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Bone density and risk of osteoporosis in Klinefelter syndrome

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Short title: Osteoporosis in Klinefelter syndrome

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Abstract

Different mechanisms in Klinefelter syndrome contribute to reduced bone mass and osteoporosis, which have a precocious onset and are detected in up to 40% of patients, irrespectively of testosterone levels. Androgen receptor, X chromosome inactivation, and INSL3 levels are hypothesized to cooperate with and modulate the effect of testosterone on the bone. In conclusion, new perspectives on genetic topics are opening exciting areas of research on the pathophysiology of reduced bone mass in Klinefelter patients.

Introduction

Osteoporosis is defined as reduced bone density and strength predisposing to an increased risk of fracture. It is an underestimated, underdiagnosed and undertreated condition in men (1), and most cases (40%) are classified as idiopathic (1). Interestingly, osteoporosis seen later in life may originate in childhood or adolescence (2).

Osteoporosis is defined according to measurement of bone mineral density (BMD) using dual-energy X-ray absorptiometry (DEXA). The commonly used BMD-based operational definition of osteoporosis has been validated for white postmenopausal women only and there is no consensus BMD-based definition of osteoporosis in the male. It is generally accepted that a BMD-T score at or below 2.5 standard deviations (SD) below normal peak values for young adults defines osteoporosis, whereas a T score between -1 and -2.5 SD defines osteopenia (Table 1). For younger men both T score and Z score could be used for the diagnosis of low BMD, with a Z score < 2 SD below the gender- and age-specific population mean identifying osteoporosis (3) (Table 1). Nevertheless, it is currently not possible to define osteoporosis in children on the

basis of BMD measurements alone and the relationship between BMD and fracture risk in children with chronic diseases is unknown (4). Thus, it is not possible to define thresholds below which there is an increased fracture risk in children, so that the diagnosis of osteoporosis in these subjects requires the presence of clinically significant fracture history and the low mineral content or density based only on the Z-score (2, 3), while some Authors suggest that the phrase “low bone density” is preferred instead of osteopenia or osteoporosis (2).

In the USA the prevalence of osteoporosis and osteopenia is estimated to be respectively about 6% and 47% (5) in men, but data on the prevalence in children and adolescents are lacking. It has been reported that 42% of boys sustain a fracture before 16 years of age and many children, apparently healthy, suffer repeated fractures during growth (6). In these subjects site specific bone weakness was found by DXA measurements (6).

Although there is increasing recognition of the problem of male osteoporosis, there remain considerable gaps in knowledge regarding this disorder as well as in the care of these patients.

Generally, men loose less bone mass than women during lifetime (7), and they are protected against bone loss and osteoporosis with a lifetime risk for fragility fractures of 15% compared to 40% in women (8). During puberty, young men and women present the same peak vertebral bone density, but bone width is greater in men, and therefore they have greater bone strength (9). Therefore, bone loss in men appears later in life compared to women (10).

Male osteoporosis is a heterogeneous entity, with multiple underlying causes and risk factors, which are generally divided into primary (age-related and idiopathic osteoporosis) and secondary causes. Many systemic diseases can affect bone mass in males, directly or indirectly (10, 11). In particular, genetic disorders that can impair

bone health in male children and during adolescence are: Klinefelter syndrome (KS), cystic fibrosis, Duchenne muscular dystrophy, osteogenesis imperfecta, hemophilia and thalassemia.

Androgens, hypogonadism and bone

Androgens have an important role in bone metabolism and different conditions associated with hypogonadism are associated with low bone mass. Hypogonadism (total testosterone < 10.4 nmol/L) (12) is found in about 15% of men with osteoporosis, although this proportion varies both across populations and with the definition of hypogonadism. The main causes of hypogonadism are: KS, orchiectomy, testicular failure from primary testiculopathies, radiotherapy or chemotherapy, treatment with GnRH agonists, and late-onset hypogonadism in the elderly men.

Testosterone regulates male bone metabolism both directly on osteoblasts through the androgen receptor (AR) and indirectly by aromatization to estrogens. The net effect of testosterone is to promote periostal bone formation mostly during puberty (13) and to reduce bone reabsorption mostly during adult life (11). The final effect of androgens on the bone is to maintain cancellous bone mass and to increase bone size by stimulation of both longitudinal and radial growth. This leads to higher bone size and bone strength compared to women.

