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REVIEW

Insights into bone health in Duchenne muscular dystrophy

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Poor bone health is a significant problem for patients with Duchenne muscular dystrophy (DMD), a progressive, disabling disease. Although the primary focus of DMD disease pathogenesis is degeneration of striated muscle, impairment of bone health likely has a role in the disease that has only been superficially examined to date. Deficiency of bone mineral density and increased incidence of bone fractures are well-recognized clinical components of the DMD phenotype. Furthermore, therapy with corticosteroids, an approved treatment for DMD that prolongs ambulation, may have multiple effects on bone health in DMD patients. This review examines the evidence in preclinical models and in human DMD disease that provides insight into the role performed by bone in the disease pathogenesis and phenotype of DMD. The information reviewed here points toward the need for mechanistic and therapeutic studies to optimize bone health in DMD patients.

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive condition characterized by progressive muscle weakness leading to premature death. It is the most common form of muscular dystrophy, affecting about 1 in 3600–6000 males.[1,2] A mutation in the dystrophin gene results in the absence of dystrophin, a muscle cytoskeletal protein.[3] The symptoms of DMD are usually first recognized in affected boys at about 3–5 years of age. The onset of proximal leg weakness impairs mobility, as evidenced by a diminished ability to run or to rise from the floor. Often by 8–12 years of age, affected boys can no longer walk independently. Progressive respiratory and cardiac dysfunction contributes to morbidity later in the disease course, ultimately culminating in early lethality.[4,5]

It is known that DMD has an impact on bone health. Published reports of these effects have appeared since 1941.[6–8] Decreased bone mineral density (BMD) and fractures are serious sequelae of DMD that have been reported repeatedly.[7–23] More recently, with the advent of routine use of corticosteroid therapy to prolong the period of ambulation in DMD patients, there is a heightened concern for bone health due to the possible osteoporotic impact of corticosteroid use.

Pathophysiological Characteristics of DMD

In normal skeletal and cardiac muscles, dystrophin is a subsarcolemmal protein critical for membrane stabilization and prevention of contraction-induced cell membrane damage. In the absence of dystrophin, muscle fibers degenerate, producing a cascade of pathophysiological changes to muscle function.[24] In skeletal muscle, muscle fiber degeneration not only activates mechanisms responsible for regeneration, but it also stimulates processes that impair that regeneration.[25] The precise sequence and exact processes by which these effects occur are not known, but a number of signaling pathways are either activated or suppressed.[26,27] Skeletal muscle regeneration is initiated by the activation and proliferation of muscle precursor cells called satellite cells (that differentiate to form myoblasts) and their fusion to damaged muscle fibers. At the same time, muscle degeneration causes the release of inflammatory mediators, such as those secreted by neutrophils, macrophages and associated cytokines that result in further tissue injury and initiation of fibrosis.[28–31]

A significant contributor to dystrophic pathology involves activation of the nuclear factor of κB pathway by tumor necrosis factor α.[32,33] In turn a nuclear factor of κB-dependent pathway induces transcriptional activation of the interleukin (IL)-6 gene and increases IL-5 production.[34,35] Deleterious alterations of calcium homeostasis, oxidative free radical damage to myofibers, the development of fibrosis and ultimately the failure of myoblast proliferation and fusion contribute to the failure of effective skeletal muscle regeneration. Studies in the *mdx* mouse model of DMD have contributed to many

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of these pathophysiological findings. Of particular interest is the recent finding of Vetrone *et al.*[36] that osteopontin (OPN) promotes fibrosis in dystrophic mouse muscle by modulating immune cells and intramuscular transforming growth factor-β. OPN was also recently shown to be a determinant of disease severity in DMD patients in that a mutation in an OPN gene promoter is associated with an earlier loss of ambulation and muscle strength.[37]

Calcium homeostasis is highly perturbed by DMD. Increased trans-sarcolemmal calcium influx, increased permeability to divalent cations that enter through channel-blocker-sensitive pathways, and entry of calcium by nonspecific cationic channels are among the alterations in calcium homeostasis observed in human patients and animal models of DMD.[38–41] Dystrophin deficiency results in an elevation of the resting intracellular calcium level in human DMD myotubes.[42] The high steady-state calcium levels are dependent on continued spontaneous calcium release and contraction of human DMD myotubes. The elevated calcium levels are not observed when contraction is diminished by tetrodotoxin, which blocks voltage-dependent sodium channels. Lipofection of full-length dystrophin in *mdx* mouse myotubes lowers the resting calcium concentration and the activity of calcium leak channels. In addition, forced expression of full-length dystrophin in a dystrophin-deficient skeletal muscle cell line (Sol8), which displays altered calcium homeostasis, rapidly restores calcium handling.[43] The expression of dystrophin reduces the abnormal resting steady-state level of calcium ions, as well as the amplitude of transient calcium release from internal stores during depolarization.[44] Providing further evidence of the disruption of calcium homeostasis in the setting of dystrophin deficiency *in vivo*, targeted inhibition of calcium/calmodulin signaling exacerbates the muscle dystrophic phenotype in the *mdx* mouse.[45] There is further evidence that dystrophin may negatively regulate sarcolemmal store-dependent calcium channels, reducing store-dependent calcium influx as well as its mitochondrial uptake.[46]

Many of these pathophysiological phenomena may also contribute to deterioration of bone in DMD patients and as such are discussed later in relation to the origins of bone loss.

