Genes and osteoporosis: time for a change in strategy

Osteoporosis is characterized by a decrease in bone mass as well as a deterioration of bone architecture, resulting in an increased risk of fracture. Although the disease is multifactorial, twin studies have shown that genetic factors account for up to 80% of the variance in bone mineral density, the best-known predictor of the risk of fractures. These studies have also shown that this high heritability is observed especially in young women (peak bone mass), before bone starts to be lost due to menopause and age. However, studies conducted so far to identify genetic variants modulating bone mass in the general population have focused mainly on women around menopause or older. This approach has likely impeded the discovery of gene variants with effects of small magnitude, which are expected to be numerous. Therefore, the search for gene variants should probably focus on women close to their peak bone mass.

KEYWORDS: bone density ■ gene ■ gene variants ■ menopause ■ osteoporosis ■ peak bone mass ■ polymorphisms

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Osteoporosis is a common disease characterized by a decrease in bone mineral density (BMD) and bone strength, leading to an increased risk of fracture. Osteoporosis in women is defined by the WHO as BMD 2.5 standard deviations (SD) or more below the mean BMD of the young-adult reference population as measured by dual x-ray densitometry. Osteoporosis is most common in women after menopause, but also develops in men. It is a major public health problem that will expand with the aging of the population [1]. Twin and family studies have shown that genetic factors are important for the risk of developing osteoporosis through their influence on BMD. Identification of genes involved in BMD regulation is believed to be important in understanding the disease and bone homeostasis molecular pathways, and to identify new molecular targets for the design of the next generation of drug treatments [2]. Also, the discovery of gene variants could provide new genetic markers, allowing the identification of individuals with a higher susceptibility to low bone density and establish, in early adulthood, focused (or even tailored) preventive programs to increase their peak bone mass and ultimately minimize their risk of bone fracture later in life. The search for genes involved in the determination of bone density started in the early nineties with a manuscript in *Nature* by Morrison *et al.* [3], reporting that common allelic variants in the vitamin D receptor gene could predict differences in bone density, accounting for up to 75% of the genetic effect in healthy individuals. However, these conclusions were later modified owing to genotyping errors [4]. Later on, it was estimated that multiple gene variants might be involved and that each gene would have a modest effect on the final phenotype [5]. Since the availability of human sequence information and the increasing performance of genotyping methods, hundreds of association studies have been published with phenotypes related to bone density and/or osteoporosis [6]. However, relatively little success has been achieved, and inconsistent results have accumulated [7–9]. The potential reasons identified for inconsistent and nonreplicable results include lack of power (small sample size), low density of single nucleotide polymorphisms (SNPs) (too few SNPs to capture the whole genetic variability in a gene), population stratification, phenotype that is not clearly hereditary, genotyping errors and finally, inappropriate statistical analysis. This paper will review recent findings that we believe are most relevant to understanding the status of our knowledge in this complex field.

Strategic approaches for finding genes associated with BMD

■ Pattern of bone density change over lifetime

Both men and women reach skeletal maturity and peak bone mass at approximately age 25 years [10]. In a large longitudinal study of 9423 participants, the Canadian Multicentre Osteoporosis Study Research Group measured BMD at the lumbar spine, total hip and femoral neck at baseline, and

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at 3-year and 5-year follow-up visits, to compute individual rates of change as a function of age among Canadians aged 25–85 years [11]. In men, femoral neck density decreases at a nearly constant rate, starting at an early age (25–29 years). Bone density at the lumbar spine declines in men aged 35–39 years but increases after age 40 years. In women, bone density at both hip and lumbar spine is very stable between 35 and 44 years. However, at age 45 years bone density begins to decline at both sites. The maximum rate of decline is reached by age 50–54 years. This age corresponds to the menopausal transition in women. Women aged 55 years and older experience a period of attenuated bone loss and by age 65–69 years a mean increase in bone density in the lumbar spine is observed. However, by age 70 years, bone loss accelerates at both sites. To better illustrate the effect of perimenopausal changes on bone loss in women, the authors examined the rate of change of bone density in the total hip by menopausal status – that is premenopausal (at baseline and at year 5), postmenopausal (at baseline) and in transition (premenopausal at baseline and postmenopausal at year 5). Women in the transition group experience the greatest loss, corresponding to 6.8% of their bone mass over a period of 5 years. The accelerated bone loss observed in women experiencing the transition between pre- and post-menopause is the major determinant of the differences in the patterns of bone loss between women and men.

