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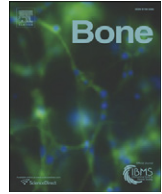

Review

New developments in biological markers of bone metabolism in osteoporosis

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ABSTRACT

Over the last 15 years several biological markers of bone turnover have been developed with increased specificity and sensitivity. In osteoporosis clinical studies, the IOF and IFCC organizations have recently recommended the measurements of serum type I collagen N-propeptide (PINP) and the crosslinked C-terminal telopeptide (serum CTX) as markers of bone formation and bone resorption, respectively. However these markers have some limitations including a lack of specificity for bone tissue, their inability to reflect osteocyte activity or periosteal apposition. In addition they do not allow the investigation of bone tissue quality an important determinant of skeletal fragility. To address these limitations, new developments in markers of bone metabolism have been recently achieved. These include assays for periostin, a matricellular protein preferentially localized in the periosteal tissue, sphingosine 1-phosphate, a lipid mediator which acts mainly on osteoclastogenesis and the osteocyte factors such as sclerostin and FGF-23. Recent studies have shown an association between the circulating levels of these biological markers and fracture risk in postmenopausal women or elderly men, although data require confirmation in additional prospective studies. Finally, recent studies suggest that the measurements of circulating microRNAs may represent a novel class of early biological markers in osteoporosis. It is foreseen that with the use of genomics and proteomics, new markers will be developed to ultimately improve the management of patients with osteoporosis.

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Introduction

Bone metabolism is characterized by an intimate cooperation of bone cells including osteoblasts, osteoclasts and osteocytes in order to maintain a regulated amount of bone tissue and the integrity of bone structure. In metabolic bone diseases such as osteoporosis, bone metabolism is altered, leading to bone loss, often accompanied by changes in the microarchitecture, leading to bone fragility. The development of serum and urinary assays for biochemical markers reflecting either enzymatic activities of osteoblasts and osteoclasts or breakdown products of bone tissue has been of high value to investigate the complex

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pathways of bone metabolism and their alterations in bone diseases, especially in osteoporosis. They have also helped the clinicians to identify patients at high risk for fracture and to monitor the efficacy of anti-resorptive therapies and bone-forming agents. In the last few years novel biological markers have been developed and studies suggest that they may be valuable research tools for investigating the mechanisms of bone metabolism, to assess the activity of osteocytes and some of them may be of value for the management of patients with osteoporosis. The aim of this paper is to review these novel developments in biological markers of bone metabolism in osteoporosis.

Established biochemical markers of bone metabolism

The structure, biology and clinical utility of conventional biochemical markers in different diseases including osteoporosis has been reviewed in several recent review papers [1–3]. At present, the most specific and sensitive markers of bone formation are serum total osteocalcin; the bone isoenzyme of alkaline phosphatase (bone ALP); and the procollagen type I N-terminal propeptide (PINP), which reflects the rate of synthesis of the main constituent of bone tissue. For the evaluation of bone resorption, most currently available assays are based on the detection in serum or urine of breakdown products of type I collagen. These include the intermolecular crosslinks pyridinoline (PYD) and deoxypyridinoline (DPD), and the cathepsin K (CTX, NTX) and matrix-metalloproteases (MMP)-generated (CTX-MMP or ICTP) type I collagen fragments. Serum 5b isoenzyme of tartrate resistant acid phosphatase (TRACP5b) is also a valuable biochemical marker reflecting mainly the number and the activity of the osteoclasts. Recently an expert group from the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry (IFCC) has performed a comprehensive review of the available data and based on this assessment has recommended the systematic use of serum PINP and serum CTX as reference biochemical markers of bone formation and bone resorption respectively in clinical studies [4]. The current biochemical markers of bone metabolism have however some limitations. These include 1) a lack of tissue specificity for bone, as type I collagen is widely distributed in different organs, 2) an inability to distinguish the metabolic activity of the different skeletal compartments, although they can be differently affected by diseases and treatments, 3) they reflect mainly the function of osteoblast or osteoclast and not the activity of osteocytes although these cells play a pivotal role in the maintenance of skeletal integrity, and 4) they are all protein-based markers, although circulating mRNA could also be of value as early biomarkers.

New biological markers of bone metabolism

Novel markers can be classified in different groups as shown on Table 1 and their involvement in bone cell biology is described on Fig. 1. These include the measurements of some non-collagenous proteins, osteoclastic enzymes other than TRACP5b, osteocyte-secreted factors, molecules involved in the coupling between osteoclast–osteoblasts, and circulating microRNAs. In this paper we will discuss in more details the most promising candidate in each category and their clinical relevance for the investigation of patients with osteoporosis.

Periostin: a matricellular Gla-containing protein as a potential marker of periosteal tissue metabolism?

The periosteum covers long bones and although in adults its metabolism is considered to be low, it plays an important for controlling the diameter of bones and thus bone strength [5]. Currently however there are no available non-invasive biological tools allowing the assessment periosteal metabolism as current bone markers reflect mainly endosteal bone remodeling [6]. The concept of developing biological markers that reflect the remodeling of a particular bone compartment has been suggested for some proteins including osteocalcin which is much more concentrated in the cortical than trabecular bone [7]. Periostin (POSTN), as suggested by its name, may be a candidate marker of periosteal metabolism. A detailed review of the structure, regulation and involvement of this protein in bone metabolism has been recently published [8], but new data have since been generated.