Bone Health

The physiological processes of bone acquisition, remodeling and turnover, and even fracture resistance are the subjects of many recent reviews.[47–54] There are reviews of bone physiology as it relates to chronic illness,[55] auto-immune-related bone loss,[56,57] obesity,[58] and bone loss associated with childhood, puberty and fragility.[47,59–61] Bone mineralization and the role of phosphate metabolism have also been recently reviewed.[62]

Later pubertal timing is predictive of low peak bone mass and increased risk of fracture in young adult men.[63] The importance of pubertal timing as a predictive factor of bone mass in young adulthood was confirmed in a multicenter study in both the genders. Delays in puberty are associated with poor bone health.[60,61,64]

Below is a brief overview of selected aspects of some of the processes that maintain bone health because they are likely to be disturbed by DMD pathophysiology and may be related to the causes of the bone loss, fragility and fractures seen in DMD patients.

Bone remodeling occurs most often in skeletal sites rich in trabecular bone, such as the vertebrae, proximal femur, calcaneus and ultradistal radius. Remodeling is a process essential to maintenance of skeletal growth and health. It provides elasticity to bone, and produces a steady source of extracellular calcium. In childhood, bone formation occurs at a faster rate than resorption until equilibrium is reached in the mid-to-late twenties or in the thirties, where bone formation and resorption occur at about the same rate. After this period, there is a normal yet progressive decline in bone density.[65–67] Remodeling is also a process essential to the prevention of and recovery from bone fracture.[54]

The bone remodeling process takes place at specific sites or skeletal lacunae in cycles of ~120 days. Bone turnover begins with the activation of surface osteoblasts. The signals for initiation include changes in the serum concentration of a number of systemic factors, such as parathyroid hormone, thyroxine, growth hormone and estrogen. Cytokines and growth factors (for example, IL-1, -6 and -11, insulin-like growth factor-1 and transforming growth factor-β) trigger the bone-synthesizing activity of osteoblasts.[68,69]

Once the osteoblast is activated, cytokine synthesis and secretion result in recruitment and differentiation of osteoclasts at the remodeling surface. These specialized bone-resorbing cells secrete proteases that dissolve the mineral matrix and break down collagen. This process releases matrix-bound growth factors that couple osteoblastic activity to osteoclastic resorption. The remodeling cycle is complete when bone mass is restored.[67,69,70] OPN is an important factor in this process.[71] OPN is thought to initiate the process by which osteoclasts develop their ruffled borders to start bone resorption. It is expressed in a range of immune cells, including macrophages and neutrophils, which are involved in DMD pathophysiology as discussed earlier. OPN is reported to be an immune modulator acting by a variety of mechanisms that include chemotactic promotion of cell recruitment to inflammatory sites and as an adhesion protein, involved in cell attachment in reaction to fracture.[72]

Properties contributing to bone strength include the rate of bone turnover, BMD, geometry, micro-architecture and the degree of mineralization. Collectively, these properties are often referred to as bone quality, which gives bone the ability to resist fracture after trivial force or trauma.[73] Bone plasticity, nanoscale structure and the cooperative deformation between mineralization and collagen are factors in fragility and fracture.[74,75] An increased risk of fractures is the strongest evidence for degraded bone quality. An imbalance in the rates of osteoblast and osteoclast activity leads to changes in skeletal structure and bone mass. Osteoporosis is a skeletal disorder characterized by compromised bone strength and increased risk of fracture due to increased bone resorption or decreased bone formation.[24,65,76]

Maintenance of appropriate nutritional status is critical to skeletal development and continuing bone health. The calcium intake level at which body retention of calcium reaches a maximal value reflects the amount required to fulfill the calcium needs of the body. It was reported to be the major predictor of calcium retention both in girls and boys 10–15 years of age. During childhood, a period of slower growth, there is a more pronounced relationship between urinary calcium and calcium intake than during the rapid growth period of adolescence. Rapidly growing

individuals retain absorbed calcium in the skeleton rather than excreting it in the urine. Urinary calcium is expected to rise only after the skeletal compartment is saturated with calcium at intakes at or above the threshold intake.[77] Renal excretion of calcium is regulated by parathyroid hormone and estrogens. It is also influenced by dietary protein and, in particular, by sodium. A high intake of salt increases urinary calcium loss.

Of the influences on calcium absorption, the most important is vitamin D, which is necessary for the active transport of calcium across the intestinal mucosa. Vitamin D status can influence absorptive performance and hence the calcium requirement.[78] To meet their high calcium requirements, adolescents must absorb calcium at higher efficiency than children and young adults.[77–79]

Evaluating Bone Quality and Fracture Risk

There are a number of non-invasive methods available to evaluate bone quality. Some, such as dual-energy X-ray absorptiometry (DXA) for BMD and biological markers for bone turnover, are in routine clinical use.[80,81] Computed quantitative computer tomography (QCT), sometimes with high resolution detection, is being used with greater frequency in the measurement of trabecular density in children. Appendicular or peripheral sites are often used for scanning as opposed to spine because of lower radiation exposure.[47] However, others, such as digital topographic analysis of magnetic resonance microimaging for bone architecture are in the research and development phase.

An important limitation of virtually all quantitative imaging techniques used to assess bone health is compromised image quality from uncontrollable motion of the patient. Motion confounds the accuracy of cortical and trabecular densitometry and structure measurements. However, there have been some recent advances in the ability to manage motion artifacts without the use of subjective criteria.[47]

DXA scanning at selected skeletal sites is the technique of choice for assessment of the effect of pathological conditions on bone in the clinical setting. It provides reference data from infancy to post-puberty, taking into account age, sex, race, maturation and size effects on BMD and bone mineral content. It allows a determination of the degree of departure from normative values in the form of *T*- and *Z*-scores.