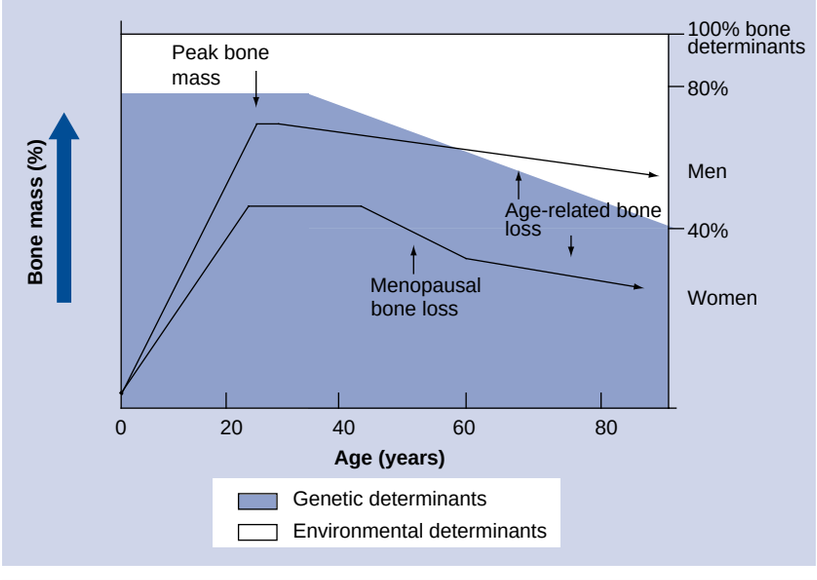

Figure 1. Bone density change with age in men and women. Bone density (lines) follows a gradual decline from the peak values achieved by early adulthood in men and women. On the right axis, genetic and environmental determinants proportion are plotted. With advancing age, environmental determinants become more important than genetic determinants.

In all, bone loss begins between ages 40 and 44 years, peaks between ages 50 and 54 years and then stabilizes among women (Figure 1). Another study by Riggs *et al.* reported rates of bone loss longitudinally [12]. These authors reported that trabecular bone loss began in young adulthood (between 20 and 29 years) in both men and women , but they also observed an accelerated loss during the perimenopause. Compared with the Canadian study, which followed-up 567 premenopausal and 1239 postmenopausal women not taking any antiresorptive agents, the study by Riggs only followed 103 premenopausal and 141 postmenopausal women. However, they measured both cortical and trabecular bone separately with quantitative computed tomography, which is a 3D measure, compared with 2D BMD by dual x-ray densitometry. However, the authors specified that quantitative computed tomography measurements had poorer precision at central position, such as lumbar spine, than at the peripheral sites, such as tibia and radius, and thus reported rates of bone loss at lumbar spine are less reliable. Some controversy may still exist concerning the stability of bone mass at lumbar spine between 20 and 40 years among women.

Peak bone mass as an intermediate phenotype

Theoretically, the best protection from osteoporosis is to reach a high peak bone mass during adolescence and subsequent growth [13,14], although trying to reduce bone loss to a minimum is also desirable. Simulation of the relative influences of peak bone mass, age-related bone loss and rapid menopausal bone loss on the development of osteoporosis revealed that a 10% increase in peak bone mass could delay the development of osteoporosis by 13 years [15]. This simulation also revealed that a similar change in the age at menopause, from 50 to 55 years, or a change in the rate of age-related bone loss results in a delay of approximately 2 years only. These authors conclude that osteoporosis is a disease caused primarily by failure to gain bone during childhood and adolescence and that peak bone mass might be the single most important factor [15,16]. Optimal peak bone mass can only be reached if there is adequate nutrition (calcium, vitamin D and proteins) and sufficient mechanical stimuli (physical activity) during growth [16]. Risk factors such as smoking, alcohol and certain medications may interfere with the amount of bone mineral mass acquired at the end of growth. However, the most important factor in the variability of peak bone mass is genetic.

Peak bone mass is highly heritable, but bone loss is not

Family and twin studies have estimated that up to 60–80% of the variance in peak bone mass is due to genetic factors [17–19]. While this trait, accounting for a large part of BMD at any age, is highly heritable, there are very few published data on the heritability of bone loss [20]. A few studies have looked at the influence of menopausal status on the genetic and environmental effects on bone density [20,21]. Hunter *et al.* used a large sample of female twins to evaluate the influence of menopause on the genetic variation in BMD. A total of 360 monozygotic twins and 885 dizygotic twins were studied. They found no evidence that different genes influenced BMD before and after menopause. However, the total variance in BMD was greater in postmenopausal women [21]. In the second study, Brown *et al.* analyzed 570 women from large Amish families. They chose this community because families tend to be very large and members are linked into a single pedigree. Moreover, they share a relatively homogeneous environment and are reluctant to use prescription medication. With the rationale that genetic variation in premenopausal women is mainly due to genetic determinants of peak bone mass, while genetic variation in postmenopausal women is due to the combined genetic effects of peak bone mass and bone loss, they evaluated and compared genetic contributions to bone density in premenopausal and postmenopausal women. This study showed, similar to the previous study with twins, that genetic factors influencing variation in BMD are common to both pre- and postmenopausal women. The total variance in BMD was higher in postmenopausal women compared with premenopausal women. In this study, genes accounted for 58–88% of the total variation in BMD in premenopausal women, compared with 37–54% of the total variation in postmenopausal women (Figure 1). Although the genetic variance was approximately the same in the two groups, the environmental variance was 3.5–4-fold larger in the postmenopausal group [20]. Although the data in this study suggested a modest genetic contribution to total hip BMD in postmenopausal women only, presumably through genetic determinants of bone loss, a longitudinal follow-up of women as they lose bone will be required to elucidate these effects.