Briefly, the mouse periostin cDNA is 3187 bp long and contains an 18-bp 5′ untranslated region, a 733-bp 3′ untranslated region, and an open reading frame of 2436 bp corresponding to a protein precursor of 838 amino acids. The protein is mainly expressed by periosteal osteoblasts and osteocytes-although osteoclasts may also express low levels [9]. At the protein level it is composed of a signal sequence, followed by an Emilin (EMI) domain rich in cysteine, 4 repeated and conserved Fasciclin-1 (FAS-1) domains, and a C-terminal hydrophilic and variable domain (Fig. 2). Alternative splicing of the C-terminal domain gives rise to at least five different human isoforms. Each FAS-1 domain is rich in glutamate residues and contains an N-terminal recognition site for the vitamin K-dependent enzyme γ-glutamyl carboxylase (γ-carboxylase recognition sites, or CRS) responsible for the posttranslational modification of glutamic residues (Glu) to γcarboxyglutamate (Gla). This protein belongs to the matricellular protein family because it contains binding sites for extra cellular matrix proteins, such as type I and type V collagens, and the cell surface receptor integrins. In adults, POSTN has been shown to be overexpressed at the periosteal surface, but also in other collagen rich tissues subjected to mechanical strain such as periodontal ligaments, heart valves and tendons [8].

The most informative data on the function of POSTN in bone metabolism have been obtained from the examination of POSTN deficient mice. These mice develop periodontis and osteoporosis with lower BMD, altered microarchitecture and decreased bone strength [10]. These studies have also shown that POSTN is an important mediator of the effects of mechanical factors and parathyroid hormone (PTH) on cortical BMD and bone strength by modulating the canonical wnt signaling pathway with a down regulation of sclerostin expression [10,

Table 1
Candidate circulating biological markers of bone metabolism in osteoporosis.

Noncollagenous protein	Osteoclastic enzymes	Regulatory molecules of osteoblasts and osteoclasts	Osteocyte molecules	Micro RNA
Periostin	Cathepsin K	OPG/RANK-L	Sclerostin FGF-23	miRNA 21 miRNA 23a miRNA 24 miRNA 25 miRNA 100 miRNA 125b miRNA 133a miRNA 148a miRNA 214 miRNA 503
		Wnt signaling molecules: Dkk-1, sFRP sphingosine-1-phosphate (S1P)		

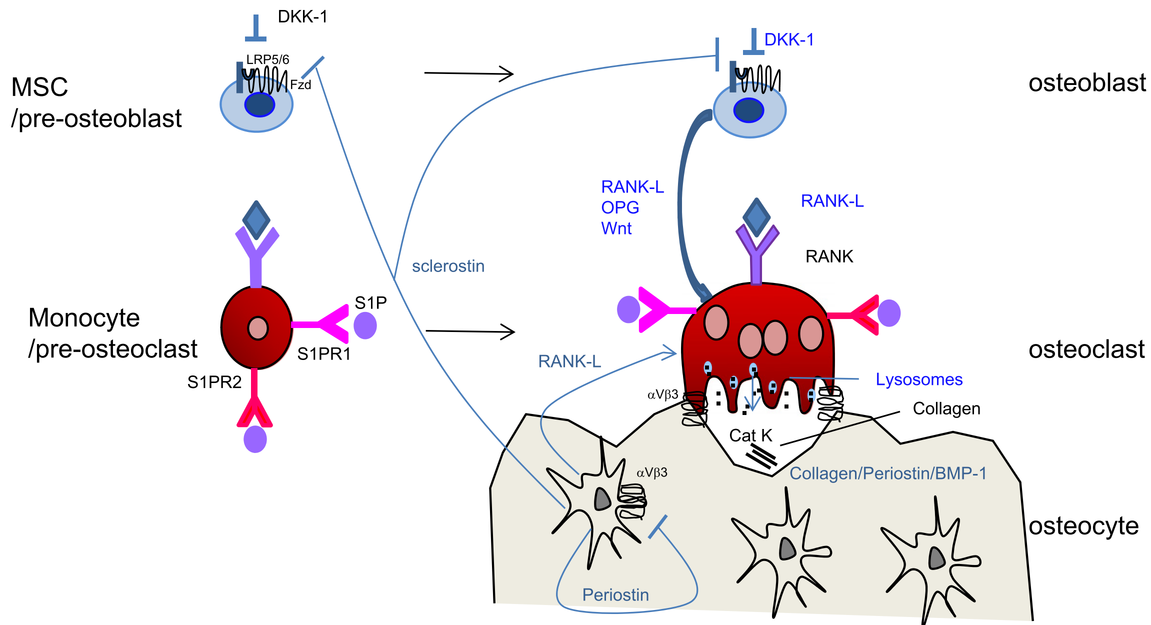

Fig. 1. Role of candidate biological markers in bone cell metabolism. Osteoblasts originate from mesenchymal stem cell (MSC) whereas osteoclasts are derived from the monocytes. Osteocytes are terminally differentiated cells embedded in bone tissue. Both precursors and mature bone cells express different receptors which play a major role in their differentiation and function. The differentiation and activity of osteoblasts are regulated by the Wnt signaling pathways through simulation of the Frizzled (Fzb) – LRP5/LRP6 – co-receptors by wnt molecules. The differentiation and activity of osteoclast is mainly regulated by the RANK-RANK-L-OPG pathway. Dickkopf-1 (Dkk-1) is a soluble factor that inhibits the Wnt signal pathway by binding on LRP5/LRP6. Osteoclast differentiation and activity is activated by RANK-L which is secreted by osteoblasts and is inhibited by the decoy receptor osteoprotegerin (OPG). Osteoclastic cells also express the spinghosine-1 phosphate (S1P) receptors, S1PR1 and S1PR2 which are modulated by the soluble factor S1P. SP1R1 exerts positive chemotaxis on monocytes to an SP1 gradient (high in serum, low in bone). Its deletion leads to the accumulation of osteoclast precursors and osteoporosis. Conversely S1PR2 induces migration in the inverse direction (chemorepulsion) leading to mild osteopetrosis. In addition S1P stimulated the expression of RANK-L by the osteoblast. Osteocytes regulate the activity of both osteoblastic and osteoclastic cells by secreting several soluble molecules including sclerostin, RANK-L and periostin. Sclerostin inhibits the Wnt signaling pathway by binding on LRP5/LRP6. Periostin negatively regulates sclerostin expression by binding on αvβ3 integrin. Periostin is also present in bone matrix in association with various proteins including type I collagen and bone morphogenic protein-1 (BMP-1) which activates the lysyl oxidase enzyme and hence promotes collagen crosslinking. Cathepsin K is a lysosomial enzyme secreted by osteoclasts in the resorption cavity that solubilizes bone collagen.

