Pannexin3 in exercise, obesity, and osteoarthritis

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Anatomy and Cell Biology

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Abstract

Pannexin 3 (PANX3), is a glycoprotein that oligomerizes to form mechano-sensitive channels expressed in musculoskeletal tissues and has been identified as a potential target for the treatment of obesity and osteoarthritis (OA). Obesity and OA are two of the most prevalent diseases worldwide, leading to disability and even death. These conditions are thought to originate from a complex interaction between genetics, aging, sex, and modifiable lifestyle factors, such as exercise. Investigating the interactions between genetic and exercise may provide a more comprehensive, context-specific understanding of gene function. This thesis aimed to understand the interactions between PANX3 and exercise interventions on relevant factors of obesity, metabolism, inflammation, and OA. Male and female wildtype (WT) and Panx3 knockout (KO) mice were bred, fed a standard chow diet or a high fat diet, and randomized to either no exercise or forced treadmill running daily for 6 weeks at either 24 weeks (young adult) or 18 months of age (aged). We discovered that, specifically in male mice, Panx3 deletion results in substantially lower body weights because of less fat mass and increased lean mass. This change in body composition was similar to that achieved by 6 weeks of forced treadmill running in WT mice. Additionally, male Panx3 KO mice had reduced inflammatory profile of their fat and muscle tissue and circulating levels of Interluekin-6. There was no additional effect of forced exercise on the body composition of Panx3 KO mice. At the knee joint, however, deleting Panx3 and forced treadmill running worked synergistically, resulting in large superficial fibrillations of the tibial cartilage and increased bone in the subchondral region. At the intervertebral discs, Panx3 KO mice had histopathological features of accelerated disc degeneration with forced treadmill running compared to sedentary WT mice. In aging, both male and female Panx3 KO mice develop spontaneous OA characterized by full thickness cartilage erosion and synovitis. This data suggests Panx3 influences fat accumulation, inflammation, and joint pathology and that these effects are dependent on the sex, age, and exercised state of the animal.
Keywords

Pannexin 3; Exercise; Obesity; OA; Aging; Fat; Cartilage; Bone; Intervertebral Disc.
Summary for Lay Audience

Obesity and osteoarthritis (OA) are two of the most common causes of disability worldwide, and current treatments are limited to lifestyle interventions. This is largely due to an incomplete understanding of the mechanisms of these diseases. Association studies in humans have identified several risk factors for developing these diseases including sex, genetics, ageing, and lifestyle factors such as diet and physical activity. How these factors interact to cause these diseases is unknown.

Pannexin 3 (PANX3) is a protein that forms large pores in our cell membranes, that are sensitive to mechanical stress, allowing the release of signalling molecules. This signalling is necessary for normal cell function, but if dysregulated, can contribute to disease progression. In fact, PANX3 has been implicated in development of fat tissue and OA. The purpose of this research was to determine the role of PANX3 in fat accumulation, inflammation, and joint health and whether forced treadmill running influences the outcomes.

In the first study, deleting the gene encoding for PANX3 resulted in large reductions in fat mass in male mice and lower fat and muscle tissue inflammation, to the same degree as 6 weeks of forced treadmill running in mice with the PANX3 gene. In the second study, mice lacking Panx3 accumulate damage to various tissues of their joints, indicative of degeneration, and that forced treadmill running in males caused further damage of the joint surface. Lastly, in the final study, as mice without Panx3 age, they develop severe tissue degeneration in the knee, indicative of OA.

Taken together, this thesis highlights the potential contribution of PANX3 to obesity and OA, and how sex, age, and exercised state of the animal influence its importance.
Co-Authorship Statement

All chapters of this thesis were written by Brent Wakefield. Editing of chapters 1-5 was done by Dr. Silvia Penuela and Dr. Frank Beier. Thesis reader: Dr. Katherine Willmore.

Chapter 1: The introductory chapter of the thesis was written by Brent Wakefield and part of it was published as an invited review (Wakefield and Penuela, J Vas Res 2022).

Chapter 2: High fat diet feeding studies were completed by Vanessa Lee prior to my PhD. Metabolic cages were run by Dr. Robert Gros at Robarts Research Institute (London, Ontario). Tissue mRNA analysis for inflammatory markers was run by Dr. Pillon (high fat diet animals) and Dr. Parastoo Boroumand at the University of Toronto (Sick Kids Hospital). Blood analysis for adipokines and inflammatory markers were done in the lab of Dr. Nica Borradaile, in the Department of Physiology and Pharmacology (London, Ontario). Brooke O’Donnell provided skin sections and Justin Tang performed the analysis of the hypodermal layer. Rafael Sanchez-Pupo provided technical advice over the course of this project. Kevin J. Barr provided mice for the high fat diet study. Danielle Johnston assisted me in the glucose tolerance testing. Dr. Samar Sayedyahossein assisted with western blot analysis. This chapter was published (Wakefield et al, Int J Obesity 2022).

Chapter 3: Scoring of the cartilage structure was done by Justin Tang and Rehanna Kanji. IVD analysis was done by Jeffrey Hutchinson and Courtney Brooks. This chapter is currently in revision (Wakefield et al, J Ortho Res 2023), and published as a pre-print (bioRxiv).

Chapter 4: Scoring of the cartilage structure was done by Geneva Harold, Rehanna Kanji, and Justin Tang. Intervertebral disc analysis was done by Jeffrey Hutchinson. Synovial analysis was completed by Dr. Patti Kiser. Tendon and enthesis analysis were performed in collaboration with Dr. Matthew Grol. This manuscript is in preparation for submission and published as a pre-print (bioRxiv).
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Thank you both from the bottom of my heart for entrusting me with this opportunity. Rest assured that you won’t be getting rid of me just yet, as I eagerly look forward to continuing our scientific collaborations and nurturing our friendships.

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<th>Full Name</th>
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<tbody>
<tr>
<td>Abt1</td>
<td>Activator of Basal Transcription 1</td>
</tr>
<tr>
<td>ACS</td>
<td>Articular Cartilage Structure</td>
</tr>
<tr>
<td>AF</td>
<td>Annulus Fibrosus</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Arg1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose-Derived Stromal Cells</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine 3',5'-monophosphate</td>
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<tr>
<td>Ccl2</td>
<td>C-C motif chemokine ligand 2</td>
</tr>
<tr>
<td>Chi313</td>
<td>Chitinase 3-like-3</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Comprehensive Lab Animal Monitoring System</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Patterns</td>
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<tr>
<td>DIO2</td>
<td>DIO2 iodothyronine deiodinase 2</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DMM</td>
<td>Destabilization of the Medial Meniscus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Eef2</td>
<td>Eukaryotic Translation Elongation Factor 2</td>
</tr>
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<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Emr1</td>
<td>EGF module-containing mucin-like hormone receptor-like 1</td>
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<tr>
<td>eWAT</td>
<td>epididymal White Adipose tissue</td>
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<td>Fetal Bovine Serum</td>
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<td>FEX</td>
<td>Forced Exercise</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>Human embryonic kidney 293 cells</td>
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<td>HFD</td>
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<td>Hipk2</td>
<td>Homeodomain interacting protein kinase 2</td>
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<td>HMW</td>
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<td>Hprt</td>
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<td>IBMX</td>
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<td>IDD</td>
<td>Intervertebral Disc Disease</td>
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<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
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<td>Abbreviation</td>
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<td>Interleukin-12a</td>
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<td>Panx</td>
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Chapter 1

Pannexins (PANX1, 2, 3) are channel-forming glycoproteins that are expressed throughout the cardiovascular and musculoskeletal system. The canonical function of these proteins is to release nucleotides that act as purinergic signalling at the cell membrane or Ca\(^{2+}\) channels at the endoplasmic reticulum membrane. These two forms of signalling are essential for autocrine and paracrine signalling in health, and alterations in this signalling have been implicated in the pathogenesis of many diseases. Many musculoskeletal and cardiovascular diseases are largely the result of a lack of physical activity which causes altered gene expression. Considering exercise training has been shown to alter a wide array of gene expression in musculoskeletal tissues, understanding the interaction between exercise training, gene function and expression in relevant diseases is warranted. With regards to pannexins, multiple publications have shown that exercise training can influence pannexin expression and may influence the significance of its function in certain diseases. This chapter further discusses the potential interaction between exercise training and pannexin biology in relevant tissues and disease models. We propose that exercise training in relevant animal and human models will provide a more comprehensive understanding of the implications of pannexin biology in disease. This chapter is adapted from an invited review in the Journal of Vascular Research:

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1 Introduction

Pannexins (PANX1, 2, 3) are channel forming glycoproteins expressed throughout the musculoskeletal system. The canonical function of these proteins is to either release nucleotides that act as purinergic signals at the cell membrane, or act as Ca\(^{2+}\) channels at the endoplasmic reticulum membrane. These two forms of signalling are essential for autocrine and paracrine signalling in health, and alterations in these activities have been implicated in the pathogenesis of many diseases. Many musculoskeletal diseases are partially due to a lack of physical activity. Considering exercise training has been shown to alter expression of a wide array of genes in musculoskeletal tissues, understanding the interactions between exercise training, gene function and expression in relevant diseases is warranted. With regards to pannexins, multiple publications have shown that exercise training can influence pannexin expression and may influence the significance of its function in certain diseases. In this thesis, using a mouse model, we investigated the effect of deleting the Pannexin 3 (Panx3) gene, which has previously been implicated in the pathogenesis of obesity and osteoarthritis (OA). Considering the importance of exercise interventions in these diseases, we subjected these mice to either sedentary or exercised conditions at different ages, to determine if there is a gene-environment interaction between Panx3 and the exercised state of the animal. Such findings will provide a nuanced picture of the function of Panx3 and determine its role in obesity and OA in various contexts.

1.1 Pannexins

The Pannexin family consists of three polytopic trans-membrane proteins (PANX1, 2, 3) with four transmembrane domains, two extracellular loops, an intracellular loop, with the N and C-terminal exposed to the cytoplasm. In their canonical function, PANXs can oligomerize and form large pore channels at cell membranes [1]. Pannexins are widely expressed in mammalian cells, with PANX1 being expressed in almost every cell type at different stages of development [2]. PANX2, originally thought to be expressed exclusively in the cells of the nervous system, is also expressed in skin, liver, skeletal muscle, and the heart to name a few [3]. PANX3 is expressed in tissues of the musculoskeletal system including adipose tissue [4], skeletal muscle [5], bone [6], and
Pannexins are involved in vital cellular signalling processes such as membrane purinergic signalling through ATP release and intracellular Ca\(^{2+}\) release from the endoplasmic reticulum (Figure 1-1) [9]. They have been implicated in a breadth of physiological and pathological processes including cell death [10-14], OA [7, 15], bone formation [6, 16], blood pressure regulation [17-19], inflammation [4, 10, 11, 17, 20-24], fat accumulation [4, 25], wound healing [26], and are

**Figure 1-1: Graphical illustration of the canonical functions of PANX3 as an ATP or calcium release channel.**
Illustration of PANX3 in a generic cell model. PANX3 can localize to the plasma membrane and act as an ATP release channel. It can also localize to the endoplasmic reticulum and act as a calcium release channel.
dysregulated in many cancers [27]. Thus, pannexins are hypothesized to be a promising therapeutic target for a variety of diseases.

Pannexins’ role as an ATP release channel has been implicated as a mechanism in many of these diseases [7, 20, 28-34]. A limited number of diseases have been linked to germline or somatic mutations of pannexins in the last 6 years [35, 36]. However, the expression levels of pannexin may be affected by, or contribute to, disease states, as much of the literature suggests an increased expression profile of pannexins during pathogenesis, potentially augmenting the release of ATP [37-39]. While variations in expression may be important, regulation of pannexin channel function is also a potential factor in their role in disease.

Pannexin channels can be opened by various molecular mechanisms, including mechanical stimulation [40, 41]. Considering the expression and function of pannexins in vascular [17] and musculoskeletal tissues [42], this creates a potential connection between mechanical loading via exercise, pannexin expression and channel activation. However, this connection has only been investigated in a few studies [4, 43, 44].

It is important to keep in mind that many of the diseases that have been linked to pannexins are multi-factorial. Pathogenesis of several of these diseases can be partially attributed to physical inactivity, and exercise can be an excellent prevention tool [45]. Exercise has been shown to be critical for preventing, managing and improving vascular function and hypertension [46], bone quantity and quality [47, 48], OA symptoms [49], and adipose tissue inflammation [50]. The benefits of physical activity are likely mediated partially by alterations in gene expression of these tissues [51]. Considering alterations in pannexin expression has been shown to contribute to these diseases, understanding the interaction with exercise is a worthy venture.

The profound effects of frequent, lifelong, exercise, including both endurance and strength training, cannot be overstated. The physiological mechanisms that drive the benefits of exercise have been studied extensively and include beneficial changes to almost every organ system [52]. These changes are largely the result of alterations in expression of numerous genes, such as seen in skeletal muscle tissue in various
transcriptomic studies [53]. Investigating the effects of exercise in various animal models can provide a comprehensive understanding of the function of genes in both a healthy and pathological state as they relate to the importance of a disease [54]. With regards to pannexins, exercise may be able to activate these mechano-sensitive channels, or regulate pannexins through changes in gene expression, phosphorylation, or other post-translational modifications. To this point, two studies to date have shown that exercise can regulate pannexin expression in bone and adipose tissue [4, 43]. Considering the transient, broad, expression of pannexins, and that they seem to be important for both healthy development and in disease states, investigating their function in healthy (exercised) and sedentary states is warranted. Therefore, we propose that using exercise models will provide novel insights to pannexin biology. This introduction will discuss the literature and potential mechanistic connections between pannexins and exercise within relevant diseases and organ systems.

1.2 Pannexins in Vasculature

Vasculature function and health is critical for maintaining blood pressure through the contraction and relaxation of smooth muscle cells lining the arteriole walls. Dysfunction of this mechanism contributes to primary hypertension [55]. The mechanisms of smooth muscle contraction in vasculature are influenced by neuroendocrine, epinephrine, and the intrinsic machinery within resident endothelial and smooth muscle cells. PANX1 has been shown to be expressed in both the smooth muscle cells [56] and the endothelial cells lining arterioles [24]. The opening of PANX1 on vascular smooth muscle cells is an intermediate step between alpha1-adrenergic receptor activation and vascular smooth muscle contraction [57] [58]. Specifically, PANX1 channels can release ATP which acts in an autocrine fashion on purinergic receptors causing muscle contraction. This finding has been shown in both preclinical models and young healthy men [59]. However, in stage 1 hypertensive male individuals, blocking PANX1 with probenecid did not seem to alter blood pressure or vascular resistance during pharmacological stimulation [60]. While these results seem to conflict with previous findings there are several potential limitations to the study that the authors acknowledge. Considering that orally administered probenecid can have off target effects, a more specific PANX1 blocker
could change the outcome of this study. Additionally, this study was completed in males, and we know that the muscle sympathetic nerve activity is regulated differently between males and females via beta-adrenergic and alpha-adrenergic receptor activity [61]. The authors also note that it could be that the effect of altering PANX1 function or expression may be more pronounced in more severe cases of hypertension.

Exercise has proven to be an effective preventative strategy against hypertension. Additionally, dose response studies have shown that even modest increases in physical activity in sedentary hypertensive individuals can have clinically significant improvements in blood pressure. Furthermore, exercise can significantly lower blood pressure in hypertensive humans – even in cases of resistant hypertension [62].

Various modes of exercise can improve endothelial function in both prehypertensive and hypertensive individuals [63]. Specifically, this is caused by an improvement in nitric oxide (NO) release via an endothelial dependent vasorelaxation mechanism [64]. Interestingly, during exercise there is a systemic wide increase in vasoconstriction via epinephrine release, however, the vasculature feeding working skeletal muscles can counter this. This phenomenon has been coined “sympatholysis”, however the mechanism by which this occurs is not clear. Interestingly, NO has been shown to inhibit ATP release from PANX1 and therefore attenuating adrenergic receptor mediated vasoconstriction [58]. It could be that during acute bouts of exercise, PANX1 is targeted by NO release from working skeletal muscle and endothelial cells to facilitate sympatholysis [13]. Understanding these mechanisms is important as it may lead to novel therapies for improving vascular function, and therefore exercise tolerance.

More recently, human intervention studies have been completed to elucidate the relationship between exercise and PANX1 [44]. Interestingly, changes in vascular resistance in the lower limb which improved blood pressure in hypertension patients was not related to changes in PANX1 function or expression. While this suggests that chronic exercise training benefits may be independent of PANX1, it would be interesting to determine if exercise and pharmacological (or genetic ablation in animal models) interventions have a compounding effect on blood pressure regulation. This would better
determine optimal treatment for those that can, and are willing to, exercise consistently and those that are unable.

The other two members of the pannexin family are also expressed in the systemic arterial network of mice, depending on the size of the arteries and in different cell types. PANX2 has been detected in smooth muscle cells, while PANX3 is predominantly expressed in endothelial cells [65]. It will be very interesting to study the function of these two additional isoforms in the vasculature and blood pressure control in both sedentary and exercise conditions.

1.3 Pannexins and Skeletal Muscle

Skeletal muscle fibers have been shown to express all three pannexin isoforms [3, 5, 66]. Previous reports have shown that PANX3 is expressed in mouse [5] and rat [67] skeletal muscle. Langlois et al., (2014) performed multiple western blots of mouse skeletal muscle at 7 months of age (sex was not reported) with our published PANX3 antibody (Panx3CT-379) that has previously been validated against KO controls in other tissues. While they correctly identified a ~43 KDa doublet band as PANX3, the lack of a KO control at that time made it impossible to verify the specificity of the antibody in skeletal muscle tissue. Pillon et al., (2014) however, found no detectable transcript levels of Panx3 in male mouse skeletal muscle on normal or high fat diet chow. We also performed qPCR analysis and found similar results to Pillon et al., 2014, in that there were no detectable levels of Panx3 transcripts in mouse skeletal muscle (Figure 1-2a). We also performed western blot analysis with this antibody on mouse skeletal muscle on both WT and Panx3 KO mouse tissues in male mice at 12 weeks of age. First, we genotyped both WT and Panx3 KO skeletal muscle tissue to ensure that the Panx3 gene was in fact deleted from the genome (Figure 1-2b). Using western blot analysis, while there were noticeable differences in the banding pattern of dorsal skin between WT and Panx3 KO mice, we observed identical banding in the WT and the Panx3 KO mouse muscle tissue near the predicted molecular weight, indicating potentially unspecific labeling in the skeletal muscle (Figure 1-2c). Since we know pannexin expression can vary among ages and between sexes [4], it is possible that PANX3 is not present in young adult murine skeletal muscle of males at 12 weeks, but that PANX3 is upregulated during
development and later in life in muscle tissue. To confirm this assumption, further studies would need to carefully examine PANX3 expression in skeletal muscle at different ages along with Panx3 KO controls.

To date, there is only data on PANX1 and a potential connection with exercise [43]. So far, PANX1 has been associated with two important processes in skeletal muscle; 1. Potentiation (contraction force) 2. Regeneration after damage.

### 1.3.1 Potentiation

During repetitive skeletal muscle contraction an influx of Ca$^{2+}$ occurs, which enhances contractile force. This process is referred to as potentiation. What is also known about this process is that extracellular ATP levels rise which can activate purinergic receptors. This purinergic signalling was found to be mediated by PANX1 channels, as they are in the T-tubules of slow and fast twitch fibers [68]. This activation of purinergic receptors is what increases Ca$^{2+}$ influx. This was further supported in KO models showing a blunted potentiation of muscle contraction to repeated electrical stimulation [69]. This would suggest that PANX1 is involved in the potentiation of skeletal muscle as needed during human physical performance. If this mechanism is conserved in human skeletal muscle, this would have implications for how patients would be affected by taking pannexin blockers, as this may attenuate skeletal muscle contractile force.