BMD remains the standard for evaluating fracture risk and is measured easily in patients.[24] BMD is reported in grams per square centimeter (a two-dimensional measurement). Thus, bone mineral content and the geometry (macro-architecture) of the site measured, both influence BMD.

Volumetric adjustments can be made to approximate bone mineral apparent density, which is less dependent on bone size. These calculations are made where the spine is assumed to be a stack of cylinders such that the projected width of the bone, obtained from the DXA scan, is equal to the approximate depth of the vertebra and consequently bone width is used to make an estimate of bone volume. This technique is only applicable to the spine. An allometric scaling approach assesses bone size and bone mass as important determinants of bone strength. The assessment is based on the principle that bone area changes in proportion to height and that bone mass scales proportionally with bone area. This interpretation requires three phases of data analysis: first assessing height for age (are the subject's bones short for his age); then assessing whether the bones are appropriate size for height (are the bones narrow), and finally assessing whether the bones are sufficiently mineralized for their size (are the bones thin).[82]

An exponential relationship exists between bone density and some measures of bone strength such that modest increases in bone density are associated with disproportionately large increases in bone strength.

Nonetheless, special considerations are needed to use DXA to assess bone mass in children. The BMD of children, when compared with the reference data of adults (*T*-scores) underestimate the BMD of children, because children have less bone mass than fully developed females (the source of data that the World Health Organization used to establish the criteria for classification of normal, osteopenia or osteoporosis). To avoid an overestimation of bone mineral deficits, and therefore an overdiagnosis of osteopenia, BMD scores are better determined by comparing BMD in children using reference data for the same gender and age (calculating a *Z*-score). Even in this calculation, age presents a confounding factor, such that in boys especially, bone age may be preferred to chronological age.[83]

Other variables can also confound the interpretation of BMD, as measured by DXA, in children. Bone size can overestimate BMD in taller boys and underestimate it in smaller ones. Other imaging technologies such as QCT are capable of measuring bone volume, and are, therefore, not susceptible to the confounding effect of bone size in the same way as DXA. Although it can cause some difficulties in interpretation, adjustments are made to BMD or bone mineral content during growth using anthropometric variables, particularly standing height and body weight. In theory, adjustment of BMD or bone mineral content for body weight is required because optimal bone mass should be adapted to body weight or leanness and muscle strength in relation to the impact of gravity on bone acquisition or remodeling. It has been shown that once peak bone mass is attained, variance in BMD and bone mineral content nearly disappears.[84]

Many studies on standing height, body composition including fat and lean mass, and pubertal stages in relation to bone mass and structure acquisition in either parameter in healthy female and male subjects in various pathological conditions are underway. These results should lead to improvements in the way we measure BMD or bone mineral content in children in the future.[47] This data, in healthy individuals as well as in certain pathological states like DMD, might also lead to a better understanding of the links between fat tissue or skeletal muscle mass/strength and bone acquisition, and possible influences of either adipocyte-produced cytokines and/or skeletal muscle mechanical forces. The age at which puberty occurs can influence the timing, development and extent of peak bone mass.[61] Corticosteroid treatment is known to delay the onset of puberty, decrease BMD and increase fracture risk.[85]

Peptides released by the bone matrix and through collagen degradation into the serum can exhibit significant changes during bone formation and resorption. Although the release of these substances reflects bone turnover and indicates abnormalities in bone and mineral metabolism, a specific marker concentration does not necessarily reflect the activity of certain cells or of the mineralization process itself. Bone resorption markers respond more rapidly to change as compared with bone formation markers, because resorption takes place over short time periods (days) and formation occurs over longer time periods

(months). Unfortunately, the variability of bone turnover markers limits their use both clinically and in research.[81]

Corticosteroid Use in DMD

Experience with the use of corticosteroids in DMD extends over more than 30 years. The initial open design studies of glucocorticoids in DMD, which suggested short-term improvement in muscle strength and/or function, were followed by randomized controlled trials to assess glucocorticoid efficacy and find the optimal dose.[86–90] In randomized studies, a beneficial effect was detected by strength measures as early as 10 days and peaked at 3 months after initiation of corticosteroids.[91–94] Controlled studies of prednisone treatment in DMD patients have only been performed during the ambulatory phase. However, prednisone and its oxazolone derivative, deflazacort, are now being used into the non-ambulatory phase of DMD. Retrospective studies with deflazacort suggest that continuing corticosteroid treatment into the non-ambulatory phase of DMD confers a treatment benefit. However, this area needs to be explored with controlled studies.[22,23,95] The potential for long-term use of corticosteroids in DMD patients underscores the need to assess bone health in this therapeutic setting.

In a trial by the Cooperative International Neuromuscular Research Group, 64 ambulatory DMD patients, ages 4–10 years, were enrolled in an equivalence trial comparing daily dosing of prednisone, with high weekend dosing of prednisone.[90] Vitamin D supplementation was included for both the groups. Although there was no significant difference in the primary safety outcome of body mass index between the groups, there appeared to be significant increases in linear growth and BMD favored by the weekend dose regimen. A longer study would be required to adequately assess the effects of corticosteroid use on bone metabolism and fracture risk.