11]. Using this animal model, we also recently reported that POSTN deficiency alters bone material properties and favors damage accumulation in response to fatigue loading, with delayed remodeling and impaired callus formation [12].

Periostin is not only involved in regulating bone formation and BMD but also could have an effect on bone strength by regulating collagen crosslinking. It has been shown that the concentration of the enzymatic cross-links PYD and DPD was decreased in the periostin −/− whole femur when compared to wild-type mice [13]. We also reported that not only the enzymatic cross-links but also the immature precursors dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) were decreased in periostin-deficient mice, and their levels correlated with bone strength [12]. Periostin interacts in bone tissue with bone morphogenic protein-1 which catalyzes the activation of lysil oxidase that promotes collagen crosslinking [14].

Because periostin is a secreted protein, it can be detected in the peripheral blood and several ELISAs, mainly in house assays, have been developed. Using such a test, it has been reported that serum POSTN is increased in several cancers including patients with breast cancer and bone metastases [15]. In mice we found that circulating POSTN – but not PINP – significantly correlates with periosteal bone formation assessed by histological studies [16]. We have also measured serum POSTN with an ELISA that detects all isoforms of the molecule in a prospective cohort of postmenopausal women and found that increased levels were associated with a higher risk of all fractures independent of BMD and prior fragility fractures [17] (Fig. 3). This association may appear counterintuitive due to the positive effects of POSTN on bone formation and bone strength in rodent. The reasons for this unexpected finding are currently unclear. One hypothesis is that circulating POSTN may reflect the adaptation of the metabolic activity of periosteum cells to the existing bone strains in order to maintain the steady state of bone quality. Women with lower bone mass and strength who are at a higher risk of fracture may have higher mechanical strain in the remaining bone that would increase POSTN expression. POSTN exists in different forms due to alternative splicing, gamma-carboxylation and dimerization [8]. It is currently unknown whether one of these isoforms

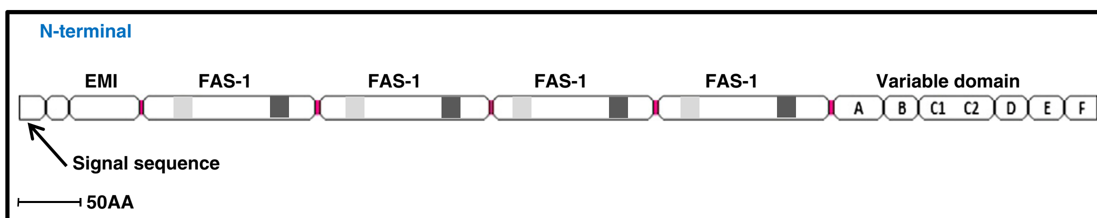

Fig. 2. Periostin protein sequence showing the signal sequence, the EMI domain, the four FAS-1 domains, and the C-terminal variable region with the 6 different cassettes (A–F) whose combination gives rise to different isoforms. Each FAS-1 domain contains an N-terminal recognition site for the γ-glutamyl carboxylase and a cell adhesion site. From Merle and Garnero [8].

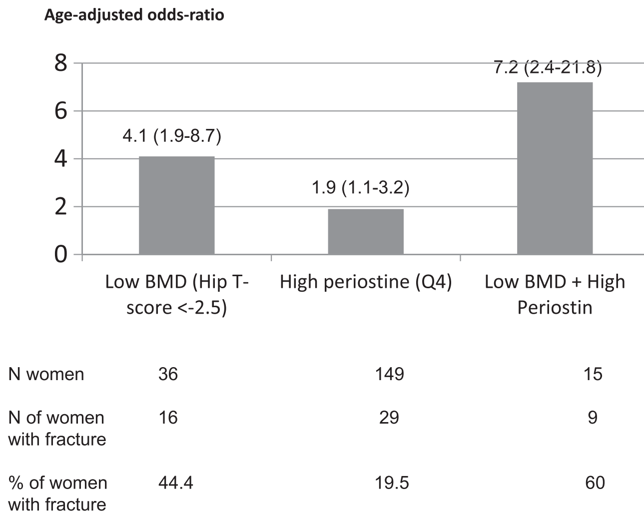

Fig. 3. Association of serum periostin and the risk of all fractures in postmenopausal women: The OFELY study 607 postmenopausal women mean age 66.6 years were followed prospectively for a median of 7 years. During followup 75 women experienced a vertebral clinical fracture or a peripheral fracture. The bars represents the relative risk for all fracture in women with low BMD (T-score < −2.5), high periostin (quartile 4 of the population) or women with both low BMD and high periostin. From Rousseau et al. [17].

could be more specific for bone tissue and can be measured accurately in the circulation. Clearly additional biological and clinical data are needed to assess the possible clinical utility of circulating POSTN as a marker of periosteal metabolism.

Serum cathepsin K: a marker of osteoclast activity?

Cathepsin K is one of the main catalytic enzymes expressed and secreted by the osteoclasts. It plays a predominant role in the degradation of bone type I collagen, and consequently is a drug target in osteoporosis [18]. It is a lysosomal enzyme synthesized as a procathepsin K, which is activated in the lysosomes through a process involving autocatalytic cleavage at low pH, and then released into the resorption lacunae [19, 20]. It may be possible that some of cathepsin K, is further released in the circulation and could be a specific biological marker of osteoclast activity. Several assays have been developed, although it remains unclear whether they detect the proenzyme, the active form or both. Using such tests it has been shown that serum cathepsin K is increased in patients with osteoporosis [21], Paget's disease [21], rheumatoid arthritis [22] and ankylosing spondylitis [23]. Increased levels have also been shown to be associated with higher bone loss in postmenopausal women [24] and levels decrease after treatment with the bisphophonate alendronate [25]. There was however no significant difference in serum cathepsin K between pre and postmenopausal women [26].