Such studies have been performed recently, with family members [22] and with twins [23]. In both studies, the authors measured a mean 5-year change in BMD and estimated heritability of bone loss to be approximately 40% at the lumbar spine and between 31 and 49% at the forearm. The study with a large number of female twins did not report any significant heritability at any hip sites [23], while the study with family members (300 men and women) reported a 44% heritability at total hip [22]. This result must be taken with caution given the small sample size. Also, measuring heritability of bone loss is challenging as bone loss is a relatively slow process and is dependent on sex, site and age [11]. Moreover, measurement error might represent an important factor, given the fact that the coefficient of variation for BMD measurement by current available instruments is 1–2% and bone loss rates are estimated to be 0.3–1.5% per year. Increasing the follow-up time will improve precision but BMD data collected over an extended period of time are likely to be measured by different instruments, which may result in larger measurement errors [24]. A different strategy was used by Yan *et al.* to investigate the existance of bone-loss-specific genes [25]. They conducted a genome-wide linkage scan in a total of 2582 white women from 451 pedigrees, including 1486 premenopausal women and 1096 postmenopausal women. By comparing linkage results for BMD obtained in the total, premenopausal and postmenopausal women, they expected to identify linkage exclusively in postmenopausal women if there are quantitative trait loci specific to bone loss, since bone mass in postmenopausal women is determined by both peak bone mass (common to both pre- and postmenopausal women) and bone loss (specific to postmenopausal women). They found no evidence for linkage that was present exclusively in postmenopausal women and, hence, no quantitative trait loci for bone loss in this large sample of women for either spine or total hip BMD [25]. Obviously, this negative result might also be due to a lack of power. Even if it is found that bone loss is hereditary, it will always remain difficult to study bone loss because peak bone mass or baseline BMD must be known. On the other hand, peak bone mass is clearly hereditary and once it is reached, it remains quite stable up to age 45 years [11] and is the most important contributor to bone mass after menopause. Therefore, a search for genetic factors is more likely to be sucessful in premenopausal women, in whom the gene effect is expected to be maximal, especially if there is no evidence for a stronger genetic component in postmenopausal women.

Premenopausal women are rarely used for genetic studies

Unfortunately, the majority of published studies on the association between gene polymorphisms and bone density-related phenotypes have been performed with women in postmenopausal years or in transition. Even recent genome-wide association studies have used women aged 18–98 years old, with an average age of 59.4 ± 14.1 years in one case and an average age of 49.7 ± 13.1 years in another case, in which the authors did not use a menopausal status term in the statistical model and BMD was only adjusted for age and weight [26,27]. These designs are likely used for their ability to link fractures with the genotype associated with low bone mass in which fractures of low trauma will be seen only in older men or women. Also, defining the menopausal status in women can be complex [28], especially in large multicentric studies where the clinical data have not necessarily been collected in a standardized fashion. The desire to link the genotype to the fracture risk is driven by clinical interest. However, fractures are thought to be due to many other factors besides bone mass [29]. Also, because the disease is currently observed in women after menopause, only menopausal women have traditionally been enrolled for genetic studies. Now, a few groups have started to study the association between genes and bone density in younger women [30–32], but unfortunately these are still too few.

What has been learned from genetic studies

Bone remodeling

Bone is a dynamic tissue, which constantly undergoes turnover through synthesis of new bone by osteoblasts and resorption of bone by osteoclasts. Therefore, bone density is dependent on the relative function of these two types of cells. One major pathway and system involving these two types of cells has been identified so far. In osteoblasts, the canonical Wnt signaling pathway is involved in the synthesis of bone. This pathway involves many players, such as the Wnt ligand, Frizzled receptor, LRP5 and LRP6 coreceptors (Figure 2) and has been the subject of numerous reviews [33–35]. The system with RANKL/RANK/OPG was discovered in the mid-1990s, which controls resorption by osteoclasts, and has been well described in some reviews [36,37] (Figure 3). In humans, rare mutations have been identified in genes involved in these two pathways, leading to different bone pathologies. Osteopetrosis is a condition in which there is a defect in bone resorption by osteoclasts, resulting in an increase in bone density [38]. An increase in bone density is also observed when genes involved in the Wnt pathway are mutated, and different mutations may lead to disease of low bone mass as well. Members of these two important pathways have been studied in the search for genetic variants responsible for the diverse bone mineral density observed in the healthy population.