### 1.3.2 Regeneration

After an injury or damage from intense exercise training, skeletal muscle stem cells called satellite cells (quiescent myoblast cells) become activated and start to proliferate, migrate, differentiate, and fuse to repair the damaged muscle fibers [70]. This process is coordinated by paracrine signalling among immune and resident cells, and until recently the role of a pannexin in this process had not been investigated. PANX1 is expressed in myoblast cells and its expression is increased upon differentiation, suggesting it is
involved in this process [66]. Using an injury model of the tibialis anterior muscle with notexin, Suarez-Berumen et al., 2021 found Panx1 transcript is upregulated after injury up until 5 days [66], suggesting PANX1 may be involved in regeneration after injury. To
support this idea, global Panxl KO mice had compromised muscle regeneration after injury, as their myoblast cells have impaired migration and fusion [66]. Next, they found that Panxl KO mice had significantly less regeneration of the myofibers and regenerated myofibers and were smaller than WT after the same injury model. The authors also determined that this was due to disrupting ATP signaling by treating cultured myoblasts with apyrase and the PANX1 channel blocker carbenoxolone, which reiterated the effects of genetic ablation.

In support of this concept, Damas and colleagues found that PANX1 expression was significantly increased 24 hours post exercise after resistance training in humans using unbiased transcriptomic analysis [71]. As our lab is interested in the effect of exercise on various pannexin isoforms, we have observed that Panxl transcript is significantly increased in mouse quadriceps after an acute bout of forced treadmill running (Figure 1-2d) with no significant changes (yet trending to increase) in protein levels (Figure 1-2e). Considering this, it would be interesting to see if Panxl KO mice have accumulated damage, or an inability to repair muscle tissues, during chronic exercise training.

1.4 Bone

Pannexin 1 and 3 are both expressed in all three bone cell types and have been implicated in the signalling responsible for bone modeling and/or remodeling [42]. Bone has a complex architecture to optimize its function as both a site for mineral storage and supporting locomotion. There are two main types of bone tissue. Cortical bone which makes up the outer shell, and trabecular, cancellous bone, which forms a lattice like structure at various internal locations, which funnels forces toward the cortical bone. This trabecular structure optimizes the bones’ ability to withstand forces acting on the skeleton, while reducing the overall weight of the bone itself [72].

Bone tissue is constantly being remodeled, which is the process of resorbing old bone and laying down new bone tissue. This process is achieved through the collaboration and communication between osteocytes responsible for maintaining bone homeostasis, and osteoblast and osteoclast cells responsible for bone formation and resorption during
remodeling, respectively [73]. Osteocytes sense mechanical loading and provide signals to osteoblast and osteoclast cells to coordinate remodeling. Specifically, apoptotic osteocytes release signalling molecules to neighboring osteocytes which triggers the release of an osteoclastogenic cytokine Receptor activator of nuclear factor kappa-B ligand (RANKL), that in turn stimulates osteoclast activity, which results in reabsorption of bone matrix. Subsequently, osteoblast cells lay down new bone tissue [74]. This process serves to adjust bone architecture in response to altered loading patterns and to prevent the accumulation of old bone.

Understanding this process is critical, as frail bones, such as during osteoporosis, is a significant health burden [75]. With the increase in aged population, morbidity rates of osteoporosis are climbing. Osteoporosis is a multifactorial disease that results in disturbances in bone metabolism, and thus, low bone quantity and deterioration in microstructure, and bone strength. While many factors can contribute to the development of osteoporosis, mechanical stimulation, via load bearing exercise, is a critical factor for maintaining, and building bone mass throughout life [76].

1.4.1 Physical Activity and Bone Health

While there are many factors contributing to the strength of our skeleton, mechanical loading seems to be the largest contributing factor [77]. Using HR-pQCT imaging on postmenopausal women to control for the effects of bone growth, more recent literature has shown that bone remodelling is primarily driven by loading patterns [78]. The authors found that bone resorption correlated with low tissue loading, while bone formation correlated with high tissue loading [78]. Additionally, a study that had postmenopausal women on an exercise routine for 6-months consisting of progressive, unilateral, high-impact exercise incorporating multidirectional hops on one leg for comparison of the contralateral control leg, showed that exercise increased femoral neck bone mineral density [48]. This suggests that load-bearing, high impact, exercise is effective at reducing risk, and even reversing bone mass loss that leads to osteoporosis. Understanding the mechanisms by which exercise stimulates bone formation is critical and may lead to the identification of new drug targets.
1.4.2 Mechanisms of exercise in bone health

Mechanical stimuli via exercise has a potent influence on bone cell signalling pathways [76]. Osteocytes are considered the primary skeletal mechanosensory cells [79]. Mechanical signals are converted into biochemical, altering phenotypic expression and mediating cell signal transduction. These signaling cascades alter the cellular activities of local effector cells (osteoblasts and osteoclasts) [73]. To trigger this signalling cascade, biological sensors must detect mechanical stimuli. Previously, it was thought that cell membrane channels are most likely important mechanotransducers in bone cells, however none had been identified. Purinergic signalling via mechanically activated membrane channels is a potential player in remodelling. *In vitro* experiments have shown that fluid flow stimulation of osteoblast cells causes the release of ATP within minutes [80]. It was then thought that this release may be mediated by pannexin channels.

1.4.3 Pannexins in Bone

Seref-Ferlengez and colleagues found that a global deletion of *Panx1* in a mouse model led to a reduction in load induced bone formation [43]. The researchers showed that this was caused by an increase in load-induced sclerostin expression, a potent inhibitor of osteoblasts released from osteocytes. This suggests that PANX1 may be acting as a mechanosensitive channel in osteocytes, modulating ATP signalling which allows osteocytes to respond to mechanical loading and initiate appropriate remodeling processes. In support of this finding, Cheung et al. 2016 found that Panx1 and P2X7R (a purinergic receptor) are required for apoptotic osteocytes, in mechanically fatigued bone, to trigger RANKL production in neighbouring bystander osteocytes. This implicates ATP as an essential signal mediating this process [81]. These results would suggest that the beneficial effects of load-bearing exercises on maintaining, and even increasing bone quantity and quality, may be mediated by PANX1 channels in osteocytes. However, Pannexin 1 expression has also been shown in osteoblasts and osteoclasts. Therefore, to understand the cell specific role of PANX1, various cell specific KO models have been developed.
Interestingly, TRAP specific *Panxl* KO mice (osteoclast cells) had higher rates of bone resorption, reduced formation, and a resultant lower trabecular bone mass in female mice [82]. This would suggest PANX1 function in osteoclasts is necessary to ensure proper coupling between bone formation and resorption. While in osteocyte specific *Panxl* KO mice, bone mineral density after reaching skeletal maturity is higher in female KO mice than in wildtype mice. Further, osteocytic *Panxl* deletion partially prevented aging effects on cortical bone structure and mechanical properties [83]. This would suggest that inhibiting PANX1 function in osteoclast cells would be detrimental for bone formation, while beneficial for bone formation if inhibited in osteocytes. This may seem at odds with Seref-Ferlengez et al. 2019 to some degree. However, upon closer look at the data, PANX1 expression increased only temporally (at two weeks and returned to baseline after 4 weeks of running) while the bone was adapting to the novel stimulus of exercise. It is possible that transient increases in PANX1 expression in osteocytes are necessary to allow for the communication to take place and ensure proper collaboration among the three main bone cells. However, considering this was a global KO model it is not possible to conclude definitively. Similar studies should compare cell specific KO models to the load-induced mechanical stress of exercise in the future.

A number of studies have shown PANX3 is a promoter of osteoblast differentiation [84-86]. Bond et al. 2011 found that mature osteoblast cells increase the expression of PANX3 during differentiation, and that its transcription is under the control of Runx2, a key transcription factor for bone formation [84]. PANX3 was shown to act as a Ca^{2+} release channel at the ER membrane in C2C12 cells, subsequently activating calmodulin signalling for differentiation [85]. *Panx3* KO mouse models have shown skeletal abnormalities and shorter long bones in newborn [87] and adulthood [88]. Using a global *Panx3* KO mouse model developed in our lab, Caskenette et al. 2016 similarly found these mice have shorter femura and humeri with larger muscle attachment sites [89]. Taken together, these reports from multiple labs suggest that PANX3 has a role in bone modelling and development. However, there are no reports to my knowledge whether PANX3 plays a role in remodeling of bone, such as after mechanical stress from exercise. Mechanistically, mechanical stimulation (such as during exercise) may be able to induce
the expression of PANX3 via activation of Wnt signalling and subsequent activation of Runx2 [90].

1.5 Cartilage

Hyaline cartilage lines the ends of long bones on articulating surfaces. Its purpose is thought to provide a smooth, lubricated surface to facilitate the transmission of loads between two bones at a low frictional coefficient. The deterioration of articular cartilage is a hallmark feature of OA. Other key features of OA include subchondral bone thickening and synovial tissue inflammation. Causes of OA are multifactorial, with a large portion associated with genetics, obesity, diet, and age, while the role of physical activity in the development of OA is not fully understood [91].

OA is one of the leading causes of disability, affecting over 520 million worldwide [92]. OA is considered a whole joint disease, and histologically presents as deterioration of the articular cartilage, subchondral bone, and changes to the synovial membrane. Additional structures that may be affected include the enthesis and the tendon that crosses the joint (Figure 1-3).

![Figure 1-3: Illustration of the major structures of the synovial joint.](image)
OA has historically been considered a “wear and tear” disease, suggesting that the more you move a joint under load the more cartilage deterioration would occur. However, it is clear this relationship is not so straightforward. While professions that require a high degree of knee bending with loads are associated with the development of OA, strong evidence suggests structured exercise routines of osteoarthritic joints improves symptoms [93], and rates of OA in athletes (not including traumatic injuries) are no different than non-athletic populations [94]. Not only does exercise seem to help with OA symptoms, but intervention studies have also shown that exercise can increase glycosaminoglycan content in femoral cartilage [95], a critical component to maintain the viscoelastic properties of cartilage. Additionally, animal models have shown that exercise training may reduce the risk of developing OA, however excessive amounts may contribute to its development [96]. This suggests a U-shaped relationship between physical activity levels and OA risk. For example, moderate levels of treadmill running in rodents have shown an improvement in joint inflammatory markers, chondrocyte phenotype, and a decrease in catabolic enzyme MMP3 [97]. However, how exercise influences OA may be genotype dependent, as variations in gene expression and function may attenuate the joints’ ability to adapt to mechanical forces.

### 1.5.1 Panx3 and OA

Panx3 is expressed in chondrocytes of both mouse and human cartilage [8]. Interestingly, PANX3 expression increases during chondrocyte hypertrophy, a phenotype shown to drive cartilage degradation during OA [7]. In a surgical model of traumatic OA in mice, we have shown that the deletion of Panx3, either globally or chondrocyte specific, has a protective effect in this model [8]. However, in an aging model of OA, Panx3 global KO mice have worse OA of the knee joint [15]. This is the first example (to my knowledge) of a gene that plays opposing roles in OA progression depending on the context. What factors are contributing to this different outcome in these models is not clear. It could be that in healthy cartilage low levels of PANX3 are necessary for proper remodeling from everyday loading. Aged animals lacking Panx3 may accumulate damage as they continue to load the joints over time. Alternatively, age associated inflammatory factors may be dysregulated in Panx3 KO mice, leading to joint inflammation, which drives the
degradation. This may be supported by the fact that these mice had significantly greater synovial inflammation [15]. To determine if this age associated OA in this model is due to a mechanical insensitivity, exercise models could help determine if Panx3 KO mice are able to respond similarly to chronic loading of their joints as WT mice. With that said, mechanically loaded aged human osteochondral explants did not find any changes in pannexin expression via RNA-seq analysis [98], suggesting pannexin expression may not be influenced by mechanical loading. Additionally, while PANX3 is expressed in cartilage and bone, there is no evidence that PANX3 or PANX1 are expressed in synovial tissue. Considering synovial inflammation is a strong driver of OA and pain [99], it would be interesting to see if 1. Pannexins are expressed and involved in this process, and 2. If the anti-inflammatory effects of exercise [100] in joints is associated with alterations in pannexin expression and/or function.

Considering PANX3 is mechanically sensitive and plays a role in OA, determining how Panx3 KO mice respond to exercise training is warranted. Mechanistically, exercise may influence PANX3 expression or function through mechanical stimulation or by altering the inflammatory microenvironment of the joint.

### 1.6 Adipose Tissue

Adipose tissue plays an integral role in metabolic health. Excessive fat accumulation which defines obesity is a global problem contributing to all causes of mortality [101]. Fat accumulation is mostly the result of adipocyte hypertrophy. This results in cell stress, insulin resistance, and the release of inflammatory cytokines [102]. The mechanisms that regulate fat accumulation are incompletely understood, and how exercise influences adipose tissue is still being investigated.

Animal and human studies have shown that exercise can reduce adipose tissue inflammation, which is one of the mechanisms by which exercise exerts its health benefits [4, 103]. Exercise can also reduce the formation of adipocytes in mouse models [104]. Additionally, exercise may convert energy storing white adipose tissue to thermogenic ‘brown’ adipose tissue [105]. These examples show the profound effects exercise can have on adipose tissue and thus whole-body metabolism. The exact
molecular mechanisms that exercise can exert these effects through are incompletely understood, and purinergic signalling via pannexins may be one such mechanism.

1.6.1 Pannexins in Adipose Tissue and Cells

PANX1 and PANX3 have both been implicated in adipocyte health [4, 25, 39, 106, 107]. Interestingly, pannexins have been shown to play a role in adipocyte metabolism, proliferation and differentiation, browning, and inflammation – all of which exercise is known to influence.

1.6.2 Panx1 in Adipose Tissue

Our lab has shown that a global deletion of PANX1 in male mice resulted in higher fat mass and lower lean mass compared to WT mice while consuming normal chow diet [25]. Using primary cultures of adipose-derived stromal cells (adipocyte stem cells) from WT mice, we found that PANX1 protein levels were elevated when these cells were pushed toward an adipogenic lineage. Additionally, cells isolated from Panx1 KO mice proliferated less and differentiated into adipocytes more easily. This suggests PANX1 plays a role in early adipogenesis.

With regards to browning of adipocytes, PANX1 channel activation has been shown to play a role in the thermogenic properties of brown adipocytes [106]. Senthivinayagam et al., 2021 found that PANX1 channel activity in brown adipocytes was induced by Beta-3 adrenergic receptor stimulation, resulting in increased thermogenesis, which was inhibited in Panx1 KO mice. In terms of exercise, aerobic exercise does not alter Beta-3 adrenergic receptor expression in adipose tissue in high fat diet fed mice, and therefore, may not be involved in this mechanism [108]. Adrenergic stimulation of white murine adipocytes causes the release of ATP through PANX1 channel [107]. The ATP release correlates with increased adipocyte metabolism and glucose sensitivity and macrophage migration. The authors found this to be regulated by a cAMP-PKA–dependent pathway. Considering exercise has no effect on cAMP in adipose tissue there may be no effect from exercise on this mechanism [109].
1.6.3 *Panx3* in Adipose Tissue

Less is known regarding the role of PANX3 in adipose tissue and cells. However, Halliwill et al. 2016 using QTL analysis revealed *Panx3* expression is correlated with BMI specifically in male mice [110]. Previously, this group had also found that *Panx3* was associated with an important gene network, *Hipk2*, for white adipose tissue development [111]. Our recent publication showed that the global deletion of *Panx3* in male mice resulted in a robust attenuation in weight gain and fat mass in adulthood [4]. Additionally, adipocyte number seemed to be reduced in *Panx3* KO mice, suggesting *Panx3* may be involved in adipocyte proliferation. *Panx3* KO mice also had significantly lower inflammatory gene expression in adipose tissue, specifically genes related to M1 macrophages. In this study, we also had sibling matched exercised mice from 24-30 weeks of age, which performed daily forced treadmill running for an hour for 6 weeks. Exercise downregulated PANX3 expression in adipose tissue of male mice. Considering many of the benefits of exercise (adipocyte proliferation, reduced tissue inflammation, improved body composition) were recapitulated in the *Panx3* KO mice, this suggests these benefits may be partially mediated through alterations in PANX3 expression and function.

1.7 Conclusion: Exercise and Pannexins

Limited research has been done to investigate the potential connection between exercise and pannexins. This may be important considering that using pannexins as potential drug targets may influence one’s tolerance, safety, or adaptation to exercise. Considering the ubiquitous expression of Pannexins, and the highly variable depending on age and sex, understanding the systemic effect of manipulating pannexins is warranted through various stresses such as exercise. Additionally, understanding the role of pannexins in healthy, exercised mice will provide a more comprehensive understanding of the health implication of these proteins. Considering exercise influences many of the pathologies and cellular mechanisms that pannexins do, more animal studies should take into consideration the potential interaction. For example, our study in Chapter 2 (Wakefield et al. 2022) would suggest there is no added benefit of exercise training on body composition if we were to block PANX3. Considering this research has implications for
obesity, understanding this interaction is critical to providing the best possible pharmaceutical therapy for a given patient, depending on exercise training status. Considering the broad, profound effects of exercise training on gene expression and overall health outcomes in humans and animals, it may be an interesting control for preclinical researchers – especially in disease models that are largely determined by physical activity. As a community, we also must consider the significant limitations we have experienced with our animal models. While mice are 90% genetically similar to humans [112], the translatability of findings from mouse research to human conditions has been less than ideal. One reason may be that lack of physical activity in these models leads to widespread changes in gene expression which provide a significant source of variation and artifact. Transgenic mouse models may be more accurate at determining the significance of certain genetic targets by incorporating an exercise control group, as it would provide a broader picture of the function of the gene in both unhealthy and healthy states. Therefore, in relevant disease models, we suggest that animal research should control and implement exercise training of their animals to determine the interaction with pannexins and other targets of interest.

1.8 Rationale, Hypothesis, and Aims

Using a mouse model with a global deletion of Panx3, this thesis will focus on examining the role of PANX3 in fat accumulation, inflammation, metabolism, and joint health, and whether environmental factors such as diet and exercise will influence these outcomes.

Aim 1 (Chapter 2)

Determine the effect of deleting Panx3 on body weight, body composition, metabolism, and inflammation in the context of forced exercise and high fat diet (HFD).

Rationale 1

Panx3 is expressed in male mouse fat tissue and is upregulated under HFD feeding conditions [67], and it has been linked to the Hipk2 pathway in white adipose tissue, suggesting it plays a role in white adipose tissue development [111]. Lastly, QTL data
from Halliwill et al. strongly correlates Panx3 with body mass index and inflammation specifically in male mice [110].

Hypothesis 1

Deletion of Panx3 will reduce body weight and fat accumulation, and concomitantly, improve glucose regulation and inflammation in male mice.

Aim 2 (Chapter 3)

Study the effect of deleting Panx3 on joint health in young adult mice subjected to forced exercise.

Rationale 2

PANX3 is a mechanosensitive, channel-forming glycoprotein [2, 40] expressed in osteoblasts [16], chondrocytes [7], and annulus fibrosus (AF) tissue [113]. Previous literature from our lab using the Panx3 KO mouse have shown that PANX3 plays a context-dependent role in OA [8, 114] and intervertebral disc disease (IDD) [115]. Specifically, the absence of PANX3 leaves mice susceptible to accelerated age-associated OA, and IDD from altered forced exercise.

Hypothesis 2

Panx3 KO mice will be susceptible to forced treadmill induced OA and IDD.

Aim 3 (Chapter 4)

Investigate the effect of deleting Panx3 on joint health in aged, forced exercised mice.

Rationale 3

Our previous report showed that while male Panx3 KO mice have accelerated OA at 18 months of age [116], they have normal intervertebral disc (IVD) compared to WT mice [115]. However, whether additional use of joints influences this outcome, and whether female Panx3 KO mice are also susceptible to age-associated OA, is not known.
Hypothesis 3

Aged *Panx3* KO mice will have greater OA than WT mice from forced exercise.
References

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Chapter 2

Previous reports have implicated \textit{Panx3} in male body composition and is linked to gene networks important for white adipose tissue development. Additionally, \textit{Panx3} expression increases in adipose tissue of male mice that are fed a HFD. In this chapter, we analyzed the body weight change from 4 to 30 weeks of age, body composition, metabolic tissue inflammation, and metabolic parameters of \textit{Panx3} KO mice under sedentary (SED) and forced exercise (FEX) conditions in adulthood. This chapter was published in the \textit{International Journal of Obesity}, doi: 10.1038/s41366-021-01037-4
2 Pannexin 3 deletion reduces fat accumulation and inflammation in a sex-specific manner.