Bone Health in DMD: Fractures, BMD and Vitamin D Nutritional Status

Highlighting the concern for bone health in DMD, a number of international workshops have convened since 2004 to address this issue.[96–100] The majority of studies addressing bone health in DMD have not used concurrent age- and sex-matched healthy controls. Because of variations in patient age, season and latitude, and the methods and sample timing, it is difficult to interpret with certainty the changes reported for many of the biochemical bone health indicators. Some of the earliest observations that dystrophin deficiency in DMD patients had accompanying effects on bone are the presence of scoliosis in DMD boys.

Scoliosis in DMD. Scoliosis is a frequent complication (68–90%) of DMD.[101] A case report study of 123 DMD boys indicates a significant association between prolonged ambulation and a reduced risk of scoliosis development. Glucocorticoid administration appears to be associated with a later onset of scoliosis, but did not alter the severity at 17 years, probably reflecting the shorter overall glucocorticoid exposure in this population.[102] There was an international workshop that reviewed the frequency, impact (with particular emphasis on cardiac and respiratory effects) and treatment of scoliosis in DMD patients with particular reference to surgical intervention and its outcome.[99] As compared with reports before the use of routine corticosteroid treatment, scoliosis seems to be of less concern.[99,103–106]

Fractures in DMD. An estimated 20–25% of boys with DMD will experience a long bone fracture and this risk increases with age and loss of ambulation.[16] For some patients, the occurrence of a long bone fracture marks the end of ambulation.[96] Before the introduction of corticosteroids, reports of vertebral fractures were relatively rare.[15,16]

McDonald *et al.*[16] reviewed the case reports of 378 boys with DMD (median age 12 years, range 1–25 years). Seventy-nine (20.9%) of these patients had experienced fractures. Forty-one percent of patients with fractures were in the age range of 8–11 years and 48% were ambulatory. Falls were the most common cause of fracture. Upper limb fractures were most common in boys using knee–ankle–foot orthoses (65%) whereas lower limb fractures predominated in independently mobile and wheelchair-dependent patients (54% and 68%, respectively). Twenty percent of ambulant patients and 27% of those using orthoses lost mobility permanently as a result of the fracture.

This study did not examine the steroid regimens used in those children who had been treated with corticosteroids, nor did it ascertain the interval between corticosteroid initiation and fracture. However, the authors reported that the fracture prevalence in DMD patients exposed to corticosteroids was similar to that of the unexposed group. Furthermore, the study references an abstract of a study in which they report that DXA scanning failed to identify differences between those DMD patients who were treated with corticosteroids and those who were not.[107]

Corticosteroid therapy appears to increase the risk of vertebral fracture. Following 8 years of corticosteroid treatment, vertebral fracture rates as high as 75% have been reported, although with a latency of about 3 years of treatment before the report of the first fracture.[17] These fractures can occur with minimal or no trauma, reflecting the fragile state of bone health in DMD. Spinal fractures cause back pain and may lead to shortening or deformity of the vertebral column.

Although vertebral fractures present certain difficulties with their diagnosis and description, their occurrence was carefully reviewed in the workshop report of Quinlivan *et al.*[100] Relatively recent studies report that vertebral fractures occurred only in corticosteroid-treated patients in their studies. In a study of 79 DMD patients, 37 of whom were treated with deflazacort, by Houde *et al.,*[108] limb fractures were similarly frequent in deflazacort-treated (24%) and untreated (26%) boys. Vertebral fractures occurred only in the treated group (7/37; compared with zero in the untreated group). In a study by King *et al.*[109] the prevalence of vertebral fractures in corticosteroid-treated DMD was 32% (21/67) compared with no vertebral fractures in steroid-naive (0/45) DMD patients. Bothwell *et al.*[17] studied 25 DMD patients who were treated with daily corticosteroids (1 prednisolone, 13 deflazacort and 11 treated with prednisolone before switching to deflazacort) for a median duration of 4.5 years. Ten of the 25 boys (40%) sustained vertebral fractures; eight were symptomatic with backache, and two had fractures detected on spinal radiographs taken because of low BMD. The first fracture occurred at 40 months into treatment. A recent study reported on the clinical management of deflazacort-treated DMD patients with *Z*-scores of less than −1.0 (spine and/or total body) with the bisphosphonate alendronate, calcium, and vitamin D. At 2 years of treatment, the mean *Z*-scores remained unchanged.[21]

BMD in DMD. A number of studies report decreases in BMD associated with DMD.[12,14,18–21,110] A profile of type of study

undertaken, the number of subjects studied, use and type of corticosteroids, the presence of bone fractures as well as BMD findings and those of other parameters when investigated are set out in the Table on BMD in DMD (**Table 1**). Recently, a study by Söderpalm *et al.*[111] examined BMD, bone turnover, body composition and calciotropic hormones in 24 boys with DMD. However, although treatment with corticosteroids was also examined, the study design did not allow examination of the impact of the corticosteroid treatment as distinct from DMD itself.