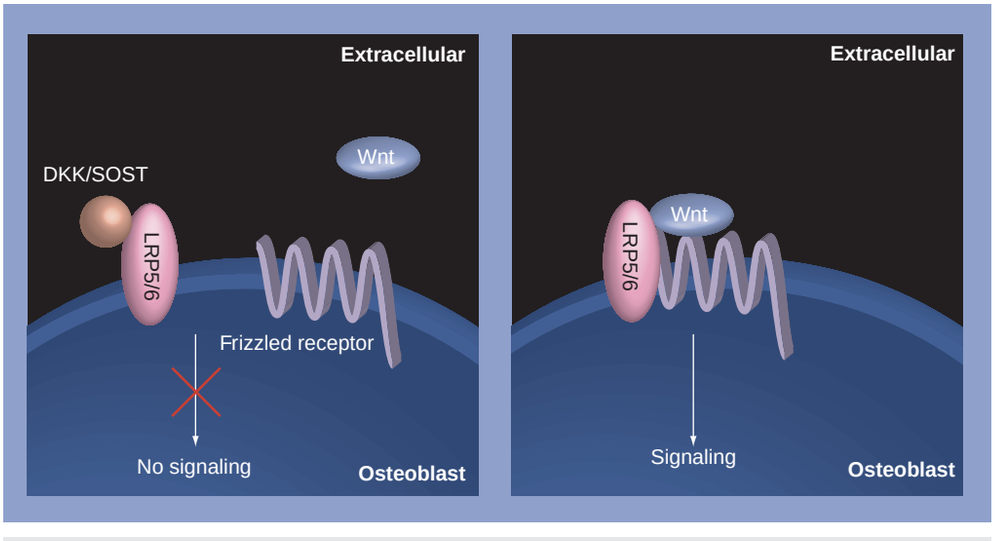

Figure 2. Wnt signaling pathway in osteoblasts. On the left, the presence of DKK or SOST proteins make the LRP5/6 coreceptor unavailable to form an active signaling complex with the Frizzled receptor and Wnt ligand, and no signal is transduced to the nucleus. On the right, in the absence of DKK or SOST proteins, and LRP5/6 is available and the signaling complex can form.

Promising candidate gene variants

LRP5

Osteoporosis–pseudoglioma syndrome is a rare autosomal recessive disorder characterized by very low bone mass and a propensity to develop fractures and deformation [39]. Using a positional candidate approach, a low density lipoprotein receptor-related family member *LRP5* was found to be the gene responsible for osteoporosis–pseudoglioma syndrome and the mutations identified were found to cause a loss of function [39]. Later, two groups reported that a gain-of-function mutation in *LRP5* was responsible for an important increase in bone mass, termed high-bone-mass phenotype [40,41]. In 2003, six novel missense mutations, all located in the amino-terminal part of the gene, were found to be associated with an increased bone density, although with different diagnoses, such as increased trabecular bone density in osteosclerosis, impaired bone resorption in osteopetrosis, increased bone formation in Van Buchem disease and a cortical bone thickening endosteal hyperostosis [42]. All these mutations were shown to prevent efficient binding of DKK1 or SOST to LRP5 and thus these mutants were not able to inhibit the canonical Wnt signaling [40,43] (FIGURE 2). The observation that diverse mutations were described in different human diseases of low and high bone mass raised the possibility that common variants altering the expression or the function of LRP5 could play a role in the variable bone density observed in the general population. From then on, many studies analyzed different SNPs in large groups of women and/or men [31,44–56]. Meanwhile, a prospective multicenter collaborative study of 37,534 individuals from 18 participating teams in Europe and North America for the Genetic Markers for Osteoporosis (GENOMOS) study was performed [57]. Two common variants of *LRP5* were studied (V667M and A1330V) and both Met^{667} and Val^{1330} were found to be associated with lower BMD at the lumbar spine and femoral neck. It was found that each Met^{667} allele copy reduced the lumbar spine BMD by 20 mg/cm^2 and femoral neck BMD by 11 mg/cm^2 and each Val^{1330} allele copy reduced lumbar spine BMD by 14 mg/cm^2 and femoral neck BMD by 8 mg/cm^2. Although the magnitude of the effect was modest, the association was highly significant and very consistent across studies. Furthermore, both variants were significantly associated with fracture risk. The two variants were in strong linkage disequilibrium but haplotype studies allowed researchers to separate the effect of each one. Haplotype 1, carrying both common alleles Val^{667} and Ala^{1330}, as used as a reference and haplotype 2, carrying the common Val^{667} and the Val^{1330} risk allele, and haplotype 3, carrying risk alleles for both Met^{667} and Val^{1330}. Both haplotype 2 and 3 were found to be associated with lower BMD, although haplotype 3 was more strongly associated, suggesting that both variants have individual effects. Also, these two polymorphisms could be in linkage disequilibrium with another causative variant not yet functionally described. Four studies reported a strong effect in young premenopausal women [31,48] or in young men [52] and women [46], which is in line with the suggested maximal genetic effect on peak bone mass. A fifth study reported only a small effect of *LRP5* variants in a sample of 588 unrelated healthy premenopausal women [44]. By contrast, in a large sample of 1377 premenopausal women, a large genetic effect due to Val667Met genotypes was observed at the lumbar spine BMD, representing 0.28 SD or 38 mg difference [31]. This is an effect much larger than the effect observed among 22,783 women of all status from GENOMOS (23 mg for the same genotype classification for women only) [57]. In the

