone density measurements and early follicular phase serum hormone values, this study showed that in both baseline and follow-up, elevated FSH levels were associated with decreased bone density, while estradiol was not [76]. In both the Australian and USA large studies, however, hormone values were only taken during the early follicular phase, a time when FSH is often elevated in perimenopausal women, estrogen is normally low, and progesterone cannot be evaluated. So, despite the large size and the power of these studies, they could only assess hormonal effects of the first week of women's menstrual cycles, excluding the remaining 75% of potentially available information [70].

Two prospective observational German studies attempted to systematically characterize the changes in bone metabolism associated with perimenopause. In the first study, serial bone turnover marker measurements were made on 64 healthy women over age 40, who had taken no exogenous hormones and were within three reproductive life phases: premenopause (mean age 43.7 years, $n = 20$), perimenopause (mean age 50.3 years, $n = 24$), and postmenopause (mean age 52.2 years, $n = 20$) [78]. These prospective, serial bone marker measurements were first made on four visits across one year (0, 3, 6, and 12 months). Parameters relating to bone resorption were the urinary excretion of pyridinoline (PYD), deoxypyridinoline (DPD), and N-terminal telopeptide (NTX), all corrected for creatinine. As well, serum bone formation markers were measured including osteocalcin (OC) and bone-specific alkaline phosphatase (BAP) [78]. In these midlife women with regular cycles, this study found a significant decrease over time for the bone formation marker BAP, leading to the conclusion that the metabolic changes in bone remodeling commonly associated with perimenopause (like the higher estradiol levels and disturbed ovulation [10]) had already begun in the late reproductive phase [30]. These participants were followed for a second year [79], as well as eventually over six [63] and nine years [80].

The six-year follow-up allowed for a longitudinal comparison of bone changes in pre- as opposed to perimenopausal and early postmenopausal women. Analysis of QCT changes over time required classification of women's reproductive status and its changes over time. Invariably for all analyses, the perimenopausal period—during which estrogen levels were still adequate—was associated with the greatest reduction of QCT, with loss rates reaching 6.3%/year. Total bone loss differed by pattern of individual women's experiences of the transition from pre- to postmenopause (one-way ANOVA $P < .05$); the average rate of loss was slower in the early postmenopausal years [63].

The second prospective observational study was initiated to further explore the drop in BAP observed in the first study and to systematically monitor bone turnover markers in the follicular and luteal phases of serial cycles in 8 women whose averaged age was 46 years. From a total of 170 cycles, 84 cycles with luteal phase serum sampling were analysed. Categorical differences were calculated to detect individual intracycle changes in bone metabolism. Figures 4(a) and 4(b) show the within-cycle change (luteal minus follicular phase levels) of serum BAP (Figure 4(a)) or HPLC-extracted urinary pyridinoline (PYD; Figure 4(b)) by the threshold progesterone level used to document ovulation [81]. These two figures show that both PYD and BAP patterns differ in ovulatory and anovulatory cycles. Further, given that positive values mean increases in the luteal phase and negative values mean luteal phase decreases, these data suggest that the higher the progesterone level the more bone formation. As mentioned earlier, decreased bone resorption markers in the luteal phase occur because resorption continues to be controlled by moderate luteal phase estradiol levels.

Seifert-Klauss and colleagues are currently conducting the ongoing "PENO-Study" (Perimenopausale Knochendichte [bone density] und Ovulation), which is a prospective observational study over the course of two years. Its purpose is to investigate menstrual cycles, hormonal values, bone turnover markers, and changes in bone mineral density (BMD) during the perimenopause to answer the following question: do perimenopausal women with a higher rate of anovulatory cycles have increased loss of bone density?

Inclusion criteria for this study are age >45 years, cycle lengths no greater than 42 days, no use of exogenous hormones during the 6 months prior to study onset, and no medical reasons for low bone mass. Lumbar spine trabecular BMD measurements are performed using QCT at baseline and after two years. Participants note the beginning and end of each cycle and used a cycle monitor to detect the day on which there was a high probability of ovulation.

Results are available so far for 54 women (mean age 48.3 ± 2.3 SD years) who have recorded 673 evaluable cycles and had 132 luteal phase blood tests suitable for analysis. QCT measurements at baseline show that 45 women had normal bone density (mean 148.2 ± 19.3 mg Calcium-Hydroxyapatite (Ca-HA)/mL^3), while nine had osteopenia (mean 103.7 ± 7.3 mg Ca-HA)/mL^3. Women with normal BMD at the beginning of the study, and those who maintained BMD levels within the normal range over two years, were more likely to experience normal ovulatory cycles with fewer ovulatory disturbances than those women whose initial or two-year QCT values showed osteopenia. During the course of this two-year study, the proportion of ovulatory cycles related to QCT bone change ($r = -0.7$, $P < .05$) (Figure 5). Also, as has previously been shown [59, 82], progesterone levels decreased before cycles without ovulation became common [77].

Although women are known to lose bone rapidly *before* as well as after they become postmenopausal [10, 83], this study adds to existing data by showing that this bone loss

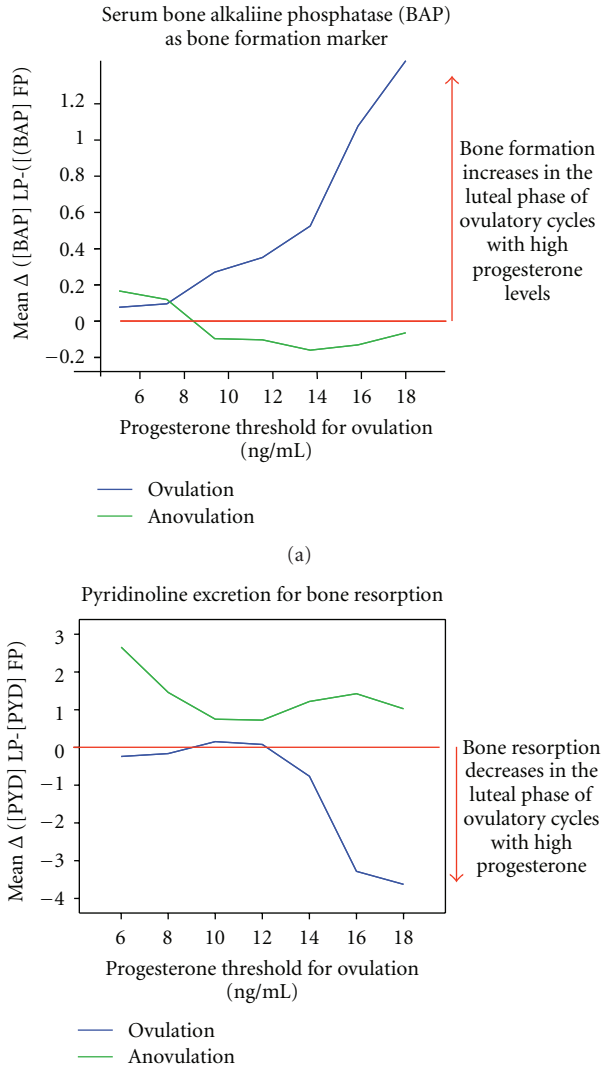


Figure 4: Intra-cycle follicular-luteal phase change in two different bone turnover markers by the serum progesterone level used as a threshold for ovulation. (a) shows the bone formation marker bone-specific alkaline phosphatase (BAP) in serum, (b) depicts changes in the bone resorption marker Pyridinoline (PYD) extracted with HPLC from urine and normalized to creatinine reprinted from [77]. Permissions provided.

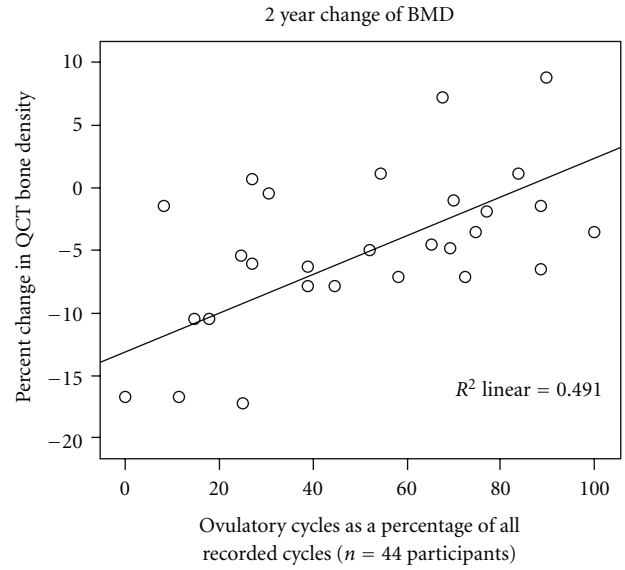


Figure 5: The 2-year-change of trabecular lumbar spine bone mineral density documented by Quantitative Computed Tomography (QCT) is shown by rate of ovulatory cycles in 28 women with complete ovulation data out of the 44 women studied prospectively in the ongoing PEKNO-Trial. Assessed by a commercially available ovulation monitor device, ovulation-likelihood was verified by luteal phase serum sampling. The graph illustrates the significant linear relationship ($r = 0.7$; $P < .05$) observed between the percentage of ovulatory cycles and BMD loss in pre- and perimenopausal women. This figure is from a presentation on the interim analysis by T. Wimmer and V. Seifert-Klauss to the Congress of the German Menopause Society (Deutsche Menopausen Gesellschaft) in Hamburg, November 6th 2009 (unpublished). The authors provide permission.

is not simply due to the increased bone resorption caused by the perimenopausal swings in estrogen levels but is also related to progesterone levels and ovulatory characteristics.

3.4. Progesterone and Bone in Postmenopausal Women. Postmenopausal women are more likely to experience fragility fractures than are pre- or perimenopausal women. This increased fracture risk is usually ascribed to estrogen deficiency—but this state also includes progesterone "deficiency." A number of published investigations have asked two questions about the relationship of progesterone to bone change in postmenopausal women. These research questions are as follows. (1) *Does progesterone therapy prevent or treat osteoporosis in postmenopausal women?* And, (2) *Does progesterone therapy add to the bone-positive effects of anti-resorptive therapies (such as estrogens, calcitonin, or bisphosphonates)?* This section and the next will review the available human data to answer these two questions.

3.4.1. Progesterone Therapy for Postmenopausal Osteoporosis. To determine whether progesterone is effective treatment for osteoporosis in postmenopausal women, changes in BMD by DXA and/or QCT in four RCTs compared treatment with progesterone or MPA with placebo. Gallagher, in the earliest study, asked whether a high dose of the non-androgenic progestin, medroxyprogesterone [MPA] effectively treated postmenopausal osteoporosis. The 20 mg dose of MPA for 23 of 28 days did not prevent bone loss by spine dual photon absorptiometry (DPA) [84]. Likewise, Table 3 shows bone change in two further RCTs of more standard MPA doses of 10 mg/d, 300 mg/d of oral micronized progesterone (OMP), or 20 mg/d of progesterone cream. The net result of the placebo-controlled trials with MPA was bone loss (−2.2% per year) despite these MPA or progesterone therapies and with no apparent difference from the bone change on placebo (−2.4%/year).

Table 3: Double-blind randomized controlled trials of percentage spine Bone Mineral Density (BMD) change per year in postmenopausal women treated with Progesterone/Medroxyprogesterone (MPA) compared with placebo.

Author/year and reference	Total number	Age	Bone site	Years	Drug and dose	Schedule	Number	% BMD Change (Active)	Number	% BMD Change (Placebo)
Gallagher 1991 [84]	81	51.7 ± 4.4	DPA* L2−4	2	MPA 20 mg	23/28 days	20	Spine −2.5	20	Spine −3.8
			Radius				20	Radius 0.0	18	Radius −2.4
Prior 1997 [85]	33	45 ± 5	QCT T12−L3 DXA	1	MPA 10 mg	Daily	18	QCT −15	NA•	NA
			WB+					WB −2.8	NA	NA
			FN++					FN −5.2	NA	NA
Leonetti 1999 [86]	102	52.5	DXA L2−4	1	*P_4 Cream 20 mg	Daily	43	Spine −1.4	47	Spine −1.0
			T Hip				43	T Hip −2.5	47	T Hip −1.0
Liu 2005 [87]	132	52.5	DXA L2−4	2	+OMP	300 mg Daily	15	Spine −1.0	23	Spine −1.0
			FN				15	FN −0.5	23	FN −0.0
					MPA	10 mg Daily	16	Spine −1.9	23	Spine −1.0
							16	FN −1.1	23	FN −0.0
Mean % Change					OMP & P_4 Cream		58	Spine −1.2	70	Spine −1.0
					MPA		36	Spine −2.2	43	Spine −2.4

*P_4: progesterone
+OMP: oral micronized progesterone
•NA: not available—this trial was controlled by conjugated equine estrogen and without a placebo.