**Background:** Pannexin 3 (PANX3), is a channel-forming glycoprotein that enables nutrient-induced inflammation *in vitro*, and genetic linkage data suggests it regulates body mass index. Here, we characterized inflammatory and metabolic parameters in global *Panx3* knockout (KO) mice in the context of forced treadmill running (FEX) and high fat diet (HFD).

**Methods:** C57BL/6N (WT) and KO mice were randomized to either a FEX running protocol or no running (SED) from 24 until 30 weeks of age. Body weight was measured biweekly, and body composition was measured at 24 and 30 weeks of age. Male WT and KO mice were fed a HFD from 12 – 28 weeks of age. Metabolic organs were analyzed for a panel of inflammatory markers and PANX3 expression.

**Results:** In females there were no significant differences in body composition between genotypes, which could be due to the low PANX3 expression in female white adipose tissue, while male KOs fed a chow diet had lower body weight, and lower fat mass at 24 and 30 weeks of age, which was reduced to the same extent as 6 weeks of FEX in WT mice. Additionally, male KO mice exhibited significantly lower expression of multiple pro-inflammatory genes in white adipose tissue compared to WT mice. While on a HFD body weight differences were insignificant, in KO mice, multiple inflammatory genes were significantly differently expressed in quadriceps muscle and white adipose tissue resulting in a more anti-inflammatory phenotype compared to WT mice. The lower fat mass in male KO mice may be due to significantly fewer adipocytes in their subcutaneous fat compared to WT mice. Mechanistically, adipose stromal cells (ASCs) cultured from KO mice grow significantly slower than WT ASCs.

**Conclusion:** PANX3 is expressed in male adult mouse adipose tissue and may regulate adipocyte numbers, influencing fat accumulation and inflammation.
2.1 Introduction

Obesity is caused by excessive fat accumulation and is a major contributor to many co-morbidities including type II diabetes and cardiovascular disease [1]. While exercise training and caloric deficit are effective treatments for obesity, many find these interventions difficult to implement and sustain [2]. Genetic factors increase susceptibility to weight gain [3], and understanding which genetic factors underlie obesity will assist clinicians in determining the best pharmacotherapeutic options for a given patient.

Pannexins (PANX1, 2, 3) are channel-forming glycoproteins that allow the passage of ions and metabolites for autocrine and paracrine signaling in a variety of cells [4]. Previous reports have shown that PANX1 is expressed in adipocytes and has a functional role in immune cell recruitment [5], adipocyte hypertrophy and fat accumulation [6], glucose metabolism [7], and thermoregulation in brown fat [8]. Recent evidence suggests that PANX3 may also play a role in adipogenesis and inflammation [9-11]. Using a systems approach involving quantitative trait loci mapping and gene expression network analysis, Halliwill and colleagues found that the Panx3 gene is linked to body mass index in male mice [9]. This group also identified Panx3 as a component of the homeodomain-interacting protein kinase 2 (Hipk2) gene network which is involved in adipocyte signaling and differentiation [10]. These studies provide indirect evidence that PANX3 may be involved in the molecular mechanism of fat accumulation.

A consequence of excessive fat accumulation is inflammation of adipose tissue [12-15]. This inflammation is thought to contribute to many comorbidities [14, 16-19]. We previously reported that the saturated-fatty acid palmitate activated cell-intrinsic pro-inflammatory programs in isolated muscle cells and concomitantly increased Panx3 expression [11]. Additionally, we demonstrated that PANX3 channels allowed adenosine triphosphate (ATP) release, attracting monocytes towards the muscle cells [11]. This would suggest that PANX3 may be a contributor to nutrient-induced skeletal muscle inflammation by acting as a conduit for ‘find me’ signals to immune cells. Lastly, we observed that HFD induced the expression of Panx3 in adipose tissue [11], which was the first published finding of Panx3 expression in mouse adipose tissue. However, its role in
diet-induced obesity, fat accumulation, inflammation and metabolism has not been investigated.

Considering the evidence above, we sought out to determine the physiological effects of a global deletion of Panx3 in mice exposed to forced exercise (FEX) and dietary excess (HFD). In males, Panx3 Knockout (KO) mice had lower body weight and fat mass, but higher lean mass corrected for body weight, and lower inflammation in adipose and quadriceps tissue compared to WT mice to the same extent as 6 weeks of forced treadmill running. This potentially beneficial loss of natural inflammatory gene expression level was still evident when challenged with caloric excess. However, there were minimal differences between female WT and KO mice, highlighting a sex-specific effect of the Panx3 deletion. This would suggest that the deletion of Panx3 attenuates fat accumulation and inflammation in males and could become a useful, sex-specific, genetic target to combat obesity and its associated inflammation.

2.2 Materials and Methods

2.2.1 Animals and ethics

Experiments performed on animals were approved by the Animal Care Committee of the University Council on Animal Care at the University of Western Ontario, London ON, Canada (UWO # 2019-069), and in accordance with relevant guidelines and regulations. Panx3 KO mice were generated as described previously [20]. Panx3 KO mice were backcrossed with C57BL/6 N mice from Charles River Canada (Saint-Constant) until a congenic line was obtained (minimum of 10 backcrossed generations). Mice were weaned at 3 weeks of age and fed either a chow diet (6.2% kcals from fat), Western (45% kcals from fat) or a HFD (60% kcals from fat, Test Diet 58Y1) as described in the respective sections. At termination, mice were sacrificed using carbon dioxide. Immediately after, blood was collected via cardiac puncture, adipose, quadriceps, and liver tissues were collected, immediately snap frozen and stored at -80°C.
2.2.2 Forced exercise (FEX) protocol

At 24 weeks of age (baseline), mice were randomized to either sedentary (SED) or FEX groups. The FEX groups were forced to run on a treadmill (Columbus Instruments, Ohio) for 6 weeks, 1 hour a day, 5 days a week, at a speed of 11 m/min, and a 10º incline. The mice were encouraged to run using a bottle brush bristle and a shock grid at the end of the treadmill as per the animal ethics protocol. Mice were acclimatized for 10 mins before each session, which consisted of being in the treadmill with no belt movement.

2.2.3 Body composition

Fat and lean mass composition were measured at baseline and 30 weeks of age using a quantitative magnetic resonance (echo-MRI) mobile unit as described previously [6]. Measurements were taken in triplicate to verify the results.

2.2.4 Blood glucose tolerance and plasma analysis

Mice were fasted 4 hours prior to testing. Fasted blood glucose was measured via a glucometer (OneTouch Ultra). Glucose tolerance testing was conducted by administration of 1 g/kg of glucose by intraperitoneal injection, and blood glucose was monitored at 0, 15, 30, 60, and 120 minutes via tail vein puncture. Glucose area under the curve (AUC) was calculated. At sacrifice, blood was collected, and plasma was isolated. ELISAs (ALPCO, New Hampshire) were performed following the manufacturer’s protocol for insulin, cholesterol was assessed by CHOD-PAP kit (Roche Diagnostics, Indianapolis, IN), and triglyceride analysis was conducted by Triglycerol/Glycerol kit (Roche Diagnostics, Indianapolis, IN) following manufacturer’s protocols.

2.2.5 Metabolic cage analysis

Metabolic analysis was assessed using the Comprehensive Lab Animal Monitoring System (CLAMS) with the Oxymax software (Columbus Instruments, Ohio) at the Robarts Research Institute. Mice were individually caged and acclimated for 24 hours prior to measuring food consumption, water consumption, energy expenditure, volume of oxygen (VO2) and carbon dioxide (VCO2), respiratory exchange ratio (RER), total activity, total ambulatory activity, and sleep duration, as described previously [6].
2.2.6 RNA extractions, cDNA synthesis and qPCR

Tissue RNA extraction were performed using total RNA isolation (TRIzol) reagent (Life Technologies) and phenol-chloroform phase separation. Samples were homogenized in TRIzol, mixed with chloroform and centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing the RNA was isolated. Isopropanol was added and samples incubated at room temperature for 30 minutes to precipitate the RNA. The extracted RNA was pelleted by centrifugation at 12,000 x g for 10 minutes at 4°C. The pellets were washed with 70% ethanol, centrifuged at 7,500 x g for 5 minutes, and then again washed with 100% ethanol and centrifuged lastly at 7,500 x g for 5 minutes. The samples were stored in -80°C.

NanoDrop 2000c spectrophotometer (NanoDrop) was used to quantify the extracted RNA concentration and its purity. SuperScript variable input linear output (VILO) kit for complementary deoxyribonucleic acid (cDNA) synthesis (Life Technologies) was used to synthesize cDNA. cDNA synthesis reaction was performed in 10 μL volume to which up to 125 ng/μL RNA was added and a final concentration of 1X VILO Reaction mix and 1X SuperScript Enzyme mix were loaded. The 96-well plate containing the samples were incubated at 42°C for 60 min, then 85°C for 5 min on a C1000 thermal cycler (Bio-Rad), then stored at -20°C.

A reaction with 20 ng of cDNA was used for each reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) along with 1X TaqMan Fast Advanced Master Mix, and predesigned TaqMan probes (Life Technologies) for the following target genes: Arg1 (Mm00475988_m1), Mrc1 (Mm00485148), IL-10 (Mm00439614), Chi3l3 (Mm00657889), Emr1 (Mm00802529_m1), IL-12a (Mm00434165), CCL2 (Mm00441242), Itgax (Mm00498701_m1), Nos2 (Mm00440502_m1), and Tnf (Mm00443258_m1) on a StepOne Plus Real-Time PCR System (Life Technologies). Samples were held at 95°C for 20 seconds, then cycled from 95°C for 1s to 60°C for 20 s for 40 cycles. Gene expression of target genes were normalized to average of the housekeeping genes Abt1 (Mm00803824_m1), Hprt (Mm03024075_m1), and/or Eef2 (Mm01171435_gH) using the ΔΔCt method. An inflammatory index score was
calculated as the ratio of the sum of pro-inflammatory gene expression over the sum of the anti-inflammatory gene expression and reflects the inflammatory status of the tissue.

For analysis of Panx3 mRNA expression in visceral fat tissue, RNA was extracted using a combination of Trizol and a Qiagen RNeasy mini kit as was previously described [21]. mPanx3 Forward: TTTCGCCAGGAGTTCTCATC, Reverse: CCTGCCTGACACTGAAGTTG, m18S Forward: GTAACCCGTTGAACCCCATT, Reverse: CCATCCAATCGTAGTAGCG and mHprt. Normalized mRNA expression levels were analyzed using the ΔΔCT method which was calculated using BioRad CFX Manager Software. Aliquots were taken from the reactions, dyed with ethidium bromide and electrophoresed on a 10% agarose gel.

2.2.7 Protein analysis

Protein lysates were extracted with lysis buffer containing: 1% Triton X-100, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40 or a RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40 (Igepal), 0.5% sodium deoxycholate). Each buffer contained 1 mM sodium fluoride, 1 mM sodium orthovanadate, and half of a tablet of complete-mini EDTA-free protease inhibitor (Roche, Mannheim, Germany). Protein was quantified by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Protein lysates (40 μg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane using an iBlot System (Invitrogen, USA). Membranes were blocked with 3% bovine serum albumin (BSA) with 0.05% Tween-20 in 1X phosphate buffer saline (PBS) and incubated with anti-mouse PANX3 antibody (1:1000; PANX3 CT-379) [22], and anti-GAPDH antibody (1:1000; Millipore Cat# MAB374). For detection,IRDye® -800CW and -680RD (Life Technologies, USA) were used as secondary antibodies at 1:10,000 dilutions and imaged using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences, USA). Western blot quantification and analysis was conducted using Image Studio™ Lite (LI-COR Biosciences). Positive controls were generated by ectopic expression of PANX3 constructs in human embryonic kidney 293T (HEK293T) cells as described before [6, 22].
2.2.8 Histological staining and subcutaneous adipocyte measurements

Dorsal skin samples from adult male WT and KO mice (12-months old) on a chow diet were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. Sections (5 µm) were deparaffinized in xylene, rehydrated in graded alcohols, and washed in PBS. Parallel tissue sections were stained with hematoxylin/eosin. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope. Measurement of adipocyte cellular size (area) and number of measured adipocytes was performed using the analytical software ImageJ (v.1.50i, National Institute of Health) by a blinded assessor. At least three tissue sections from each mouse were analyzed and individual adipocytes with complete boundaries were selected for quantification and counting.

2.2.9 3T3-L1 cell culture and adipogenic induction

Mouse embryonic fibroblast pre-adipocyte (3T3-L1) cells were purchased from ATCC and checked for mycoplasma before use. Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5 g/L glucose, 1% Pen-Strep, and 10% calf serum (Thermo Fisher Scientific) and cells below passage 10 were included in the studies. Adipogenic media for days 1-2 contained: DMEM with 4.5 g/L glucose (Thermo Fisher Scientific), 10% calf serum (Thermo Fisher Scientific), 1% Pen-Strep, 100µg/mL of isobutylmethylxanthine (IBMX), 390 ng/mL dexamethasone, and 5µg/mL insulin (Sigma Aldrich). Adipogenic media for days 3-4 contained all of the above components without IBMX or dexamethasone. Following day 4, cells were fed every 2-3 days with DMEM + 10% FBS (Thermo Fisher Scientific) until differentiation was complete at day 10.

2.2.10 Adipose-derived stromal cell isolation

ASCs were isolated as described previously [6]. WT and KO male mice were fed on the HFD, with the modification of isolating cells from the inguinal adipose depot and cells were filtered through a 100 µm filter to remove debris prior to cell seeding. Fat from up to three mice was pooled together for each separate isolation. Cells were seeded at high density (80 000 cells/cm²) and rinsed 24 hours after isolation with sterile PBS and
passaged when confluent (approximately 7 days). ASCs were grown in DMEM: Ham’s F-12 (Sigma Aldrich), supplemented with 10% fetal bovine serum and 1% Pen-Strep and growth medium was changed every 2 days. ASCs used for assays were grown to Passage 2.

2.2.11 Growth curves and adipogenic differentiation of ASCs

ASCs from WT and Panx3 KO mice were plated in 12 well plates at a seeding density of 10 000 cells/cm². Cell counts were measured in triplicate every other day up until day 7 using an automated cell counter, Countess II (Thermo Fisher Scientific). Cells were fed every other day with DMEM: Ham’s F12 media, 10% FBS, 1% Pen-Strep, (Sigma Aldrich). Adipogenic induction was conducted with WT ASCs plated in 6 well plates at a seeding density of 30 000 cell/cm². Adipogenic media as previously described [23] with the modifications of substituting 1μg/mL Troglitazone and 0.25 mM IBMX (Sigma Aldrich) for days 1-3. Media was changed every other day for 14 days.

2.2.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 9.20 (GraphPad, San Diego, CA). Outliers were removed from data sets using the outlier test from GraphPad Prism Version 9.20. Normality tests were used to determine similar variation among the groups for fat mass in males. A power analysis was conducted using the male baseline fat mass mean and standard deviation data to determine an appropriate samples size for the 30-week time point analysis. Body weight progression was analysed using a three-way repeated measures ANOVA with genotype x activity x age as factors. Single time point measures between genotypes were analysed using an unpaired t-test. A two-way ANOVA with genotype x activity as factors was used for 30-week time points, and other two-variable analyses. For blood glucose tolerance curves, a three-way factorial ANOVA was used with genotype x activity x time as factors. Data are presented as mean ± standard error (SEM). N indicates number of animals.
2.3 Results

2.3.1 Male Panx3 KO mice weigh less, have less fat mass and more lean mass than WT mice to the same extent as 6 weeks of forced exercise.

Male and female WT and KO congenic mice were bred, fed *ad libitum* on a normal rodent chow diet, and randomly allocated to either a SED or FEX protocol from baseline to 30 weeks of age (Figure 2-1a). Body weights were tracked bi-weekly, and body composition and blood glucose tolerance were analyzed at baseline and at 30 weeks of age. Postmortem, livers, skeletal muscle, and visceral fat were collected for protein and mRNA analysis.

In males, KO mice weighed significantly less than WT mice as they aged (Figure 2-1b). When analyzing the baseline and 30-week body weights with and without exercise, KO mice weighed significantly less at baseline, and at 30 weeks compared to SED WT mice, but were not significantly different from the FEX WTs as exercise attenuated weight gain in WT mice (Figure 2-1c).

Considering that Panx3 may be involved in adipogenesis [10], is linked to body mass index [9], and resulted in lower body weight in the present study, we then sought to determine if this lower body weight in KO mice is due to differences in fat and/or lean mass. KO mice had significantly less fat mass (Figure 2-1d) and fat mass corrected for body weight (Figure 2-1e) than WT mice at baseline. At 30 weeks of age, SED and FEX KO mice had significantly lower fat mass (Figure 2-1d) and fat mass corrected for body weight (Figure 2-1e) compared to SED WT mice. Interestingly, FEX significantly decreased fat mass in WT mice, while FEX had no additional effect on fat mass in KO mice (Figure 2-1d & e). This suggests that the deletion of Panx3 alone has a profound effect on fat mass that is not further decreased by FEX. While there were no significant differences in raw lean mass among the groups (Figure 2-1f), when lean mass was corrected for body weight, KO mice had significantly more lean mass compared to WT mice at baseline (Figure 2-1g). Additionally, at 30 weeks of age, SED KO mice had significantly higher lean mass corrected for body weight compared to SED WT mice (Figure 2-1g). However, the FEX WT mice had similar lean mass when corrected for
body weight compared to KO mice. These results suggest that the deletion of *Panx3* reduces fat mass and increases lean mass to the same extent as 6 weeks of FEX in male WT mice.

**Figure 2-1:** Male *Panx3* KO mice weigh less, have less fat mass and more lean mass than WT mice to the same extent as 6 weeks of forced exercise. Visual graphic of the experimental design. Male and female WT and *Panx3* KO (KO) mice were randomly allocated to either SED or FEX groups from 24 (baseline) to 30 weeks of age (6 weeks) (a). Body weights were measured biweekly, and blood glucose and body composition were measured at baseline and 30 weeks of age. After which, blood and metabolic organs were collected for *ex vivo* analysis. Male body weight development from 4 to 30 weeks of age (b). Baseline comparisons between (checkered
Using individual metabolic cage analysis, we found that there were no significant differences in O2 Volume (Supp. 2-1a), CO2 Volume (Supp. 2-1b), Respiratory Exchange Ratio (Supp. 2-1c), Energy Expenditure (Supp. 2-1d), Water Consumed (Supp. 2-1f), Total Activity (Supp. 2-1g), Ambulatory Activity (Supp. 2-1h), or Sleep Time (Supp. 2-1i) between male WT and KO mice. However, there was a main effect for Food Consumption suggesting that KO mice ate more food overall (Fig. S1e), indicating that the reduced body weight and fat mass in KO mice was not due to increased activity or reduced food consumption.

2.3.2 Male Panx3 KO mice have lower inflammatory index in quadriceps and visceral fat tissues compared to WT mice.

Changes in adiposity and lean mass could be correlated to inflammatory activation of adipose, liver, and skeletal muscle tissue [24]. Therefore, we next determined if Panx3 deletion influences inflammatory gene expression in these metabolic tissues. Liver, quadriceps muscle and visceral white adipose tissues were collected from male WT and KO mice from both SED and FEX groups for analysis of inflammatory genes and an inflammatory index was calculated. Macrophage markers Emr1 and Itgax (CD11c), pro-inflammatory genes Tnfα, Nos2, Il12a, Ccl2 and Il6 and anti-inflammatory markers Arg1, Mrc1, Il10 and Chi3l3 were analyzed in these tissues using RT-qPCR. An inflammatory index was calculated as the ratio of the sum of the pro-inflammatory markers over the sum of the anti-inflammatory markers and reflects the inflammatory status of the tissue. In quadriceps, SED WT mice had significantly higher Emr1 compared to all other
Figure 2-2: Male Panx3 KO mice have lower inflammatory index in quadriceps and visceral fat tissues compared to WT mice.