Vitamin D status. It is very difficult to analyze vitamin D and calcium nutritional status as it relates to the deterioration of bone health of DMD patients. In general, there is a lack of systematic, quality data on normal vitamin D and calcium status in healthy children as it relates to bone health and growth, especially for boys in the age range of DMD patients.[112–115] Another important factor is the difficulty in interpreting the literature on the normal serum level of vitamin D. Although the serum 25-hydroxy vitamin D level is thought to be the best indicator of vitamin D status, the widely used commercial assays may not offer comparable sensitivity and accuracy.[116–118] More recently a consensus has emerged on the best methods available.[119]

Nonetheless, it is clear that many of the boys with DMD are vitamin D insufficient and even deficient.[12,14,19,96, 97] Very recently, an informative paper by Bianchi *et al.*[120] followed up on earlier work. The authors carried out a prospective study of 33 children with DMD already being treated with a fixed dose of prednisone (1.25 mg kg^{-1} every 2 days). The patients were followed for 3 years: one year of observation and two years of treatment with vitamin D_3 (0.8 mcg kg^{-1} per day) plus adjustment of dietary calcium to the internationally recommended daily allowance (the exact value was not reported by the authors). During the observation year, bone mineral content and BMD decreased in all patients. After supplementation with vitamin D_3 and calcium, bone mineral content and BMD significantly increased in over 65% of patients. The authors also observed that bone metabolism parameters and bone turnover markers normalized in most patients (78.8%). The authors concluded that the combination of vitamin D_3 supplementation and adequate dietary calcium intake was effective treatment for most DMD patients demonstrating correction of vitamin D deficiency and increases in bone mineral content and BMD. Those who did not respond to this therapy appeared to have persistently high bone turnover.

The Bianchi *et al.* results are consistent with the view that the bone disorder in DMD patients presents with aspects of both osteomalacia and osteoporosis. The component of osteomalacia is suggested by observed improvement of low bone mineral content and BMD as well as bone formation, as indicated by the changes in the bone turnover marker osteocalcin, in response to vitamin D_3 supplementation and calcium enrichment. In addition, the failure to fully restore BMD for all patients and the presence or absence of change in bone markers for osteoclastogenic activity suggest certain aspects of osteoporosis are present and not fully corrected by improved vitamin D and calcium nutritional status.

The recent study of Rufo *et al.,*[121] aimed at exploring the mechanism responsible for the deterioration of bone health in DMD, is one of the most definitive studies of bone health and DMD to date. The study uses a well-conceived, thorough and wide-ranging design that employs multiple outcome measurements in both the humans and the *mdx* mouse model. It compares corticosteroid-treated DMD patients to concurrent aged-matched controls. It determines many indicators of bone health including: micro-QCT, BMD, bone turnover markers, indicators of vitamin D nutritional status such as, 25-hydroxy vitamin D and parathyroid hormone, as well as a panel of pro-osteoclastogenic cytokines. Its results are summarized in various sections of this review. The study also employed *in vitro* cell cultures of osteoclast precursors and primary osteoblasts. The results certainly point to the possibility of multiple causes of the effects of DMD on bone health.

Animal Models of DMD and Bone Health

Some of the pathophysiological findings in the *mdx* mouse model of DMD were described in the section above on the pathophysiology of DMD. Most of the information on the mechanisms underpinning the disease have been elucidated using mammalian models of the disease. A recent review provides an excellent overview of these models and the effects of DMD in them.[122]

There are three recent studies of the effects of DMD on bone in the *mdx* mouse model of the disease.[121,123,124] The primary purpose of the study by Novotny[123] was to determine if tibial bone strength is compromised in *mdx* and if so, what geometric and material properties of the bone are involved. Results of three-point bending tests showed that tibia of *mdx* and *dko* (dystrophin- and utrophin-deficient) mice had up to 50% to 80% lower strength and stiffness compared with wild-type mice. This suggests that DMD effects on muscle have downstream functional effects on bone. Micro-CT scanning showed that dystrophic tibia had varying reductions (between 6–57%) in cortical cross-sectional moment of inertia and cross-sectional area. Metaphyseal trabecular bone morphometry was also altered up to 78% in *mdx*. Therefore, low bone strength in dystrophic mice was mostly attributed to altered bone geometry and bone mass more so than alterations in BMD or other intrinsic material properties of bone.

Bone-to-muscle functional ratios (that is, three-point bending measures to muscle strength) indicated that bone strength was relatively high in 7-week-old *mdx* compared with muscle strength, but ratios were similar to wild-type mice by 24 months of age. This means that the functional capacity of the tibial bone of young *mdx* and *dko* mice is greater than that of the adjacent EDL muscle as indicated by high bone to muscle functional capacity ratios relative to wild-type mice, but following improvements in muscle function in *mdx* mice with age the bone-to-muscle relationships in *mdx* mice become equivalent to those in wild-type mice. Combined, these data highlight that there are clear decrements in both bone and muscle tissues of dystrophic mice, as well as a distinct relationship between muscle disease and overall bone health.

As observed in DMD patients, bone tissue abnormalities are found in *mdx* mice as they age. It appears that the loss of strength in muscle, caused by cycles of muscle degeneration and regeneration, is probably one of the factors promoting these changes to bone. To test the hypothesis that factors, other than muscle weakness, inherent to DMD might be associated with the deterioration of bone, a study done by Nakagaki *et al.*[124] investigated the changes that occur in the femur of *mdx* mice at 21 days of age when muscle damage is still not significant. The results demonstrated a lower strength, stiffness

and energy absorption capacity in the femur of the *mdx* mouse. Higher values for the structural (load and stiffness) and material (stress, elastic modulus and toughness) properties were observed in the wild-type normal mouse as compared with the *mdx*. *Mdx* femurs were shorter and were characterized by a smaller cortical area and thickness, and a smaller area of epiphyseal trabecular bone. The hydroxyproline content was similar in the two groups, but there was a significant difference in the Ca/P ratios. Thermogravimetry measurements showed a higher mineral matrix content in cortical bone of control animals.