One of the RCTs of MPA alone and bone change was a unique one-year randomized blinded comparative study versus conjugated equine estrogen (CEE) in 41 premenopausal women who had just undergone premenopausal abdominal hysterectomy with bilateral ovariectomy for benign problems [85]. CEE (0.6 mg/day) was compared with MPA (10 mg/day) over one year [85]. All 41 women began study participation after fasting blood and urine samples were obtained on the morning they were discharged following their surgery [85]. Results showed highly significant rates of spinal cancellous QCT bone loss in women on both therapies (−15% MPA, −8.3% CEE, $P = .04$). MPA also did not prevent significant bone loss in the whole body (−2.8%) and femoral neck (−5.2%).

The results of this MPA versus CEE randomized comparative trial may provide clues to causes for major bone loss despite progesterone/MPA therapy with their osteoblast differentiating and bone formation effects. On average at seven days following surgery, bone resorption markers were three to five *standard deviations* higher than premenopausal levels. These bone resorption markers did not decrease across a year of MPA therapy despite the fact that all women were supplemented with 600 mg of additional calcium/day and gained approximately 2.5 kg in weight [85]. These data suggest that MPA does not decrease bone resorption. One further randomized 2-year placebo-controlled trial of a progestin and bone change is available. This study treated early postmenopausal women with promegestone, a 19 norprogestin, or placebo and showed some prevention of bone loss (−1.3% versus −4.5% on placebo, $P = .05$) [88]. Urinary calcium excretion was significantly decreased in the promegestone group, however, suggesting it may decrease resorption rather than acting through the progesterone receptor.

Taken together from this meta-analysis, the answer to the first question is as follows *MPA/Progesterone alone is not effective therapy for postmenopausal osteoporosis* because it has no or little effect to control bone resorption, the driving force in human bone loss and osteoporosis.

3.4.2. Progesterone as Co-Therapy with Antiresorptives for Postmenopausal Osteoporosis. Yearly percent bone change on co-therapy with an antiresorptive and progesterone or MPA compared with the antiresorptive agent alone has been studied in five randomized, double, blind controlled trials to answer the second research question named above (Table 4). Two of these were major studies in the bone field (Postmenopausal Estrogen Progestin Investigation [PEPI] [89] and the Women's HOPE trial) [90]. A more recent study combined MPA 10 mg/d with oral micronized estradiol in

Table 4: Comparison of randomized double-blind controlled trials of bone change in postmenopausal women with osteoporosis comparing combined estrogen [Conjugated Equine Estrogen (CEE) or Estradiol (E_2)] plus progesterone or medroxyprogesterone (MPA) and documenting percentage (%) change per year in Bone Mineral Density.

Author	Type	Number	Age ± SD	Bone sites	Years	Anti-resorptive mg/d	Progesterone/ MPA mg/d	Number	Combined % bone change	Number	Anti-R*% bone change
Gallagher 1991 [84]	RCT not blinded	81	52 ± 4	DPA L2−4 SPA	2	0.3 CEE 23/28 days	MPA 10 mg 23/28 days	16	Spine +0.25	18	Spine +1.0
				Radius					Radius +0.0		Radius −0.05
PEPI 1996 [89]	DB-RCT	875	56	DXA L2−4	3	CEE 0.625	MPA 2.5 mg/d	174	Spine +1.6	175	Spine +1.4
				Total Hip (TH)				174	TH +0.5	175	TH +0.5
Adachi 1997 [91]	DB-RCT	98	54	DPA	1	CEE 0.625	MPA 10 mg for 15 d/mo.	33	Spine +2.7	34	Spine +1.9
Lindsay 2002 [90]	DB-RCT	695	58	L2−4 DXA	2	CEE 0.625	MPA 2.5 mg/d	81	Spine +1.7	84	Spine +1.2
				Total Hip (TH)					TH +1.3		TH +1.4
						CEE 0.45	MPA 2.5 mg/d	87	Spine +1.5	91	Spine +1.1
									TH +1.1		TH +1.0
Liu 2005 [87]	DB-RCT	132	53	DXA L2−4	2	E_2 1 mg/d	MPA 10 mg/d	20	Spine +2.3	23	Spine +1.3
				FN		OME[#]			FN +0.9		FN +1.0
Totals^								330	Spine +1.7	425	Spine +1.3

•DPA: dual photon absorptiometry, SPA: single photon absorptiometry, DXA: dual energy X-ray absorptiometry.
*Anti-R: antiresorptive therapy, #OME: oral micronized estradiol.
^Note that the two estrogen-dose arms of the Lindsay study were considered as two different studies in the mean spine bone change.

a dose of 1 mg/d, versus the estradiol alone [87]. The results of these RCTs show the mean change in spinal BMD on co-therapy with daily low-dose MPA, and an antiresorptive was slightly more positive (+1.7%/year) than with the antiresorptive alone (+1.3%/year), a difference of about 24%. In both of the largest studies [89, 90], estrogen-progestin spine results were noted to be significantly more positive than those related to the antiresorptive alone [89, 90]. This, however, did not appear to occur when the progestin was given cyclically [91], suggesting that daily progesterone is needed to increase BMD in menopausal women. Therefore the answer to the second question is as follows. *Progesterone daily co-therapy with estrogen is more effective than estrogen alone for postmenopausal osteoporosis.*

The non-randomized clinical studies combining MPA with an antiresorptive therapy show similar but even greater increases in BMD on MPA and antiresorptive co-therapy compared with the antiresorptive alone [92–94]. It is very difficult to compare studies in women who have had hysterectomy with/without ovariectomy because women with hysterectomy, and certainly following ovariectomy, are much more likely to be treated with estrogen than are women with natural menopause; this represents confounding by indication. The New Zealand studies examined older postmenopausal women with high rates of bone loss who were treated with CEE plus 5 mg/d of MPA or with CEE alone—this one-year study saw a 65% greater spine BMD increase on combined therapy (6.6%) compared with CEE alone (4.0%) [93]. A prospective study also from New Zealand and in a similar population looked at the rates of bone change in the hip and spine when either CEE or transdermal estradiol was paired with MPA [92] and compared with placebo. One-year results were quite positive in the spine (+7.1%) and in the femoral neck (2.9%) but there was no anti-resorptive alone comparison in this descriptive study. Finally, a small pilot study on a random sample of ($n = 20$) clinical patients treated with MPA combined with an early bisphosphonate (intermittent cyclic etidronate, Didrocal) compared their bone change data with that from a meta-analysis of RCTs of etidronate alone [95]. In this comparison, spine increases were greater on MPA-antiresorptive co-therapy (+2.6%) than on antiresorptive therapy alone (+1.8%); femoral neck BMD increases on MPA co-therapy were also more positive (+1.5% versus +0.5%) [94].

So far in this paper, the fracture prevention shown in the CEE-MPA and the CEE only arms of the Women's Health Initiative (WHI) trials has not been discussed [5, 96]. These are not only the largest studies of bone in women randomized to postmenopausal hormone therapy or placebo ($n = 27,000$ in both studies together), they are also the only randomized, placebo-controlled studies to show fracture prevention with ovarian hormone therapy. These data are especially important since the study population was not selected for low bone mass or osteoporosis risk factors. Ideally we could compare the rates of fracture in the co-therapy with the CEE-only arms but this is not possible because each was in a different randomization scheme and had its own placebo group. We have requested collaboration with WHI investigators in such an adjusted analysis.

3.4.3. Breast Cancer and Other Issues Relating to Progesterone/MPA Therapy in Postmenopausal Women. Data so far suggest that progesterone or MPA co-therapy with estrogen is more effective for osteoporosis; however breast cancer is an important clinical concern [96]. Combined hormone therapy containing MPA has been repeatedly associated with increased breast cancer risk [97–99]. The prospective E3N observational study, following 80,000 French women over eight years however, saw an increase in breast cancer risk only with estrogen alone or with synthetic progestins, but not with oral micronized progesterone combined with estrogen [99, 100]. Randomized controlled research on the breast cancer risks with estradiol plus progesterone is needed. Likewise, effects of progesterone and various progestins on intramammary estradiol metabolism are needed before combined estradiol and progestin would be considered safe or acceptable.

Other important non-bone issues in women with postmenopausal osteoporosis are sleep disturbances and hot flushes and night sweats (vasomotor symptoms, VMS) that have been linked to increased bone loss [101–103]. Oral micronized progesterone is clinically useful for insomnia and in a dose of 300 mg at bedtime significantly increased total sleep time, decreased time required to fall asleep, and increased early night REM sleep in a cross-over RCT [104]. That trial also showed that, after 21 days of treatment, progesterone caused no lack of alertness or any cognitive impairment in the morning [104]. Vasomotor symptoms, that occur in about 70% of postmenopausal women and are severe in almost 10 percent, are effectively treated by MPA [105, 106] and also by oral micronized progesterone treatment [100].

3.5. Conclusions. Although the dominant osteoporosis paradigm for women is, and should remain, centred on estrogen, progesterone is emerging as an important partner hormone that collaborates with estrogen. *In vitro* studies of human osteoblasts in culture, prospective studies in adolescent, premenopausal, perimenopausal, and postmenopausal women all indicate that progesterone—likely working through bone formation pathways—plays an active role in maintaining women's bone and in osteoporosis prevention.

Finally, although progesterone or MPA therapy does not prevent bone loss when bone turnover is high, evidence from a number of randomized controlled trials suggests that progesterone as co-therapy with an antiresorptive agent may have promise. Data on progesterone co-treatment and fracture prevention are urgently needed, as is more information about the microarchitectural and histomorphometric changes during progesterone therapy.

Progesterone, a physiological ovarian steroid that is normally secreted in high levels for two weeks per menstrual cycle in ovulatory menstruating women, appears to have complementary bone actions with estrogen and antiresorptive therapies. Progesterone deserves to be studied more as a new and emerging agent for achieving and preserving peak bone mass, for prevention of pre- and perimenopausal bone loss, and, with an antiresorptive therapy, in increasing BMD and potentially decreasing fractures in postmenopausal women.

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Review Article

Scientific Basis for the Potential Use of Melatonin in Bone Diseases: Osteoporosis and Adolescent Idiopathic Scoliosis

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The objective of this paper was to analyze the data supporting the possible role of melatonin on bone metabolism and its repercussion in the etiology and treatment of bone pathologies such as the osteoporosis and the adolescent idiopathic scoliosis (AIS). Melatonin may prevent bone degradation and promote bone formation through mechanisms involving both melatonin receptor-mediated and receptor-independent actions. The three principal mechanisms of melatonin effects on bone function could be: (a) the promotion of the osteoblast differentiation and activity; (b) an increase in the osteoprotegerin expression by osteoblasts, thereby preventing the differentiation of osteoclasts; (c) scavenging of free radicals generated by osteoclast activity and responsible for bone resorption. A variety of in vitro and in vivo experimental studies, although with some controversial results, point toward a possible role of melatonin deficits in the etiology of osteoporosis and AIS and open a new field related to the possible therapeutic use of melatonin in these bone diseases.

1. Introduction

Bones are structures under a continuous process of remodelating by the coupled activity of cells with resorptive functions (osteoclasts) and cells responsible for the formation of new bone (osteoblasts). The balance between the activities of both cell types is under the control of systemic hormones including parathyroid hormone (PTH), estradiol (E2), and growth hormone as well as of cytokines and growth factors produced in the bone marrow [1]. A major disease of bone, osteoporosis, has been defined as "a systemic disease characterized by low bone mass and micro architectural deterioration of bone tissue, with consequent increase in bone fragility and susceptibility to fracture". This is a disease predominantly associated with aging, with a special prevalence among women [2]. Adolescent idiopathic scoliosis (AIS) is the most common type of scoliosis and also is more prevalent among females, especially during prepuberal and puberal growth, when bone acquisition is highest [3].

Melatonin is an indoleamine secreted primarily by the pineal gland but also synthesized in other organs such as retina, gastrointestinal tract, and bone marrow. Melatonin plays a regulatory role in many physiological processes including bone physiology [4–10]. Nocturnal plasma melatonin levels significantly decline after the age of 50 in both genders [11, 12]. Since the time course of the reduction of melatonin production and the progression of bone deterioration run in parallel, the possible role of melatonin in osteoporosis has been considered worthy of study. Regarding AIS, the fact that experimental pinealectomy in different animal models [13–22] results in scoliosis which closely resembles the human pathology opened a new field of research on the role of melatonin in the AIS.

The objectives of the current paper are (a) to review the data supporting the possible role of the age-dependent decrease of melatonin in the development of osteoporosis and the therapeutic value of melatonin as a treatment for this disease, and (b) to analyze the evidence related to the role of melatonin in the etiology and treatment of the AIS.

Before doing so, we will describe the effects of melatonin on bone physiology, as the basis to understand the participation of this indoleamine in bone pathology.