WT (blue bars) and KO (red bars) mice were allocated to either SED (clear bars) or FEX (solid bars) groups from 24 (baseline) to 30 weeks of age. Quadriceps (a & b), visceral fat (c & d), and liver (e & f) tissues were collected, and mRNA expression was analyzed by RT-qPCR for macrophage markers Emr1 and Itgax (CD11c), pro-inflammatory genes Tnfα, Nos2, Il12a, Ccl2 and Il6 and anti-inflammatory markers Arg1, Mrc1, Il10 and
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Chi3l3. An inflammatory index score was calculated as the ratio of the sum of the pro-inflammatory over the sum of the anti-inflammatory markers and reflects the inflammatory status of the tissue (b, d, f). A two-way ANOVA was conducted with genotype x activity as factors. n = 3-5. mean ± SEM. Different letters indicate significantly different means (p < 0.05). ns: non-significant. a.u.: arbitrary units. WT SED: wildtype sedentary, KO SED: Panx3 knockout sedentary, WT FEX: wildtype forced exercise, KO FEX: Panx3 knockout forced exercise.

groups, while there was a main effect for KO mice having significantly higher Nos2, Tnfa, and Il12a compared to WT mice (Figure 2-2a). When analyzing the inflammatory index for the quadriceps, no group was significantly different than SED KO mice (Figure 2-2b). In visceral adipose tissue, KO mice had significantly lower pro-inflammatory markers Emr1, Itgax, Ccl2, Tnfa, and anti-inflammatory markers Mrc1 (Figure 2-2c).

KO mice had significantly lower inflammatory indexes in visceral fat compared to both SED and FEX WT mice (Figure 2-2d). However, there were no significant differences among the groups for any genes in the liver or the overall inflammatory index (Figure 2-2e & f). These results suggest that the deletion of Panx3 results in a potential shift of inflammatory tone in skeletal muscle and white adipose tissue, comparable to the effects of FEX. Despite these changes in body composition and potential anti-inflammatory effect in skeletal muscle and adipose tissue from a global deletion of Panx3, there were no significant differences in blood glucose tolerance between genotypes (Supp. 2-2a, b, & c). However, when analyzing the blood glucose tolerance curves for the 30-week time point the p–value for genotype approached significance (p = 0.0528) (Supp. 2-2b). There were no significant differences in other circulating measures of metabolic health such as insulin (Supp. 2-2d), cholesterol (Supp. 2-2e), and triglycerides (Supp. 2-2f).

When analyzing circulating measures of inflammation, we found that SED KO mice had significantly lower levels of total adiponectin compared to SED WT mice (Supp. 2-2g). However, there were no significant differences in heavy molecular weight (HMW) adiponectin (Supp. 2-2h) or the ratio of total/HMW adiponectin (Supp. 2-2i). Additionally, there were no differences in serum amyloid A (SAA) between genotypes (Supp. 2-2j). However, there was a significant main effect for Genotype suggesting KO mice have significantly lower circulating levels of IL-6 compared to WT mice regardless of activity (Supp. 2-2k).
Females

a

Genotype x Activity x Age: p = 0.5554
Genotype x Activity: p = 0.7393
Genotype x Age: p < 0.0001
Genotype: p = 0.0714

b

Genotype x Activity x Age: p = 0.7268
Genotype x Activity: p = 0.5320
Genotype x Age: p = 0.7498
Genotype: p = 0.0068

Genotype x Activity x Age: p = 0.3843
Genotype x Activity: p = 0.7742
Genotype x Age: p = 0.6661
Genotype: p = 0.7656

Genotype x Activity x Age: p = 0.2294
Genotype x Activity: p = 0.5047
Genotype x Age: p = 0.4880
Genotype: p = 0.2225

Genotype x Activity x Age: p = 0.9242
Genotype x Activity: p = 0.6229
Genotype x Age: p = 0.4466
Genotype: p = 0.9038

Genotype x Activity x Age: p = 0.5554
Genotype x Activity: p = 0.7393
Genotype x Age: p < 0.0001
Genotype: p = 0.0714

Begin Exercise
Female WT (blue) and KO (red) mice were randomly allocated to either SED (clear bars) or FEX (solid bars) group from 24 (baseline) to 30 weeks of age (6 weeks). Body weights were measured biweekly and body composition was measured at baseline and 30 weeks of age. Female body weight measurements from 4–30 weeks age (a). Female body weight comparison at baseline (checkered bars) and 30 weeks of age (N = 7-13) (b). Fat mass (c), fat mass normalized to body weight (d), lean mass (e), and lean mass normalized to body weight (f) was determined by echo-MRI. Results are expressed as mean ± SEM. An unpaired t-test was conducted to assess significant differences between genotypes at baseline of age (N = 7-8). A three-way ANOVA was conducted with activity x genotype x age as factors to determine significant differences between the genotypes (N = 3-4).

WT SED: wildtype sedentary, KO SED: Panx3 knockout sedentary, WT FEX: wildtype forced exercise, KO FEX: Panx3 knockout forced exercise. ns: non-significant. Different letters indicate significantly different from each other (p < 0.05).

2.3.3 Female Panx3 KO mice weigh slightly less than WT mice with no significant differences in body fat or lean mass.

To determine if these differences between genotypes are seen in females, we next compared WT and KO female mice under both SED and FEX conditions. Interestingly in females, KO mice weighed significantly less as they aged, indicated by a significant Genotype x Age interaction (p < 0.0001) (Figure 2-3a). When analyzing baseline and 30-week-old data, there was a significant main effect for Genotype, suggesting female KO mice weighed significantly less than WT females, however the effect size was much smaller than in males (Figure 2-3b). Despite differences in body weight, there were no significant differences between genotypes in body fat (Figure 2-3c), body fat corrected for body weight (Figure 2-3d), lean mass (Figure 2-3e), or lean mass corrected for body weight (Figure 2-3f) at baseline or with and without FEX at 30 weeks of age between genotypes, but there was an effect for Activity in reducing fat mass regardless of genotype. This would suggest that PANX3’s role in fat accumulation in females is not as pronounced as in male mice.
Figure 2-4: Female Panx3 KO mice have higher inflammatory index in quadriceps and liver tissues compared to WT mice.

WT (blue) and KO (red) mice were allocated to either SED (clear bars) or FEX (solid bars) groups from 24 to 30 weeks of age. Quadriceps (a & b), visceral fat (c & d), and liver (e & f) tissues were collected, and mRNA expression was analyzed by RT-qPCR for macrophage markers Emr1 and Itgax (CD11c), pro-inflammatory genes Tnfα, Nos2, Il12a, Ccl2 and Il6 and anti-inflammatory markers Arg1, Mrc1, Il10 and Chi3l3. An inflammatory index score was calculated as the ratio of the sum of pro-inflammatory over anti-inflammatory markers.
the sum of anti-inflammatory markers and reflects the inflammatory status of the tissue (b, d, f). A two-way ANOVA with genotype x activity as factors was conducted. N = 3-5. ns: not significant, mean ± SEM. arbitrary units (a.u.). Different letters indicate significantly different means (p < 0.05). WT SED: wildtype sedentary, KO SED: Panx3 knockout sedentary, WT FEX: wildtype forced exercise, KO FEX: Panx3 knockout forced exercise.

2.3.4 Female Panx3 KO mice have higher inflammation in quadriceps and liver tissues compared to WT mice.

Female WT and KO mice from both SED and FEX groups were sacrificed, and skeletal muscle, visceral adipose, and liver tissues were excised for analysis as described above. In quadriceps, FEX KOs had significantly higher expression of Nos2 expression compared to SED WT and KO mice, while WT mice had significantly higher expression of Mrc1 (Figure 2-4a). KO mice had significantly higher overall inflammatory index in quadriceps compared to WT mice (Figure 2-4b). In visceral fat, both SED and FEX KO mice had significantly lower expression of Tnfα compared to SED WT mice and significantly higher expression of Arg1 regardless of activity (Figure 2-4c). However, there were no significant differences among the groups when assessing the inflammatory index for visceral fat (Figure 2-4d). In liver, KO mice had significantly higher expression of TNFα regardless of activity group, and lower Arg1 and Chi313 compared to SED WT animals (Figure 2-4e). For the overall liver inflammatory index, KO mice had a significantly higher score compared to WT mice regardless of activity (Figure 2-4f). This would suggest that in female mice the deletion of Panx3 leads to a higher inflammatory tone in the quadriceps and liver tissues. When assessing blood glucose tolerance in females, there was no significant difference at baseline (Supp. 2-3a), however at the 30-week timepoint there was a significant Genotype x Activity interaction (Supp. 2-3b). While there was no significant effect of exercise on AUC in KO mice, WT mice seemed to have improved glucose handling with FEX (Supp. 2-3c). Additionally, there was an overall main effect of Genotype for AUC, suggesting KO mice have improved glucose handling (Supp. 2-3c). There were no significant Genotypic effects on insulin (Supp. 2-3d), triglycerides (Supp. 2-3e), or cholesterol (Supp. 2-3f). However, there was a main effect of Activity for insulin (Supp. 2-3d). When assessing circulating levels of inflammatory markers there were no significant differences in total adiponectin (Supp.
2-3g), HMW adiponectin (Supp. 2-3h), total/HMW adiponectin (Supp. 2-3i), SAA (Supp. 2-3j), and IL-6 (Supp. 2-3k).

Figure 2-5: PANX3 expression is higher in male visceral fat tissue compared to females, and is regulated by FEX and dietary fat intake.
Male and female WT mice were fed a normal chow diet and allocated to either SED or FEX groups, and their visceral fat was isolated and analysed for PANX3 protein expression (a & b). Protein from animals fed a Western diet (45% kcal from fat) was used as a positive control. mRNA from visceral fat of male mice fed a chow or Western diet and subjected to either the SED or FEX protocol was analysed for Panx3 expression (c & d). Male WT and Panx3 Knockout (KO) mice were fed a high fat diet (HFD, 60% kcal from fat) from 12 to 28 weeks of age and epididymal fat was analysed for PANX3 protein expression (e & f). KO mouse tissues were used as a negative control (e & f). ns: not significant, N = 3, n = 3. Different letters indicate significantly different means (p < 0.05). GAPDH was used as a loading control for Western blots, while 18s and Hprt was used for housekeeping genes for qPCR. Body weight (g) and body weight % change (h) was measured in male mice to determine differences in weight gain between genotypes on a HFD. A two-way repeated measures ANOVA with genotype x age was conducted (N = 13–16). Results are expressed as mean ± SEM

2.3.5 Panx3 expression is higher in male visceral fat and is regulated by FEX and dietary caloric excess.

Considering Panx3 deletion is producing sex differences in fat and lean mass, we next wanted to determine if PANX3 expression is different between male and female visceral fat. Protein from visceral fat of both SED and FEX male and female WT mice was isolated and ran on a Western blot (Figure 2-5a & b). Males had significantly higher expression of PANX3 compared to females regardless of activity levels. Interestingly, FEX seemed to increase PANX3 expression, but this did not reach significance (p = 0.0607) (Figure 2-5a). Next, considering Pillon et al. previously showed that HFD significantly increases Panx3 mRNA expression in fat [11], we wanted to confirm these results, and determine if FEX was able to counter this effect. Visceral fat mRNA was isolated from male WT mice that ate either chow or a Western diet (45% kcal from fat) and were subjected to either SED or FEX conditions (Figure 2-5c & d). Western diet significantly increased Panx3 expression compared to chow fed animals, however, FEX attenuated this expression in Western fed animals (Figure 2-5c & d). Next, we fed male WT and KO mice a HFD (60% kcal from fat) from 12 to 28 weeks of age. Like Pillon et al. [11] and our mRNA results, HFD significantly increased PANX3 expression in fat compared to chow fed animals (Figure 2-5e & f). Next, we wanted to determine if deleting Panx3 would have a protective effect on body weight under HFD feeding as seen in chow fed male mice. However, there were no significant differences in raw body
Male Panx3 KO mice fed a HFD have less inflammation in epidydimal adipose and skeletal muscle tissue.

Considering HFD regulated PANX3 expression in adipose tissue, and PANX3 may mediate nutrient-induced inflammation [11], we then set out to determine if KO mice are protected from diet-induced inflammation. At sacrifice, HFD fed KO and WT mice had liver, quadriceps muscle and epidydimal white adipose tissues (eWAT) collected for analysis of inflammatory markers, as described above. KO mice had significantly higher expression of anti-inflammatory genes Arg1 and Il10 compared to WT mice (Figure 2-6a), resulting in a significantly lower inflammatory index in quadriceps (Figure 2-6b). In eWAT tissue (Figure 2-6c & d) KO mice had lower expression of pro-inflammatory genes Ccl2 and Il6, and significantly lower Arg1 and higher Chi313 anti-inflammatory expression, resulting in significantly lower inflammatory index (Figure 2-6d). However, there were no significant differences in individual gene expression (Figure 2-6e) or overall inflammatory index in the liver (Figure 2-6f) between genotypes. These results suggest that male KO mice have lower skeletal muscle and fat tissue inflammatory tone compared to WT mice while on an HFD.

Panx3 KO mice have fewer adipocytes, and their ASCs grow slower than WT mice.

Considering we found that male KO mice have significantly lower fat mass than WT mice, we wanted to determine if this was the result of less adipocytes or a reduction in adipocyte hypertrophy. While there were no differences in the size of subcutaneous adipocytes between KO and WT mice (Figure 2-7a & b), KO mice had significantly fewer adipocytes (Figure 2-7a & c). This suggests that the deletion of Panx3 may reduce the total number of adipocytes in subcutaneous tissue. Considering there is no published literature on PANX3’s role in adipose-derived stromal cells (ASCs) or early adipocyte development, we wanted to determine what role Panx3 may be playing in cell
Figure 2-6: Male *Panx3* KO mice are protected from HFD induced inflammation compared to WT mice.

WT and *Panx3* KO (KO) mice were fed a HFD (60% kcal from fat) from 12 to 28 weeks of age. Quadriceps (a & b), epididymal white fat (eWAT) (c & d), and liver (e & f) tissues
were collected, and mRNA expression was analyzed by RT-qPCR for macrophage markers \textit{Emr1} and \textit{Itgax} (CD11c), pro-inflammatory genes \textit{Tnfa}, \textit{Nos2}, \textit{Il12a}, \textit{Ccl2} and \textit{Il6} and anti-inflammatory markers \textit{Arg1}, \textit{Mrc1}, \textit{Il10} and \textit{Chi3l3}. An inflammatory index score was calculated as the ratio of the sum of pro-inflammatory over the sum of the anti-inflammatory markers and reflects the inflammatory status of the tissue. An unpaired t-test was conducted to determine significant differences between genotypes. N = 5, * = p < 0.05. Results are expressed as mean ± SEM. ns: non-significant, arbitrary units (a.u.).

proliferation and viability. ASCs were isolated from the inguinal fat pads of WT and KO male mice on a HFD, as described previously [6]. ASCs cultured from KO mice grew significantly slower (Figure 2-7d) at 4 and 7 days compared to WT ASCs. When ASCs were cultured to induce differentiation to adipocytes, there was a non-significant trend for \textit{Panx3} protein expression to increase (Figure 2-7e & f). In a pre-adipocytes cell line (3T3-L1), \textit{Panx3} significantly increased during induction to terminal adipocyte differentiation (Figure 2-7g & h). These results suggest that \textit{Panx3} deletion reduces total fat cell number in adult male mice, reduces ASC growth, and may be involved in adipocyte development as its expression is increased during induction.

2.4 Discussion

A number of studies have shown that \textit{Panx3} has a role in the development and pathophysiology of skin [9, 25-28], bone [25, 29, 30], and cartilage [20, 31, 32], and there have been indirect reports of its involvement in body mass index [9] and adipogenesis [10]. In previous publications we examined weight and fat mass differences between WT and \textit{Panx3} KO mice at 12 weeks of age [20] or at later ages (18- and 24-months) [32], and we saw no significant differences between genotypes. In this study we observed large significant differences in weight and fat mass in male KO mice at 24 and 30-weeks of age on a chow diet. We also showed that diet and exercise are regulators of \textit{Panx3} expression in mouse adipose tissue, and it is significantly more expressed in male adipose tissue. While there was a genotype effect for female KO mice to weigh less at later time points, the deletion of \textit{Panx3} resulted in much larger weight reductions in males. Most of this body weight difference can be accounted for by the lower fat mass in KO mice. This lower fat mass was to the same extent as 6 weeks of
Figure 2-7: Panx3 KO mice have fewer adipocytes and their primary adipose stromal cells (ASCs) grow slower than those isolated from WT mice.
Representative images of the subcutaneous fat of male wildtype (WT) and Panx3 knockout (KO) mice (Scale bar = 100 μm) (a). Adipocyte size (normalized to WT size) (b) and the number of cells (normalized to the standardized area of view) (c) were quantified. ASCs were isolated from WT and Panx3 KO mice and placed in growth media (d). Western blot and quantification showing PANX3 protein expression in ASCs under controlled and induced conditions (for adipocyte differentiation) (e & f). PANX3 expression in terminal
differentiated 3T3-L1 pre-adipocytes as shown by Western blots of 3T3-L1 cells cultured under controlled and induced conditions (g) and the quantification of PANX3 protein expression (h). N = 3, n = 3, p < 0.05. Results are expressed as mean ± SEM. * p < 0.05, ****p < 0.0001. ns: non-significant. Overexpressing HEK293 cells were used as positive controls (+).

FEX in WT mice, however, there were no significant differences in body weight between genotypes when challenged with a HFD. Upon further investigation to determine why Panx3 deletion may reduce fat mass, we found that these mice have a reduced number of adipocytes in their subcutaneous fat. Furthermore, KO mice had lower levels of multiple pro-inflammatory genes in white adipose and skeletal muscle tissue under both regular chow and HFD feeding. These results suggest that PANX3 is expressed at higher levels in male adipose tissue, and may regulate adipocyte cell proliferation, body fat accumulation and inflammatory gene expression in male mice. Differences in obesity rates between males and females is the result of a complex interaction between chromosomal, hormonal, gender and behavioural factors [33]. While there were significant differences in body weight between WT and KO females as they aged, deleting Panx3 in males had a much more profound effect on body mass and composition. Considering male C57BL/6 mice are much more susceptible to weight gain and fat expansion under a variety of dietary conditions [34], this may explain why we observed a greater effect in males. Furthermore, quantitative trait loci data linking Panx3 to body mass index were specific to male mice [9] which further supports the findings in this study. Additionally, we found that PANX3 expression was significantly higher in male adipose tissue compared to females, supporting the notion that Panx3 plays a role in male but not female adiposity.

Both estrogen and testosterone play a role in metabolic disease and obesity [35]. We did not measure sex hormones in this study, and there are no published reports of estrogen and testosterone levels in Panx3 KO mice. However, PANX3 is expressed in Leydig cells [36] and therefore may influence testosterone production. Interestingly, the female Panx3 KO mice in this study had significantly higher inflammatory indices in quadriceps and liver tissues. This dichotomy in inflammatory changes between male and female KOs is perplexing, however PANX3 may influence inflammation differently between sexes due
to gonadal white adipose tissue, which contributes to differences in lipid metabolism and inflammation between sexes [37].

We have previously shown, in cultured myotubes, that PANX3 contributes to the cell-intrinsic pro-inflammatory effects of the dietary fatty acid palmitate [11]. Blocking of PANX3 channels reduced the capacity of cultured skeletal muscle cells to recruit monocytes. While in the present study we did not quantify immune cells, male Panx3 KO mice had significantly lower expression of Emr1, a macrophage marker, in quadriceps and adipose tissue. Additionally, KO mice had reduced expression of pro-inflammatory relative to anti-inflammatory genes, suggesting that the deletion of Panx3 may attenuate diet and sedentary behaviour induced adipose tissue inflammation [38]. While these results support our previous observations regarding PANX3’s role in inflammation, we are unable to determine which cell type is responsible for the altered inflammatory expression.

Exercise has been shown in both animal and humans to have anti-inflammatory effects systemically and in adipose tissue [39-41]. Studies in mouse models show that exercise attenuates visceral white adipose tissue inflammation caused by HFD [42], specifically, the recruitment of M1-like macrophages and CD8+ T cells upon exposure to HFD [43]. In this study, while we did not assess markers of CD8+ T cells, we found that FEX in WT mice resulted in significantly lower levels of macrophage markers in skeletal muscle and adipose tissue. Interestingly, the deletion of Panx3 also reduced macrophage markers, and resulted in lowering of multiple pro-inflammatory genes that exercise had no effect on. This would suggest that the deletion of Panx3 has an even greater impact on inflammatory gene expression than 6 weeks of daily FEX.