Recently an assay detecting only the active form of cathepsin K was generated [27]. No change in serum active cathepsin K levels could be observed in postmenopausal women treated with anti resorptive therapy such as estrogen and bisphosphonate or in patients with ankylosing spondylitis [27]. A major difficulty in measuring circulating cathepsin K is related to the fact that levels are very low and because it can be degraded by other enzymes, including cathepsin S. Also, it remains unclear which circulating forms is the most clinically relevant to measure. Whether assays for urinary cathepsin K will demonstrate higher sensitivity than serum tests remains to be investigated [28]. In the meantime, the measurement of serum CTX-MMP is an indirect marker of cathepsin K activity – its inhibition leads to a dramatic and very rapid increase in CTX-MMP – which has been particularly useful in clinical trials of cathepsin K inhibitors.

Novel findings on the osteoblast–osteoclast regulating factors (RANK-L, Dkk-1, S1P)

Measurements of the regulators of osteoclastic and osteoblastic differentiation and activity have been suggested to be useful in assessing bone metabolism in osteoporosis, arthritis and metastatic bone diseases. The receptor activator of *NF-κB ligand (RANKL)/RANK/osteoprotegerin (OPG)* system is one of the main regulators of osteoclast formation and function, as demonstrated by a series of preclinical and clinical studies (for a review [29]). Recently it has been shown that serum RANK-L levels were increased in patients with systemic autoimmune diseases before glucocorticoid therapy compared to healthy controls [30]. Patients with high baseline RANK-L levels demonstrated an increase of BMD after therapy whereas BMD decreased in those with lower RANK-L values. This suggests that serum pre-treatment RANK-L could be useful to predict response to glucocorticoid in patients with systemic autoimmune diseases [30]. In order to increase the sensitivity of systemic measurements, OPG and RANK-L expression have recently been measured in peripheral blood mononuclear cells (PBMC). No changes in OPG or RANK-L expression could be demonstrated in PBMCs of postmenopausal women after treatment with either ibandronate or strontium ranelate [31]. Measuring circulating RANK-L and OPG remains challenging, especially RANK-L, because their levels are very low. It is also unlikely that circulating levels of OPG and RANK-L adequately reflect local bone marrow production. These limitations explain the conflicting data on the association of circulating OPG and RANK-L with BMD and biochemical markers of bone turnover in postmenopausal women and elderly men [32].

The Wingless (Wnt) signaling pathway plays a pivotal role in the differentiation and activity of osteoblastic cells [33]. The primary receptors of Wnt molecules are the seven-transmembrane Frizzled-related proteins (FRP), each of which interacts with a single transmembrane low-density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6). Different secreted proteins, including soluble FRP-related proteins (sFRP), Wnt inhibitory factor-1 (WIF1), and Dickkopfs (Dkk) 1–4 prevent ligand-receptor interactions and consequently inhibit the Wnt signaling pathway. Alterations of the Wnt signaling pathway and its regulatory molecules including Dkk-1 and sFRP play an important role in bone turnover abnormalities associated with osteoporosis, arthritis, multiple myeloma, and bone metastases from prostate and breast cancer. Immunoassays for circulating Dkk-1 have been developed. Serum Dkk-1 levels have been reported to be increased in clinical situations characterized by depressed bone formation such as multiple myeloma [34] but also in diseases characterized by focal osteolysis such as multiple myeloma [34], bone metastases from breast or lung cancer [35], and rheumatoid arthritis [36]. Conversely, in patients with osteoarthritis of the hip, a clinical situation characterized by focal sclerosis of subchondral bone, lower serum Dkk-1 levels have been associated with a decreased risk of joint destruction [37,38]. In postmenopausal osteoporosis a negative association of Dkk-1 with BMD has been reported in two recent studies [39,40]. Dkk-1 levels have also been reported to increase after treatment with PTH [40] and were decreased after 12 months of glucocorticoids [41]. Interestingly, serum Dkk-1 has been reported to increase after 12 and 18 months of teriparatide treatment in women with postmenopausal osteoporosis which may explain the waning of the anabolic effect of this drug [42]. As for the assessment of OPG and RANK-L, circulating Dkk-1 might not adequately reflect local bone contribution. One way to increase the sensitivity of circulating Dkk-1 measurements may be to use an assay based on the binding of Dkk-1 on LRP-6, so-called functional Dkk-1. A recent study showed that circulating functional Dkk-1 was associated with radiological progression in patients with ankylosing spondylitis, whereas total Dkk-1 was not predictive [43].

From a practical point of view, measurements of Dkk-1 in plasma are recommended, as platelets activated during the clotting process to obtain serum are major contributors to circulating Dkk-1 [44].

Sphingosine-1-phosphate (S1P), is a lipid mediator which acts on different functions of cells through S1P receptors (SP1R, SP1R1 and SP1R2) which are in the G protein coupled receptor family. Although S1P may affect proliferation, survival, and migration of osteoblasts [45–47], the dominant effect of this protein on bone metabolism seems to be on osteoclastogenesis. Indeed in vitro and in vivo experiments have shown that S1P significantly potentiates osteoclast differentiation by increasing RANK-L in osteoblasts [48]. When substantial gradients exist between the S1P concentration in blood and bone, the migration of osteoclast precursors from blood (high S1P state) to bone (low S1P state) is facilitated [49,50]. The deletion of S1PR1 in monocyte cells leads to an accumulation of osteoclast precursors and a resultant increase in bone resorption [49]. Conversely, S1PR2-deficient mice exhibit moderate osteopetrosis as a result of a decrease in osteoclastic bone resorption [50]. Thus SP1R1 exerts positive chemotaxis to an SP1 gradient, whereas S1PR2 induces migration of in the inverse direction-so called chemorepulsion. Increased serum S1P levels were found to associate with high levels of bone resorption – but not bone formation – markers, low BMD and a higher risk of prevalent vertebral fractures in postmenopausal women (Fig. 4) [51,52]. These data suggest that the measurement of circulating S1P levels could be a new biological marker of fracture risk, although these cross-sectional data obtained in small number of Asian subjects require confirmation in larger prospective studies.