2. Melatonin and Bone Physiology

The effects of melatonin on bone physiology were reviewed in an excellent article by Cardinali et al. [4]. The possible influence of melatonin on bone metabolism was repeatedly proposed by different authors during the last four decades [23–27]. These proposals were made on the basis of evidence for the pineal control of the secretion of parathyroid hormone and calcitonin, demonstrable by the ultrastructural and functional changes observed in parathyroid glands after pinealectomy. The earliest experiments examined the influence of the pineal on calcemia. It was observed, for example, that the inhibition of melatonin synthesis by exposure of newborn rats to white fluorescent light reduced the concentration of calcium in the serum [28]. This effect was prevented by exogenous melatonin administration. Light-induced hypocalcemia may result from augmented calcium uptake by bone when melatonin levels are reduced after inhibition of its synthesis by light [28]. Likewise, when melatonin secretion was inhibited in rats by the administration of β-adrenoceptor blockers, serum concentrations of calcium dropped [29] an effect which was also prevented by the administration of melatonin. The conclusion from these experiments is that suppression of melatonin causes hypocalcemia and additionally suggesting that melatonin would normally upregulate the blood levels of calcium.

More recently, Ostrowska et al. [30] re-examined, in male rats, the effects of the exposure to different lighting conditions not only on calcemia but also on bone physiology. They did this by evaluating the influence of alterations in the light:dark cycle on biochemical markers of bone metabolism (serum alkaline phosphatase, concentration of carboxyterminal propeptide of type I procollagen, cross-linked carboxyterminal telopeptide of type I collagen, inorganic phosphorus, urinary excretion of hydroxyproline and calcium). They reported that short days (LD 0.5:23.5 h) had a stimulatory effect on the level of these markers, while exposure to long days (LD 23.5:0.5 h) was inhibitory. Anomalies in daily oscillations of these markers with a negative correlation with the changes in endogenous melatonin concentrations and a positive correlation with daily fluctuations of IGF-I and triiodothyronine (T_3) were also described. These results led the authors to conclude that lighting conditions influence bone metabolism in rats, and that melatonin likely plays an important role in these photoperiodic effects. Secondary changes in daily IGF-I and T_3 oscillations, caused by short- and long-day conditions, also result in altered rhythmicity of daily bone resorption [30]. This experiment demonstrated the possible influence of melatonin on bone metabolism but not its concrete effects on bone formation and resorption. However, positive effects of melatonin on osteoblastic activity were deduced from the increases in the formation of cortical bone in mice treated with intraperitoneal injections of the indoleamine [31].

One interesting finding potentially related to melatonin and bone health is the demonstration of high concentrations of melatonin in bone marrow cells from mice and humans [32, 33], with the concentrations being approximately twice as high as nighttime levels in peripheral blood [32]. The cells in question contain aryl-alkyl-N-acetyltransferase activity and express the mRNA encoding hydroxyindole-O-methyltransferase, indicating the ability of the cells to synthesize melatonin *de novo* [33]. Moreover, human osteoblasts express MT1 melatonin receptors, and its expression level decreases with the age of the host [31]. The presence of melatonin in bone marrow may be protective against oxidative damage in the proliferating hematopoietic cells or involved in bone development through osteoblast differentiation [34, 35].

A variety of in vitro studies support the hypothesis of stimulatory effects of melatonin on both osteoblast differentiation and activity. Preosteoblasts cultured in the presence of melatonin underwent early cell differentiation and a major expression of bone marker proteins compared to control cells incubated without melatonin [36]. These effects are prevented by the melatonin receptor antagonist luzindole [36]. The age-related decrease of melatonin production could shift the bone marrow cells differentiation from osteoblastic differentiation toward an adipocytic line of cell, which could explain the development of osteoporosis during aging [37]. Melatonin also promotes the osteogenic differentiation of bone marrow stem cells whereas it has negative effects on differentiation of adipose-derived stem cells [38, 39].

In cultures of human osteoblasts [31, 40], melatonin, at pharmacological doses (μM range), (a) stimulates the proliferation and alkaline phosphatase activity of these cells; (b) promotes the expression of type I collagen, osteopontin, bone sialoprotein, and osteocalcin; (c) stimulates the formation of mineralized matrix. The signaling mechanisms mediating the melatonin actions on osteoblasts are still unknown although the role of the MAPK pathway seems relevant [35].

The activity of osteoclasts is under the control of paracrine factors produced by the osteoblasts. PTH and 1,25-dihydroxycholecalcipherol stimulate the expression of an osteoclast differentiating factor (ODF) by the marrow stromal cells and osteoblasts. ODF binds to the receptor activator of nuclear factor-κB (RANK) on the surface of the osteoclast activating bone resorption [4, 41]. In mouse osteoblasts, melatonin, at micromolar doses, decreases the expression of RANK mRNA and increases both the mRNA and protein levels of osteoprotegerin, a member of the super family of TNFR (tumor necrosis factor receptor) which inhibits the differentiation of osteoclasts by binding to ODF and preventing the binding of this factor to RANK [42]. Via this mechanism, melatonin could cause an inhibition of bone resorption and an increase in bone mass.

One important component of the osteoclasts activity is the generation of free radicals which contribute to the process of bone degradation and resorption [43]. Melatonin, due to its ability to directly neutralize free radicals and to

stimulate the activity of antioxidative enzymes [44, 45], may reduce osteoclastic activity.

Figure 1 summarizes the principal mechanisms reviewed above related to melatonin's effects on bone function. These actions include: (a) the promotion of the osteoblastic differentiation, activity and expression of osteoprotegerin which prevents the differentiation of osteoclasts, and (b) scavenging of free radicals generated by osteoclastic activity and responsible for bone resorption.

There are, however, data contrary to the hypothesis of the effect of melatonin on bone-forming osteoblasts. Ostrowska et al. [46] found, in male Wistar rats, that high plasma concentrations of melatonin correlated with low levels of bone forming markers, and that pinealectomy elevated the levels of bone metabolism biomarkers and altered the phase and amplitude of its circadian rhythm. In another interesting study, Suzuki and Hattori [47] cultured osteoblasts in the presence of osteoclasts, analyzing the effects of melatonin on both, that is, osteoblastic and osteoclastic activity, by the changes on specific biomarkers in each cell type. They observed an inhibition by melatonin of the activity of both cell types. These authors emphasize the importance of the cell-to-cell interactions between osteoblasts and osteoclasts to understand their physiologic function as well as in the response to melatonin. Since melatonin inhibits both osteoblasts and osteoclasts, the final outcome of their effects could be the balance between the actions of these cellular elements. In postmenopausal women, bone resorption increases more than bone formation, thus resorption becomes the major determinant of bone mass [48]; in these cases, even therapies like melatonin, that may inhibit both osteoclasts and osteoblasts activity, should have positive effects on bone mass.

An interesting question is the interaction of melatonin with estrogens at the level of the osteoblast. Estrogens have a positive impact on bone growth. Melatonin, according to the studies summarized above, has similar effects. Under many other circumstances, however, estrogens and melatonin usually have opposing effects. Thus, it is well known that estradiol modulates the function of the melatonin receptors in rat ovary [49] and Chinese hamster ovary cells [50], and that melatonin suppresses transcriptional activation of the ERα in MCF-7 cells by mechanisms involving calmodulin [51–53]. A study in goldfish scales showed that melatonin suppresses the activity of osteoblasts by downregulating the ER [47]; however, melatonin seems to enhance the effects of estradiol in the prevention of bone loss in ovariectomized rats [54]. The nature of the interactions of estradiol with melatonin on bone could be dependent of the estrogen concentration. In the above mentioned experiment, the prevention of the postovariectomy disruption of bone remodeling with pharmacological doses of melatonin required adequate concentrations of estradiol.

3. Melatonin and Osteoporosis

Osteoporosis is a prolonged structural deterioration of the skeletal system, usually associated with age, and with a major prevalence in women. Antiosteoporosis therapies include the use bisphosphonates, estrogen, and calcitonin to inhibit bone-resorbing osteoclasts preventing further bone breakdown. However, these therapies are insufficient in cases of individuals suffering from severe osteoporosis. Drugs that stimulate bone-forming osteoblasts (e.g., teriparatide) are expensive and with important associated side effects [5]. These facts and the above described effects of melatonin on bone physiology prompted studies on their possible utility as a complementary therapy for osteoporosis. Melatonin has been shown at the cell and tissue levels to promote osteogenesis and prevent bone deterioration in mammals [55], birds [56, 57], and fishes [21].

At present, no clinical trials have focused on the possible therapeutic value of melatonin in the treatment of osteoporosis. Some epidemiologic studies re-enforce the possible etiologic role of melatonin in osteoporosis. This is the case from a recent study of Feskanich et al. [58]. This group reported that in a sample of more than 38,000 postmenopausal women, compared with women who never worked night shifts, twenty or more years of night shift work significantly increased the risk of wrist and hip fractures over 8-year follow-up period. Night shift work causes disturbances in the patterns of melatonin secretion as well as severe circadian rhythm disruption [59].

Experimental studies carried out mostly in ovariectomized rats (as a model of menopause) suggest, in general, a protective role of melatonin in preventing bone degradation and promoting bone formation most probably through an action that involves melatonin receptors [4, 5]. Among these studies are those of Oktem et al. [60], suggesting that melatonin's prevention of osteoporosis could be related with its ability to inhibit inducible nitric oxide synthase (iNOS). iNOS plays a critical role in the pathogenesis of osteoporosis since it promotes the generation of nitric oxide, a free radical which contributes to bone resorption caused by estrogen depletion. By using the ovariectomized rat as a model, these authors demonstrated that melatonin treatment markedly reduced the expression of iNOS and the number of apoptotic cells in nucleus pulposus and epiphyseal cartilage of the spinal column, which increased after ovariectomy. Using the same animal model, Uslu et al. [61] described how trabecular thickness and trabecular area of vertebra and femur and cortical thickness of femur, which were significantly reduced after ovariectomy, increased after treatment with melatonin. Recently, Suzuki et al. [62, 63] developed a synthetic melatonin derivative, 1-benzyl-2,4,6-tribromomelatonin (bromomelatonin) which augmented the total bone mineral density of ovariectomized rats more efficiently than melatonin, suggesting its potential use in the treatment of osteoporosis.

4. Melatonin and Adolescent Idiopathic Scoliosis (AIS)

Although the etiology of the AIS is unclear, histomorphometric data on iliac crest biopsies and vertebrae of scoliosis patients showed an impaired function of both osteoblasts and osteoclasts [64, 65]. The persistent osteopenia in patients with AIS [64–66] and the effects of melatonin in bone

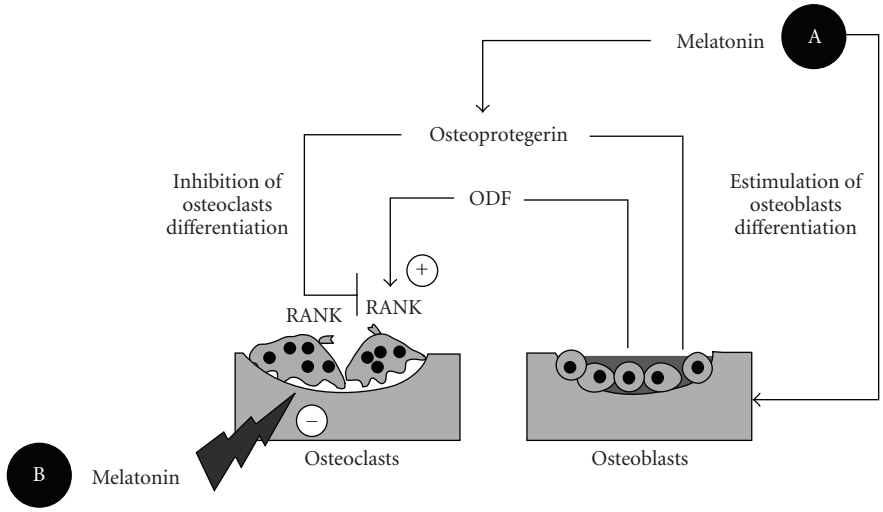

Figure 1: Effects of melatonin on bone metabolism. (a) Melatonin promotes the osteoblast proliferation and the synthesis osteoprotegerin, which inhibits the differentiation of osteoclasts by preventing the binding of ODF (osteoclast differentiation factor) to RANK on the differentiating osteoclasts. (b) Melatonin through its free radical scavenging properties impairs osteoclast activity on bone. Based on Cardinali et al. [4].

metabolism stimulated several studies in animal models and humans related to the possible relationship between melatonin deficits and scoliosis [18, 19, 67–72].

The neuroendocrine hypothesis involving a melatonin deficiency as the source for AIS has generated great interest and controversy. This hypothesis, represented in Figure 2 (modified from Moreau et al. [73]), stems from the fact that experimental pinealectomy in the chicken [13, 14, 18–20, 74, 75], rats, and mice with genetic deficiency of melatonin forced into a bipedal mode of locomotion [16, 17, 22, 76], rabbits [77], and Atlantic salmon [21] results in scoliosis that closely resembles the AIS. Pinealectomy in chickens induces histomorphometric changes in the vertebral column. In particular, the loss of melatonin induces a scoliotic curvature and reduces mean weight and length of cervical vertebrae, possibly due to a reduction in the total number of osteocytes. These results were interpreted to mean that melatonin may act to enhance osteocyte proliferation in the cervical vertebrae [57].