Previously we reported that Panx1 KO mice have more fat mass, less lean mass and weigh more than WT mice [6]. This suggests an opposite effect than what was observed in the present study with the deletion of Panx3. The potential opposing role of Panx1 and Panx3 in adipose tissue is not certain, however this may be due to differing functions of the two pannexin isoforms during early development and their involvement in pre-adipocyte fate. While both ASCs from Panx1 and Panx3 KOs have reduced proliferation
compared to WT ASCs, Panx1 KO ASCs have enhanced adipogenic differentiation. We did not perform any further assays to assess differentiation fate of Panx3 KO ASCs, and future research is necessary to study the function of PANX3 in these cells.

Consistent daily exercise is necessary for health, however much of the literature suggests that exercise alone cannot reduce adiposity in people with obesity, and dietary interventions are necessary [44, 45]. In mouse models, the extent to which exercise can influence body weight may be dependent on the age, sex, diet, and the nature of the exercise intervention (voluntary versus forced) [46, 47]. We found that FEX attenuated weight gain in WT mice because of reduced fat mass and increased lean mass to body weight ratio. FEX had no additional effect on body weight in Panx3 KO mice however, as these mice do not gain a significant amount of weight or fat mass between 24 and 30 weeks of age. This suggests the presence of the Panx3 gene is necessary for the natural weight gain that occurs in adult male WT mice under sedentary conditions. What is striking is the magnitude of difference in body weight (difference between means: 7.117g ± 0.6830) and fat mass (difference between means: 4.727g ± 1.238) between genotypes. This equates to an approximately 46.8% reduction in fat mass, which is like the effect of FEX in this experiment.

While we saw drastic effects on body and fat mass from the deletion of Panx3 in males under SED and regular chow fed conditions, there were no significant differences in body weight during HFD feeding. This finding is in line with multiple previous reports that obesity is mainly the result of excess caloric intake [48, 49]. However, we know individuals can vary in how much weight they gain while in a similar caloric excess [50] which would suggest genetic and behavioural factors are also at play. Our findings highlight the importance of taking into consideration environmental and behavioural factors that can interact with genetics when investigating multifactorial diseases such as obesity. Manipulating Panx3 may not be effective when consuming an excessive caloric diet, however it may be an effective target for patients who are also engaging in healthy caloric consumption.
While Panx3 levels were low in adipose tissue of chow fed WT animals, it was significantly elevated in mice fed a Western or HFD. This suggests that Panx3 expression is sensitive to dietary factors, as gleaned from previous work in cell culture models [11]. Moreover, FEX was able to counter this diet induced Panx3 upregulation. This would suggest that exercise is able to inhibit the signalling responsible for PANX3 expression caused by dietary factors. Future studies will be needed to determine what signaling pathways are responsible for the induction and suppression of PANX3 expression by diet and exercise. However, our previous data along with those reported by others indicate that the toll-like receptor 4 (TLR4)/nuclear factor -κB (NF-κB) pathway is activated by the saturated fatty acid palmitate [51]. We previously showed that this pathway mediated the expression of Panx3 mRNA [11]. Conversely, moderate aerobic exercise is known to downregulate TLR4, and consequently the proinflammatory NF-κB pathway, thus, potentially inhibiting Panx3 expression [52].

2.5 Conclusion

We have shown that the deletion of Panx3 attenuates body weight gain because of lower fat mass in male mice. Additionally, skeletal muscle and adipose tissue of KO mice shift to a more anti-inflammatory phenotype in males. This effect was equivalent to the reduction in body weight gain and fat mass reduction caused by 6 weeks of daily FEX. This suggests PANX3 plays a significant role in fat accumulation and inflammation in adult male mice. This phenotype may be the result of PANX3’s role in adipocyte proliferation in early life. Considering this study used a global KO model, future research is needed to determine if PANX3 functions in other cell types involved in this phenotype. Manipulating PANX3 channel function or expression may be a potential therapeutic target in conjunction with dietary and exercise interventions to manage obesity and associated inflammation in males.
2.6 Supplemental Figures

Supp. 2-1: *Panx3* KO mice eat more food in a metabolic cage but have similar metabolic activity and sleep.

WT and *Panx3* KO male mice fed *ad libitum* on a normal chow diet were placed in metabolic cages to assess metabolism and activity during their sleeping period (light) and the active period (dark). O2 (a) and CO2 consumption (b), respiratory exchange ratio (c), energy expenditure (d), food consumption (e), water consumption (f), total activity (g), ambulatory activity (h), and sleep time (i). A two-way ANOVA was conducted with genotype x time as factors. N = 4. Results are expressed as mean ± SEM.
Supp. 2-2: No overt differences in markers of metabolism or circulating markers of inflammation in male SED or FEX WT and KO mice.

Male WT and Panx3 KO (KO) mice were fed a chow diet and allocated to either SED or FEX conditions. Blood glucose curves for 24 weeks (baseline) (a) and 30 weeks for SED
and FEX animals (b). N = 3–4. Blood glucose area under the curve (AUC) from baseline and 30 weeks of age (c). A three-way ANOVA with genotype x activity x age as factors was conducted. Plasma insulin (d), cholesterol (e) triglycerides (f), total adiponectin (g), heavy molecular weight (HMW) adiponectin (h), ratio of HMW/total adiponectin (i), serum amyloid A (SAA) (j), and interleukin 6 (I\(\text{Il6}\)) (k). A two-way ANOVA with genotype x activity as factors. N = 4–5.
Supp. 2-3: *Panx3* KO female mice have lower blood glucose under SED conditions with no differences in circulating measures of inflammation.
Female WT and Panx3 KO (KO) mice were fed a chow diet and allocated to either SED or FEX conditions. Blood glucose curves for 24-week (baseline) (a) and 30-week timepoint for SED and FEX animals (b). Blood glucose area under the curve (AUC) from baseline and 30 weeks of age (c). A three-way ANOVA with genotype x activity x age as factors was conducted. N = 3–4. Plasma insulin (d), triglycerides (e) cholesterol (f), total adiponectin (g), heavy molecular weight (HMW) adiponectin (h), ratio of HMW/total adiponectin (i), serum amyloid A (SAA) (j), and interleukin 6 (IL6) (k). A two-way ANOVA with genotype x activity as factors was conducted. N = 4–5.
References


Chapter 3

Male \textit{Panx3} KO mice have shown to be protected from surgically-induced OA, while they have accelerated OA in aging. At the lumbar IVD, these mice are also showing protection to surgically induced IDD, while sensitive to altered biomechanics in the disc. These data suggest a context-dependent role of \textit{Panx3} in joint health. In this chapter, we assessed the joints of young adult \textit{Panx3} KO mice under SED and FEX conditions, to determine if these mice are susceptible to joint damage from forced use via treadmill running. This chapter is currently in revision in the \textit{Journal of Orthopaedic Research} and published as a preprint in bioRxiv: https://doi.org/10.1101/2023.03.20.532801
3  Pannexin 3 deletion in mice results in knee OA and intervertebral disc degeneration after forced treadmill running.

**Background:** Pannexin 3 (Panx3) is a glycoprotein that forms mechanosensitive channels expressed in chondrocytes and AF cells of the IVD. Evidence suggests Panx3 plays contrasting roles in traumatic versus aging OA and IDD. However, whether its deletion influences the response of joint tissue to forced use is unknown. The purpose of this study was to determine if Panx3 deletion in mice causes increased knee joint OA and IDD after forced treadmill running.

**Methods:** Male and female WT and Panx3 KO mice were randomized to either a no exercise group SED or FEX from 24 to 30 weeks of age. Knee cartilage, tibial secondary ossification center and IVD histopathology were evaluated by histology.

**Results:** Both male and female Panx3 KO mice developed larger superficial defects of the tibial cartilage after forced treadmill running compared to SED WT mice. Additionally, both male and female Panx3 KO mice developed greater bone area of the tibial secondary ossification center with running. In the lower lumbar spine, both male and female Panx3 KO mice developed histopathological features of IDD after running compared to SED WT mice.

**Conclusions:** These findings suggest that the combination of deleting Panx3 and forced treadmill running induces OA and causes histopathological changes associated with degeneration of the IVDs in mice.

3.1  Introduction

OA [1] and low back pain [2] are two major causes of disability worldwide. While a subset of these conditions is caused by trauma, they are also associated with genetic [3] and environmental factors (ex. physical activity [4]), developing spontaneously. Most studies of genetically modified animal models (mice) induce joint stress using injury or surgical interventions, which may not simulate primary OA or spontaneous IDD in humans. Indeed, the molecular mechanisms of traumatic versus idiopathic joint pathology
may differ, highlighting the need to use multiple models of OA or IDD to investigate the role of target genes associated with disease pathogenesis.

Pannexin 3 (PANX3), a membrane bound channel-forming glycoprotein expressed in osteoblasts [5], chondrocytes [6], and annulus fibrosus cells of the IVD [7], has been identified as a potential target for OA [8, 9] and IDD [10, 11]. PANX3 expression is induced by the transcription factor RUNX2, which drives chondrocyte hypertrophy [12]. Hypertrophic chondrocytes produce catabolic enzymes such as matrix metalloproteinase (MMP13) which contribute to the breakdown of cartilage extracellular matrix [13]. Our lab has shown that global and chondrocyte specific deletion of Panx3 in male mice is protective against OA caused by destabilization of the medial meniscus (DMM) surgery (a model of post-traumatic OA) [8]. Paradoxically, we subsequently found that Panx3 KO mice had worse knee cartilage degradation and sclerotic subchondral bone when aged to 18 months [9]. These data were amongst the first to show completely opposite roles of the same gene in different models of OA – Panx3 appears to promote OA in a post-traumatic model but protects joints during aging.

Regarding IVD health, Panx3 KO discs were protected from trauma-induced degeneration and did not develop spontaneous age-associated IDD [10]. IVDs adjacent to the site of injury experience altered mechanics, and these IVDs of Panx3 KO mice showed accelerated degeneration compared to WT discs. This suggests the absence of PANX3 is beneficial in traumatic models, while its presence is necessary for the adaptive cellular responses to altered or accumulated loading.

### 3.2 Methods

#### 3.2.1 Mice

Animals used in this study were bred in-house and euthanized in accordance with the ethics guidelines of the Canadian Council for Animal Care. Animal use protocols were approved by the Council for Animal Care at Western University Canada (AUP 2019-069). Mice were housed in standard shoe box-style caging and exposed to a 12-hour light/dark cycle and ate regular chow ad libitum. Body weights were taken bi-weekly and body composition was assessed at 24 and 30 weeks of age [14]. C57BL/6 N WT and
Panx3 KO mice were congenic. DNA was collected from ear clippings of each mouse to determine the genotype using polymerase chain reaction as previously described [8, 15]. At sacrifice, mouse knees and spines were collected and immediately processed for histological analysis.

### 3.2.2 Forced Exercise Intervention

At 24 weeks of age, mice were randomized to either a SED or FEX group. The FEX groups ran on a treadmill (Columbus Instruments, Ohio) for 6 weeks, 1 hour a day, 5 days a week, at a speed of 11 m/min, and a 10° incline, an adapted protocol that has previously been used to induce OA in C57BL6 mice [16]. The mice were encouraged to run using a bottle brush bristle and a shock grid at the end of the treadmill as per the animal ethics protocol.

### 3.2.3 Histopathological Assessment of the Knee Joint

Knee joints were fixed in 4% paraformaldehyde at room temperature for 24 hours on a shaker, and then decalcified in 5% EDTA for 12 days at room temperature. Knees were processed and embedded coronally in paraffin, and 5-μm–thick sections were cut from front to back. Sections were stained with toluidine blue. Six sections spanning the width of the knee were scored by 2 blinded reviewers using a 12-point system developed by McNulty et al. [17]. The average max score of each sample was then used for statistical analysis. Cartilage area of the load bearing region was analyzed using the OsteoMeasure (OsteoMetric) software. Unmineralized and mineralized cartilage was manually segmented at the tidemark.

### 3.2.4 Histopathological Assessment of the Lumbar Intervertebral Discs

Lumbar spines were fixed overnight with 4% (w/v) paraformaldehyde, followed by 7 days of decalcification with Shandon’s TBD-2 (Thermo Scientific, Waltham) at room temperature. Tissues were embedded in paraffin and sectioned in the sagittal plane at a thickness of 5 μm. Mid-sagittal sections were deparaffinized and rehydrated as previously described [18] and stained using 0.1% Safranin-O/0.05% Fast Green. Sections were imaged on a Leica DM1000 microscope, with Leica Application Suite (Leica).
Microsystems: Wetzlar). To evaluate IVD degeneration, spine sections were scored by two observers blinded to age, exercise, sex, and genotype using a previously established histopathological scoring system for mouse IVDs [19]. Modifications were made in Part (I) (“score 4: mineralized matrix in NP” was omitted). Compartment scores (NP, AF, and NP/AF Boundary) were summed for each IVD and reported for each lumbar level. To report on degeneration across the lumbar spine, scores for individual lumbar IVDs (L2-L6) were summed, and the total score plotted for each individual mouse.

3.2.5 Measurements of Tibial Subchondral Bone and L5 Vertebral Body

OsteoMeasure (OsteoMetric) software was used to measure the tibial secondary ossification center and the L5 bone area (cortical and trabecular) and marrow area. Three randomly selected slides per animal were chosen, and for the subchondral tibia, the bone tissue was segmented from the overlaying articular cartilage of the tibia and the underlying growth plate. Bone area and marrow area were divided by the total area.

3.2.6 Statistics

The department of Epidemiology and Biostatistics at Western University was consulted to determine the appropriate statistical analysis. Data are presented as means ± CI. Prism (GraphPad Software Inc.) was used to run all statistical tests including one-way analysis of variance (ANOVA) or three-way ANOVA for comparison. For articular cartilage structure scores and histopathological scores of the IVD, males and females were analyzed separately within their respective genotypes and activity group. A Kruskal-Wallis test was used with an uncorrected Dunn’s test for multiple comparisons to determine statistical differences from the SED WT control group. For the cartilage area, subchondral bone, and L5 bone measures, a three-way ANOVA followed by Sidak’s multiple comparisons test was performed to determine the effect of activity within each genotype for each sex. All applicable data met assumptions for homoscedasticity or normality of residuals. Based on the recommendations of the editorial entitled: Moving to a World Beyond “p < 0.05” [20] we did not set a threshold for significance, and we refer to the data as supported by little to no evidence (p = 0.01-1), weak (p = 0.05-0.1),
moderate ($p = 0.01-0.05$), strong ($p = 0.01-0.001$) or very strong ($p = 0.001-0.0001$) evidence based the recommendations previously published [21].

### 3.3 Results

#### 3.3.1 Forced treadmill running and \textit{Panx3} deletion increases features of tibial knee OA.

To better understand how \textit{Panx3} deletion influences OA pathogenesis, we examined the knee joints of male and female WT and \textit{Panx3} KO mice under SED and FEX conditions (Figure 3-1A). Histological analysis revealed that male \textit{Panx3} KO mice have higher articular cartilage structural (ACS) scores, indicating damage in the superficial zone of the unmineralized cartilage across the whole tibia compared to SED WT mice (9.361; mean rank difference; $p = 0.0165$) (Figure 3-1B). This is evident by the surface erosion and fibrillation on the medial and lateral tibia. With forced treadmill running, \textit{Panx3} KO mice had larger surface erosions and fibrillation across the joint compared to SED WT mice (16.11; $p = 0.0003$) (Figure 3-1B). When analyzing the medial compartment alone, there was weak evidence that SED \textit{Panx3} KO mice had higher ACS scores compared to SED WT mice (5.278; $p = 0.1590$) (Figure 3-1C). However, when subjected to treadmill running, \textit{Panx3} KO mice had larger surface defects compared to SED WT mice (14.78; $p = 0.0005$) (Figure 3-1C). In the lateral compartment, SED \textit{Panx3} KO mice develop superficial cartilage erosion and fibrillation compared to SED WT mice (8.972; $p = 0.0171$), and strong evidence suggesting differences between FEX \textit{Panx3} KO and SED WT mice (13.60; $p = 0.00015$) (Figure 3-1D). Taken together, this suggests the deletion of \textit{Panx3}, in combination with forced treadmill running, causes greater surface cartilage damage compared to SED WT mice.
For female mice, across the whole tibial joint, statistical and scientific inference would suggest forced treadmill running did not influence ACS scores in either genotype (Figure 3-1E-G). There was moderate to strong evidence that both SED (14.44; p = 0.0013) and FEX (13.04; p = 0.0076) Panx3 KO mice had larger surface defects across the joint compared to SED WT mice (Figure 3-1E). In the medial compartment, the ANOVA resulted in a p-value = 0.2428, suggesting there were no structural differences among the groups, and thus we did not run a post-hoc comparison (Figure 3-1F). In the lateral compartment, there was moderate evidence that SED Panx3 KO mice have higher ACS scores compared to SED WT mice (9.125; p = 0.0469), while there was moderate to strong evidence that FEX Panx3 KO mice had higher ACS scores compared to SED WT (14.71; p = 0.0022) (Figure 3-1G).

Next, we analyzed the tibial cartilage area and thickness in males (Figure 3-2B-E) and females (Figure 3-2G-J). Using a three-way ANOVA to analyze the effect of genotype, exercise and sex, we found that there was moderate evidence to indicate that the deletion of Panx3 reduced the medial tibia unmineralized cartilage area [F (1,56) = 6.108, P = 0.0165] and thickness [F (1,56) = 5.493, p = 0.0227] in both males (Figure 3-2B&D) and females (Figure 3-2G&I). In the mineralized cartilage of the medial tibia, there was moderate evidence [F (1,56) = 5.229, p = 0.0276] suggesting FEX WT mice have greater mineralized cartilage area compared to SED WT mice (0.0077, 0.0148 to 0.00, p = 0.0276) (Figure 3-2B). In the lateral tibia, there was weak evidence for differences in

**Figure 3-1:** The tibial cartilage of Panx3 KO mice shows features of OA that are compounded by forced treadmill running. Representative Tolidine Blue staining of knee joints from male and female wildtype (WT) and Panx3 knockout (KO) mice under sedentary (SED) and forced treadmill running (FEX) conditions, as indicated (A). Images on the left show 4x magnification of whole joints in the frontal plane, 20x magnification of tibial cartilage in the medial (middle panel) and lateral (right panel) surfaces. Black arrows indicate cartilage defects, while red arrows indicate loss of staining. Scale bars, 100µm. (B – D) Total (B), medial (C), and lateral (D) tibial articular cartilage structure (ACS) score of male mice for WT SED (N = 8), WT FEX (N = 7), KO SED (N = 6), and KO FEX (N = 4), as indicated. (E – G) Total (E), medial (F), and lateral (G) tibial articular cartilage structure (ACS) score of female mice for WT SED (N = 14), WT FEX (N = 8), KO SED (N = 9), and KO FEX (N = 7), as indicated. For statistical comparisons among the groups, a Kruskal-Wallis test was performed. All data are shown as means ± CI.
Figure 3-2: Medial unmineralized cartilage area and thickness of the tibia are reduced in Panx3 KO mice.

Tibial cartilage of the medial and lateral surface was segmented from the underlying subchondral bone and divided into unmineralized and mineralized cartilage for male (left) and female (right) mice (A). Images show 10x magnification of the medial (left) and lateral (right) load bearing regions of the tibial surface. Scale bar = 100µm. Black arrows indicate tidemark. Medial (B) and lateral (C) unmineralized area and thickness (D & E) for male mice. Medial (F) and lateral (G) unmineralized area and thickness (H & I) for female mice. WT SED (N = 8 males, N = 14 females), WT FEX (N = 8 males, N = 8), KO SED (N = 10
unmineralized cartilage area (Figure 3-2C), however, there was moderate evidence for a main effect for activity, suggesting forced treadmill running increased thickness \([F (1,53) = 7.992, p = 0.0066]\) in both males (Figure 3-2E) and females (Figure 3-2J). Lastly, there was no evidence of differences in the lateral mineralized cartilage area (Figure 3-2C & H) or thickness (Figure 3-2E & J). Collectively, this data indicates that Panx3 KO mice have less unmineralized cartilage of the medial tibia, regardless of sex, and develop cartilage damage under SED conditions, with males have more damage from forced treadmill running.