Table 1 Summary of literature reports of bone mineral density in DMD patients

First author	*Corticosteroid treatment*	*Number of patients*	*BMD finding*	*Fracture finding*	*Comments*
Larson[12]	None	40/36 (with DXA scans)	Ambulatory, mean *Z*-score, −0.8 Non-ambulatory, mean *Z*-score of 1.7; progressively decreasing to a mean *Z*-score of −3.9	18 (44%)	
Aparicio[14]	No previous history of use	10 (all ambulatory)	Using *Z*-scores as a measure, 8 of the 10 boys had osteoporosis of the proximal femur; the remaining 2 had significantly lower bone mass (osteopenia); at the spine 5/10 decreased BMD, 2 were osteoporotic, 3 osteopenic	None reported	Tanner scale used to determine pubertal status. All the boys were at the appropriate pubertal stage for their age
Louis[18]		12 (three with Becker muscular dystrophy)	BMD increased 3% in five, still ambulatory, patients Urinary excretion of collagen type I cross-linking *N*-telopeptide declined to about one-third ($P<0.001$)		Study of the effects of creatine supplementation
Bianchi[19]	22 long-term dosage of 0.75 mg kg^{-1} per day (mean duration of 38.5±6.8 months; mean cumulative dose of 13 998±9 829 mg	32	Bone mineral density was lower (*Z*-score) than normal for age in all patients, even lower in the group of steroid-treated children[a]	Six patients (18%) had fractures that occurred at least 6 months before entering the study. During the study there were no new fractures	A well-controlled study, comparing corticosteroid-treated and corticosteroid-naïve patients
Douvillez[20]	Not reported	22 mean age 11.4±4.0 years	The BMC was lower, especially in the lower limbs, it decreased before the loss of ambulation and was correlated with muscular weakness	The fracture prevalence was high, especially in young patients	Observational study
Hawker[21]	Deflazacort (starting dose of 0.9 mg kg^{-1} per day at age 6–7 years. As they get older, and their weight increases, the mean daily dose per kilogram decreases)	42 assessed 11/16 in the Alendronate phase were prepubertal	23/43 boys (before alendronate treatment) had reduced BMD (*Z*-scores, <1.0) at the spine and/or total body Further scanning was technically impossible for 7 of 23 boys (treated with alendronate, 2.5 mg per day). At 2 years, mean *Z*-scores were unchanged for 16 boys. Mean bone age, 9.2 years (5.5–14.5)[b]	Two fractures before alendronate treatment; none thereafter were reported	Before-after trial of alendronate in low bone mass, CS-treated boys
Palmieri[111]	None reported	19	BMD was reduced in DMD (*Z*-score was −1.0 There was a negative correlation between age and Z-scores of BMD indicating a reduced development of bone mass in DMD	Not reported	Observational study
Soderpalm[112]	16/Prednisolone (between 0.22 and 0.35 mg kg^{-1} per day) The medication was withdrawn or not provided to four patients because of weight gain or because the patients and their parents chose to terminate the prednisolone treatment. However, before the study the four had received a median dose of prednisolone (281 mg kg^{-1} over a median 2.2-year period)	24/DMD (2.3–19.7 years) 11/aged-matched, healthy controls	There was lower BMD in the DMD group for total body, spine, hip, heel and forearm. The differences between DMD patients and controls increased with increasing age. DMD/spinal BMD was −2.5±1.9 NC/spinal BMD was −0.1±1.1 Biochemical markers of both bone formation and resorption revealed reduced bone turnover in DMD patients. The DMD group had low vitamin D levels but high leptin levels in comparison with the control group	6 of 24 DMD patients (25%) had sustained 11 fractures during their lifetime; all occurred at least 6 months before inclusion in the study. In the control group, a higher number of fractures occurred—the difference was not statistically significant	Cross-sectional study examining bone mineral density, bone turnover, body composition and calciotropic hormones

Table 1 Continued

First author	*Corticosteroid treatment*	*Number of patients*	*BMD finding*	*Fracture finding*	*Comments*
Bianchi[125]	Prednisone (1.25 mg kg^{-1} every 2 days)	33[b]	During the year of observation, both BMC and BMD (*Z*-scores) progressively decreased, especially at lumbar spine, with treatment, on average; they increased in a statistically significant way. After the 2-year treatment period, spinal BMC increased ($P<0.01$) with respect to the pre-treatment values in 22 patients; 8 had non-significant increases, whereas only 3 continued to worsen. These changes BMC and BMD[c] correlated with the altered 25-OH D levels ($r=0.53$; $P<0.01$) and with changes in NTx excretion ($r=-0.58$; $P<0.01$)	During the observation year, there were four fractures in four patients, while during the 2-year treatment period there were two fractures in two patients	3-year study, observation followed by 2 years of treatment with 25-OH vitamin D_3 (calcifediol) plus adjustment of dietary calcium to the recommended (in Italy) daily dose
Rufo[126]	None	16/DMD 11/aged-matched, healthy controls	In six patients (37%) the BMD *Z*-score was less than −1; in three it was between −1.1 and −2; and in the other three, it was below −2. BMD was adjusted and expressed as a *Z*-score of BMAD based on the approximate bone volume calculated, considering lumbar vertebral bodies as cylinders. The *Z*-score was calculated, selecting a reference sample of age-matched healthy Italian boys	None reported	Mechanistic study
Crabtree[82]	Prednisolone intermittent regime (10 days on, 10 days off) at a dose of 0.75 mg kg^{-1} per day of prednisolone	25 (mean age 7.4 years)	Three approaches were used to adjust the data: a volumetric adjustment; allometric scaling ('the Molgaard algorithm') and a functional approach.[27] At baseline, L2L4 bone mineral content was significantly low for projected bone area although appropriate for reduced lean body mass. Subcranial bone area for height and subcranial BMC for area and LBM were all significantly reduced. After 30 months of steroid therapy there was a significant increase in subcranial bone area for height but a significant reduction of subcranial BMC for area. At the lumbar spine, there were no significant changes in bone area but small increases in L2L4 BMC both for bone area and LBM	Before starting steroids, four boys reported six fractures. One subject lost ambulation during his second year of treatment, and two boys experienced fractures (one finger and one femur)	Study the functional and skeletal effects of 30 months of steroid treatment in DMD