In bipedal pinealectomized rats a reduction in melatonin, as a consequence of the pineal ablation, was found to cause scoliosis [14]. Recently, the possible role of calmodulin (CaM) as a mediator of the melatonin antiscoliosis effects has been proposed [78–80]. Melatonin is an inhibitor of calmodulin [81, 82] and, the loss of this inhibition, due to the lack of melatonin, could be the cause of scoliosis in these animal models. Since tamoxifen is working not only through estrogen receptor but act also as a CaM antagonist, pinealectomized chickens were treated with tamoxifen, and the incidence of scoliosis decreased, presumably due to CaM antagonism of this drug, although measures of CaM activity were not made. In a similar study, carried out on C57BL6 mice (which are genetically melatonin deficient), it was observed that they develop scoliosis when rendered bipedal; in these animals as well, tamoxifen improved the scoliosis deformities. In humans, Acaroglu et al. [78] compared the content of CaM and melatonin in muscle and platelets of scoliotic and healthy populations. The patients suffering with AIS had asymmetric distribution of CaM in the paraspinal muscles, with its concentration being higher at the convex side and lower at the concave curvatures of the spinal column, whereas neither platelet melatonin nor platelet CaM was found to be representative of the muscle protein values.

Not all data support the hypothesis of the reduction of melatonin as the cause of scoliosis. Melatonin therapy after pinealectomy in young chickens had no effect on the development or progression of scoliosis [83], and cutting of the pineal stalk of the chicken, without removal of the pineal gland, also resulted in scoliosis, whereas suppression of melatonin secretion by exposure of the chickens to constant light did not induce spinal curvature [84]. This suggests that the cause of the scoliosis is more related with the surgery than with the changes in melatonin secretion. Furthermore, although melatonin receptors are present in the spinal cord of the chicken, the changes detected in melatonin receptor binding after pinealectomy cannot explain why scoliosis develops in some chickens after pinealectomy, while it does not in others [85].

Bipedal ambulation in mammals is required, associated to low levels of melatonin, to generate scoliosis [22, 86]. The disturbance of equilibrium and other postural mechanisms secondary to a deficiency of melatonin may promote development of lordoscoliosis with vertebral rotation especially in the bipedal posture [86]. However, pinealectomized young rhesus monkeys (8–11 months old) do not develop scoliosis. This fact suggests that the possible etiologic factors producing idiopathic scoliosis in lower animals may be different from primate, and findings in birds and rodents cannot necessarily be extrapolated to human beings [87, 88]. Since monkeys in captivity, placed in cages that greatly restrict their

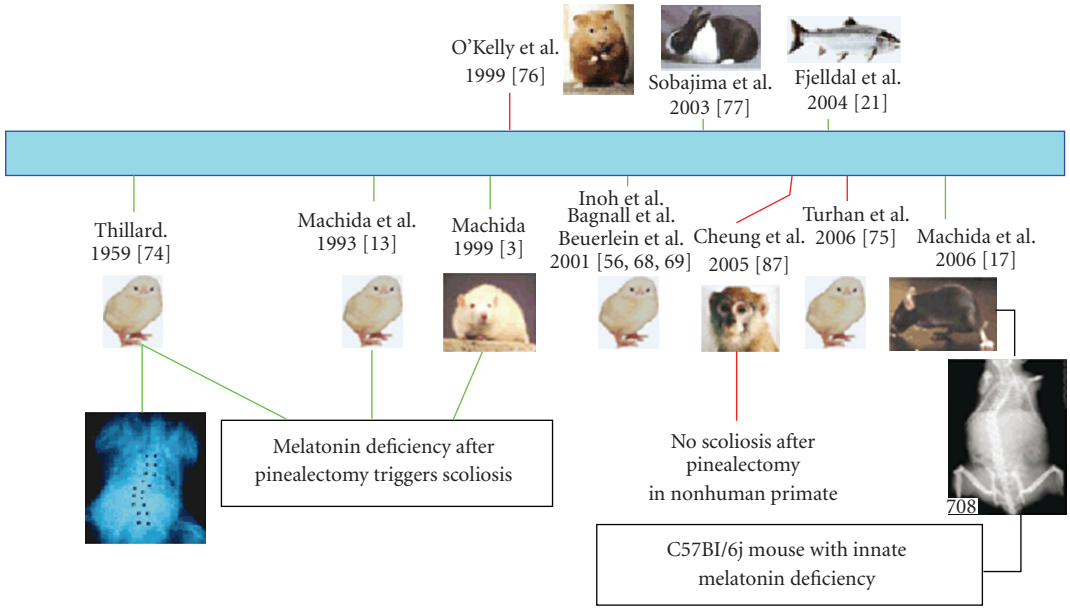

Figure 2: Summary of the experiments focused on the hypothesis involving a melatonin deficiency as the source for AIS. Effective (green lines) and noneffective (red lines) results are indicated. Modified from Moreau et al. [73].

mobility, spend most time in quadrupedal position, whether or not posture and gravity are determinants in the response to pinealectomy in terms of scoliosis is still unclear.

In humans, the question of the possible role of melatonin in scoliosis has been addressed using different analytical approaches (see Figure 3, modified from Moreau et al. [73]). One of these approaches was the detection of the possible changes in melatonin production in scoliotic patients. In this regard, Sadat-Ali et al. [89] found serum melatonin levels significantly lower in AIS patients than in healthy controls these results support the hypothesis that serum melatonin levels may contribute to the pathogenesis of idiopathic scoliosis. However, no significant difference between patients with AIS and controls regarding in serum concentration of melatonin or levels of urinary excretion of 6-sulfatoxy-melatonin was found by other authors; they concluded that a permanent melatonin deficiency is not a causative factor in the etiology of AIE in humans [67, 71, 90–92].

Genetic studies have screened AID and healthy patients looking for gene variants or single nuclear polymorphism in genes involved in the control of melatonin synthesis or in the expression of melatonin receptors. The screening of the MT2 receptor gene polymorphism in AIS patients and controls [93] suggests that this is a gene involved in the predisposition for AIS. However, the promoter polymorphism of the MT1 gene was not associated with the occurrence or curve severity of AIS, thus, indicating that MT1 gene may not be involved in the etiopathogenesis of AIS [94]. Polymorphisms of the arylalkylamine N-acetyltransferase (AANAT) gene were not associated with AIS whereas single nuclear polymorphism of tryptophan hydroxylase 1 gene (TPH1) seems closely related with the dysfunction of melatonin in AIS [95]. Other authors did not observe mutations in the coding region of the gene for human melatonin receptor in patients with familiar AIS [96].

A third category of studies have focused on the possible changes in melatonin receptors in AIS patients. The expression of MT2 melatonin receptors in bilateral paravertebral muscles in AIS and congenital scoliosis is asymmetric, being higher in muscles on concave side than that on convex side of the spinal column in AIS, but MT1 expression was not significantly different [97, 98]. These differences in the expression of melatonin receptors have been considered as secondary to the bilateral asymmetry due to force exerted on the scoliotic spine and not important in the pathogenesis of AIS [97, 98].

A different and interesting approach presented by Moreau et al. [99] could clarify the discrepancies regarding the role of melatonin in AIS. These authors consider that instead of changes in melatonin production or expression of melatonin receptors, the problem may be in the specific response of the osteoblast to melatonin in AIS patients. They demonstrated a melatonin signaling dysfunction occurring in osteoblasts isolated from AIS patients but not in similar cells isolated from healthy subjects. In most cells, melatonin inhibits the forskolin-stimulated adenylyl cyclase activity and decreases cAMP. In contrast, osteoblasts from patients with AIS showed a lack or a marked inhibition by melatonin of the forskolin-stimulated adenylyl cyclase activity [99]. The cause is an increased phosphorylation of serine residues affecting the activity of G-inhibitory proteins normally associated with melatonin surface receptors [99]. In response to estradiol, osteoblasts from a specific group of AIS patients treated with melatonin decreased the cAMP abnormally increased by the indoleamine [100]. From the findings of Moreau et al. [99], a preliminary molecular classification of AIS patients based on the cellular response to melatonin (changes in cAMP), has been proposed [101]. Recently, the same group [73] have developed the first blood test to detect children without symptoms who are at risk of developing scoliosis. This test

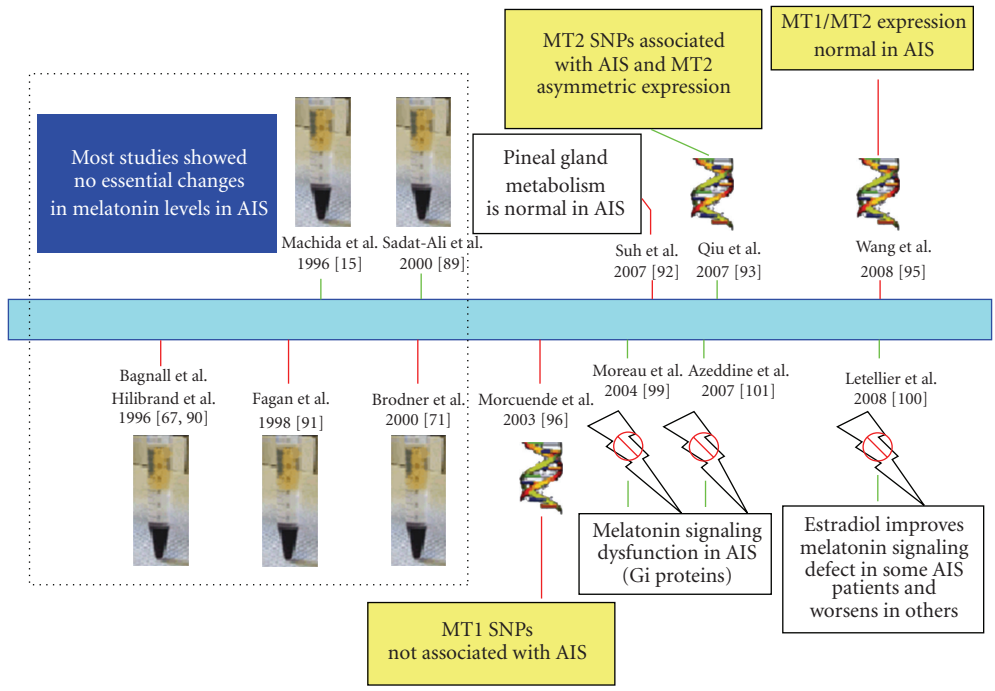

Figure 3: Summary of the main experimental approaches carried out in humans to clarify the role of melatonin on the AIS. Left, (doted rectangle), studies of changes in melatonin production. Yellow labels are the screening of polymorphisms in genes related with pineal function. White labels identify studies of possible changes in melatonin metabolism or response of target tissues. As in Figure 2, effective (green lines) and noneffective (red lines) results are indicated. Modified from Moreau et al. [73].

is based on the cellular reaction to melatonin. The most recent clinical study on the relationship between melatonin and AIS has been a prospective analysis on the correlation of serum melatonin levels (monitored yearly for 3–6 years) and curve progression in 40 patient with moderate to severe AIS [102]. From 22 patients with normal melatonin levels (similar to healthy age-matched controls), 16 had stable scoliosis whereas 6 had progressive scoliosis. The 16 patients with low melatonin levels were treated with oral melatonin (3.0 mg 1.5–2.0 hour before the desired sleep time). Twelve of them developed stable scoliosis, whereas four continued to have progressive course. This is the first description of the therapeutic application of melatonin for this disease and suggests that melatonin supplementation could prevent the progression of the scoliosis, especially in mild cases. Obviously, more clinical trials are required to strengthen on the evidence regarding the benefits of melatonin and treatment for scoliosis.

5. Concluding Remarks

From the above analyzed data, and despite some controversial results which demand further clarification, the following conclusions are proposed. (a) Melatonin seems to promote bone formation and prevent bone resorption via several mechanisms which include the increase in the osteoblastic activity and differentiation, as well as the reduction in osteoclastic differentiation and activity, and by increasing osteoprotegerin expression and scavenging the free radicals responsible of bone resorption. (b) Melatonin may be an etiologic factor in the postmenopausal osteoporosis, and a therapeutic tool for this pathology, as an adjuvant with conventional treatments such as the administration of estrogens. (c) The recent data concerning the association of melatonin and AIS point toward their possible usefulness as both a diagnostic and therapeutic tool. (d) The experimental evidence on animal models suggests the value of clinical trials to assess the therapeutic possibilities of melatonin in bone diseases.