3.3.2 Panx3 KO mice have greater bone area in the proximal tibia after forced treadmill running compared to SED Panx3 KO mice.

It has been established that subchondral bone changes of the tibia occur early on in OA [22], and PANX3 is expressed in bone cells and regulates osteoblast differentiation and bone modelling [23]. However, there is no published research investigating the role of Panx3 in bone remodelling, such as during exercise. Considering that the Panx3 KO mice develop signs of cartilage erosion, which is exacerbated with treadmill running, we next wanted to determine whether the secondary ossification site in the tibia showed any pathological changes (Figure 3-3A). We performed histomorphometry analysis of the proximal tibial secondary ossification site, which involved segmenting the bone from the marrow within the region of interest as explained in the methods section. For male mice, there was no evidence that forced treadmill running altered the total area of interest for either WT \((p = 0.8212)\) or Panx3 KO \((p = 0.5849)\) mice (Figure 3-3B). There was strong evidence for a two-way interaction of genotype x activity \([F (1, 47) = 8.660, P = 0.0050]\) for bone area, resulting in Panx3 KO mice having increased bone area with forced treadmill running \((0.06441; 0.1281, 0.00072); p = 0.0465)\), while there was no change in WT mice \((p = 0.9571)\) (Figure 3-3C). Additionally, there was strong evidence for a two-way interaction of genotype x activity \([F (1, 47) = 8.696, P = 0.0050]\) for marrow area,
Figure 3-3: Forced treadmill running results in greater bone of the tibial secondary ossification center in Panx3 KO mice.

Bone and marrow were segmented from the overlaying articular cartilage and the underlying growth plate of the proximal tibia. Representative Tolidine Blue staining from male (left panel) and female (right panel) wildtype (WT) and Panx3 knockout (KO) mice under sedentary (SED) and forced treadmill running (FEX) conditions, as indicated (A). Images show 4x magnification in the frontal plane, Scale bars, 100µm. (B-D) Total area (B), bone area corrected for total area (C), and marrow area corrected for total area (D) of male WT and Panx3 KO mice. WT SED (N = 9), WT FEX (N = 8), KO SED (N = 7), and KO FEX
resulting in moderate evidence that FEX Panx3 KO mice had less marrow area compared to SED Panx3 KO mice (0.06442; 0.0006, 0.1282; p = 0.0469), while there was no evidence this change happened in WT mice (p = 0.9571) (Figure 3D).

In female mice, there was moderate evidence that the total secondary ossification site area of the proximal tibia of the WT females increased with forced treadmill running (0.1707, 0.3237, 0.01774; P = 0.0230) (Figure 3E). Like the male mice, strong evidence suggested that female Panx3 KO mice had higher bone area with forced treadmill running (0.08566; 0.1472, 0.02413; p = 0.0030) while there was no evidence for this in the WT mice (p = 0.9674) (Figure 3F). Similarly, there was strong evidence that Panx3 KO mice have less marrow area with forced treadmill running (0.08538; 0.02376, 0.1470; p = 0.0032) (Figure 3G). These data suggest that Panx3 KO mice, regardless of sex, have an altered response of the proximal tibia secondary ossification site to forced treadmill running that may contribute to the development of OA.

3.3.3 Both WT and Panx3 KO mice have greater bone area of the L5 vertebral with forced treadmill running compared to their SED counterparts.

We next examined the effects of Panx3 deletion and forced treadmill running on the axial skeleton in this model. We analyzed the L5 vertebra as we had done for the tibial secondary ossification site (Figure 3-4). There was no evidence that genotype or activity influenced the total area of the L5 vertebrae (Figure 3-4B&E). However, there was strong evidence that activity increased bone area (F (1,54) = 14.22, p = 0.0004) (Figure 3-4C&F) and decreased marrow area (F (1,54) = 14.58, p = 0.0004) (Figure 3-4D&G) in males and females of both genotypes. This would suggest that the forced treadmill
Figure 3-4: WT and Panx3 KO mice L5 vertebra respond similarly to forced treadmill running.
L5 vertebral bone and marrow was segmented from each other for analysis. Representative Safranin O and Fast Green staining of the L5 vertebra from male (left
Forced treadmill running caused accelerated IVD degeneration in Panx3 KO mice.

We previously elucidated the potential role of PANX3 in maintaining disc homeostasis in both injury and aging models of IVD degeneration [10]. To better understand the role of PANX3 in mediating the response to forced exercise, we performed histopathological scoring to assess features of disc degeneration within each compartment of the IVD across the lumbar spine (Figure 3-5). While no overt signs of degeneration were detected in the upper lumbar spine, degenerative changes were observed in the lower lumbar regions in both male and female Panx3 KO mice following forced treadmill running (Figure 3-5B&E). In males, there was moderate to strong evidence that under forced treadmill running conditions, Panx3 KO mice had increased degeneration at the L5/L6 IVD compared to SED WT mice (13.60, p = 0.0078) (Figure 3-5B). The increase in histopathological scores were primarily driven by degenerative changes in the annulus fibrosus and interzone, including increased accumulation of hypertrophic cells as well as widening and reversal of the lamellar structure (Figure 3-5A). When histopathological scores were combined to assess the entire lumbar spine, there was weak evidence for differences in scores among the groups (p = 0.6454) (Figure 3-5C). In females, loss of Panx3 was likewise associated with IVD degeneration in the lower lumbar spine following forced treadmill running (Figure 3-5D). Specifically, moderate to strong evidence suggests FEX Panx3 KO mice have increased IVD degeneration.
degeneration at L6/S1 compared to FEX WT (9.889, p = 0.0231), and SED WT mice (13.50, p = 0.0029) (Figure 3-5E). Additionally, there was moderate evidence that SED Panx3 KO mice had increased degeneration compared to SED WT mice (8.438, p = 0.0244) (Figure 3-5E). When histopathological scores were combined to assess the entire lumbar spine, there was moderate evidence that FEX Panx3 KO mice had increased degeneration compared to SED WT mice (13.25, p = 0.0108) (Figure 3-5F). Following forced exercise, IVDs from Panx3 KO mice showed hallmark degenerative changes in the annulus fibrosus and interzone, such as hypertrophic cells and widening and reversal
of the lamellar structure compared to SED WT mice. This data suggests that forced treadmill running in Panx3 KO mice causes accelerated IVD degeneration in both male and female mice.

### 3.4 Discussion

Here, we characterized the effects of forced treadmill running on knee joints and lumbar spines of male and female Panx3 KO mice. Both male and female Panx3 KO mice have thinner unmineralized cartilage of the medial tibia compared to WT mice. With the addition of forced treadmill running, Panx3 KO mice showed large surface defects of the unmineralized cartilage and alterations of the secondary ossification center in the tibia. In addition to these changes to the knee, the forced treadmill running stressed the axial skeleton, as evidenced by the greater bone area in the L5 compared to sedentary mice for both WT and Panx3 KO mice. This stress, in both male and female Panx3 KO mice, was associated with accelerated IVD degeneration at L5/L6 and L6/S1 disc levels, respectively, while there was weak evidence for this effect in WT mice. Collectively, data indicate that the loss of Panx3 in mice, in combination with 6 weeks of forced treadmill running, induces pathological changes to joint tissues.

We have previously shown that male Panx3 KO mice are protected from a traumatic model of OA [8], but demonstrate worse OA during aging [9]. In our previous study of DMM surgery in Panx3 KO mice, we only used male animals, and therefore the results may not be representative of females. Additionally, considering the severity of OA in DMM models, we did not observe the subtle differences in cartilage structure that may have been present in the non-surgical knees of the Panx3 KO mice. For the present study, we used the 12-point articular cartilage structure scoring system which seems to be more sensitive to the subtle differences that may be present in OA models such as forced treadmill running [17]. It seems that while Panx3 KO mice are protected from post-traumatic OA, the present data, and our aging study [9] would suggest these mice develop accelerated spontaneous OA and are sensitive to forced treadmill running.

Forced treadmill running offers an interesting intervention to study the effects of genetic factors on OA and IDD, as there is a complex relationship between mechanical use and
the development of pain and joint radiographic pathology. This relationship seems to be U-shaped in nature in humans, as OA is more prevalent in sedentary populations [24], and there is an increased risk associated with frequent high volumes of loading, as seen in the workplace [25]. The degree to which one is susceptible to mechanical “overuse” could be influenced by genetic differences. A limited number of genetic studies have used forced treadmill running interventions in OA [16, 26-28], as most studies use more traumatic models [29]. Bomer et al., however, determined that forced treadmill running caused differential expression of multiple OA associated genes in cartilage, validating its use as a model of OA [16]. Subsequently, they determined that mice lacking the DIO2 gene (previously identified as a susceptible locus for OA) were protected from treadmill running induced OA. This suggests genetic factors may in fact influences one’s susceptibility to exercise induced OA. Matsuzaki et al., performed aging, DMM and treadmill running in a Col2Cre-FoxO KO mouse model to determine the importance of this transcription factor in various OA contexts [27], while Rellman et al. showed that in protein disulfide isomerase ERp57 KO, to induce chondrocyte ER stress, mice are susceptible to age but not forced treadmill induced OA [28]. Studies like these are important as they highlight the molecular differences between various factors that influence OA progression. Considering studies have shown that exercise can induce knee OA [30-32], and paradoxically, studies also show exercise reduces OA development [26, 33] in mice, it would be imperative to elucidate the genetic factors, and also the exercise parameters that elicit these contrasting results. Here we implemented a modified forced treadmill running protocol previously reported to induce knee OA in mice [16]. We observed weak to moderate evidence that this protocol induced OA in our WT mice. Additionally, in female mice, our model suggests forced treadmill running had little to no effect on knee cartilage structure within each genotype. We are unaware of any previous studies investigating the effect of forced treadmill running on female C57BL/6 mouse cartilage to compare these findings. Considering that we saw the greatest development of OA in the Panx3 KO mice that were forced to run, these data may suggest an interaction or additive effect between activity and genotype. However, the nature of this data does not allow for the performance of statistical tests to determine such relationships.
With regards to IVDs, we have previously examined the influence of *Panx3* deletion on IDD in both aging and IVD puncture (traumatic) models [10]. While there were no obvious differences in pathological IVD changes between *Panx3* KO and WT mice during aging, following IVD puncture, AF tissue architecture appeared better preserved in IVDs from *Panx3* KO mice compared to WT mice. This would suggest that the absence of *Panx3* in traumatic injuries is beneficial. Interestingly, IVDs adjacent to the site of puncture, which experience increased mechanical stress, showed signs of accelerated nucleus pulposus degeneration [10]. These data suggest that *Panx3* KO mice are prone to accumulating damage of the lumbar IVD when mechanically stressed. Considering we saw increased IVD pathology in our forced treadmill running KO mice, this supports this previous finding. Interestingly, Belonogova et al. found that *PANX3* rare polymorphic non-coding variants in humans are strongly associated with back pain, which suggests *PANX3* may be involved in human IDD associated pain [11].

Considering our findings using our *Panx3* KO mouse model, and the findings of Belonogova et al. in humans [11], further investigation of *PANX3* in IVD health and disease is warranted.

To our knowledge, there are no forced treadmill running studies investigating its effects on IVD health in mice, as all the rodent studies are in rats [34-36]. Rat models of forced treadmill running have shown to be anabolic for IVDs [35], and dynamic compressive forces to IVDs in an *in vitro* model stimulated an anabolic response by increasing gene expression for types I and II collagen and aggrecan [37]. Additionally, other researchers have shown that the effect of hydrostatic pressure on IVD response is dose dependent, with higher forces eliciting a catabolic response [38]. These data suggest dynamic, cyclical, loading (such as during running) may be anabolic for IVDs, which is supported by cross-sectional studies in humans [39, 40]. In our model, *Panx3* KO mice forced to run develop histological features of IDD while there was weak evidence for this in WT mice. Like the knee data, this would suggest that the *Panx3* KO mice are sensitive to forced running. What molecular mechanism is mediating this potentially pathological response is to be determined.
Unfortunately, our model had low n values for multiple groups which increased the range of our confidence intervals. Considering the effect of the forced treadmill running on OA and IDD measured is small (if any in the female WT mice), our experiment provides weak statistical evidence to determine, the precise effect of forced treadmill running within each genotype. We had lost 2 male FEX Panx3 KO samples during processing which ended up being the rate limiting group for the statistical analysis. However, considering we analysed multiple tissues and sites at the knee joint, and IVDs, in both males and females, which all provided similar relationships among the groups, our data suggests the combination of deleting Panx3 and forced treadmill running worsened OA and IDD measures. Additionally, the OA and IDD differences we observed in our analysis are relatively mild compared to phenotypes observed in other genetic and traumatic injury-induced models. This could be due, in part, to the parameters of the treadmill running protocol. Nevertheless, the clinical impacts of these findings are therefore uncertain, and further studies investigating permutations of deleting Panx3 under spontaneous models are needed. It is possible that older mice may be more susceptible to the effects of forced treadmill running, which may exacerbate the differences between genotypes.

A strength of our model was the consideration of both male and female mice. While WT and Panx3 KO mice were not littermates, littermate siblings within each genotype were divided between activity groups. We believe this is an important control to determine the effect of the forced treadmill running within each genotype. Lastly, while our model determined that Panx3 KO mice forced to run had superficial cartilage lesions in the tibia of the knee and IVD histopathology, it is known that structured exercise is effective at reducing OA pain and improving function, which we did not measure. Additionally, we did not measure voluntary activity of the mice while in the cages, and it is possible that there were differences in activity between genotypes that was not documented.

3.5 Conclusion

Panx3 deletion results in superficial cartilage erosion in adulthood for both males and females and forced treadmill running has an additive effect producing larger cartilage erosion, subchondral bone changes, and moderate evidence for synovitis in male mice. At
the lumbar IVDs, forced treadmill running in *Panx3* KO mice resulted in histopathological changes in the AF. These findings indicate that PANX3 is important for the response of musculoskeletal tissues to the stress of forced treadmill running in mice, which could be further explored as new targets for disease interventions.
3.6 Supplemental Figures

Supp. 3-1: Male Panx3 KO mice show signs of synovial lining thickening with forced treadmill running.
Representative coronal sections of the lateral compartment of knee stained with toluidine blue from WT and Panx3 KO mice under SED or FEX conditions. 20x magnification images of the synovium just lateral of the lateral tibial plateau (scale bar 100µm). Black arrows indicate synovial thickening. Histopathological scoring of hyperplasia (number of cells thick) for males (B) and females (C). Scores represent summed scores of the medial and lateral compartments across 3 slides per animal. Moderate evidence suggests male FEX Panx3 KO mice have synovial lining thickening compared to SED Panx3 KO mice. N = 4-9 mice/group. A Kruskal-Wallis test with an uncorrected Dunn’s test was used for analysis. All data are represented as mean with 95% CI.
References


Chapter 4

Our findings in Chapter 3 indicated that adult Panx3 KO mice accumulated superficial damage of the cartilage surface, subchondral bone changes, and potential synovial changes to the knee that are indicative of early OA. In this final data chapter, we wanted to determine if these mice develop age-associated OA and the potential impact of forced treadmill running later in life. This chapter is in preparation for submission, pending additional u-CT data from collaborators, and is a bioRxiv pre-print (https://doi.org/10.1101/2023.07.04.547676).
4 Aged male and female Panx3 KO mice develop severe osteoarthritis independent of forced exercise.

Background: Osteoarthritis (OA) is a multi-factorial disease that is strongly associated with aging. As the molecular mechanisms underpinning the pathogenesis of this disease are partially unclear, there are no disease-modifying drugs to combat OA. The mechanosensitive channel Pannexin 3 (PANX3) has been shown to promote cartilage loss during posttraumatic OA. In contrast, the ablation of Panx3 in male mice results in spontaneous full-thickness cartilage lesions at 24 months of age. Additionally, while protected from traumatic intervertebral disc (IVD) degeneration, Panx3 knockout (KO) mice show signs of IVD disease with altered disc mechanics. Whether the deleterious effects of ablating Panx3 in aging is the result from accumulated mechanical damage is unknown.

Methods: Male and female wildtype (WT) and global Panx3 KO C57Bl6 mice were aged to 18 months of age. Mice were then randomized to sedentary (SED) or forced treadmill running (FEX) for 6 weeks (N = 5-14). Knee joint tissues including patellar tendon, quadriceps and distal patellar enthesis, and synovium were analyzed histologically, along with lumbar spine IVDs.

Results: Approximately half of male and female Panx3 KO mice developed full-thickness cartilage lesions, severe synovitis, and ectopic fibrocartilage deposition and calcification of the knee joints. Additionally, Panx3 KO mice with severe OA show signs of quadriceps and patellar enthesitis, characterized by bone and marrow formation. Forced treadmill running did not seem to exacerbate these phenotypes in male or female Panx3 KO mice; however, it may have contributed to the development of lateral compartment OA. The IVDs of aged Panx3 KO mice displayed no apparent differences to control mice, and forced treadmill running had no overt effects in either genotype.

Conclusion: Aged Panx3 KO mice show histological features of late-stage primary OA including full-thickness cartilage erosion, subchondral bone thickening, and severe synovitis. This data suggests the deletion of Panx3 is deleterious to synovial joint health in aging.


4.1 Introduction

Globally, knee osteoarthritis (OA) affects millions—23% of the population over 40—causing disability that results in enormous personal and socioeconomic burden [1]. While several risk factors exist for developing OA including sex, obesity, previous joint injury, joint shape and alignment, aging is the single greatest risk factor [2]. The molecular mechanisms that underpin this age-associated destruction of synovial joints are poorly understood.

Pannexin 3 (PANX3) is a channel-forming glycoprotein expressed in osteoblasts where it can act as a Ca$^{2+}$ release channel at the endoplasmic reticulum [3] and in chondrocytes where it can act as an ATP release channel at the cell membrane [4]. PANX3 is also expressed in AF cells of the IVD [5, 6]. In an early rat model of traumatic OA (anterior cruciate ligament transection), Panx3 mRNA expression was upregulated in OA cartilage compared to control knees [7]. Additionally, in a 30-week-old mouse model, PANX3 was upregulated in cartilage following destabilization of the medial meniscus (DMM) surgery to induce post-traumatic OA [8]. This increased expression of PANX3 in diseased cartilage suggests its mechanistic involvement in traumatic OA development in rodent models. In fact, Moon et al. performed the DMM surgery on global and chondrocyte specific Panx3 KO mice and found that these mice were strongly protected from OA compared to WT mice [8]. Similarly, in an IVD injury model, Panx3 KO mice had fewer hypertrophic cells of the AF, and the AF structure was largely preserved compared to WT mice [6]. These two models suggest that the absence of PANX3 is protective in traumatic/injury-induced joint disease. In humans, PANX3 is upregulated in OA cartilage tissue [8], and noncoding intronic single nucleotide polymorphisms (SNPs) of PANX3 are strongly associated with chronic low back pain [9]. These data suggest that PANX3 function in cartilage is conserved across rodents and humans, and may be an important molecular player of OA.

Previous studies have shown that aging influences the genetic response of joint tissues to stress/injury [10-12], and therefore, aged models are required to better understand the specific mechanism of age-associated OA. To this point, male Panx3 KO mice at 18 or 24 months of age showed accelerated cartilage erosion, subchondral sclerosis, and
synovitis of the knee joint [13], which was in contrast to the previously seen protective effects in the DMM model in adult mice [8]. An important difference between the two studies is that the aged mice were given a running wheel in their cage for environmental enrichment, which could have contributed to the different effects of Panx3 KO on joint tissues. In the IVD, we have shown that uninjured IVDs are sensitive to aberrant biomechanical loading [6], again highlighting the context-dependent function of PANX3 in joint health.

In this study, we investigated how aging and forced treadmill running influence joint pathology in Panx3 KO mice. Panx3 KO mice demonstrated a bimodal distribution in which roughly half of the animals, regardless of forced running, had full-thickness cartilage erosion down to the subchondral bone and expanded synovium containing ectopic calcification and fibrocartilage with scattered lymphocytes, while the other half had mild synovitis. In contrast, WT mice had mild signs of superficial cartilage erosion and synovitis. Male Panx3 KO mice also displayed cartilage, bone, and bone marrow in the quadriceps enthesis reminiscent of enthesitis. The degree to which these mice had enthesitis or tendinopathy of the knee was correlated with OA scores. At the lumbar spine, Panx3 KO mice IVDs were histologically similar to WT mice. These results suggest that aged Panx3 KO mice, regardless of sex and activity, develop severe knee joint pathology including OA in aging.