Abbreviations: BMAD, bone mineral apparent density; BMC, bone mineral content; BMD, bone mineral density; CS, corticosteroid; DMD, Duchenne muscular dystrophy; DXA, dual-energy X-ray absorptiometry; LBM, lean body mass; 25-OH, 25-hydroxy vitamin D.
[a]NTx, the marker of bone resorption, was approximately three times the normal value; and osteocalcin, the marker of bone formation, was at the upper limit of normal; 25-OH D levels were low, and significantly lower in the CS-treated children ($P<0.02$). 1,25 (OH)2D was normal in both groups. PTH levels were within the normal range, even if, in the CS-treated children, at the upper limit of normal.
[b]Bone age was compatible with chronological age in all children.
[c]At baseline and after the year of observation, CTx and NTx (markers of bone resorption) were much higher than normal, and OC (a marker of bone formation) was at the upper limit of normal. After 2 years of treatment, CTX and NTx decreased significantly, even if the level remained a little higher than normal: OC always remained within the normal range, but was decreased at the end of the 2-year treatment. 1,25(OH)2 vitamin D levels did not change. Serum 25-OH D was normal at baseline. At the end of the year of observation, it was also normal in 13 children (39.4%), but was lower than normal in 20 children (60.6%). (In particular, in a total of 40 measurements in these 20 children: in 15 measurements serum 25-OH D was <12 ng ml^{-1}; in 25 measurements it was between 12 and 19 ng ml^{-1}. Following calcifediol treatment, the serum 25-OH levels normalized). PTH levels were at the upper limit of normal at baseline (>50 pg ml^{-1}) in 14 children (42.4%); in particular it was above 60 pg ml^{-1} in 13 measurements and between 50 and 59 pg ml^{-1} in 15. The PTH levels showed a statistically significant decrease after the normalization of 25-OH D. As the authors expected, 25-OH D and PTH levels before treatment showed a significant negative correlation.

The authors found that the femurs of *mdx* mice had impaired mechanical and biochemical properties as well as changes in collagen organization in the extracellular matrix. The *mdx* mice developed femoral osteopenia even in the absence of significant muscle fiber degeneration. This led to a conclusion by the authors that weakness of the *mdx* femur is probably due to genetic factors that are directly or indirectly related to dystrophin deficiency.

The study of Rufo,[121] summarized in the table on BMD in DMD, also used the *mdx* mouse model, but in conjunction with human, *in vivo* and *in vitro* analyses and cell culture to investigate possible mechanisms underlying DMD-induced bone loss. Micro-QCT and histomorphometric analyses showed reduced bone mass and higher osteoclast and bone resorption parameters in *mdx* mice compared with wild-type mice, whereas osteoblast parameters and mineral apposition rate were lower. In a panel of circulating pro-osteoclastogenic cytokines evaluated in the *mdx* a serum, IL-6 was increased compared with wild-type mice. Circulating IL-6 also had a dominant role in osteoclast formation because *ex vivo* wild-type calvarial bones cultured with 10% sera of *mdx* mice showed increase osteoclast and bone-resorption parameters that were diminished by treatment with an IL-6 antibody.

Etiology of the Deterioration of Bone Health in DMD

The pathophysiology of DMD, discussed previously, has a number of consequences that could be the cause of the bone loss observed in DMD patients. Paramount among the adverse effects of DMD that might be related to bone loss are greater fragility of the sarcolemma of muscle fibers, increased susceptibility to mechanical stress, attempted regeneration and abnormal cellular metabolism. This leads to a state of chronic inflammation characterized by infiltration of neutrophils and macrophages, and the presence of their many associated cytokines and chemokines. The inflammatory response dominates the molecular signature of dystrophin-deficient muscle not only in humans but also in the *mdx* mouse.[121]

There are a number of possible causes of the deterioration of bone health seen in DMD patients including: loss of muscle strength; side effects of corticosteroid treatment (that might also lead to many other phenomena including delayed puberty or impaired bone mineralization;[125] the effects of the cytokines and chemokines released as a result of the inflammatory response in dystrophin-deficient muscles or other inflammatory responses; activation of osteoclastogenesis by altered metabolism in the muscle (for example, activation of nuclear factor of κB pathways); altered calcium homeostasis either by corticosteroid treatment, cytokines, immobility or poor calcium absorption from the intestine or into bone, and changes in vitamin D nutritional status.

Muscle tension on bone is essential for proper bone growth and geometry.[76,80] It is commonly assumed that a significant component of the effect of DMD on bone health results primarily from decreased muscle strength, which is ultimately so severe that it results in the loss of ambulation and exclusive use of a wheelchair for mobility. Although it seems likely that the loss of muscle tension that occurs in DMD is a cause of poor bone quality, decreased BMD and osteopenia or osteoporosis, there are other pathophysiological effects of DMD that are also likely to contribute significantly to the deterioration of bone health or the failure of adequate bone development and maintenance.