The AR pathway is particularly effective in the trabecular bone where androgens preserve or increase trabecular numbers, suppress trabecular reabsorption, and reduce trabecular spaces, therefore increasing trabecular bone volume (14). A decrease in trabecular bone is common in AR knockout mice (15) and patients with AR mutations show a reduced bone mass (16). Studies suggesting that one year after castration in men vertebral BMD is reduced by at least 5-10% (8) lend support to this hypothesis. On the other hand, cortical bone is affected when both AR and estrogen receptor disruption

occur (14, 15), whereas bone loss from estrogen deficiency is mostly evident at the cortical level (17).

Testosterone is fundamental in a critical stage of bone maturation to reach the peak bone mass at the end of puberty and to keep it during adult life. Thus, an early onset of testosterone deficiency during puberty is an important risk factor for precocious male osteoporosis. In fact, premature male osteoporosis is usually associated with hypogonadism, as observed in KS, idiopathic hypogonadotropic hypogonadism or delayed puberty, and in hyperprolactinaemia.

A positive correlation between BMD and testosterone levels has been demonstrated in normal men, osteoporotic men and in KS (18, 19). It has also been reported that both serum levels of testosterone and luteinizing hormone (LH) show a significant association with osteoporosis or fractures (20).

Moreover, there are connection between testosterone and the vitamin D pathway. Testosterone acts indirectly on the PTH-Vitamin D axis, because testosterone deficiency is related to a reduction in renal 1α-hydroxylase activity with a subsequent decrease in $1,25\text{-}(OH)_2D$ concentration, the active form of Vitamin D (21).

Bone turnover in the male is influenced also by estrogens and a large part of testosterone action on bone metabolism is mediated by its aromatization to estrogens. Estrogens are important enhancer of bone mass during growth and maturation, they thicken bone cortices, maintain BMD, retard bone loss and are essential in periostal bone expansion during puberty. As testosterone, estrogens gradually decrease in men during aging (22) and rare causes of isolated hypoestrogenism in men or estrogen receptor disorders associated with bone loss have been described (23).

Pathogenesis of osteoporosis in Klinefelter syndrome

KS is characterized by primary hypogonadism due to a progressive testicular failure initiating during the pubertal development. Patients with KS have a high risk of developing osteoporosis and osteopenia and a consequent increased risk of fractures (24, 25). In fact, KS is associated with decreased bone mass in 25-48% of cases (26) and with osteoporosis in 6-15% (27) and it is due to both reduced bone formation and higher bone reabsorption (28). The annual decrease in bone mass rate in KS has been calculated in 1.18±0.53% at the lumbar level and 1.03±0.43% at the femoral neck level (29).

Young KS patients have normal bone density in childhood and at the beginning of pubertal development (25). During the later stages of puberty, KS patients develop a progressive testicular failure leading to primary hypogonadism. Such a deficiency in testosterone production during puberty represents therefore the most important risk factor for reduced bone mass and osteoporosis in KS. Supporting this hypothesis testosterone plasma levels have been shown to positively correlate with BMD in these patients (18, 19, 29). Similarly to that observed in hypogonadal non KS patients, bone histology of KS subjects demonstrated loss of cancellous tissue, profound depression of osteoblast activity, decreased osteoid seam width, and slowing of the apposition rate (30). These findings have not been documented in KS patients with normal testosterone levels who have a normal cortical bone mass (18).

However, several studies showed that testosterone replacement in KS men with low testosterone levels and low BMD does not reverse the decreased bone mass (31). This was more evident when testosterone replacement therapy was started after puberty, also after many years of therapy (27). On the contrary, other studies showed that androgen replacement therapy starting in young age (i.e. before 20 years) can lead to normal BMD (32).

Of particular interest is the finding of reduced bone mass also in KS patients with normal testosterone levels (19), suggesting that bone loss in KS might be, at least in part, independent from the presence of hypogonadism. It is well known that testosterone levels can be normal in a high proportion (40-50%) of KS patients (33) but it should be noted that in KS patients testosterone levels might not reflect the actual androgen status, as signs of hypogonadism may be present also in men with apparently normal T levels, whit LH levels invariably high and disproportionately elevated in these individuals. Based on this, it has been suggested that in KS hypogonadism might be better defined by a bivariate LH and testosterone chart because more complex nonlinear relationships between LH and testosterone levels may be present (34).

Unfortunately, there are no definitive data in the literature clearly showing the prevalence of osteoporosis in KS patients with low or normal testosterone levels, or with/without other signs of hypogonadism. Moreover, it was recently highlighted that testosterone could not be a predictor of BMD in KS (35).