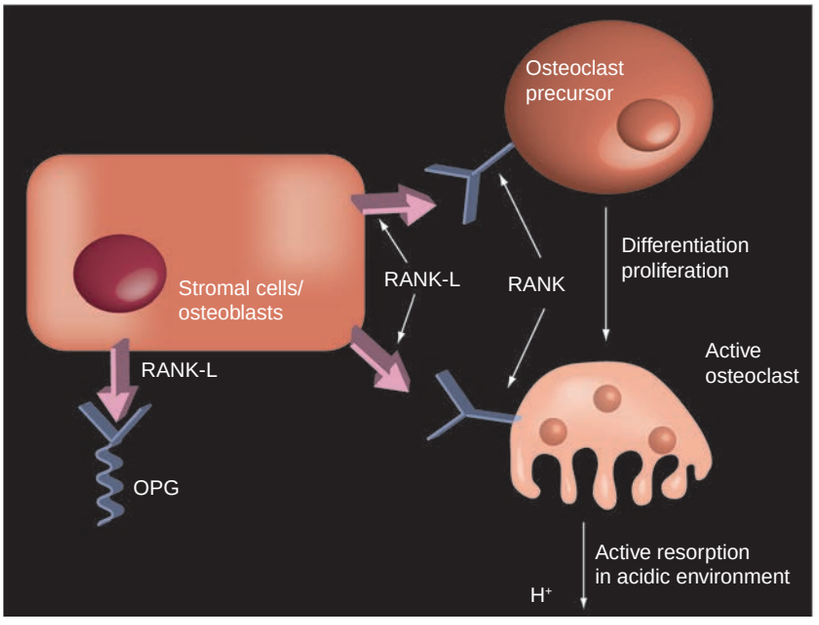

Figure 3. Roles of RANK, RANKL and osteoprotegerin in osteclast differentiation and activation. Osteoblasts and marrow stromal cells express the cytokine RANK ligand which bind to RANK on the surface of osteoclast precursor. This binding triggers a cascade in the osteoclast leading to differentiation and proliferation. Differentiated osteoclasts will produce ruffled borders and acidic medium to dissolve bone. OPG is produced by osteoblasts (and other cell types) and functions as a soluble decoy molecule to counter balance the activation of osteoclasts by RANKL.
OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor-κB; RANKL: Receptor activator of nuclear factor-κB ligand.

study of premenopausal women, the authors performed a successful replication, essentially as described by Chanock *et al.* [58]. In the first sample of 709 premenopausal women, a gene effect of 0.27 SD, with a p = 0.013 at lumbar spine BMD, was observed, and in the replication set of 668 premenopausal women from a different metropolitan area, a gene effect of 0.28 SD with a p = 0.015 was observed. Combined together, the gene effect remained 0.28 SD at lumbar spine, with a $p < 5 \times 10^{-4}$ [31]. Three studies by Giroux *et al.* and Ferrari *et al.* reported that Met^{667} was more likely to be a causative variant than Val^{1330} [31,46,48]. However, a functional *in vitro* assay with a *LRP5* coding sequence containing the three major haplotypes has shown that Val^{1330} was sufficient to reduce the Wnt-signalling capacity of LRP5 [53]. The identity of the causative variant(s) thus remains uncertain. Another large study on fracture risk performed in 6752 women during a mean follow-up of 14.5 years did not report any association with *LRP5* gene with 658 vertebral fractures [59]. Unfortunately, the variant studied was not analysed in previous reports and was located close to Val667Met (Glu644Glu) but was uncorrelated with it or with Ala1330Val in the HapMap sample [60]. Therefore, this later study does not shed new light on the validity and identity of the causative variants.

TNFRSF11B

The *TNFRSF11B* gene codes for osteoprotegerin, a secreted protein discovered in 1997 and named after its bone-protecting abilities [61]. Osteoprotegerin is a member of the superfamily of TNF receptors on the basis of sequence homology. The function of this protein was revealed by the creation of transgenic mice expressing the protein, which showed a dramatic increase in bone density, characteristic of osteopetrosis. Simonet also demonstrated that recombinant osteoprotegerin blocked osteoclastogenesis *in vitro* and *in vivo* and protected mice from bone loss after ovariectomy [61]. Later, targeted loss-of-function mutations in the mouse osteoprotegerin gene [62,63] have shown a marked bone loss and a reduced bone strength. Osteoprotegerin was defined as a key factor, acting as a negative regulator against osteoclastogenesis. Osteoclastogenesis is the process responsible for bone resorption and osteoclasts are cells derived from hematopoietic lineage and are the unique players in the process. Osteoclast precursors express the receptor activator of nuclear factor-κB (RANK) on their surface and upon the binding of its ligand (RANKL, a transmembrane protein produced by osteoblasts or stromal cells) activate differentiation into functioning osteoclasts and bone resorption. Osteoprotegerin acts as a decoy receptor by binding to RANKL and thus preventing its action on osteoclasts [64] (**Figure 3**). A rare disease described as 'juvenile Paget's disease' was identified in two unrelated Navajo patients and the cause of the disease was found to be the homozygous deletion of the *TNFRSF11B* gene [65]. The disease was characterized by rapidly remodeling woven bone, osteopenia, fractures and progressive skeletal deformity. Together, all these observations suggested that common polymorphisms affecting the expression or function of the *TNFRSF11B* gene could explain bone density variation in the general population, as was seen for the *LRP5* gene.