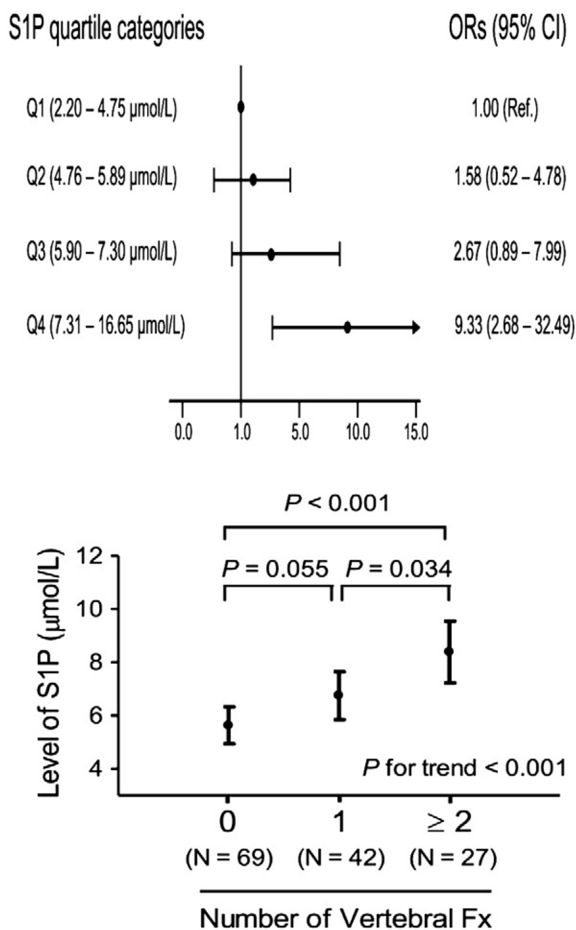

Fig. 4. Association of sphingosine-1-phosphate (S1P) with vertebral fracture in postmenopausal women. The upper panel shows the odds-ratio (95% confidence interval, x-axis) for osteoporotic fracture according to quartile of plasma S1P levels (y-axis). On the lower panel are shown the plasma S1P levels (y-axis) according to the number of vertebral fractures (x-axis). All results shown on the two panels were adjusted for age, BMI, current smoking, alcohol intake, regular outdoor exercise, parental history of osteoporotic fracture and spine BMD. From Kim et al. [52].

Osteocyte factors: sclerostin and fibroblast growth factor-23 (FGF-23)

Osteocytes which are terminally differentiated cells embedded within a mineralized matrix plays important roles in bone remodeling. These cells comprise a network of canaliculae which are believed to participate in the regulation of the targeted remodeling process by sensing micro fractures. This mechanism is crucial for replacing altered bone tissue by a new collagen matrix with optimal biochemical competence. Osteocytes are also important in the termination of the remodeling cycle by secreting factors such as sclerostin which inhibits osteoblast activity and promote their apoptosis and fibroblast growth factor-23 (FGF-23) which suppresses osteoblast differentiation and bone matrix mineralisation.

Sclerostin

Sclerosing dysplasias, sclerosteosis and van Buchem disease has led to the identification of important osteocyte signaling pathways that regulate bone formation. These bone disorders are characterized by progressive generalized osteosclerosis [53–56]. The genetic defect that leads to sclerosteosis was identified in a gene called *SOST*. Although *SOST* mRNA is expressed in many tissues during embryogenesis, sclerostin protein has been reported only postnatally in osteocytes, mineralized hypertrophic chondrocytes, and cementocytes. In vivo, analysis of *SOST* knockout mice showed significant increases in BMD, cortical and trabecular bone volume, bone formation rate, and bone strength [57]. All together these in vitro and in-vivo animal data support a negative effect of sclerostin on bone formation. Sclerostin can also stimulate the secretion of osteocyte derived RANK-L, thereby activating directly osteoclast activity [58,59]. Sclerostin has been shown to bind LRP5 and LRP6 and, thereby, could inhibit this pathway [60,61]. Placebo-controlled studies in postmenopausal women demonstrated that injection of the monoclonal antibodies against sclerostin Romosozumab and Blosozumab transiently increased bone-formation markers and BMD and was associated with decreased bone resorption markers [62,63].

Different immunoassays including three commercially available tests have been developed to detect changes of sclerostin in blood. The 2 ELISAs from Biomedica and TECOmedical and the Meso Scale Discovery (MSD) electrochemiluminescence assay use antibodies recognizing different epitopes on the sclerostin molecule. As recently reported they are likely detecting different circulating immunoreactive forms [64]. The MSD test recognizes only the intact molecule, whereas the 2 ELISAs also measure fragments from sclerostin and possible other structurally similar but unrelated molecules. It has been shown that although all 3 assays were highly correlated especially the 2 ELISAs [65], absolute levels can differ markedly and their association with bone turnover and PTH may also vary [64]. Circulating sclerostin is correlated with bone marrow plasma values, suggesting that serum level is likely to reflect local bone production [66]. Sclerostin is cleared from the body by the kidney and its renal excretion increases with declining kidney function [67].

Circulating sclerostin in healthy subjects and association with BMD and bone structure. Sclerostin levels are slightly higher in the girls prior to puberty, tended to decrease in both sexes during puberty, but remained significantly higher in boys compared to girls after puberty [68].

Sclerostin was found to be significantly higher in postmenopausal women than in premenopausal controls [69]. In women and in men, sclerostin levels increased over-life by an average of 2.4 and 4.6 fold [70], respectively. A study in 1235 premenopausal women and 568 postmenopausal women showed a linear increase of sclerostin with age [71]. These data suggest that increased sclerostin production by osteocytes may be involved in the age-related impairment of bone formation.