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Research Article

Superparamagnetic Bifunctional Bisphosphonates Nanoparticles: A Potential MRI Contrast Agent for Osteoporosis Therapy and Diagnostic

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A bone targeting nanosystem is reported here which combined magnetic contrast agent for Magnetic Resonance Imaging (MRI) and a therapeutic agent (bisphosphonates) into one drug delivery system. This new targeting nanoplatform consists of superparamagnetic γFe_2O_3 nanoparticles conjugated to 1,5-dihydroxy-1,5,5-tris-phosphono-pentyl-phosphonic acid (di-HMBPs) molecules with a bisphosphonate function at the outer of the nanoparticle surface for bone targeting. The as-synthesized nanoparticles were evaluated as a specific MRI contrast agent by adsorption study onto hydroxyapatite and MRI measurment. The strong adsorption of the bisphosphonates nanoparticles to hydroxyapatite and their use as MRI T2* contrast agent were demonstrated. Cellular tests performed on human osteosarcoma cells (MG63) show that γFe_2O_3@di-HMBP hybrid nanomaterial has no citoxity effect in cell viability and may act as a diagnostic and therapeutic system.

1. Introduction

Bisphosphonates exhibits a powerful binding affinity to bones and are routinely used for treatment in bone resorption and other bone disorders like Paget's disease, osteoporosis, or tumor induced osteolysis [1]. The binding to bone mineral depends upon the P-C-P structure and is enhanced by including a hydroxyl group (hydroxy methylene bisphosphonate, called HMBP in the text). This was probably due to tridendate binding hydroxyl substituted bisphosphonates to calcium. In contrast, bisphosphonates lacking a hydroxyl group, that provide a bidendate binding to calcium crystals, had significantly lower binding affinities [2]. Hence HMBP molecules, such as Alendronate (4-amino-1-hydroxybutylidene bisphosphonic acid), inhibit osteoclast-mediated bone resorption [3]. With the recent developments in magnetic resonance, in vivo studies showed that patients with, and without, osteoroporotic fractures could better be separated with parameters of bone architecture obtained by MRI than BDM [4]. For molecular imaging, the use of nanoparticles emerge as very exiting nanoobjects in that many functionalities can be added to the surface of the particle. More specifically, superparamagnetic iron oxide [5] (SPIO, hydrodynamic diameter >50 nm) and ultrasmall superparamagnetic iron oxide (USPIO, hydrodynamic diameter <50 nm) particles have been introduced as an MRI contrast agent after the gadolinium chelates and appear to be currently a more relevant agent than Gd chelates due to the high MR signal per unit of metal. As these particles are made of thousands iron atoms, they defeat the inherent low contrast agent sensitivity of MRI and thus can be detected at micromolar concentration of iron. Moreover the iron ions are much less toxic than the gadolinium ones and can be reused or recycled by cells using normal

biochemical pathways for iron metabolism [6, 7]. Our previous studies have shown that bisphosphonate such as 1-phenyl-1-hydroxymethylene-1,1-phosphonic acid (HMBP-COOH) [8] or 1-hydroxy-2-(imidazol-1-yl)ethylidene-1,1-bisphosphonic acid (zoledronate) [9] act as very efficient ligand for iron oxide nanoparticles. In the case of quaternary ammonium bisphosphonate coated iron oxide nanocrystals, it has been shown that this hybrid nanocrystals [10] presented adequate performance for blood remanance and weak liver capture. No significant desorption of the coating molecules was observed on steel plates. In recent work [11] it has been demonstrated that pretreatment of metal alloy surface with an aqueous polyallylamine bisphosphonate solution (BP-... NH_2) result in the formation of a molecular bisphophonate layer that permit the attachment via the amine terminated function of vector binding agent for therapeutic gene delivery. After 30 day incubation, the layer is not altered indicating that a mechanism of desorption reabsorption of BP molecules seems to be highly unlikely. In this article, an innovative approach is presented, leading to the optimization of the nanoparticle structure to achieve selective targeting for osteoporosis imaging and therapy. Superparamagnetic nanoparticle surface are passivated using a bifunctional passivating agent such as 1,5-dihydroxy-1,5,5-tris-phosphono-pentyl-phosphonic acid (call di-HMBP in the text, Scheme 1). One HMBP function complexes the nanocrystal surface and the other one at the outer surface allows bone targeting. A stable ferrofluid (γFe_2O_3@di-HMBP) is obtained on large concentration and pH range. The large numbers of HMBP functionalities on the magnetic core of the particle have a strong affinity for hydroxyapatite and can be used for bone targeting. The feasibility of such process is demonstrated by the complexation of the hybrid nanomaterial to calcium ions and hydroxyapatite and imaged using MRI.

2. Materials and Methods

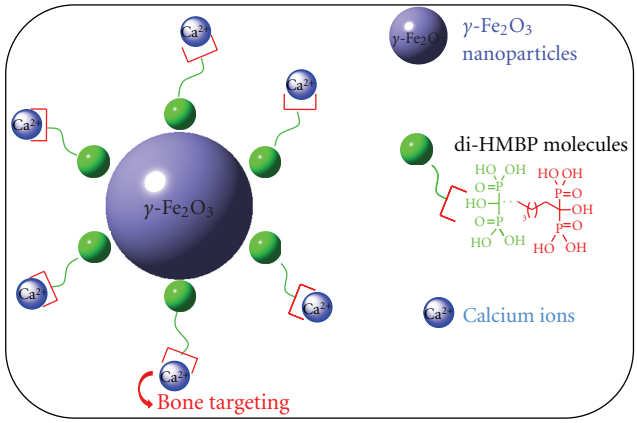
2.1. Materials and Reagent. IR spectra were recorded on a Thermo Electron Corporation Nicolet 380 FTIR (KBr pellet). UV-visible spectra were recorded on a Varian Cary 50 Scan UV-Visible spectrophotometer. Transmission electron microscopy (TEM) measurements were carried out using a Philips CM10. ^{1}H-NMR spectra were obtained on a Varian Gemini spectrometer at 200 MHz with chemical shifts being reported as ppm from trimethylsilane as internal standard. The size and the zeta potential of the nanocomplex were determined by dynamic laser light scattering (DLS) on a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK. All chemicals products used for nanoparticles and bisphosphonate molecules were purchased from Sigma-Aldrich (St Louis, MO). Millipore H_2O was employed for the preparation of all aqueous solutions.

2.2. Synthesis of (1,5-Dihydroxy-1,5,5-Tris-Phosphono-Pentyl)-Phosphonic Acid [12] (Di-HMBPs). In a 50 mL round-bottom three-neck flask equipped with a thermometer, glutaryl chloride (18 mmol) was added dropwise, under argon, at −5°C, to tris(trimethylsilyl) phosphite (72 mmol). When addition was completed, reaction mixture was allowed to stand at room temperature for 1 hour. The evolution of the reaction was monitored by ^{31}P{1H} NMR. Then, volatile fractions were evaporated under reduced pressure (0.1 Torr) before methanolysis (20 mL). After evaporation, crude products were precipitated in diethylether and lyophilized. The pure product was obtained in 95% yield. ^{31}P NMR {^{1}H} (161.9 MHz, D_2O) δ 19.3, ^{1}H NMR (400.1 MHz, D_2O) δ 1.78–2.05 (m, 6H, C(OH)-$(CH_2)_3$-C(OH)), ^{13}C NMR {^{1}H} (100.6 MHz, D_2O) δ 18.1 (-CH_2-CH_2-CH_2-), 34.0 (-CH_2-CH_2-CH_2-), 73.2 (t, $^1J_{P-C}$ = 143.7 Hz, P-C(OH)-P).

2.3. Synthesis of γFe_2O_3@Di-HMBP Nanocrystals. To prepare noncoated γFe_2O_3 particles, the first step is to add a solution of dimethylamine 40% in water ($(CH_3)_2NH$, 10.5 mL) to an aqueous micellar solution of ferrous dodecyl sulfate ($Fe(DS)_2$) (0.61 g, 10^{-3} mol). The solution is stirred vigorously for 2 hours at 28.5°C and the resulting precipitate of uncoated nanocrystals is isolated from the supernatant by centrifugation. In the second step, this precipitate is washed with an acidic solution (HCl 10^{-1} mol · L^{-1}) and a solution of di-HMBPs molecules ($n = 10^{-4}$ mol in 30 mL of water) is added. The solution is stirred for two hours at room temperature. The precipitate that appears is washed with an acidic solution (HCl 10^{-1} mol · L^{-1}). Free HMBP are isolated from the coated particles thanks to a magnetic field and by centrifugation. The magnetic nanocrystals coated with di-HMBP molecules are dispersed in water. The initial pH is equal to 4 and then progressively increased to pH 7.4 by addition of sodium hydroxide NaOH (10^{-1} mol · L^{-1}). The iron concentration is deduced from UV-vis absorption.

2.4. Nanocrystal Surface Characterization. FTIR spectroscopy is used to demonstrate nanocrystal surface complexation via phosphonate groups. The average number of molecules per nanocrystal is deduced with ^{31}P NMR spectroscopy. A range of concentrations of free di-HMBP (NMR ^{31}P{1H} (80.9 MHz): 19.17 ppm solution added with NaH_2PO_4 (in capillary, 10^{-1} mol · L^{-1}; NMR ^{31}P{1H} (80.9 MHz): 0 ppm) was prepared for calibration. The di-HMBP molecules are removed from magnetic γFe_2O_3 nanoparticles by addition of sodium hydroxide NaOH (1 mol · L^{-1}) in order to avoid shifting of the ^{31}P NMR signal. The supernatant is analyzed with ^{31}P NMR and the concentration (number of molecules per nanocrystal) of di-HMBP into the sample is deduced from this calibration plot.

2.5. Analysis of the Size and Surface Charges of the γFe_2O_3@Di-HMBP Nanocrystals. The mean particle size was determined by transmission electron microscopy. Colloid suspensions were deposited directly onto a carbon-coated copper grid. The size and the zeta potential of the nanocomplex was determined by dynamic laser light scattering (DLS) on a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK. Each sample was



Scheme 1: Superparamagnetic γFe_2O_3@di-HMBPs for bone targeting.

analyzed at room temperature with diluted ferrofluid ([Fe] = $5 \cdot 10^{-4}$ mol $\cdot$ L^{-1}) at pH = 7.4.

2.6. Calcium Complexometric Titration. Standard procedures with Eriochrome black *T* (EBT) was used to quantify the amount of calcium ions in solution. The EBT was mixed to γFe_2O_3@di-HMBP (or free di-HMBP) aqueous solution ([Fe] = $1,47 \cdot 10^{-2}$ mol $\cdot$ L^{-1}) at pH 10. Then this solution is titrated with calcium solution ([Ca^{2+}] = $1,44 \cdot 10^{-4}$ mol $\cdot$ L^{-1}) until the color solution change from blue to pink for free di-HMBP and from green to brown for γFe_2O_3@di-HMBP particles solution. The variation of color is due to the complexation between EBT and calcium ions. Then the amount of calcium ions complexed with the HMBP functionality is deduced.

2.7. Magnetic Properties and Magnetic Resonance Imaging. The magnetic behavior of the as-synthesized nanoparticles is characterized using the MIAplex^R reader (Magnisense). The MIAplex reader [13] measures the nonlinear response of the magnetic labels when they are exposed to a multi-frequency alternating magnetic field. This specific signature [14] is based on $d^2B(H)/dH^2$.

MR imaging of the test tubes was performed using a 4.7 T MR scanner (Bruker). For measurements of T1 relaxation times, axial spin echo (SE) sequences were obtained with TR values of 10,000 ms as well as TE of 16 ms at 4.7 T. For measurements of T2* relaxation times, axial T2*-weighted SE images were obtained with a TR of 800 ms and TE of 6.4 ms at 4.7 T.

2.8. In Vitro Hydroxyapatite Targeting. The lyophilized hydroxyapatite [15] with a ratio Ca/P equal to 1,64. HA (10 mg/mL) was suspended in a 5 millimeter γFe_2O_3@di-HMBP sol 0,4 mg/mL (Fe = $5 \cdot 10^{-3}$ M). Then nanoparticles are incubated and shaken with HA at 37°C during 24 hours. After filtration and water washing with a syringe filter with 0.45 μm pore size, HA is resuspended in sol and lyophilized for infrared spectroscopy. The concentration of nanoparticles remained in the water suspension was measured by UV/VIS spectrophotometer at 350 and 480 nm for the calculation of the amount bound to HA.

2.9. Cell Viability. Human osteosarcoma cells (MG63) line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% calf serum. MG63 osteoblast-like cells used in the present study were obtained from the American Type Culture Collection (ATCC N° CRL 1427).

Cell viability was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at day 1, day 3, and day 5. Cells were seeded at a density of 20×10^3 cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 1, 3, and 5 days. Then, medium was removed and replaced by 10% FCS-medium containing increasing concentrations γFe_2O_3@di-HMBP nanocrystals. After 1, 3, and 5 days of incubation, cells were washed with phosphate buffered saline (PBS, Invitrogen) and incubated with 0.1 mL of MTT (2 mg/mL, Sigma-Aldrich) for additional 4 hours at 37°C. The insoluble product was then dissolved by addition of DMSO (Sigma-Aldrich). Optical density was measured at 570 nm using a Labsystems Multiscan MS microplate reader. Each in vitro experiment was performed three times, with four wells per sample per experiment.