Interestingly, two genome-wide association studies reported that polymorphisms close to the *TNFRSF11B* gene were significantly associated with BMD [26,27]. In the first study, 301,019 SNPs of 5861 Icelandic subjects were analyzed [27] and positive findings were repeated in three replication sets, one of 4165 Icelandic men and women, one of 2269 postmenopausal Danish women and one of 1491 Australian men and women. Two correlated SNPs on 8q24, rs6469804 and rs6993813 reached genome-wide significance for both spine and hip BMD. These SNPs are 7 kb apart and are located some 81 kb from the osteoprotegerin gene start codon in a common linkage disequilibrium block. The two SNPs were associated with spine bone density with high significance in the two Icelandic sets but only with moderate significance in the Danish and Australian sets. The p value for all sets combined was around 10^{-14}. For the hip, the association was slightly weaker. No strong association was observed with 4406 low-trauma osteoporotic fractures with both SNPs. However, in this large study, the association between BMD and *LRP5* variants was not observed.

In the second study, 314,075 SNPs were analyzed in a sample of 3680 white women of European ancestry [26]. Positive associations were replicated in three groups of 690 women from the Chingford cohort [66], 1692 women from the TwinsUK cohort [67] and 2497 men and women from the Rotterdam cohort [68]. Two SNPs reached genome-wide evidence, one on chromosome 8 near the *TNFRSF11B* gene and the other on chromosome 11 in the *LRP5* gene (Ala1330Val), which can be considered a positive control. Three SNPs, rs4355801, rs6469792 and

rs6469804, which are close to the *TNFRSF11B* gene, were associated with BMD. Two are located in the 5′ region while rs4355801 is in the 3′ untranslated region and is the SNP with the strongest association ($p = 7.6 \times 10^{-10}$ for lumbar spine BMD and $p = 3.3 \times 10^{-8}$ for femoral neck BMD). Each copy of the risk allele (allele A) was associated with a decrease of 0.09 SD at the lumbar spine. Also, the authors used cis-associated allelic expression studies to determine whether the risk allele (allele A) that is associated with low BMD affected expression of the transcript in lymphoblast cell lines. They found a twofold overexpression of the G allele compared with the A allele at rs4355801. This would suggest that the A allele is associated with a lower expression of the gene and therefore produces less decoy receptor and thus is less effective at repressing bone resorption by RANK and RANKL during osteoclastogenesis, leading to a lower BMD. These results are consistent with the biological mechanisms. This polymorphism was not significantly associated with an increased risk of fracture.

Several smaller studies have examined the association between *TNFRSF11B* gene polymorphisms and BMD and/or fracture risk [69–76]. The most studied polymorphisms are a change of amino acids in exon 1 Lys3Asn (rs2073618) and rs3102735 (163 A/G) in the 5′ region. Rs2073618 was significantly associated with bone mass more often than the polymorphism in the 5′ region, and the CC genotype (Asn–Asn) was consistently associated with a higher bone mass. Conversely, some studies did not find associations between bone mass and the coding SNP in Irish [77] and Japanese postmenopausal women [78]. This coding SNP is located in the signal peptide region of the gene and the prediction tools Sift [201] and SNPeffect [202] both predicted a damaging or deleterious effect while Polyphen predicted a benign effect [203]. A meta-analysis such as the one performed for *LRP5* gene should be undertaken to clarify and validate the association. The analysis of haplotype could help to identify the causative variant(s) in a very large sample or at least identify the block in which that variant is located, given that exon 1 and the promoter region have been reported to be in different haplotype blocks [60,69]

Other candidate genes studied

Other candidate genes involved in rare human diseases

Osteopetrosis is a heterogeneous group of heritable conditions in which there is a defect in bone resorption by osteoclasts [38]. A total of 60% of patients with severe autosomal recessive osteopetrosis have a mutation in the *TCIRG1* gene coding for a proton pump and approximately 15% of patients have a mutation in chloride channel 7 (*CLCN7)* gene [38]. Both genes are necessary for the osteoclasts ruffled border to generate acidification in the extracellular environment [79]. Also, heterozygous missense mutations of the *CLCN7* gene cause autosomal dominant osteopetrosis [38]. These observations prompted some groups to look for common polymorphisms in these genes for association with BMD in normal individuals. One variant in the promoter region of *TCIRG1* gene was found to be significantly associated with lumbar spine and femoral neck BMD in premenopausal women [80]. They studied a relatively small group (308 premenopausal women or 591 pre- and postmenopausal and hormone therapy users) and thus the findings must be replicated in other samples to exclude the possibility of a false-positive result. The *CLCN7* gene was also studied for association with BMD in the general population. The first study reported a significant association with a single coding SNP Val418Met and femoral neck BMD in a sample of 1077 women [81]. This sample was mainly composed of postmenopausal women (88%), of whom 36.1% were current users of hormone therapy and 17.2% were past users. Another study with 425 postmenopausal women reported no association with Val418Met but a slightly significant association between intron 8 variable number tandem repeat and femoral neck Z-score but not with lumbar spine Z-score [82]. A more recent study analyzing 1692 premenopausal sisters and 715 brothers did not report any positive association with Val418Met nor with the intron 8 variable number tandem repeat or with any of the five remaining SNPs they analyzed [30]. Therefore, evidence for a true association with this gene appeared slim.