Serum sclerostin was found to be positively associated with whole body BMD in elderly women and men [72,73]. Sclerostin was also shown to be associated with improved trabecular bone quality assessed by high resolution pQCT (HR-pQCT) in older men [73]. In Chinese-American women, a small study found a positive association of serum sclerostin with cortical and trabecular volumetric bone density at the tibia in premenopausal but not postmenopausal women [74]. In elderly women participating in the Study of Osteoporotic Fracture (SOF) with the highest sclerostin levels had significantly higher areal and volumetric BMD, and larger bones than women with the lowest sclerostin levels [75]. Women with the highest serum sclerostin levels had pQCT parameters of bone strength 9.4% to 15.3% higher than those with the lowest concentrations, but only at the 66% site — and not at the 33% site of the tibia [75]. The reasons for these differences between the tibial sites are unclear. The 66% site is subjected to lower mechanical loading than the 33% site and because sclerostin production is upregulated in animal and human models of unloading, this may explain this selective association. It is also possible that measurements of bone tissue by pQCT reflect the number of osteocytes, which may be why there was a positive association between sclerostin and pQCT parameters. Two studies in Asian postmenopausal women and men reported a positive association of circulating sclerostin with fat mass [76,77].

In postmenopausal women, sclerostin correlated negatively with total and free estradiol and with intact PTH [69]. Finally a recent family study in Afro-Caribbean subjects showed that circulating sclerostin is genetically determined and 3 independent SNPs accounting for part of this effect were identified [78].

Circulating sclerostin and fracture risk in healthy women and men and in patients with type II diabetes. The association of sclerostin with fracture risk has recently been investigated in elderly women and men. In the SOF study, a nested case control analysis showed that elderly women over the age of 65 years with sclerostin in the highest quartile had a higher risk of hip fracture than women in the lowest quartile. The odds-ratio was however not significant after adjustment for age, body mass index, prior fracture and estrogen use [79]. An association between sclerostin and fracture risk was also reported in the CEOR study [80]. It was shown that women with levels in the highest quartile of baseline sclerostin had a 15 fold increase in the risk of all fractures compared to women in the lowest quartile which was independent of BMD and other risk factors (Fig. 5). This impressive risk associated with sclerostin needs to be confirmed in other studies. Actually in the French OFELY study there was no significant association between baseline sclerostin levels and the risk of all nonvertebral and clinical vertebral fractures which occurred in 64 women over a median follow-up of 6 years [81] Counter intuitively, high sclerostin was associated with a lower risk of all fractures in elderly men participating in the MINOS study [82]. The reasons for these discrepant findings between studies are unclear but could include difference in population characteristics, type of fracture, follow-up duration and assays. Indeed the 2 positive studies were using the Biomedica test whereas the OFELY and male studies utilized the TECOmedical assay.

Type II diabetes is characterized by skeletal fragility associated with increased risk of fracture despite a normal or even higher BMD than healthy controls. Histological studies have shown that patients with type II diabetes are characterized by low bone turnover associated with depressed osteoblastic activity [83].

Four crossectional studies reported that patients with type II diabetes are characterized by an increase of circulating sclerostin [84–87]. In these subjects, serum sclerostin was negatively associated with markers of bone turnover and positively with BMD. In postmenopausal women with type 2 diabetes, it was also shown that subjects with increased serum sclerostin had a higher risk of prevalent fractures independent of BMD and bone turnover [86,87]. In the former study, patients with high serum sclerostin and low IGF-1 had the highest risk of vertebral fractures. These findings suggest that sclerostin could be a

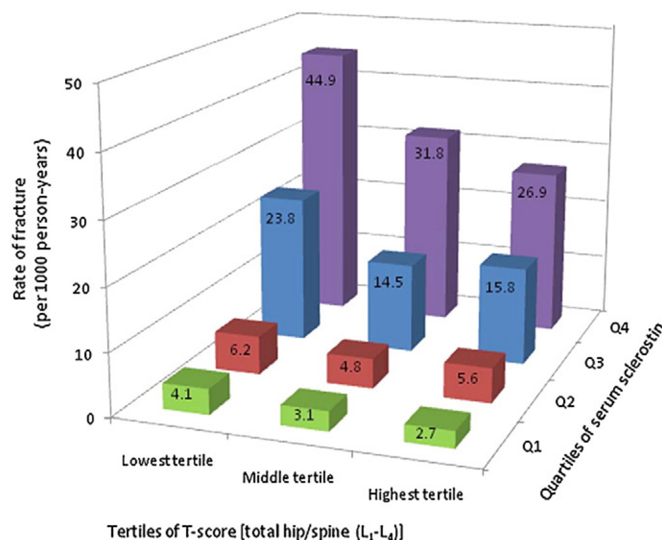

Fig. 5. Association of baseline sclerostin and BMD with the rate (per 1000 person-days) of all fractures in postmenopausal women. The CEOR study. Subjects were categorized in quartiles of sclerostin and tertiles of BMD (total hip/spine) T-score. From Ardawi et al. [80] with permission.

determinant of skeletal fragility in patients with type 2 diabetes, although they required to be confirmed by longitudinal studies.

Effect of bone active treatments on circulating sclerostin. The effect of osteoporosis treatment on serum sclerostin has been investigated in several clinical studies. A four week treatment of postmenopausal women with transdermal 17 beta estradiol resulted in a 27% decrease of sclerostin [72]. Sclerostin was also decreased after 19 months of raloxifen therapy in postmenopausal women [88]. The effect of bisphosphonate on sclerostin remains controversial. Gatti et al. reported a dose dependent increase of sclerostin after 12 months of monthly intramuscular neridronate [89]. Conversely, a retrospective study in postmenopausal women reported no significant effect of a 19 month treatment with alendronate or risedronate [88]. More recently a placebo controlled study showed that IV injection of zoledronic acid induced a transient increase of serum sclerostin and its changes were associated with the reduction in the bone formation marker bone alkaline phosphatase [90]. Although the mechanisms by which bisphosphonate could alter sclerostin levels remain unknown, it is possible that sclerostin plays a role in the inhibition of bone formation observed during bisphosphonate treatment. A randomized placebo controlled study in postmenopausal women recently reported that denosumab given every 6 months for 36 months was also associated with a significant increase of circulating sclerostin [91].