There are other possible hormonal modulators of bone metabolism in KS. Even if estradiol levels are generally normal or high in this syndrome, low estrogen levels have been related to decreased bone mass also in these patients by some authors (36), and estradiol levels are inversely related to the rate of bone loss in KS (29). However, these data have not been replicated and conclusions on this possible pathogenetic mechanism can not be made. Similarly, only two studies examined the vitamin D levels in KS. Stepan et al showed that 25-OH and 1,25(OH) vitamin D are in the low-normal range in KS patients and that the rate of gain of BMD in the femoral neck after ibandronate therapy was inversely related to 25-hydroxyvitamin D, highlighting the importance of vitamin D insufficiency or deficiency in the response to therapy (29). Besides, Bojesen and colleagues observed that signs of secondary hyperparathyroidism are present among

KS, with plasma levels of 25-OH vitamin D significantly lower in KS patients compared to healthy subjects (35).

Another possible mechanism involved in the development of bone loss in KS might be related to the unfavourable fat/muscle ratio caused by increased fat mass and reduced muscle mass (24). However, it is not clear whether such an altered ratio is caused exclusively by the low testosterone levels or by other mechanisms related to the genetic defect. In fact, recent studies suggested that the unfavourable fat/muscle ratio is already present in young adolescents, whereas bone mass defects appear in late puberty or later (25), while muscle strength represents an independent predictor for BMD in KS patients (35).

Recent researches suggested other possible pathogenetic mechanisms of reduced BMD in KS, related in particular to the AR function and Insulin-like factor 3 (INSL3) levels. The first exon of the AR gene encodes for the transactivation domain of the AR protein. It contains the highly polymorphic CAG repeat, the length of which is inversely correlated with sensitivity to androgens (37). Although the length of CAG repeat has been associated with different disorders (male hypogonadism, cryptorchidism, prostate cancer, testicular cancer), conflicting data have been published on the relation between it and bone metabolism. The CAG polymorphism of the AR has been reported to be negatively and independently associated with BMD, in particular in young men (38). Another study found on the contrary a positive relation, explained with the negative feed-back of CAG related AR sensitivity on testosterone concentrations and thus on higher estrogen levels, with a global positive effect on BMD (39).

A certain degree of androgen resistance has previously been reported in KS (26), with a decreased activity of bone 5-α-reductase (40) and a lower peripheral AR expression on lymphocytes (41), testis (42) and smooth muscle cells (43). However, the AR expression in the bone has never been studied in KS.

Another important aspect of AR is that the AR gene is located on the X chromosome (and therefore present in double copy in KS) and there is evidence of non-random X inactivation in men with more than one X chromosome (44). In KS the CAG polymorphism length depends on the inactivation rate of the two X chromosomes by methylation. Therefore, the effective CAG repeat value in heterozygous KS men for the CAG polymorphism of the AR gene is calculated after the analysis of the methylation rate in the two X chromosomes in order to obtain a X-weighted biallelic mean, not an arithmetic mean (45). As a consequence, a relation with different clinical outcome and response to testosterone therapy was found, with a statistically significant negative correlation between BMD evaluated by phalangeal ultrasound and the X-weighted biallelic mean of CAG repeats (45), as previously shown in normal men (44). Moreover, it has also been described a higher inactivation rate of shorter alleles, thus determining a less sensible AR (45).

This was the first important finding about the evidence that reduced testosterone levels are not the unique cause of decreased bone mass in KS patients and that additional factors related to the androgenic status might contribute to the altered bone metabolism in patients with KS. A non-random X inactivation and lower androgen function could therefore be, at least in part, responsible for or contribute to decreased bone mass in KS, particularly evident in those patients with normal testosterone concentration. Thus, this mechanism could explain not only the high prevalence of decreased BMD also in eugonadal KS patients, but also the frequent ineffectiveness of testosterone replacement therapy in improving BMD in KS.

Another important aspect related to testicular failure and bone metabolism in KS is the circulating level of INSL3. INSL3 is a protein hormone produced almost exclusively by pre- and postnatal Leydig cells of the testis (46-48). The major known endocrine role of INSL3 is related to the regulation of testicular descent during foetal development by

acting on gubernaculum via its specific receptor RXFP2 (Relaxin Family Peptide 2) (49). As a consequence, *Insl3* and *Rxfp2* knockout mice have bilateral cryptorchid testes (49) and mutations in the *INSL3* and *RXFP2* genes have been associated with testis maldescent also in humans (50-53). In addition to the prenatal role for INSL3, further possible endocrine and paracrine actions in adult males have recently gained particular attention (46, 54, 55). These studies showed that INSL3 is produced constitutively but in a differentiation-dependent manner by the Leydig cells under the long-term Leydig cell differentiation effect of LH. On this basis INSL3 has been proposed as a specific marker of Leydig cell differentiation status (46, 48). Testosterone and INSL3 provide different information on the status of the Leydig cells: testosterone better reflects the steroidogenic activity that is acutely sensitive to LH, whereas INSL3 seems to be uncoupled from this rapid stimulation of steroidogenesis and better reflects the differentiation status and general wellness of the Leydig cells (46, 56).