Sclerosteosis and van Buchem disease are two similar autosomal recessive craniotubular hyperostoses caused by loss-of-function mutations in the gene coding for sclerostin (*SOST* gene) [83] and deletion of a *SOST*-specific regulatory element [84,85]. Three studies have reported analysis of polymorphisms in the *SOST* gene in association with BMD [56,86,87]. Balemans and Sims both used a case–control study; Sims included a total of 344 postmenopausal women and found a significative association [56] while Balemans included 619 perimenopausal women and did not find any SNP associated with BMD [86]. Uitterlinden *et al.* used a sample of 1016 women and 923 men in which they observed different associations for

each sex [87]. In men, they observed an association between both lumbar spine and femoral neck BMD and a SNP located in the 3′region of *SOST* gene, while in women they observed an association with a small deletion of three base pairs in the upstream region of the gene. However, this association could be a false-positive and should be analyzed in more and larger samples to accumulate more evidence.

Classical candidate genes

Four candidate genes have been intensively studied from the very beginning of association studies on BMD. Vitamin D receptor (*VDR*) gene, estrogen receptor-α (*ESR1*) gene, α1 chain of type 1 collagen (*COL1A1*) gene and *TGFB1* gene have all been widely studied but the results have been varied and in some cases contradictory. In each gene, only a small number of variants were analyzed and their selection was based only on availability and not on functionality. A screen with a high-density SNP should be performed before ruling out any potential role of these genes in BMD variation. The GENOMOS consortium has revisited many claimed associations by analyzing five common polymorphisms in the *VDR* gene among 26242 men and women from nine European teams [88], three common polymorphisms in the *ESR1* gene among 18917 men and women from eight European teams [89], one polymorphism in the *COL1A1* gene among 20786 men and women from nine European teams [90] and five common polymorphisms in the *TGFB1* gene among 28924 men and women from ten European teams [91]. Only the *COL1A1* gene was found to be associated with BMD. The single polymorphism studied is within a putative Sp1 binding site, which was thought to influence the expression of the gene [92]. Homozygotes for the rare allele had significantly lower BMD than the two other genotype groups at lumbar spine and femoral neck. However, the homozygotes represent only 3.4% of the population (minor allele frequency of 18%). Sex-specific analyses showed similar results for females at lumbar spine and femoral neck, but results for males were not significant for lumbar spine and heterogeneity was observed between studies. However, the results were significant at femoral neck for males, without heterogeneity between studies. The association with a higher risk of fracture could not be formally demonstrated, although a modest association with incident vertebral fractures among women was observed. The effect size was similar to that observed with *LRP5* Val667Met, that is, 21 mg/cm^2 at lumbar spine and 24 mg/cm^2 at femoral neck but involving at least threefold fewer individuals (3.5 vs 11.5%) with low bone mass genotype.

While the consortium could not confirm any genetic contrast with the three polymorphisms studied (intron 1 *Xba*I, *Pvu*II and promoter TA repeats microsatellite) in the *ESR1* gene with BMD [89], they found significant reduction in fracture risk with the *Xba1* polymorphism (rs9340799). Carriers of genotype XX versus Xx and xx (CC vs CT and TT) had a 20% reduction in fracture risk ($p < 0.001$). The reduction was even greater (30%) for vertebral fractures and these results were consistent for women and men. There is no formal explanation for the protection against fracture, with no association with higher bone density; however, possibilities include effects on bone quality, bone geometry, bone turnover or other risk factors for fracture [89]. An effect on bone turnover (especially bone resorption) could be plausible given the function of estrogen or hormone replacement therapy in counteracting the excess of bone resorption that occurs in the postmenopausal years [11]. In the context of low concentration of estrogen associated with the postmenopausal status, a slight difference in the number of estrogen receptors expressed could make a difference in the level of bone resorption, which in turn, would impact the risk of fracture. To test this hypothesis, bone markers for resorption should be evaluated and compared between *Xba1* genotypes in a group of postmenopausal women. If this association is significant, it would be the first example of a genetic marker for bone resorption.

The previously reported associations between *VDR* and *TGFB1* polymorphisms and BMD did not remain significant in these large studies.

Other candidate genes

Genes involved in the synthesis of estrogen, such as *CYP17A1* and *CYP19A1* genes, are attractive candidates. *CYP19A1* gene codes for the enzyme aromatase, which converts androgenic precursors into estrogens, and *CYP17A1* codes for a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens and estrogens. Both have been studied [93–97] but generally only in samples of small size. Thus, no firm validation of any of these associations has been performed so far. Genes involved in calcium and vitamin D regulation, such as those coding for the parathyroid hormone receptor, calcitonin receptor, klotho and vitamin D binding protein were also studied [97–99]. Some genes, such as *ALOX12* and

ALOX15, have been shown to play an important role in the acquisition of bone mass in mouse models and were then studied in the general human population [100–102]. We also analyzed many uncorrelated SNPs in these two genes in a sample of 707 premenopausal women and did not find any associations. None of these gene variants has been validated in replication sets of very large samples.