The effect of PTH was also investigated in postmenopausal women, elderly men and subjects with anorexia nervosa. Acute PTH infusion induces a rapid decline of serum sclerostin within 6 h in healthy men [92]. Circulating sclerostin levels also decreased significantly in postmenopausal osteoporotic women receiving PTH 1–34 for 14 days [61]. In contrast no change in serum sclerostin was observed in other studies investigating women with postmenopausal osteoporosis [42,93] or patients with anorexia nervosa [94].

In summary, serum sclerostin appears to adequately reflect local bone production, increases with age and is positively associated with BMD and bone structure in healthy adult women and men. The association of serum sclerostin with fracture risk remains controversial and consequently its measurement cannot be recommended in this clinical situation at the present time. The main factors influencing circulating sclerostin levels are summarized in Table 2.

Table 2
Main factors associated with circulating sclerostin.

Factors associated with	
Increased sclerostin	Decreased sclerostin
Age	PTH
BMD	Teriparatide treatment?
Fracture risk?	Estrogen and raloxifen treatment
Mechanical unloading	Mechanical loading
Low bone formation diseases	Hyperparathyroidism
- Hypoparathyroidism	Ankylosing spondylitis
- Multiple myeloma	
- Type 2 diabetes	
High bone remodeling diseases through compensatory mechanisms	
- Bone metastases	
- Paget's disease	
Bisphosphonate treatment?	
Denosumab treatment	

FGF-23

FGF-23 is a circulating factor expressed predominantly in osteocytes that negatively regulates serum levels of inorganic phosphorous and 1,25-dihydroxyvitamin D_3 [95–97]. The release of FGF-23 by both young and old osteocytes may be a mechanism whereby the osteocytes can control mineralization [98] and phosphate homeostasis. Several clinical skeletal disorders that result in mineralization abnormalities and elevated FGF-23 serum levels have been reported and include autosomal dominant hypophosphatemic rickets, tumor-induced osteomalacia, and X-linked hypophosphatemic rickets [98,99]. Moreover, mice overexpressing FGF-23 have low cortical and trabecular BMD [100]. Given the importance of FGF-23 as a regulator of phosphate homeostasis it is thus possible that this osteocyte factor could be associated with bone strength and fracture risk.

FGF-23 is synthesized as a molecule of 251 amino acids, including a leader sequence coding for a signal peptide of 24 amino acids. During secretion, FGF-23 can be proteolytically cleaved between Arg179 and Ser180 to generate N-terminal and C-terminal fragments. Thus, in circulation, FGF-23 can be present as the intact form and the C and N-terminal fragments [101]. Circulating FGF-23 can be measured by immunoassays detecting only the intact molecule or with a C-terminal assay that detects both intact FGF-23 and C-terminal fragments (Fig. 6). Currently it remains unclear whether the C-terminal assay will give comparable results than the intact one in different clinical situations.

Two prospective studies have analyzed the relationships between serum intact FGF-23 and fracture risk in elderly men. Mirza et al. [102] measured baseline intact FGF-23 in 2868 elderly Swedish men (mean age 75 years) followed prospectively for more than 3 years. They found that increased baseline FGF-23 was associated with an increased risk of all fractures and vertebral fractures. For nonvertebral and hip fracture the relationship was not linear and only patients with serum levels of FGF-23 in the highest quartile have an increased risk of fracture. Interestingly the association remained significant after adjustment for