4.2 Methods

4.2.1 Mice

Animals used in this study were bred in-house and euthanized in accordance with the ethics guidelines of the Canadian Council for Animal Care. Animal use protocols were approved by the Council for Animal Care at Western University Canada (AUP 2019-069). Mice were housed in standard shoe box-style caging, and exposed to a 12-hour light/dark cycle and ate regular chow ad libitum. C57BL/6 N WT and Panx3 KO mice were congenic [8]. DNA was collected from ear clippings of each mouse to determine the genotype using polymerase chain reaction (PCR) as previously described [8, 14]. At
sacrifice, mouse knees and spines were collected and immediately processed for histological analysis.

4.2.2 Forced treadmill running

At 18 months, mice were randomized to either a no exercise group (sedentary, SED) or a forced treadmill running (forced-exercise, FEX) group. FEX groups ran on a treadmill (Columbus Instruments, Ohio) for 6 weeks, 1 hour a day for 5 days a week, at a speed of 11 m/min, and a 10° incline—an adapted protocol that has previously been used to induce OA in male C57BL/6 mice [15]. Per the animal ethics protocol, the mice were encouraged to run using a bottle brush bristle and a shock grid at the end of the treadmill.

4.2.3 Histopathological assessment of the knee joint

At the experimental endpoint, knee joints were fixed in 4% paraformaldehyde at room temperature for 24 hours on a shaker and then decalcified in 5% EDTA for 12 days at room temperature. Knees were processed and embedded in the sagittal plane in paraffin, and 6-μm–thick sections were cut from front to back. Sections were stained with toluidine blue. Three sections from the medial and lateral compartments were scored by 2 blinded reviewers using the Osteoarthritis Research Society International (OARSI) recommendations for histological assessments of OA in the mouse [16]. The average max score of each sample was then used for statistical analysis.

4.2.4 Synovial tissue pathological assessment

Considering the severity and bimodal distribution of the phenotype in these animals, we opted to take a more descriptive approach when describing the synovial changes that were occurring within these animals versus a semi-quantitative analysis. Dr. Kiser, a pathologist with experience describing joint disease in animal models investigated the occurrence of specific pathological features observed in the synovium of the animals. The pathologist was blinded to all genotypes, sex, and activity. One hematoxylin and eosin (H&E)-stained section of the medial load-bearing region per animal was selected for analysis.
4.2.5  Enthesis and patellar tendon analysis

Mid-tendon sections were stained with toluidine blue and scored for distal quadriceps tendon enthesitis, distal patellar tendon enthesitis and patellar tendinopathy. Enthesitis was scored using the following parameters: 0 = normal; 1 = ectopic cartilage; 2 = ectopic cartilage/bone; 3 = ectopic cartilage/bone with a marrow cavity; 4 = ectopic bone with a marrow. Tendinopathy was scored using the following system: 0 = normal; 1 = increased cellularity; 2 = cell rounding/clustering; 3 = chondrogenesis (i.e., proteoglycan-rich matrix, hypertrophy); 4 = ectopic cartilage/bone.

4.2.6  Histopathological assessment of the lumbar intervertebral discs

Lumbar spines were fixed for 24 hours with 4% paraformaldehyde, followed by 7 days of decalcification with Shandon’s TBD-2 (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. Tissues were embedded in paraffin and sectioned in the sagittal plane at a thickness of 5 μm. Mid-sagittal sections were deparaffinized and rehydrated as previously described [17] and stained using 0.1% Safranin-O/0.05% Fast Green. Sections were imaged on a Leica DM1000 microscope, with Leica Application Suite (Leica Microsystems: Wetzlar, DEU). To evaluate IVD degeneration, spine sections were scored by two observers blinded to age, exercise, sex, and genotype using a previously established histopathological scoring system for mouse IVDs [18]. Alterations to the scoring system were made, in which we excluded a score of 4 for the NP: “mineralized matrix in the NP”. To report on degeneration across the lumbar spine, scores for individual lumbar IVDs (L2-L6) were averaged, and the total score plotted for each individual mouse.

4.2.7  Statistics

The Department of Epidemiology and Biostatistics at Western University was consulted to determine the appropriate statistical analysis. Data are presented as stated in the respective figure. Prism 9 (GraphPad Software Inc.) Version 9.4.1 (458) was used to run
all statistical tests including one-way analysis of variance (ANOVA) or two-way ANOVA for comparison. For OARSI scores, histopathological scores of the IVD, enthesitis and tendinopathy scores, males and females were analyzed separately within their respective genotypes and activity group. A Kruskal-Wallis test was used with an uncorrected Dunn’s test for multiple comparisons to determine statistical differences among the groups. Correlations using Pearson’s r were performed on medial tibial OA scores and scores of enthesitis and tendinopathy. Max scores for each animal was used. 

All applicable data met assumptions for homoscedasticity or normality of residuals. Based on the recommendations of the editorial entitled: Moving to a World Beyond “p < 0.05” [19] we did not set a threshold for significance, and data is referred to in terms of weak, moderate or strong statistical evidence.

4.3 Results

4.3.1 *Panx3* KO mice have comparable body weights to WT mice under sedentary and forced treadmill running conditions within their respective sex.

Since we have previously published large reductions in body weights of *Panx3* KO mice compared to WT mice in adulthood [20], we first investigated the effect of deleting *Panx3* on body weight in aged animals (Figure 4-1) and found that those genotypic differences were diminished with age. There was weak statistical evidence for differences in body weight between genotypes in both male (Figure 4-1A) and female (Figure 4-1B) mice. While there was weak statistical evidence for body weight differences between activity groups in males, there was moderate statistical evidence for female mice to have lower body weights when forced to run on a treadmill for 6 weeks compared to SED female mice. This data suggests aged WT and *Panx3* KO mice have similar body weights under SED and FEX conditions.
Male Panx3 KO mice develop full-thickness cartilage erosion of the tibia and femur surface in aging.

Next, we assessed whether male Panx3 KO mice develop histopathological OA compared to WT mice in aging, and whether forced treadmill running would influence these outcomes. Toluidine blue-stained, paraffin-embedded sections from the knee joints of 18-month-old WT and Panx3 KO mice under SED or FEX conditions were analyzed. Histologically, full-thickness cartilage lesions were observed in half of the Panx3 KO mice in the medial tibial and femoral surfaces but not in any of the WT mice (Figure 4-2A, B, D). Additionally, this subset of Panx3 KO mice displayed thickening of the subchondral bone plate (Figure 4-2A). In the lateral compartment, there was weak statistical evidence for differences between SED WT and SED Panx3 KO mice (Figure
Regarding the effect of forced treadmill running, there was weak statistical
evidence that the addition of forced treadmill running influenced the cartilage structure in
both knee compartments of WT mice. In the lateral compartment, there was moderate
statistical evidence that FEX Panx3 KO mice had worse OARSI scores compared to SED
WT mice, as 3 mice had full-thickness lesions of the tibia (Figure 4-2C); however, there
was weak statistical evidence for differences in femur OARSI scores among the groups
(Figure 4-2E). Taken together, this suggests male Panx3 KO mice develop histological
features of severe OA in the medial compartment, while the combination of Panx3
deletion and forced treadmill running results in lateral compartment erosion.

### 4.3.3 Female Panx3 KO mice develop full-thickness cartilage
erosion of the tibia and femur medial compartment in aging.

Next, we performed the same histological analysis of female knees from aged WT and
Panx3 KO mice under SED and FEX conditions. Note, female mice had not been
included in our earlier aging study [21]. Like in males, several female Panx3 KO mice
developed full-thickness cartilage erosion in the medial compartment (6 mice in total),
along with thickening of the subchondral bone, while no WT mice developed such
erosion (Figure 4-3A, B, C). Forced treadmill running seemed to have little to no effect
on cartilage structure in the medial compartment (Figure 4-3A, B, C). In the lateral
compartment, while some SED Panx3 KO mice showed signs of cartilage erosion, full
thickness erosion was only observed in some Panx3 KO mice (N = 3) that were forced to
run, but there was weak statistical evidence to suggest differences between these groups
(Figure 4-3D, E). This data suggests, like male Panx3 KO mice, female mice exhibit a
bimodal distribution, with a subset of mice developing full-thickness cartilage erosion,
and the addition of forced treadmill running may influence development of cartilage loss
of the tibia in the lateral compartment.
Figure 4-2: Half of male Panx3 KO mice develop full-thickness cartilage erosion of the medial tibia and femur regardless of forced treadmill running.

Representative toluidine blue staining of knee joints from male wildtype (WT) and Panx3 knockout (KO) mice under sedentary (SED) and forced treadmill running (FEX) conditions, as indicated (A). Whole joint images were taken at 4x magnification (scale bar = 500 µm) and 10x magnification of the cartilage (scale bar = 100 µm) of the medial (left) and lateral (right) compartments. Double black arrows show cartilage erosion to
the subchondral bone. Green arrows show superficial cartilage erosion, and red arrows show erosion to the calcified cartilage. (B – E) Violin plots showing the distribution/grouping of medial tibia (B) and femur (C) and lateral tibia (D) and femur (E) OARSI max scores of male mice for WT SED (N = 10), WT FEX (N = 10), KO SED (N = 10), and KO FEX (N = 9), as indicated. For statistical comparisons among the groups, a Kruskal-Wallis test was performed.
Figure 4-3: A subset of female Panx3 KO mice develop full-thickness cartilage erosion of the medial tibia and femur regardless of forced treadmill running. Representative toluidine blue staining of knee joints from female wildtype (WT) and Panx3 knockout (KO) mice under sedentary (SED) and forced treadmill running (FEX) conditions, as indicated (A). Whole joint images were taken at 4x magnification (scale bar = 500µm) and 10x magnification of the cartilage (scale bar = 100µm) at the medial (left) and lateral (right) compartments. Double black arrows show cartilage erosion to the subchondral bone. Green arrows show superficial cartilage erosion, and red arrows show erosion to the calcified cartilage. (B – E) Violin plots showing the...
distribution/grouping of medial tibia (B) and femur (C) and lateral tibia (D) and femur (E) OARSI max scores of female mice. WT SED (N = 12), WT FEX (N = 9), KO SED (N = 5), and KO FEX (N = 11), as indicated. For statistical comparisons among the groups, a Kruskal-Wallis test was performed.

4.3.4  Panx3 KO mice develop mild to severe synovitis under both sedentary and forced treadmill running conditions.

Considering the bimodal distribution seen in our KO mice, and upon observation of the severe synovial changes in the mice with full-thickness erosions, we chose to take a descriptive approach for the synovial analysis. Synovial tissue was assessed by Dr. Kiser, a blinded pathologist with extensive experience describing animal model synovial tissue. H&E-stained sections in the medial load-bearing zone were assessed. Three distinct joint morphological phenotypes were observed across the groups: 1) no lesions (Figure 4-4A); 2) mild acute synovitis (Figure 4-4B); 3) and severe diffuse synovial fibrosis with ectopic ossification (Figure 4-4C). All samples with mild to severe synovitis were from Panx3 KO mice, except for one WT sample. Synovium of Panx3 KO mice with intact cartilage consisted of acute lymphocytic synovitis, where the synovium was expanded by lymphocytes and few macrophages (Figure 4-4B mild synovitis). Full-thickness cartilage erosion in the Panx3 KO mice coincided with severe diffuse synovial fibrosis, ulceration, and ectopic ossification (Figure 4-4C severe synovitis). These mice had locally extensive to complete effacement of the synovium by collagen, fibrocartilage and in some cases, bone interrupted by areas of acellular basophilic material. These findings suggest that aged Panx3 KO mice develop mild-to-severe synovitis of the knee joint, which coincides with the severity of cartilage erosion.
Figure 4-4: *Panx3* KO mice develop mild to severe synovitis of the knee in aging.
Sagittal sections of the medial compartment were stained with H&E and assessed by a blinded pathologist. Slides were assessed for signs of immune cell infiltration of the synovial lining (black arrows), indicating mild synovitis, which was a phenotype of *Panx3* KO mice (one WT mouse was characterized to have mild synovitis).
Male Panx3 KO mice display ectopic cartilage and bone deposits containing marrow in the enthesis of the quadriceps and patellar tendons.

Considering the enthesis originates from fibrocartilage cells that are highly responsive to mechanical loading [22], we next analyzed the patellar tendon for signs of tendinopathy and enthesitis at the distal patella and quadriceps tendons (Figure 4-5A). In male mice, there was moderate statistical evidence suggesting SED Panx3 KO mice develop enthesitis of the quadriceps tendon enthesis, consisting of cartilage and bone deposits, often with a marrow cavity (Figure 4-5B). Throughout the patellar tendon, there was weak statistical evidence for histopathological cellular changes in any of the groups (Figure 4-5C). Like the quadriceps enthesis, the distal patellar tendon enthesis of SED Panx3 KO mice showed signs of enthesitis, including deposits of cartilage and bone, often with a marrow cavity, compared to SED WT mice (Figure 4-5D). In female mice, we performed the same semi-quantitative analysis of the enthesis and patellar tendon (Figure 4-6A). At the quadriceps enthesis, there was moderate statistical evidence that Panx3 KO mice develop enthesitis with forced treadmill running (Figure 4-6B), with all the mice showing ectopic cartilage and bone, often with marrow formation, within the quadriceps tendon enthesis (Figure 4-6E). Throughout the patellar tendon, there was weak statistical evidence for histopathological changes to cellular shape or distribution among the groups (Figure 4-6C). At the patellar tendon enthesis, there was moderate statistical evidence that WT mice develop enthesitis with forced treadmill running, with weak statistical evidence of histological differences in Panx3 KO mice (Figure 4-6D). This data suggests that Panx3 KO mice develop enthesitis of the quadriceps and patellar tendon entheses during aging, while forced treadmill running in female WT mice produced enthesitis at the distal patellar tendon enthesis.
Figure 4-5: Sedentary male Panx3 KO mice show signs of patellar and quadriceps enthesitis.

Male WT and Panx3 KO knee sections were stained with toluidine blue, sectioned in the sagittal plane and scored for quadriceps enthesitis (A1), patellar tendinopathy (A2), and patellar enthesitis (A3). Sedentary (SED), and forced treadmill running (FEX). Violin plots showing distribution/grouping of histological scores for quadriceps enthesitis (B), tendinopathy (C), and patellar enthesitis (D).
Representative toluidine blue sagittal sections (E). 10x magnification. Scale bar = 100 µm. Black arrows point to cartilage, yellow arrows point to bone and marrow. WT SED (N = 8), WT FEX (N = 10), KO SED (N = 12), and KO FEX (N = 11). For statistical comparisons among the groups, a Kruskal-Wallis test was performed.
We next ran a Pearson’s correlation between medial OA scores and the enthesitis and tendinopathy scores to determine if there was a relationship between mice that develop OA and those that develop enthesis and tendon pathology. There was strong statistical evidence suggesting that higher enthesitis and tendinopathy scores coincided with higher OA scores (Table 4-1).

Table 4-1: Medial tibial OARSI scores correlate with tendinopathy and enthesitis scores

<table>
<thead>
<tr>
<th></th>
<th>OARSI Score</th>
<th>Tendinopathy</th>
<th>Quadriceps Enthesitis</th>
<th>Patellar Enthesitis</th>
</tr>
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<tbody>
<tr>
<td>OARSI Score</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tendinopathy</td>
<td>0.38 $p = 0.001$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadriceps Enthesitis</td>
<td>0.38 $p = 0.002$</td>
<td>0.27 $p = 0.029$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Patellar Enthesitis</td>
<td>0.58 $p &lt; 0.001$</td>
<td>0.32 $p = 0.008$</td>
<td>0.19 $p = 0.127$</td>
<td>-</td>
</tr>
</tbody>
</table>

4.3.6 *Panx3* KO mouse IVDs age normally in males and females even when forced to treadmill run.

To investigate the role of PANX3 in age-associated IVD degeneration, and whether forced treadmill running in aging influences disc health, we next analyzed the IVDs for histological changes. WT and *Panx3* KO mice were aged to 18 months and lumbar spines were analyzed histologically as described in the Methods section. Under both SED and
FEX conditions, male (Figure 4-7A, B) and female (Figure 4-7C, D) Panx3 KO IVDs appeared normal relative to WT controls with weak statistical evidence for differences in histopathological features of degeneration in the nucleus pulposus (NP) and the AF at any disc height (Figure 4-7A, C) or when averaged across the lumbar spine (Figure 4-7B, D). This data suggests that the IVDs of both male and female Panx3 KO mice, age and respond to forced treadmill running similarly to their WT counterparts.

Figure 4-7: No overt differences in histopathological scores of the IVDs between aged WT and Panx3 KO mice.
Wildtype (WT) and Panx3 KO (KO) mouse lumbar spines were stained with Safranin O Fast Green. Histopathological scores for males by disc height (A) and average disc score across the lumbar spine (B). WT SED (N = 8), WT FEX (N = 10), KO SED (N = 12), and KO FEX (N = 11). Female histopathological scores by disc height (C) and average disc score across the lumbar spine (D). WT SED (N = 14), WT FEX (N = 8), KO SED (N = 4), and KO FEX (N = 11). For statistical comparisons among the groups, a Kruskal-Wallis test was performed. All data are shown as means ± CI.
4.4 Discussion

In this study, we found that 18-month-old male and female Panx3 KO mice demonstrate severe OA of the knee, while their IVDs seem to be histologically comparable to the WT mouse controls. The addition of forced treadmill running does not exacerbate this phenotype in the medial compartment, while a subset of FEX Panx3 KO mice had full-thickness lesions in the lateral compartment, suggesting a compartment-specific effect of forced treadmill running in Panx3 KO mice. Additionally, Panx3 KO mice develop mild to severe synovitis consisting of lymphocyte infiltration, and ectopic fibrocartilage and calcification of the knee joint. Male Panx3 KO mice also appeared to have histological features of quadriceps and patellar tendon enthesitis under SED conditions, whereas female Panx3 KO mice developed quadriceps enthesitis tendon with forced treadmill running. Within lumbar spine IVDs, both male and female Panx3 KO mice had similar histopathological features to the WT controls. Even with the stress of forced treadmill running, there was weak statistical evidence for histopathological differences among the groups, suggesting running later in life is not detrimental to disc structure in either genotype.

Full-thickness cartilage erosion is not a normal histological feature of knee joints in aged mice [23]. In this study, the full-thickness cartilage loss observed in our animals was accompanied by erosion and fibrillation of adjacent cartilage surfaces. In addition to cartilage erosion, we saw mild to severe synovitis in the Panx3 KO animal knees, which included extensive fibrocartilage and calcification deposition within the synovium. Whether PANX3 is expressed in synovial tissue has not been determined; however, in silico data suggests PANX3 should be expressed in human synovial fibroblasts [24]. Additionally, considering the severe inflammatory phenotype, we are unaware of any indication that macrophages or lymphocytes express Panx3. Future studies should investigate the periarticular expression and function of Panx3 in joint tissues.

Considering the evidence showing PANX3’s role in cartilage, it is possible that the synovial phenotype is initiated by cartilage degradation and subsequent synovitis. Chronic release of damage-associated molecular patterns or other catabolic signals (e.g. cytokines) from degrading cartilage into the synovial fluid space may activate synovial
lining macrophages [25]. In our previous report challenging 30-week-old Panx3 KO mice, we showed superficial cartilage erosion which was exacerbated by forced treadmill running and resulted in moderate evidence of synovial lining thickening, suggesting early OA development (Wakefield et al, in revision). A lifetime of cartilage erosion may be chronically stimulating synovial macrophages leading to these pathological changes. Previously, we found that aged Panx3 KO mice had low lubricin expression in the superficial zone of the articular cartilage [13]. Lubricin is an essential lubricating protein for the joint surface [26], and in vitro models have shown that lubricin has anti-inflammatory effects on synovial lining fibroblasts by binding to toll-like receptors 2 and 4 [27]. Taken together, the superficial erosion in adulthood of Panx3 KO mice may lead to reduced lubricin levels in aging, which could be chronically activating synovial lining cells, and thus producing the severity of synovitis we observed in the present study.