Conclusion & future perspective

Although advances have been made in understanding the role of genetic factors in the regulation of BMD in the last decade, a lot of additional research is needed to identify every single gene variant influencing bone density in the general population. So far, only two gene candidates (*LRP5* and *COL1A1*) are promising because they have been associated in more than one study and in very large sample sizes. The *LRP5* gene appeared to be a true associated gene, given all the evidence accumulated, but the causative variant(s) have not yet been confirmed. *COL1A1* was also shown to be associated in a very large sample, but it affects only a small percentage of the population (3.4%) and therefore will not be so useful in the context of public health. *TNFRSF11B* also appeared to be a promising target, although it needs to be validated in more samples and the causative variant(s) needs to be identified. Interestingly, LRP5 and TNFRSF11B are important players in the two major pathways described for bone remodeling.

Recent drug development has focused on the osteoclastogenesis system and Wnt signaling in bone. Novel therapies with antiresorptive properties include denosumab, a human monoclonal antibody against RANKL that mimicks the endogeneous effects of osteoprotegerin, the *TNFRSF11B* gene product. A randomized, blinded clinical test evaluated denosumab in comparison with alendronate, the drug currently used to treat osteoporosis, in a total of 1189 postmenopausal women [103]. The denosumab treatment resulted in significantly greater increases in BMD at all measured skeletal sites and significantly greater reduction of bone turnover markers compared with alendronate therapy. This therapy can prevent bone loss but cannot activate bone formation. The recent accumulation of knowledge on the role of the Wnt signaling pathway in regulating bone formation (FIGURE 2) has triggered an effort by the pharmaceutical and biotech industry to develop therapeutic products that would increase bone formation in osteoporotic and osteopenic patients [2]. So far the only anabolic treatment for bone is the use of injectable parathyroid hormone [104]. New targets are preferably those that are expressed only in the skeleton, and thus new therapy could increase bone formation without affecting Wnt signaling in other organs. This challenge may be achievable by targeting DKK1 or SOST with monoclonal antibodies because these two proteins are essentially restricted to osteoblasts and/or osteocytes in the adult mouse skeleton [105,106]. Both proteins have been shown to inhibit the Wnt signaling pathway and therefore blocking the inhibitor will lead to increased bone formation. Indeed it was shown that anti-DKK1 displayed a bone-anabolic activity by increasing the femur BMD in mice and that sclerostin-neutralizing monoclonal antibodies had bone anabolic activity in mice and rats [2]. These compounds are in early-stage discovery or reaching Phase I clinical trials.

The discovery of the multiple genetic variants responsible for low peak bone mass may help to develop a genotype score to predict the risk of osteoporosis later in life. In addition, this could be performed at an early age, allowing time for changing at-risk lifestyles (e.g., smoking) and adopting healthier measures such as exercising regularly and having good nutrition. Some individuals may also benefit more than others from quitting smoking or increasing exercise, and this can only be revealed by gene–environment interaction studies, which are still scarce. Very large samples and well-established gene variants would be needed to explore this aspect more deeply, as well as carefully collected lifestyle data. Studies on Type 2 diabetes suggest that genetic risk determinants may be useful in younger individuals, before obvious risk factors have developed [107,108], which is in line with the approach we propose.

We expect that in the future, large groups of premenopausal women, in whom we believe that the gene effect is maximal, will be enrolled and analyzed with a high-density polymorphism screen to capture genes with small effect. To confirm or validate an association as not being a false-positive, replications performed according to the guidelines published by Chanock *et al.* should always be pursued [58]. Rather than setting a stringent significance level, replication studies in different populations are the most important requirement for establishing a positive association [109]. Variation in copy number in the human genome has also been recognized as a new form of genetic variation, which deserves to be fully investigated [110]. Recently,

a genome-wide copy number variation study identified UDP glucuronosyltransferase gene (*UGT2B17*) as a susceptibility gene for osteoporosis [111]. *UGT2B17* copy number was associated with osteoporotic fractures in two independent Chinese samples, as well as with hip bone density and femoral neck bone geometry in two samples of 689 unrelated Chinese subjects and 1000 unrelated white subjects. More similar studies should soon appear in the literature.

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Executive summary

Osteoporosis

- Osteoporosis is characterized by a reduced bone mineral density and represents an increased risk of fracture.
- Bone mineral density in the healthy population at any age is mainly due to the peak bone mass reached during young adulthood.

Peak bone mass

- Peak bone mass is the phenotype presenting the most important heritable part, up to 80%.
- Menopause transition in women is synonymous with accelerated bone loss and increasing age is also associated with bone loss.
- Bone loss has not convincingly been associated with genetic factors.

Identification of genes

- The *LRP5* gene, involved in the Wnt canonical pathway, is the first candidate to be clearly associated with bone mineral density in rare autosomal diseases and in the variation of bone density in the general healthy population.
- *TNFRSF11b* gene, which is involved in the regulation of bone resorption, is a promising target, but more evidence is needed.
- Genes with smaller effects could be identified in large samples of younger women close to their peak bone mass.
- The most frequently investigated candidate gene variants for osteoporosis (*TGFB1, VDR* and *ESR1)* were discarded, thanks to the large prospective study GENOMOS.

Conclusion

- Although genetic factors are important for bone mineral density, few gene variants have been clearly identified so far. A lot of work remains to be done, but the use of larger sample and younger women closer to their peak bone mass, where the highest heritability is found, should help to identify genes with small effect.

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http://snpeffect.vib.be/index.php

203 Polyphen SNP Data
http://genetics.bwh.harvard.edu/pph/data