2.10. Cell Labelling. The labeling of living cells is evaluated using Prussian blue staining for γFe_2O_3@di-HMBPs nanocrystals. The principle of Prussian blue staining is that the ferric iron (Fe^{3+}) in the presence of ferrocyanide ion is precipitated as the highly colored and highly water-insoluble complex, potassium ferric ferrocyanide, Prussian blue. The cells were cultivated for 24 hours in eight-well chamber slides in the presence or not of γFe_2O_3@di-HMBPs nanocrystals. The cells were then washed three times with PBS, fixed with acetone (10 minutes) and dried at room temperature for 20 mn. The attached cell monolayer was incubated with 5% potassium ferrocyanide (5 minutes), washed with PBS and then incubated again with solution containing 5% potassium ferrocyanide and 10% hydrochloric acid for 10 minutes and washed with distilled water three times. The iron particles in the cells were observed as blue dots using an optical microscope with phase contrast.

3. Results and Discussion

Nanoparticles functionalization plays a major role within nanotechnologies applications. Scheme 1 describes the procedure to design a new MRI nanoparticle for targeted drug delivery to bone. Small γFe_2O_3 nanocrystals were chosen for their superparamagnetic behavior and their high T2 contrast agent sensitivity for MRI. The 1,5-dihydroxy-1,5,5-tris-phosphono-pentyl-phosphonic acid (di-HMBPs) was chosen for the two HMBP functionalities: one HMBP moiety as anchoring agent for γFe_2O_3 surface and the second as targeting function due to strong affinity for bone. Our approach requires the two HMBP functions of

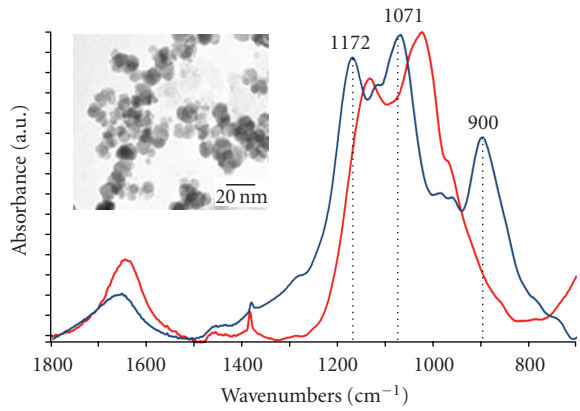


FIGURE 1: IR spectra of di-HMBP free molecules (blue curve) and γFe_2O_3@di-HMBP (red curve). Insert: transmission electron microscopy image taken of a nanoparticle solution at pH 7.

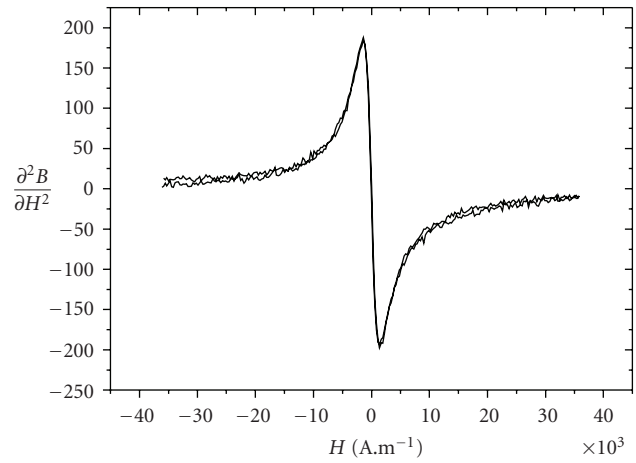


FIGURE 2: γFe_2O_3@di-HMBP second derivative of the magnetization recorded at pH 7.4, in H_2O solutions.

the molecule to be separated by a short spacer, to avoid the nanoparticles anchoring with the two HMBP moieties, leading to nanoparticles aggregation and lost of the specific bone targeting.

Maghemite γFe_2O_3 nanocrystals were prepared as described previously [16] by soft chemistry. At the end of the synthesis, a solution of di-HMBP in water at pH 4 is added to the bare nanoparticle dispersion. The pH was then progressively increased to pH 7.4 by the addition of sodium hydroxide NaOH, thus achieving a stable dispersion of nanoparticles.

After dialysis, the dispersed solution is lyophilized. The powder is easily dispersed in water and the nanoparticles sols are stable over a broad range of pH (4–12) and concentration (over 40 wt%), in suitable ionic strength (<0.6 mol · L^{-1}) and in various biological buffers such as PBS and Hepes. The TEM image (insert Figure 1) of deposited nanocrystals indicates an average diameter and a polydispersity, respectively, equal to 11 nm and 20%.

IR spectroscopy analysis (Figure 1) shows that the phosphonate groups are highly interaction with the nanoparticle surface.

For the free HMBP-COOH molecules (blue curve), within the P–O stretching region (1200–900 cm^{-1}), the spectrum exhibits two sharp peaks at 1172 and 900 cm^{-1}, assigned to P=O and P–OH, respectively [17]. The broad band at 1071 cm^{-1} is characteristic for the vibrational mode for the PO_3 group [18].

Comparing the γFe_2O_3@di-HMBP nanocrystals (red curve) with the free di-HMBP solution (blue curve), the large changes observed within the P–O stretching region (1200–900 cm^{-1}) show that a strong interaction between the phosphonate headgroup and the Fe_2O_3 surface is present. These results are consistent with phosphonate binding to the oxide surface [19] and we can suggest that the Fe atoms within the particle surface are coordinated by oxygen atoms from the phosphonate groups [20].

^{31}P NMR titration is used in order to quantify the average number of molecules per nanocrystal. An average number of 2100 ± 100 di-HMBP molecules per nanoparticle is obtained, corresponding to 0.1 equivalent per Fe ions (around 0.3 per surface Fe ions).

Dynamic light scattering was used to characterize zeta potential and hydrodynamic diameter. This measurement is an indication of surface charge on a particulate species, which plays an important role in determining solution stability, susceptibility to aggregation and precipitation problems, as well as protein and cellular surface binding in vivo. At physiological pH, the γFe_2O_3@di-HMBPs particles exhibit a negative zeta potential (−54 mV) and a hydrodynamic diameter of 36 nm suggesting the presence of few aggregates (mean crystalline core of 11 nm). The negative charge surface suggests the presence of free HMBP functionalities on the magnetic core of the particle (Scheme 1). To determine the number of free HMBP, we used standard procedures of colorimetric tests to deduce the number of calcium ions complexed per γFe_2O_3@di-HMBP nanoparticles. For free di-HMBP molecules, we found 3.8 calcium ions complexed per molecule meaning that each HMBP functionality may complex about 2 calcium ions. The amount of calcium ions complexed per nanoparticle is found equal to 3100 ± 200. Considering that each HMBP functionality may complex 2 calcium ions, an amount of 1550 free HMBP per nanoparticle is deduced. This result is consistent with NMR measurements leading to 2100 HMBP per nanoparticle. Hence, the free HMBP functionalities at the outer of the nanoparticles surface should allow their bone targeting and the increase of bone mineral density.

The magnetic properties of these nanoparticles have been studied using a MIAplex^R reader.

The second derivative of magnetization $d^2B(H)/dH^2$ (Figure 2), presents one maxima and one minima with no hysteresis loop. This specific magnetic signature is characteristic of superparamagnetic behavior of particles with low dipolar interaction [21]. This superparamagnetic behavior allows to use these particles as contrast agent for MRI.

To investigate the MR signal enhancement effects, the aqueous as-prepared nanoparticles at different Fe concentrations were measured on a 4.7 T MRI scanner. As shown

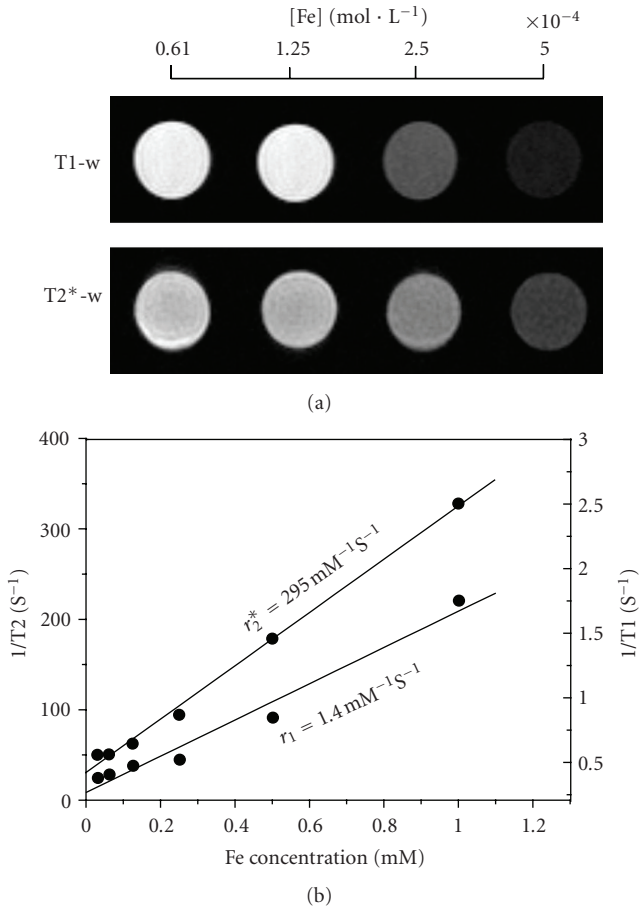


FIGURE 3: (a) T1 weight MR images and T2* weight MR images of aqueous solutions of as-synthesized nanoparticles at different Fe concentrations; (b) T1 and T2* relaxation rates (1/T1, 1/T2*) plotted against the Fe concentration for the various aqueous solutions.

in Figure 3(a), both T1 and T2* weighted images change drastically in signal intensity with an increasing amount of nanoparticles, indicating that as synthesized nanoparticles generated MR contrast on both longitudinal (T1) and transverse (T2*) proton relaxation times weighted sequences. Figure 3(b) shows the relaxation rates 1/T1 and 1/T2* as a function of the iron concentration. The relaxation rates varied linearly with the iron concentration, as expected. The longitudinal r_1 and transverse r_2* relaxivities (corresponding to the slopes of the lines) are found to be 1.40 Fe mM^{-1}s^{-1} and 295 Fe mM^{-1}s^{-1}, respectively. Such values for r_1 and r_2* suggest that HMBP coated nanoparticles can act as both T1 and T2* contrast agents taking into account their small size, but seem to be more favourable as T2* contrast agents due to their much larger r_2* value.

One of the factors that makes HMBP most potent BP drugs is its high skeletal uptake and retention, which is directly related to its affinity towards hydroxyapatite [22] (HA). To demonstrate the specific targeting of γFe_2O_3@di-HMPBs nanocrystals to bone, standard in vitro assay [12] were performed to demonstrate the strong affinity

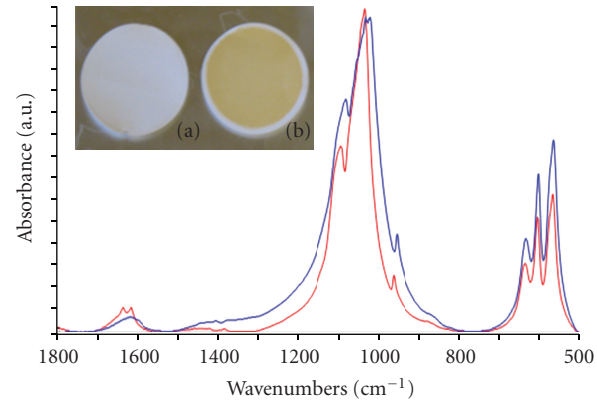


FIGURE 4: IR spectra of HA (red curve) and HA incubated with γFe_2O_3@di-HMBP (blue curve) for 24 hours and separated from free nanoparticules. Insert: optical image taken from HA (a) and HA incubated with γFe_2O_3@di-HMBP (b).

of those new MRI contrast agent with hydroxyapatite. A γFe_2O_3@diHMBP sol ([Fe] = 5 · 10^{-3} M) have been incubated with HA at 37°C, and then separated and washed using a 0.45 μm filter. The binding capacity of the as-synthesized nanocomplexes has been studied using UV-vis and infrared (Figure 4) spectroscopies. As shown insert Figure 4, the change of HA color from white to brown indicates γFe_2O_3@di-HMBPs binds HA with very high affinity due to the high amount of iron nanoparticles within HA. The concentration of nanoparticles remained in the water suspension was measured by UV-vis spectrophotometer at 350 and 480 nm for the calculation of the amount bound to HA. The deduced bound amount is equal to 0.05 ± 0.01 mg of nanoparticles per mg of HA (eq. 0.19 mM HMBP per mg HA). Figure 4 displays the IR spectrum of HA (blue curve) and incubated HA with γFe_2O_3@diHMBP nanoparticles (red curve).