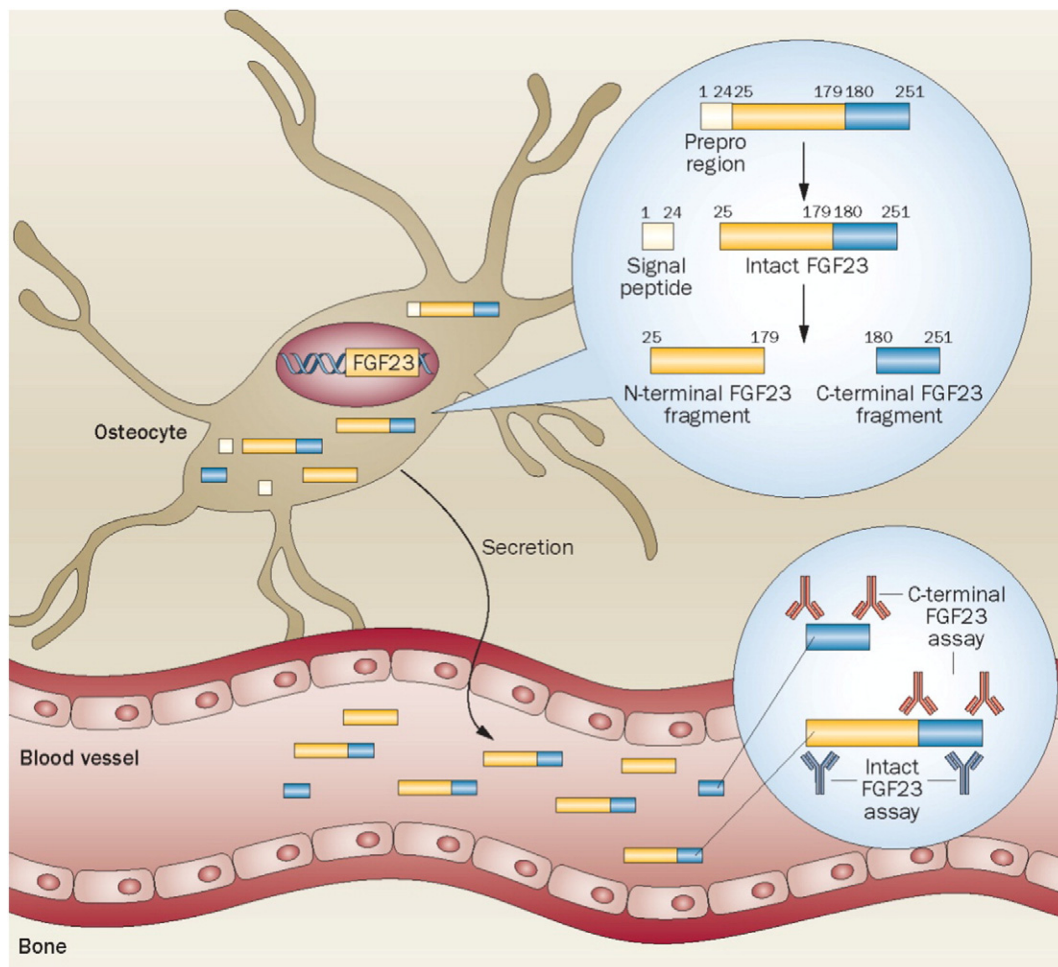

Fig. 6. Synthesis, secretion and structure of fibroblast growth factor-23 (FGF-23). FGF-23 is produced as a peptide with 251 amino acids, including a leader sequence coding for a signal peptide of 24 amino acids. During secretion, FGF-23 can be proteolytically cleaved between Arg179 and Ser180 to generate N-terminal and C-terminal fragments. Circulating FGF-23 can be measured with either an intact FGF-23 assay or with a C-terminal assay that detects both intact FGF-23 as well as C-terminal fragments. From Komaba H, Fukagawa, M [101].

several potential confounding factors such as BMD, PTH and glomerular filtration rate (GFR). Lane et al. [103] more recently performed a case control investigation in 387 men with incident nonvertebral fractures including 75 hip fractures and a random sample of 1385 elderly men (>65 years, mean 74 years) participating to the US MrOS study. In contrast to the findings in Swedish men, overall they found no significant association of serum FGF-23 analyzed in quartiles and the risk of nonvertebral or hip fracture. When subjects were stratified according to renal function, there was however a twofold higher risk of nonvertebral fracture for patients with levels of serum FGF-23 in the highest quartile, only among those with a GFR <60 mL/min/1.73 m^2. The reasons for the discrepancy between these two studies remain unclear and additional data in prospective studies in men and postmenopausal women with various degrees of renal function would be helpful.

Micro RNA as a novel class of biological markers in osteoporosis?

MicroRNAs (miRNAs) are a class of small (~22 nucleotides), single-stranded noncoding RNAs. They induce either translational repression or cleavage of the target mRNAs by imperfect or perfect base pairing with specific sequences in the 3′ untranslated regions of target mRNAs. Several in vitro and a few in-vivo animal studies have shown that miRNAs play important roles in osteoblast [104] and osteoclast [105] differentiation and function. As recently reviewed, miRNA are not only involved in normal osteoblast and osteoblast function but also can be deregulated in bone disease states including osteoporosis [106]. A study showed that miRNA-214 levels are elevated in bone tissue specimens from old individuals with fracture and that levels correlated with several biological and histological indices of bone formation [107]. The target gene of miRNA-214 was identified; it is *ATF4* which codes for an important transcription factor required for osteoblast function [108]. From a diagnostic point of view, measuring circulating miRNA would be very attractive as they may provide valuable information on the alterations of bone metabolism, possibly earlier than protein-based biomarkers. Because miRNAs are resistant to RNAase activity in the peripheral blood, circulating miRNA may serve as excellent biomarkers [109, 110].

Although many studies have analyzed circulating miRNA in different diseases including cancer, the data in osteoporosis are very limited. Recent studies reported that the levels of miRNAs can be measured in PBMCs which are a circulatory reservoir of osteoclast precursors. It was reported that patients with Lupus who are characterized by a low BMD had a higher expression of miRNA-148a than age matched healthy controls [111]. The overexpression of miRNA-148a in vitro or in vivo increased whereas its inhibition depressed osteoclastogenosis by targeting the V-maf musculoaponeutrotic fibrosarcoma oncogene homologue B. Wang et al. [112] showed that miRNA-133a was upregulated in peripheral monocytes from 10 patients with low BMD compared to 10 healthy controls. CXCL11, CXCR3 and SLC39A1 which are involved in osteoclast differentiation were identified as putative target genes. Conversely levels of miRNA-503 were reported to be decreased in CD14 + PBMC of 10 untreated postmenopausal women with osteoporosis compared to 10 women with normal BMD [113]. In ovariectomized mice, the authors showed that silencing of miR-503, using a specific antagomir, increased RANK protein expression, promoted bone resorption and decreased bone mass. Conversely, the over-expression of miR-503 with agomir inhibited bone resorption and prevented bone loss. Finally a recent study investigated the levels of free miRNAs in the serum of 60 patients with hip fracture who were categorized as osteoporotic and nonosteoporotic based on DXA assessments [114]. Nine miRNAs were found to be upregulated in patients with osteoporosis, among those 5 (miR-21, miR23a, miR24, miR25, miR100, mir125b) were also significantly increased in the corresponding hip bone tissue.

Before these candidate miRNAs can be used clinically to investigate bone metabolism, data need to be confirmed in larger studies. It is however likely that in the future this field will markedly expand. This task may lead to the discovery of a signature of circulating miRNAs demonstrating a clinical utility for the investigation of patients with osteoporosis. Such signature has been recently reported in patients with osteoarthritis another age-related skeletal disease [115].

Conclusion

During the last 5 years new developments have been achieved in the field of biological markers of bone metabolism, especially with the investigation of the clinical utility of serum sclerostin in osteoporosis and other metabolic bone diseases. Clinical data on these novel markers remain however limited and sometimes are controversial. The next years will be important to confirm the potential clinical utility of some of these biological markers and it is likely than novel candidates will be developed with an optimal use of genomic and proteomic approaches.

Disclosure

P. Garnero is an employee of Cisbio Bioassays.

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