Early Onset of Bone Pathology in a Murine Model of Duchenne Muscular Dystrophy

Daniel March, M.D.1, Clifford Voigt, MD1, Hongshuai Li, M.D. & Ph. D.1, Xueqin Gao, M.D. & Ph.D.1, Aiping Lu1, Ying Tang1, Bing Wang, MD,PhD1, Johnny Huard, Ph.D.2.
1University of Pittsburgh, Pittsburgh, PA, USA, 2University of Pittsburgh, Pittsburgh, PA, USA.


Introduction: Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder causing muscle weakness and deterioration in affected children. The inherited defect in DMD leads to a failure to express dystrophin, a critical component of the protein complex that links the myocyte cytoskeleton to the extracellular matrix, thus stabilizing the sarcolemma. As muscle wasting progresses, patients with DMD are typically rendered wheelchair bound by the second decade of life and then expire in the third decade. In addition to muscular degeneration, there are disturbances of osseous structure and function that impact the clinical course of the disease, but these have been far less extensively explored in the literature [1]. Clinical studies have demonstrated an increased risk of long bone fracture in affected patients that is independent of the concurrent muscle pathology, and such injuries result in a loss of mobility in as many as 20% of previously independently mobile DMD patients [2]. Traditionally, fractures in DMD patients were thought to result from osteopenia caused by reduced mechanical forces related to muscular degeneration; however, dystrophin/utrophin “double knockout” (dKO) mice, a severe murine model of DMD, have been shown to exhibit a spectrum of musculoskeletal abnormalities, with degenerative changes in bone, articular cartilage, and intervertebral discs [3]. With multiple tissues of mesenchymal origin affected, it is questionable whether this musculoskeletal pathology is secondary to muscle sarcopenia, or if an intrinsic mechanism may be responsible. Degenerative changes in skeletal muscle are detectable in homozygous dKO mice at as early as 5 days of life, but the exact temporal relationship between muscle and bone pathology remains unclear [4]. The purpose of this study was to determine whether bone pathology occurs before skeletal muscle pathology at 5 days of life in this animal model of DMD, which would indicate that these osseous abnormalities are intrinsic in nature rather than a secondary effect of muscle sarcopenia.

Methods: Wild type C57 mice were obtained from Jackson Laboratories (Bar Harbor, ME), while mdx (dystrophin/-, utrophin+/+), dKO-heterozygous (dystrophin/-, utrophin+/-), and dKO-homozygous (dystrophin/-, utrophin/-) mice were provided by our laboratory colony. The Stem Cell Research Center (SCRC) has previously investigated the pathophysiology of DMD using this murine model, wherein mdx mice have mild skeletal muscle defects, dKO-homo mice display a severe DMD-like phenotype and have a greatly shortened life span, and the dKO-hetero mice have a similar phenotype to mdx mice. Animals were sacrificed at 2 days and 1 week of life. Skeletal samples were harvested including skull, lumbar spine, tibia, and femur. Mouse skulls, trabecular bone in the vertebral bodies and proximal tibia, and cortical bone of the femoral diaphysis were imaged with a VivaCT micro-CT scanner. Measurements of the ratio of bone to total volume, trabecular number, trabecular thickness, trabecular space, and bone mineral density were made for each sample and compared among the groups. After scanning, the bone was sectioned and histological analysis performed with von Kossa, Alcian blue, and Herovici’s staining.
Diaphragm and gastrocnemius muscles were stained with hematoxylin and eosin (H&E) and analyzed for signs of skeletal muscle degeneration.

**Results:** Micro-CT analysis of the lumbar vertebrae revealed a significant reduction in the average bone volume and trabecular thickness in the dKO-homo mice when compared to the dKO-hetero mice at day 2 of life (p <0.05); this difference could also be appreciated histologically with Herovici’s staining (Figure 1). Skulls of 2 day old dKO-homo mice demonstrated significantly wider open cranial sutures that their dKO-hetero counterparts, suggesting delayed suture closure (n=2, Figure 2). While preliminary micro-CT analysis of the proximal tibia showed no statistically significant difference at 2 days of age, histological analysis suggests a shortened proliferative zone in dKO-homo relative to dKO-hetero mice (n=2, Figure 3). Proximal tibia trabecular bone and femoral shaft cortical bone micro-CT analysis are still ongoing.

**Discussion:** Although the statistical power is low, these preliminary results demonstrate that osseous abnormalities are detectable in dKO-homozygous mice as early as 2 days of life, and that they occur at least simultaneously with, if not before observable muscle pathology at 5 days. This indicates that the bone pathology in DMD patients is most likely intrinsic in nature, and not a secondary effect of reduced mechanical forces resulting from muscle sarcopenia. In support of this contention, we have reported that bone healing in dKO mice is delayed when compared to mdx mice [3]. Moreover, these findings strongly suggest that factors other than a lack of dystrophin expression are involved in the pathophysiology of DMD, as dystrophin is not expressed in bone. Stem cell dysfunction represents a plausible alternative explanation for the degeneration seen in muscle, bone, articular cartilage, and intervertebral discs, and the order in which these tissues begin to show degenerative changes may relate to the time that it takes for their tissue-specific stem cell populations to be exhausted. Further reinforcing this concept, our lab has previously detected a significant decrease in the number of bone marrow mesenchymal stem cells (BMSCs) in dKO mice relative to age-matched mdx mice [5]. This argues strongly in favor of further research into the role of stem cells in the pathophysiology of DMD, and how modulating their function could affect the natural history of this uniformly fatal disease.

**Significance:** In concluding that pathologic changes in bone are intrinsic rather than secondary to muscle sarcopenia in this murine DMD model, we offer preliminary evidence that the underlying pathophysiology of DMD is driven by mechanisms other than the mere absence of dystrophin expression and resulting muscle degeneration. Further research will investigate these mechanisms, and we postulate stem cell dysfunction as a potential focus of this investigation.
Figure 1: dKO-homo mice showed early onset of bone pathology at 2 days after birth. (A) Herovici’s and (B) H&E staining of spine; red color in Herovici’s indicates major bone matrix collagen type 1. (C) Micro-CT histomorphometric analysis of lumbar vertebral bodies. *P<0.05.

Figure 2: dKO-homo mouse skulls showed signs of delayed cranial suture closure at 2 days after birth.
Figure 3: dKO-homo mice showed a shortened proliferative zone in the proximal tibial physis at 2 days old. (A) Alcian blue and H&E staining of proximal tibia; (B) Micro-CT histomorphometric analysis of proximal tibia.