The HA spectrum (red curve) exhibits different bands between 1250–600 cm^{-1} that are characteristic of the P–O stretching region within HA [10]. For the HA nanocomplex, the analysis of the P–O stretching region is complicated due to strong background absorbance of the HA matrix (ν(PO4)) [23]. The HA incubated with γFe_2O_3@di-HMBP (blue curve), the P–O stretching region is broadened compared to initial HA. This is very difficult to clearly assign this effect. Obviously, more experiments are needed to elucidate the exact mechanism of nanoparticle surface bonding on HA.

The magnemite nanocrystals deduced from UV-vis spectroscopy and the brown color (insert Figure 4) of incubated HA with γFe_2O_3@di-HMBP nanocrystals are suggesting selective interaction of the nanocomplex with HA and then potential targeting to bone.

In order to assess cell viability we performed viability tests on osteosarcoma MG-63 cells, a cancer line, but a pertinent model to study efficiently the behavior of osteoblastic cell line [24]. The as-synthesized nanocrystals were incubated with MG63 osteosarcoma cells precultured for 24 hours, 3 days and 5 days for various extra cellular

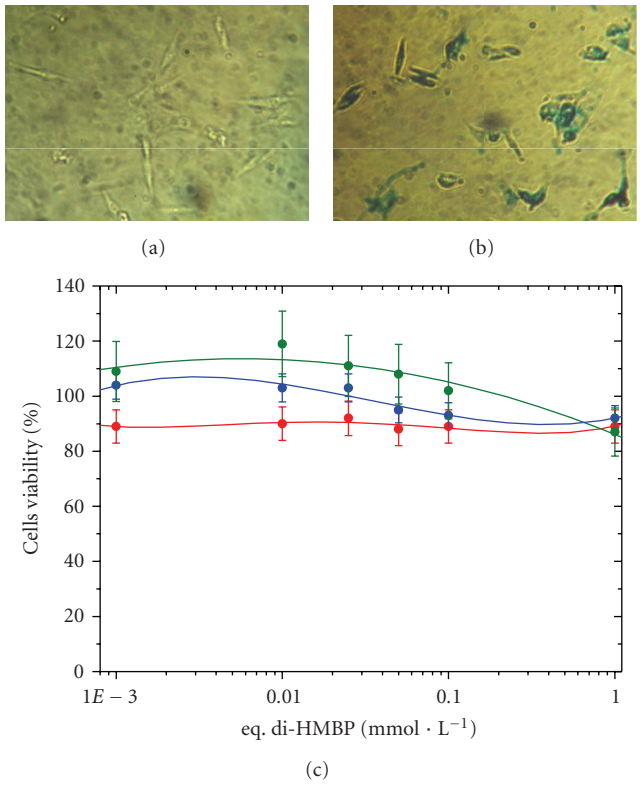


FIGURE 5: Optical blue prussian images of MG63 cells control (a) and MG63 cells incubating 24 hours with γFe_2O_3@di-HMBPs at 100 μM (b). Comparative effects of γFe_2O_3@di-HMBPs on MG-63 osteoblast cells proliferation (c) for 24 hours, (red curve), 3 days (blue curve) and 5 days (green curve).

iron concentrations up to 3 mmol · L^{-1} (1 eq. mmol · L^{-1} di-HMBP). The proliferation of MG63 cells was indicated by the MTT assay as shown in Figure 5).

For the three times of incubation, MTT proliferation assay showed normal growth of osteoblast cells. No cytoxicity was observed. To determine the intracellular uptake of γFe_2O_3@di-HMBPs nanocrystals, blue prussian imaging was performed on human MG63 cells (Figures 5(a) and 5(b)). The iron particles into the cells were observed as blue dots using an optical microscope with phase contrast (Figure 5(b)). This picture indicates massive and uniform internalization of nanocrystals within the cells. Hence, the γFe_2O_3@di-HMBPs nanocrystals may act as a diagnostic and therapeutic system. A full biological study is in progress to understand the mechanism of such nanoparticles for osteoporosis treatment and diagnostic. The aim of this work is to describe influences of nanoparticles on protein expression patterns related to the differentiation and mineralization of bone-forming cells, viability, remodeling of cell architecture, cell adhesion, and assembly of extracellular matrix in human normal cells.

4. Conclusion

A bone-targeted MRI contrast agent have been designed with superparamagnetic nanoparticles and bisphosphonate moieties. HMBP functionalities exhibits highly iron and calcium complexing effects. To test feasibility of such nanosystem, this system have been complexed to hydroxyapatite to demonstrate bone targeting and increasing bone mineral density to reduce the incidence of major osteoporotic fracture. Moreover, the superparamagnetic behavior of such nanoparticle allows them to be used as MRI contrast agent in order to improve the therapeutic diagnostic for osteoporosis.

Acknowledgments

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Review Article

Osteoporosis Syndrome in Thalassaemia Major: An Overview

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Osteoporosis in thalassaemia major (TM) represents a prominent cause of morbidity. The mechanism of pathogenesis of bone disease (BD) in TM is multifactorial and complicated. Peak bone mass is achieved shortly after completion of puberty and normally remains stable until the third decade of life when age-related bone mass begins. Growth hormone (GH) and sex steroids play a crucial role in bone remodeling and in the maintenance of skeletal architecture during adult life. GH and insulin growth factors (IGFs) have anabolic effect in bone formation. Sex steroids act probably by increasing the expression of RANKL by osteoblastic cells and alterations in the RANK/RANKL/OPG system in favor of osteoclasts. Impaired GH secretion and lack of sex steroids in thalassemic patients due to pituitary damage, contribute to failure of achieving optimal peak bone mass. Other endocrine complications such as hypoparathyroidism and vitamin D deficiency have also a detrimental role on bones in TM. It is still questionable whether the international criteria for defining osteopenia and osteoporosis are relevant to patients with TM; also a question arises for the diagnostic methods such as DEXA scan and management of osteoporosis with known treatment protocols, in the thalassaemic patient.

1. Introduction

Osteoporosis is a universal medical problem, affecting both genders. It is generally accepted that its main causes are aging, genetic disorders of osteogenesis, lack of certain nutritional elements or physical activity, and endocrine disorders mainly estrogen deficiency. Other causes include neoplastic disorders, gastrointestinal disorders causing malabsorption, liver diseases, inflammatory conditions, and drugs. Osteopenia and osteoporosis represent prominent causes of morbidity in patients of both genders with thalassaemia [1]. During the last decade, the presence of osteopenia and osteoporosis in well-treated thalassaemics has been described in different studies with high prevalence up to 50% [2]. The pathogenesis of osteoporosis in thalassaemia major (TM) is complicated and differs from the pathogenesis of bone deformities characteristically found in nontransfused patients who develop bone distortion mainly due to ineffective haemopoiesis and progressive marrow expansion [3].

Several factors are implicated in reduction of bone mass in TM. Delayed sexual maturation, growth hormone (GH) and insulin growth factor-(IGF)-1 deficiency, parathyroid gland dysfunction, diabetes, hypothyroidism, ineffective haemopoiesis with progressive marrow expansion, direct iron toxicity on osteoblasts, as well as liver disease have been indicated as possible etiological factors for thalassaemia-induced osteoporosis [2, 3]. Furthermore, iron chelation has correlated with growth failure and bone abnormalities, and high desferrioxamine dosage has been associated with cartilage alterations [2, 4, 5].

2. The Role of RANK/RANKL/OPG System

Two distinct cell types are involved in the maintenance and renewal of bone: (A) osteoblasts—cells of mesenchymal lineage responsible for bone formation and (B) osteoclasts—cells of hematopoietic lineage responsible for bone resorption and remodeling. In thalassemia patients, progressive "aging" of the bone starts even in childhood by the gradual development of an imbalance between augmented osteoclastic resorption and insufficient osteoblastic bone formation.

The bone marrow stromal cell molecules which are members of the tumor necrosis factor (TNF) receptor superfamily, receptor activator of nuclear factor-kappa B ligand (RANKL), receptor activator of nuclear factor-kappa B (RANK), and osteoprotegerin (OPG), play a central role in bone remodeling via conjunction with various cytokines and calciotropic hormones [6]. GH and IGF-1 stimulate the production of OPG and its accumulation in the bone matrix. Sex steroids act probably by increasing the expression of RANKL by osteoblastic cells [6]. Alterations in the RANK/RANKL/OPG system in favor of osteoclasts are characteristic in thalassaemia due to complicated mechanisms involving chronic anemia, iron toxicity, and endocrine complications.

In patients with Thalassaemia, elevated markers of bone resorption such as serum alkaline phosphatase, osteocalcin, urinary levels of N-telopeptides of collagen type I (NTX), serum levels of tartrate resistant acid phosphatase isoform 5b (TRACP-5b), pyridinoline, and deoxypyridinoline are found. Such elevated markers reveal increased osteoclastic activity and enhanced osteoblastic dysfunction [7–12].

Several studies have recently proven that the ratio of sRANKL/OPG is increased in patients with TM and low bone mineral density (BMD), providing evidence for the role of RANKL/OPG system in the pathogenesis of osteoporosis in thalassaemia. The increase of RANKL, followed by unmodified OPG levels, with the consequent increase of RANKL/OPG ratio may represent the cause of uncoupling on bone turnover observed in thalassaemia patients [2, 9, 12]. A negative correlation between the sRANKL/OPG ratio and free testosterone in male thalassaemia patients and between 17-b oestradiol in female thalassaemia, which has also been proven, speculates the role of RANKL/OPG system on the action of sex steroids on bone [12].

3. Hypogonadotrophic Hypogonadism

It is well known that sex steroids regulate skeletal maturation and preservation in both men and women. The impact of gonadal insufficiency on skeletal integrity has been widely recognized in adult men and women ever since. Androgens can be converted into estrogens within the gonads and peripheral tissues and both are present in men and women. Sex steroid signaling via sex steroid receptors found in bone possibly affects bone formation and preservation. The exact role of steroids in the pathogenesis of osteopenia is not completely clear. However, it is suggested that sex steroids may act by altering the normal balance of the OPG/RANKL system. Sex steroids increase the expression of RANKL by osteoblastic cells [12]. Oestrogen and progesterone appear to inhibit osteoclastic activity and promote osteoblastic activity [13] while testosterone has a direct stimulatory effect on osteoblast proliferation and differentiation [14]. Thus in the lack of steroids, the osteoclastic activity increases resulting in osteopenia.

Association between hypogonadotrophic hypogonadism and osteoporosis in adult patients with TM has been reported in the past [15, 16]. The contribution of sex steroids in addition to other factors on the development of bone disease in TM has been unequivocally proven [2, 17, 18]. Iron deposition on gonadotrophic cells leads to disruption of gonadotrophin production and consequently leads to delayed puberty and hypogonadotrophic hypogonadism. More than 50% of TM females fail to attend menarche and present with Primary Amenorrhea while Secondary Amenorrhea will invariably develop with time especially in patients poorly compliant to chelation therapy [19–21]. Male patients develop hypogonadotrophic hypogonadism and secondary gonadal failure resulting in low testosterone secretion [22, 23]. Primary gonadal failure may also present due to iron deposition on the testes and ovaries [23, 24].

Patients carrying certain genetic defects have an increased degree and rate of iron overloading through transfusions over the years and their Ferritin levels poorly correlate with their total body iron concentrations as proven by the increased frequency of hypogonadism. The role of the genotype as an independent risk factor for the development of endocrine complications in patients with TM on chelation therapy can be explained by the differences in the actual and realistic haemosiderosis, because the patients with the more severe defects have a greater rate of iron loading through higher red cell consumption. [15]. This observation is further supported by the finding of our previous study, which showed that gonadal function was the only factor strongly associated with decreased BMD when the patients were grouped based on genotype [16].

Throughout childhood, BMD normally rises at a steady rate until the age of about 12 years and then there is a sudden acceleration of bone mineral accretion, which coincides with the onset of puberty and the pubertal growth spurt. In patients with thalassaemia, BMD which is already low in childhood [25] decreases further during and after puberty especially in patients with absent or delayed puberty [26]. Bielinski et al. have proved that thalassemic adolescents who failed to progress normally through puberty also failed to preserve adequate bone mineralization and achievement of peak bone mass [27]. Other studies have shown suboptimal bone accrual, regardless normal or induced puberty [1, 28, 29].

Reduced bone mass is definitely more common among adult patients with TM due to hypogonadism which has been proposed as a mechanism for osteopenia on this condition. In adult TM patients, the prevalence of osteoporosis and osteopenia is above 50%, and vertebral fractures have been reported in up to 20% of patients [8]. However many other factors including the severity of the hemoglobinopathy, concomitant treatments, and gender differences in bone mass acquisition and maintenance are implicated in bone metabolism in hypogonadal thalassemics. Regarding the hormonal replacement therapy (HRT), there is conflicting evidence on its clinical effectiveness in maximizing bone mass in TM patients. Some studies have shown beneficial effect of HRT especially in younger patients [8, 11] while some others have shown little or no effect of HRT on BMD in thalassaemics who presumably have not achieved peak bone mass [30].