The onset of puberty is delayed in DMD patients,[126] which is associated with poor bone health.[60,61,63,64] However, there are no published reports that delay of puberty onset was a common symptom of DMD before the introduction of corticosteroid therapy, but there were a number of reports of bone loss. Nonetheless the effects of sex hormones on bone health are well established.

As a result of inadequate dietary intake or supplementation and low levels of sun exposure, vitamin D deficiency may contribute significantly to poor bone health.[112] Further putative contributors are a disturbance of calcium homeostasis and the increased activity of inflammatory cytokines, such as OPN or tumor necrosis factor-α.

Glucocorticoid therapy may exacerbate loss of bone density and quality. Glucocorticoids are one of the few medications that are effective in treating DMD patients. It is thought that their potent anti-inflammatory activity reduces the inflammatory response in dystrophin-deficient muscle, which in turn delays the loss of muscle strength prolonging ambulation. Unfortunately, they have almost equally potent side effects on bone health. The possible adverse side effects of glucocorticoid therapy directly on bone are difficult to separate from the probable indirect beneficial effects on bone by the positive effects of glucocorticoid therapy on muscle strength.

There is a report that the cytokine IL-6 may also be involved in the bone loss seen in DMD patients.[121] The authors report higher than normal serum levels of IL-6 in DMD subjects, as well as in the sera of *mdx* mice compared with wild-type mice. Furthermore, human primary osteoblasts from healthy donors incubated with 10% sera from DMD patients showed decreased nodule mineralization. Many osteogenic genes were downregulated in these cultures, including *osterix* and *osteocalcin*, by a mechanism blunted by an IL-6-neutralizing antibody. In contrast, the mRNAs of osteoclastogenic cytokines IL-6, IL-11, inhibin-βA and transforming growth factor-β were increased.

The presence of other cytokines, such as, OPN and tumor necrosis factor-α may also contribute to bone loss by stimulating osteoclastogenesis.[36,49,71,127–129]

There are numerous studies that demonstrate that calcium homeostasis is perturbed by the effects of dystrophin deficiency (see the section on DMD pathophysiology). Calcium homeostasis and bone mineral metabolism have been studied in healthy children and in children with a variety of diseases.[130] Although there is clear evidence of decreased BMD and alterations in bone turnover in DMD, no data exist on calcium homeostasis, including calcium absorption from the intestinal tract.[111] Corticosteroid treatment, cytokines and immobility have all been shown to disrupt normal calcium homeostasis in children.[130] As corticosteroid treatment improves mobility in DMD children, corticosteroids may have complex effects on both calcium homeostasis and bone health.

Vitamin D deficiency and disturbances of calcium homeostasis (particularly calcium absorption from the gut and incorporation into bone) may cause significant bone demineralization, a characteristic of osteomalacia.[62,73,131] Therefore, in DMD patients, it is likely that the bone disorder seen in DMD is not only osteoporotic in nature but also presents aspects seen in osteomalacia or, in most cases, a mixture of both. This adds complexity to the choice of possible therapeutic approaches and emphasizes the importance of gaining a better understanding of the causes of the DMD bone loss in DMD patients.

Therapeutic Approach to Bone Health in DMD

There are virtually no clinical trials of potential therapies aimed at preventing or slowing deterioration or restoring bone health in DMD patients. There are a few studies of bisphosphonate use in DMD. An example is the study by Hawker[21] summarized

in the table above. Forty-two patients with DMD receiving daily deflazacort were screened. Only 16 were deemed eligible to participate in the study due to limitations of performing BMD assessments. A common limitation to the study of bone health in DMD is the physical disabilities it induces that preclude evaluation of BMD and the inability to swallow oral medications or, in the case of bisphosphonate, to sit upright for at least 30 min. The 16 bisphosphonate-treated DMD boys received 0.08 mg kg^{-1} per day of alendronate orally, with 750 mg of daily calcium and 1000 IU of vitamin D. Over the 2-year follow-up no patient suffered a vertebral fracture and two patients had a long bone fracture. Whole-body BMD scores remained stable and total body and lumbar *Z*-scores improved in the younger children who had been taking deflazacort for a shorter time period.[21] A chart review attempted to examine the association of bisphosphonate therapy with survival of DMD patients, born between 1963 and 2006, treated with corticosteroids and seen in a single Canadian center. Bisphosphonate therapy was started in 16 of the 44 patients (36%) receiving corticosteroids for more than 1 year. A possible therapy-duration effect was present.[132] No mention was made of intake or serum levels of either vitamin D or calcium in this study.

Studies of Soderpalm, Bianchi and Rufo, described elsewhere in this review, demonstrated positive benefits of vitamin D and calcium supplementation. It is clear that further controlled, prospective clinical trials of bisphosphonate use in DMD patients are needed before this therapy can be recommended more broadly.

Conclusions

Bone disease is a significant concern in DMD patients and can contribute to loss of ambulation and adversely affect quality of life and possibly even longevity. The bone disorder in DMD may have components of both osteomalacia and osteoporosis, leaving unanswered questions related to etiology, diagnosis and treatment. Studies using outcome measures other than BMD, and without resorting to bone biopsy, based on newer technologies such as peripheral QCT, micro-magnetic resonance imaging, vertebral morphometry, non-radioactive calcium isotopes coupled with kinetic modeling, and validated measurement of vitamin D, its metabolites and of intact parathyroid hormone, are required to fully assess bone quality.

Conflict of Interest

The authors declare no conflict of interest.

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