The dynamic of circulating levels of INSL3 is very similar to that of testosterone. After birth, INSL3 increases at about 3 months of age under the increased levels of LH (minipuberty) (57). Soon after, INSL3 declines to undetectable levels and remains low during infancy (57) and then progressively increase throughout puberty (55). Reduced plasma concentrations of INSL3 are seen in situations of undifferentiated or altered Leydig cell status or reduced Leydig cell number, such as in anorchid men, men with hypogonadism, infertility, or obesity (46, 55).

Although the exact role of postnatal INSL3 is not fully understood, the general hypothesis is that reduced INSL3 activity (caused by altered testicular function, *INSL3* or *RXFP2* gene mutations) could cause or contribute to some symptoms and signs of hypogonadism, such as reduced BMD, currently ascribed to testosterone deficiency. We showed that human and mouse osteoblasts express the INSL3 receptor and that young adult men carrying the T222P mutation of the *RXFP2* gene and with normal

testosterone levels are at significant risk of reduced bone mass and osteoporosis (58). Consistent with the human phenotype, bone histomorphometric and μCT analyses at the lumbar and femoral sites of *Rxfp2*$^{-/-}$ mice showed decreased bone volume, alterations at the trabecular bone, reduced mineralizing surface, bone formation, and osteoclast surface (58). These data suggested a functional osteoblast impairment causing a negative balance between bone formation and bone resorption in mice knockout for *Rxfp2* and in humans with mutations in *RXFP2*.

Only one study examined INSL3 levels during puberty in boys with KS, showing a normal increase in serum INSL3 at initial stages of puberty and then a levelling off (59). Few studies examined INSL3 in adult men with KS. We reported that adult KS with reduced testosterone levels had also very low levels of INSL3 (46). These data have been confirmed in another study (60), which showed also that KS patients with the highest INSL3 levels were those who were not in need of testosterone substitution. Taken together these findings, although preliminary, would suggest that: i. INSL3 seems more appropriate than testosterone to assess the Leydig cell function in KS; ii. the low INSL3 levels observed from mid-puberty onward in KS could have a role in the reduced BMD and osteoporosis in these patients; iii. the limited efficacy of testosterone replacement therapy in KS patients with osteoporosis could be justified by this alternative pathogenetic mechanism.

Table 2 summarizes the possible mechanisms involved in reduced bone mass in KS. The combined effect of all these factors at the end of puberty and during young adulthood could therefore represent the pathogenetic mechanisms leading to the precocious decrease in bone mass in KS patients. Unresolved questions are related to the choice of treatment in osteoporotic KS patients with normal testosterone levels. Future pharmacogenomic approaches based on AR sensitivity and the possible development of drug with INSL3 properties could be of extreme interest.

List of abbreviations

KS: Klinefelter syndrome

BMD: Bone mineral density

DEXA: Dual-energy X-ray absorptiometry

AR: Androgen receptor

LH: Luteinizing hormone

INSL3: Insulin-like factor 3

RXFP2: Relaxin family peptide 2

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Table 1. Diagnostic criteria for osteoporosis in men. SD: standard deviation.

Age	Criteria
< 50	The diagnosis should be made on the basis of both T-score and Z-score. Some Authors recommend to use only the Z-score (low BMD when Z score < -2 SD, osteopenia if Z score < -1 SD).
50-64	T-score ≤ - 2.5 SD at both spine and hip plus risk factors for fracture
≥ 65	T-score ≤ - 2.5 SD
any age + secondary causes of low BMD	T-score < -1 SD

Table 2. Possible mechanisms contributing to reduced bone mass in Klinefelter syndrome.

Mechanisms affecting the whole bone	
Low vitamin D levels Unfavourable fat/muscle ratio	
Trabecular bone	**Cortical bone**
Low testosterone levels Low androgen receptor expression Non random X chromosome inactivation and AR CAG length Low INSL3 levels	Testosterone and estrogen deficiency AR defects plus estrogen deficiency