4. GH-IGF Axis

Although sex steroids are essential for the preservation of BMD during adolescence and adulthood, insulin growth factors (IGFs) and GH play a crucial role on bone formation during childhood and bone maintenance later in life. A number of clinical studies have provided evidence for potential effect of GH/IGF system on bone mass. However, our understanding of the regulation of production and actions of GH/IGF system on bone is incomplete. The anabolic effects of GH and IGF-1 in bone are important for the acquisition of bone mass during adolescence and possibly for the maintenance of skeletal architecture during adult life. The changes in GH and IGF-I secretion that occur with aging are paralleled by a progressive loss of muscle mass and strength, a decline in physical performance, an increase in body fat, and a decrease in BMD [31]. The skeletal effects of GH and IGF are modulated by complex interactions between circulating IGF-I and IGF-binding proteins (IGFBPs) and the locally produced IGF-1 and IGFBPs.

Growth hormone stimulates the proliferation of cells of the osteoblastic lineage [32] although IGF-1 is required for selected anabolic effects of GH in osteoblasts [33]. Specifically, GH affects the fate of mesenchymal precursors favoring osteoblastogenesis and chondrogenesis and opposing adipogenesis [33]. In addition, it stimulates the expression of bone morphogenetic proteins, which are important for the differentiation of osteoblasts, bone formation [34] and the production of OPG and its accumulation in the bone matrix [35]. Via these mechanisms, GH stimulates longitudinal bone growth, either directly or through an effect mediated by the local IGF-1 [35].

The fundamental role of IGF-1 is the stimulation of osteoblastic function and bone formation. IGF-1 has modest effects on the proliferation of cells of the osteoblastic lineage and enhances the function of the mature osteoblast [36]. Additionally, IGF-1 upregulates collagen synthesis and decreases its degradation, which is important for maintaining the appropriate levels of bone matrix and bone mass. Less clear is the function of IGF-1 on osteoclasts. Osteoclasts express IGF-1 receptors and IGF-1 has direct effects on their function [37].

Impaired GH secretion is not a rare occurrence in adult thalassaemics, which contributes to osteopenia and osteoporosis. However, as it was proven in the past the majority of TM patients have the same GH secretion as in the normal population [38]. Some studies have shown impaired GH response to GH-challenge tests in TM patients aged 10–23 years [39, 40]. Others have reported 3.1% of thalassemics to have GHRH-GH-IGF-1 axis dysfunction [41], which is attributed to multiple mechanisms such as neurosecretory dysfunction, hypothalamic GH-releasing hormone (GHRH) deficiency, and increased somatostatin activity [42, 43]. Additionally, thalassemic patients with delayed puberty do not exhibit normal growth spurt; their GH peak amplitude is reduced as well as their nocturnal GH levels [42, 44]. Thalassemics are also proven to have low IGF-1 levels in high prevalence regardless normal or subnormal GH secretion [45]. Recently, it has been reported that defective GH secretion and diminished serum IGF-1 levels may contribute to femoral demineralization in TM patients [45]. In the favor of further investigation, it is valuable that GH status should be retested in thalassaemic patients with childhood onset of GHD [46]. If the diagnosis of adult GHD is established, GH treatment is worth considering as it could contribute to improve bone mineral density.

There are no data of GH treatment in adults with thalassaemia and how this treatment may contribute to the improvement of BMD of these patients. In a study of Sartorio et al., one year of GH treatment in thalassemic children is able to increase, but not normalize, bone turnover; however it is insufficient to improve BMD values [47]. Prolonged periods of GH therapy are probably requested to positively affect both bone turnover and BMD values in GH deficient thalassaemic patients, as it occurs in children and adults with GH deficiency.

5. Hypoparathyroidism and Vitamin D Deficiency

Hypoparathyroidism is another endocrine complication in thalassaemia, which may develop in late adolescence and contribute to osteopenia and subsequently osteoporosis. A recent study has reported prevalence up to 13.5% with no sex differences [48]. Iron overload with deposition on parathyroid cells and tissue fibrosis are the main causes of hypoparathyroidism while chronic anemia is an additional factor causing parathyroid dysfunction [49]. The condition presents with the typical biochemical picture of hypoparathyroidism of low calcium and high phosphate levels. PTH may be normal or low and vitamin D is low. Low calcium and phosphorus are found in 24-hour urine collection. Bone X-rays are characteristic for osteoporosis. Abnormal cerebral CT findings are reported to be related with hypoparathyroidism in thalassemics [48, 49].

Vitamin D deficiency may start early in thalassemics, before hypoparathyroidism is established. Vitamin D deficiency potentially contributes to low bone mass in thalassaemia. Notably, TM patients progressively develop iron overload, and it is possible that a deficiency in liver hydroxylation of vitamin D, or in vitamin D absorption, appears in older thalassemic patients. However, studies in children [50, 51] and in adult thalassemic patients [52, 53] have shown contradictory results. Voskaridou et al. evaluating 45 adult TM patients reported that serum vitamin D (25-OH and 1,25-OH-vitamin D) levels were within normal limits in almost all patients [8]. Conversely, Praticò et al. [53] observed that 32 of 113 thalassemic patients (including children and adults) had low serum levels of 25-OH-vitamin D. Other studies have proved a disturbance in the circulating levels of 25-OHD in thalassaemic patients which is aggravated with increasing age [54]. It is difficult to explain these differences due to the multiple factors implicated in the mechanism of vitamin D deficiency. Additionally in countries with poor sunlight, vitamin D deficiency is more common even in the normal population. It is, though,

important to consider solar irradiation as a vital factor taking action in the metabolism of vitamin D.

Inaddition to vitamin D, vitamin C and trace elements such as zinc and cooper are involved in the bone metabolism. Vitamin and trace mineral deposition is proven to be inhibited by desferrioxamine in thalassemic patients who receive inappropriately high doses of this chelating agent. Mineral depletion may result in decrease of alkaline phosphatase activity, a zinc-dependant enzyme. Direct toxicity of desferrioxamine by inhibiting cell proliferation, DNA synthesis, and collagen formation cause bone damage resulting in platyspondylosis with flattening of the vertebral bodies and consequently shortening of the spinal height [5].

On the other hand iron deposition in bone may impair osteoid maturation and inhibit mineralization locally, resulting in focal osteomalacia. Iron interferes in osteoid maturation and mineralization by the incorporation into crystals of calcium hydroxyapatite which consequently affects the growth of calcium hydroxyapatite crystals and increases osteoid in bone tissue [28]. A damaged bone with small size may simply contribute to the markedly low BMD seen in the thalassemic patients.

6. Gender Differences in Bone Disease in TM

The impact of gonadal insufficiency on skeletal integrity has been widely recognized in both genders. It is though unclear whether there are gender differences in bone disease in TM. Some studies support that there is a gender difference not only in the prevalence but also in the severity of osteoporosis syndrome in TM. A recent study by our institution showed that male patients were more frequently and more severely osteopenic/osteoporotic than females (Figure 1) [55]. Other studies also showed male predominance on osteoporosis although some other reported no gender variation [56–60]. On the other hand, primary amenorrhea and, furthermore, hypogonadism are proven to have a greater impact on osteoporosis in females rather than in males [56]. Additionally, eugonadal females are less severely affected, on spine, compared with eugonadal males most likely due to the beneficial effect of HRT in women with TM (Figure 2) [55]. Alternatively, the bones of male patients are more vulnerable to the adverse effect of other contributing factors, which operate with a complicated and still unknown mechanism. However, some studies showed no gender differences in hypogonadal patients with TM [59, 60].

7. Diagnostic Methods of Osteoporosis Syndrome in TM

BMD is generally measured by the Dual Energy X-ray Absorption (DEXA) method. However, it is known that TM patients have spinal degenerative skeletal changes, which can be detected only by MRI and more likely interfere with BMD values, resulting in false diagnosis of bone disease (BD). Therefore, DEXA scans may fail to provide accurate and precise information on osteoporosis in thalassaemic patients. This probably explains the discrepancy between the findings

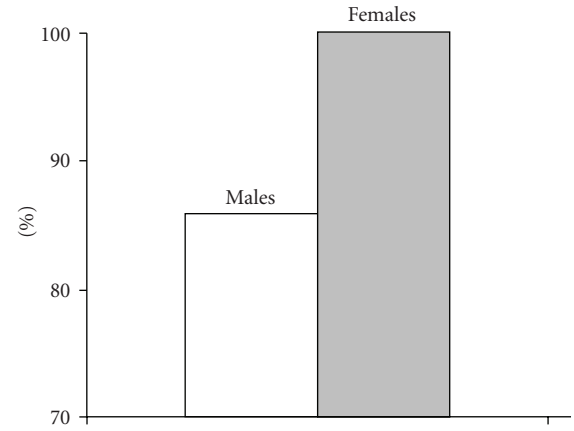

Figure 1: Prevalence (%) of osteoporosis osteopenia syndrome in hypogonadal male and female patients with TM.

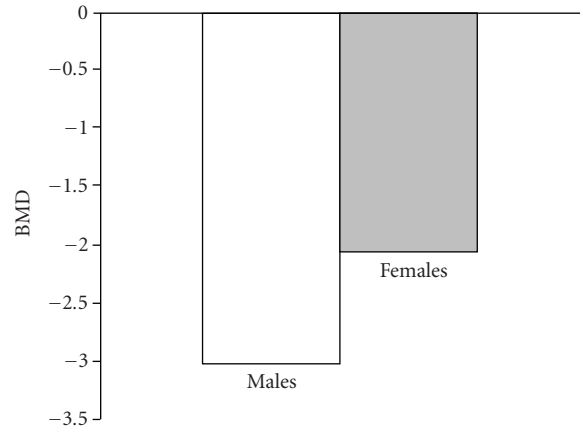

Figure 2: Mean BMD (spine) values in non-hypogonadal male and female patients with TM.

of osteoporosis in TM in different studies. For example in a recent Iranian study by Shamshirsaz et al. the prevalence of osteoporosis was found in 44% of the patients by using the DEXA method whereas only 6% of the same population were osteoporotic based on the Quantitative Computed Tomography (QCT) [59].

One additional contributing factor that interferes with BMD readings in DEXA method is short stature of thalassaemia patients, attributed mostly in the shortening of the spine. The reason is that DEXA measurements are influenced by size and that BMD represents a measurement of bone area rather than volume, leading to underestimation of bone density in such individuals. In summary, since DEXA may fail to provide accurate information of BD in thalassaemia, other methods, such as QCT, high resolution computed tomography and single energy quantitative computed Topography (SEQCT) should be considered as being more sensitive and reliable to detect bone disease in this condition.

8. General Aspects in Management of Osteoporosis in TM

The gold key in the management of osteoporosis in patients with TM, in whom, loss of bone mass starts early, is prevention. Treatment of anemia with regular transfusions and the management of iron overload with prompt chelation are mandatory for every patient with thalassaemia in order to avoid adverse events of the disease on bones. Additional lifestyle measures should be encouraged such as physical activity and smoking quitting. Adequate calcium and vitamin D intake during skeletal development can increase bone mass in adult life with the final goal to prevent bone loss and fractures. Early recognition and management of endocrine complications of thalassaemia are essential for eliminating the risk of BD. Induction of puberty at a proper age and treatment of hypogonadism with HRT seem to be the most effective ways for preventing osteoporosis and other bone deformities in thalassaemia [17]. Calcitonin, a potent inhibitor of osteoclasts, has also been tried in combination with calcium and vitamin D [60]. Bisphosphonates are worldwide used in patients with postmenopausal osteoporosis to increase BMD and prevent bone fractures [61]. The reduction in fractures may be related not only to the increase in bone mass arising from the inhibition of bone resorption and reduced activation frequency of bone-remodeling units but also to enhanced osteomineralization. Alendronate, pamidronate, and zoledronic acid seem to have greater efficacy in osteoporotic patients with TM either with normal or impaired gonadal function [8, 9, 62, 63]. Since the origin of bone disease in TM is multifactorial and some of the underlying pathogenic mechanisms are still unclear, further research in the therapeutic trials with bisphosphonates is needed to allow definite conclusions.

9. Conclusion

It is obvious that osteoporosis in thalassemia is of multifactorial etiology and of complex mechanism which need to be clarified. Endocrine complications mentioned in this paper inaddition to progressive marrow expansion, iron and desferrioxamine toxicity on bones, as well as liver disease contribute to the complex mechanism of osteoporosis in thalassaemia patients. Thus, osteoporosis in TM represents a unique clinical entity, which adversely affects the life expectancy of patients with TM. It is still questionable whether the international criteria for defining osteopenia and osteoporosis are relevant to patients with TM; also a question arises for the diagnostic methods and management of osteoporosis in the thalassaemic patient as multiple factors are involved in its pathogenesis. Close follow-up early recognition of osteopenia, as well as proper management are crucial for every thalassemic patient giving him the right to live a better life.

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