Inflammation is associated with age-related pathologies [28] including primary OA, and nuclear Factor Kappa β (NF-κβ) is a proposed central pathway of inflammation in OA [23]. Interestingly, through mechanotransduction pathways, chondrocytes can release ATP, and this extracellular ATP has been shown to activate NF-κβ signalling and contribute to OA [29, 30]. While some ATP release is required to maintain normal cartilage homeostasis [31], abnormal mechanical loading of cartilage increases chondrocyte ATP release [32, 33]. This suggests that there are physiologically healthy levels of ATP release required for cartilage maintenance, but dysregulation of this mechanism could contribute to inflammation and OA. Our previous reports showed that aging WT mouse cartilage maintains similar PANX3 protein expression at 6, 18, and 24 months of age [13], suggesting PANX3 is required to maintain cartilage health well into aging. Deletion of Panx3 may dysregulate this ATP signalling given its canonical function as a mechanosensitive, plasma membrane ATP release channel in cells such as chondrocytes [4, 34].

In our previous report, Panx3 deletion did not significantly impact the progression of age-associated histopathological IVD degeneration in male mice at 18 and 24 months of age compared to WT mice [6]. The present study also determined that the aged female Panx3 KO mice IVD histopathological analysis matched that of males. The contrasting
The difference between the knee joint and IVDs of Panx3 KO mice is interesting considering the similar mechanism of disease progression between OA and IVD diseases [35]. It appears that PANX3 is not essential to IVD health during normal aging, as our previous report showed low transcript expression of Panx3 in IVDs from 6 to 24 months of age relative to levels at 2 months of age [6]. It is possible that PANX3 is utilized in early life and development of the IVD, while dispensable in aging. Interestingly, forced treadmill running seemed to have no effect on histological features of the IVDs regardless of genotype or sex. This was surprising, considering Panx3 KO mice developed histopathological features in the AF of IDD with forced treadmill running in adult mice (Wakefield et al, in revision). It was reasonable to hypothesize that aging would have rendered these mice more susceptible to forced treadmill running-induced changes to the IVD. However, considering aging alone results in relatively severe spontaneous IDD in mice [36], any potential impact of forced treadmill running, positive or negative, may have been undetectable with histopathological scoring.

Future studies should involve a time course of Panx3 KO mouse OA development. Specifically, analyzing earlier time points (for example, 6, 9, and 12 months) to determine when distinct OA phenotypes arise, and to characterize its progression. This will allow for the analysis of the early cellular changes that may be driving this severe OA. Additionally, considering that obesity is a strong risk factor for OA, and coincides with aging, high-fat diet studies in Panx3 KO mice are also warranted. Lastly, tissue-specific KO models using various Cre driver lines are also warranted to determine the cell type responsible for this severe OA development.

### 4.5 Conclusion

Aged male and female Panx3 KO mice develop severe OA under both SED and FEX conditions, and this occurs spontaneously as in human primary OA. Additionally, Panx3 KO mice have enthesitis of the quadriceps and distal patella more often than WT mice, but at the lumbar IVDs, Panx3 KO mice have similar histological features as WT mice in aging. Collectively, our data suggests chronic suppression of PANX3 throughout life may be contraindicated and cause severe OA. Potential therapeutic interventions could include...
agonists of PANX3 to overcome the lower expression or reduced functionality of PANX3 channels in aged joint tissues.
References


15. Bomer, N., et al., *The effect of forced exercise on knee joints in Dio2(-/-) mice: type II iodothyronine deiodinase-deficient mice are less prone to develop OA-like*


Chapter 5

5 General Discussion

5.1 Summary of main findings

Pannexins, in their canonical function, act as mechanosensitive channel-forming glycoproteins facilitating ATP and Ca\(^{2+}\) signaling [1]. PANX3, the focus of this thesis work, is expressed in various cells of the MSK system including chondrocytes [2, 3], osteoblasts [4], AF cells of the IVD [5, 6] and adipocytes (as observed in Chapter 2 of this thesis). This wide expression profile makes them an interesting, yet complex, area of study, and previous reports have implicated their role in adiposity [7, 8] and joint pathology [3, 9, 10]. Previous work from Pillon et al. has shown that palmitic acid, a known proinflammatory fatty acid, can induce the expression (and function) of Panx3 in rat muscle cells and recruit monocytes, suggesting changes in expression profile may be influencing its role in pathogenesis [11]. Additionally, they found that Panx3 transcripts were upregulated in fat tissue of male mice fed an HFD. This suggests the expression of Panx3 can be regulated by environmental factors. Considering this, and the fact that these channels are mechanosensitive, we sought to investigate the physiological effects of ablating Panx3 on adiposity, inflammation, metabolism and joint health, and whether the effect would be context dependent on environmental factors such as diet and forced exercise. To do this, we used the established global Panx3 KO mouse model in our laboratory, and a modified treadmill running protocol that has been shown to induce the expression of OA genes in cartilage and histological features of early OA of the knee [12].

5.2 Panx3 deletion reduces fat accumulation while preserving lean mass in male mice.

In the first data chapter of this thesis, Chapter 2, we found that global germline deletion of Panx3 resulted in large reductions in body weight gain in adult male mice. Interestingly, this lower body weight was almost exclusively explained by less fat mass, as male Panx3 KO mice had 46% lower fat mass on average compared to WT mice.
Additionally, there was an increase in lean mass when normalized to body weight between WT and Panx3 KO mice, suggesting preservation of lean mass in this model. This is a potentially important finding, as maximizing fat loss while maintaining or increasing lean mass is a principal strategy during healthy weight loss. It is widely accepted that up to one-quarter of weight loss experienced is due to loss of lean mass [13], as has been observed in dietary (hypocaloric) interventions [14] and in response to the new GLP-1 agonist drugs [15]. For example, a randomized, double-blind, placebo-controlled trial over 12 weeks with a weekly administration of semaglutide, a GLP-1 agonist drug, in participants with obesity had an estimated loss of 20% lean mass [16].

Considering weight regain is most likely to occur after cessation of a hypocaloric diet [17] or a GLP-1 agonist [18], the resultant weight regain will predominantly be fat mass, resulting in an obesogenic-sarcopenic-phenotype [19]. If PANX3 were to become a target for obesity, in theory, it would preserve lean mass while reducing fat accumulation based on these findings. This alone makes PANX3 an interesting target for obesity treatment or prevention.

While we observed significantly lower body weights in the female Panx3 KO mice, we were unable to detect differences in fat mass or lean mass compared to their WT counterparts. Considering our analyses did not investigate various fat pads, it may be that there were changes in specific fat depots, such as subcutaneous fat, that would not have been detected when analyzing overall fat mass. In fact, our recent publication showed that female Panx3 KO mice do have smaller hypodermal areas [20]. Additionally, it is worth mentioning that female C57BL/6 mice have less than half the fat mass of male mice at this age. Therefore, females would have less fat mass to lose to any such intervention. However, our results fall in line with previous QTL data suggesting Panx3 plays a role in male body mass index but not female [7].

### 5.3 PANX3 and metabolic tissue inflammation

In addition to the lower fat mass in male Panx3 KO mice, their adipose tissue had lower expression of multiple pro-inflammatory genes. Interestingly, this phenotype was observed even when these mice were fed an HFD compared to WT controls. Considering the evidence suggesting visceral fat tissue inflammation may be contributing to the
development of other diseases [21] such as cardiovascular diseases [22], glucose intolerance [23], and OA [24], the fat tissue of Panx3 KO mice may display a ‘healthier’ phenotype. Interestingly, in female mice, we did not see a similar alteration in gene expression. In fact, female Panx3 KO mice had a higher pro-inflammatory gene expression overall in skeletal muscle and liver tissue. This may be due in part to sex differences in immune cell function [25] and/or muscle inflammation [26].

5.4 *Panx3* KO ASCs grow slower compared to WT in culture.

A potential mechanism explaining this lower fat mass in Panx3 KO mice could be its role in adipose-derived stromal cell (ASC) proliferation. We showed that PANX3 expression is upregulated in differentiated 3T3-L1 cells, a pre-adipocyte cell line. Additionally, we found that ASCs isolated from male Panx3 KO mice proliferate slower than WT cells in culture. This suggests that Panx3 may be a promoter of adipocyte development.

Figure 5-1: Graphical abstract of Chapter 2 results.
In fat tissue of male wildtype (WT) mice, a high fat diet (HFD) increased the transcript and protein expression of PANX3. When mice were subjected to 6 weeks of forced treadmill running, this blunted the expression of Panx3, suggesting diet and exercise
modulate PANX3 levels. When \textit{Panx3} was ablated in male mice, it resulted in lower body weights, with no changes in lean mass, but large reductions in fat mass and an anti-inflammatory phenotype of the fat and muscle tissue. Lastly, ASCs from \textit{Panx3} KO mice grow slower than WT mouse ASCs in culture.

5.5 \textit{Panx3} deletion and forced treadmill running work synergistically to cause superficial cartilage erosion and subchondral bone changes indicative of OA

In Chapter 3, we found that 30-week-old \textit{Panx3} KO mice, both male and female, have small cartilage defects in the superficial zone of the proximal tibial cartilage. Additionally, forced treadmill running in male mice was associated with larger defects of the cartilage surface, while there were no changes in the depth of the lesions. This suggests that \textit{Panx3} KO mice spontaneously develop cartilage defects over time [12]. As discussed in Chapter 3, OA is a whole joint disease that affects not only the articular cartilage but also the subchondral bone and synovial tissue. While under sedentary conditions, when \textit{Panx3} KO mice developed small cartilage defects, there was weak evidence for changes to bone and synovium. However, with forced treadmill running, \textit{Panx3} KO mice had greater subchondral bone area and moderate evidence for thickening of the synovial lining (synovitis) in male mice. Previous reports have suggested that subchondral bone [27] and synovitis [28, 29] occur before cartilage degradation. This thesis would suggest that cartilage erosion, albeit subtle, precedes changes to the subchondral bone and synovium in this model. Alternatively, it could be that previous reports did not use a sensitive enough scoring system to detect subtle cartilage changes that may precede periarticular tissue changes. This is important, as we first scored the knees using the commonly used OARSI system [30], however, we were unable to detect differences between genotypes, even though they were visibly different at 20x magnification. We then switched to the ACS scoring system [31], which allowed us to capture the surface defects of the cartilage in the \textit{Panx3} KO mice and from forced treadmill running. It is possible that the combination of \textit{Panx3} ablation and forced treadmill running results in changes that occur early in OA compared to other models that produce more severe phenotypes.
5.6 *Pannexin 3* deletion resulted in subtle superficial cartilage defects in male and female mice, while forced treadmill running only influenced male cartilage.

Another interesting finding in this chapter was the sex-specific differences. While the deletion of *Panx3* resulted in similar superficial cartilage defects in males and females, forced treadmill running seemed to produce cartilage defects exclusively in male mice. Unfortunately, there are no published studies that have looked at forced treadmill running on knee cartilage in female mice to compare these findings. While the subchondral bone changes were seen in both sexes of *Panx3* KO mice with forced treadmill running, only in males was there moderate evidence for synovitis after forced treadmill running. This may be because the addition of forced treadmill running results in more cartilage damage in males, producing more DAMPS to activate synovial lining immune cells [32].

![Figure 5-2](image.png)

*Figure 5-2: Graphical abstract of Chapter 3 results.*

In females, *Panx3* knockout (KO) mice develop mild erosion of the superficial zone with no additional effect of forced treadmill running. However, subchondral bone changes occurred with forced treadmill running. In males, the deletion of *Panx3* and forced treadmill running worked synergistically on joint changes, including larger erosion, thickening of the subchondral bone, and synovial lining thickening (not shown).
5.7 **Panx3 deletion and forced treadmill running produce histological features of disc degeneration in adult mice.**

Considering PANX3 is expressed in AF cells of the IVD [5], and that Panx3 KO mouse IVDs showed signs of IDD with altered biomechanics [6], we next wanted to investigate whether forced treadmill running had a negative effect on Panx3 KO mouse IVDs. Histologically, we observed that both male and female Panx3 KO mice had histological signs of AF pathology in the lower lumbar spine IVDs. Specifically, within the lower lumbar region, Panx3 KO mice had hypertrophic cells and reversal of the lamellar structures in the AF [33]. Running in rodent and human models has generally shown positive effects on IVD structure [34, 35] and cell number [36], which would suggest running exercise in particular is healthy for IVDs. Here we show that Panx3 KO mice develop histopathological signs in the IVD in response to forced treadmill running, suggesting the presence of PANX3 is necessary for the protective response of the AF to running. Taking into consideration previous reports using our Panx3 KO mouse, data further highlights the context-dependent role of PANX3 in controlling joint tissue response to various stresses.

5.8 **Panx3 deletion in ageing is associated with severe joint tissue pathology in male and female mice.**

In chapter 4, we wanted to confirm and refine our previous findings in aged male Panx3 KO mice. We had found that male Panx3 KO mice have severe cartilage erosion, sclerotic subchondral bone, and mild synovitis at 18 months of age [10]. However, at 12 months of age these mice were provided a cage wheel for environmental enrichment, and we did not know if Panx3 KO mice may respond differently to wheel running compared to WT mice. Considering our findings in chapter 3, and that genetics can influence mouse knee OA response to voluntary running in aging [37], we wanted to control for this variable, allowing us to confirm if this was spontaneous or induced through forced exercise.
Both male and female Panx3 KO mice had full thickness cartilage defects, mild to severe synovitis and patellar and quadriceps enthesitis indicative of severe joint pathology. This phenotype occurred in roughly half of the mice, while the other mice had seemingly normal aging of the joint tissue. In other words, Panx3 KO mice showed a bimodal distribution of OA phenotype. Considering this distribution, it was difficult to determine the effect of forced treadmill running in the Panx3 KO mice. Data confirms our previous findings of accelerated cartilage erosion during aging in Panx3 KO mice, and adds that this occurs in both male and female mice. The bimodal distribution is an interesting phenomenon that we have seen in other transgenic models. Unfortunately, the mechanisms that could explain these results are not known. It is possible that various environmental factors (e.g. social hierarchy causing stress, dermatitis, aggressive behaviour, or differences in activity between mice) could explain why some Panx3 KO mice develop severe OA while others do not.
A central theme of this thesis is that the deletion of Panx3 in this mouse model had various effects on measures depending on the sex and/or activity of the animal. This provides a nuanced look at the potential role of PANX3 as a target for preventing or treating obesity and OA. Related to obesity, if PANX3 were to be a target for reducing fat accumulation, if one is engaging in sufficient physical activity, there may not be any benefit to such a drug, considering the forced exercise was just as effective as deleting Panx3 for body composition. In regard to joint health, the ablation of Panx3 resulted in worse knee joint OA for both sexes, while in males, it worked synergistically with forced treadmill running to produce features of OA. Considering Panx3 KO mice had severe OA with aging, this suggests that long-term targeting of Panx3 may not be beneficial for joint health. Taken together, this data suggests context-dependent roles of Panx3, and emphasizes the need for precise targeting of Panx3 to limit potential negative side effects.

5.10 Limitations

As with many human tissues, musculoskeletal tissues are difficult to obtain, and intervention studies to investigate the causality of disease in humans are not ethical. For
this reason, animal models, such as mice, are often used to study genetic and environmental factors for diseases such as obesity and OA. Mice are particularly useful as we can manipulate their genome, they have a short lifespan, and they are cost-effective. However, in general, a major limitation of animal models is their translatability to human disease [38]. Particularly for this thesis, studying transcriptomic response to inflammation in disease using mice has been shown to be poorly correlated with human response [39]. Additionally, it is possible that Panx1 may be compensating for the loss of Panx3 in various tissues, and may explain the sex specific differences [40]. However, mouse models can serve as proof-of-concept to then justify the retrieval of human tissue, if possible, to study a gene of interest.

Transgenic mouse models can also have potential off target effects that may influence findings in such models. Germline deletion may alter the expression of many genes to compensate for the loss of said gene. This may have downstream effects on the disease pathway being investigated which could create artifacts in the analysis. Additionally, global KO models consist of deleting the gene from every cell in the mouse from germline. This makes it impossible to determine which cell type is responsible for the phenotype you are observing, making it difficult to determine which tissue to investigate further to uncover the underlying mechanism. In summary, it is important for researchers, or anyone reading the findings of this thesis, to keep in mind the overall limitations of mouse models and their translatability to humans.

In Chapter 2, we show that male Panx3 KO mice had less fat mass compared to WT mice, which caused a reduction in overall body weight. This analysis only allowed for determining overall fat mass, and did not tease out differences in specific fat depots. Therefore, we are unable to confirm if Panx3 was regulating fat accumulation in specific depots or if it regulates all fat. This may be particularly important for understanding if Panx3 does regulate fat accumulation in female mice.

Considering this is a global KO mouse model, and that many bodily systems can influence fat accumulation, we are unable to conclude which cell type is responsible for
this phenotype. Our data in ASCs would suggest that Panx3 KO mice have fewer adipocytes to store fat, however, and therefore explain our results.

With regard to the inflammatory data, we did bulk tissue RNA analysis. Considering there are many different types of cells in fat tissue that could be the source of these RNA molecules, we cannot conclude which cells are contributing to the change in expression. Additionally, we are unable to determine if there are changes in the number and phenotype of specific immune cells that contribute to adipose tissue inflammation. Additionally, animals were sacrificed roughly one week after the forced treadmill protocol was completed. This could cause some detraining effect and is a limiting factor when analyzing the effect of forced treadmill running on inflammatory markers.

In Chapter 3, we found that adult Panx3 KO mice have early signs of OA, consisting of superficial cartilage defects in the tibia. These defects were larger in male mice but not female mice after forced treadmill running. However, both male and female Panx3 KO mice had greater subchondral bone area in the tibia, which could be changes indicative of OA. Additionally, there was moderate statistical evidence for thickening of the synovial lining in male mice after forced treadmill running. The use of 2D histology slides for semi-quantitative scoring systems limit our ability to completely capture the structural changes of the cartilage that occur. Contrast imaging using µ-CT would be a better option to allow for 3D visualization of the cartilage. Additionally, in vivo, repeated measures pre and post forced treadmill running, would have been a much more powerful method to assess the subchondral bone changes that occur with forced treadmill running. Cartilage thickness and area were analyzed using histology slides. It is possible that there is variation in the sectioning angle between the samples which could influence cartilage thickness. Therefore, data should be interpreted with caution.

Considering the chief complaint for patients with OA is pain, understanding how a model of OA influences pain is important. Here we did not perform any behavioral assays to determine if these mice experience any pain which could coincide with features of OA. This would be interesting, considering exercise training has been shown to improve pain and function in patients with knee OA [41]. Considering our Panx3 KO mice seem to be
developing features of OA with forced treadmill running, this form of ‘exercise’ may be deleterious for pain symptoms as well, however we cannot make any conclusions regarding pain.

In Chapter 4, we investigated how aged (18-month-old) Panx3 KO mice develop joint pathology with and without forced treadmill running. Considering aging is strongly associated with the development of OA, understanding the mechanisms associated with age-associated OA is warranted. It seems at this time point we have captured end stage OA considering the full thickness cartilage lesions, synovitis, fibrosis, and ectopic calcification and fibrocartilage formation. This makes it impossible to analyze the cartilage for molecular differences between the Panx3 KO and the WT mice. Additionally, we are unable to determine the effect of forced treadmill running on these mice. Future experiments using a time point in which these mice still have intact cartilage, and then force them to run, would allow us to determine how these mice fair under forced treadmill running conditions later in life. We also analyzed the patellar and quadricep tendon histologically. This analysis can be influenced by sectioning angle variation and therefore should be interpreted with caution.

5.11 Future Directions

This thesis provides a broad description of the potential role of PANX3 in musculoskeletal tissues, creating more questions than answers. We have provided a nuanced look at the potential interaction between Panx3 and environmental factors such as diet and exercise. First, from a methods perspective, exploration of different forced treadmill running protocols, including downhill running, time of day, and progressing the intensity over time, would be useful to determine the most appropriate protocol to influence health of different joint tissues. Additionally, the use of in vivo repeated measures μ-CT will provide a much powerful model to study the effect of forced treadmill running on bone. Also, contrast imaging of cartilage and other soft tissue structures of the joint will provide improved structural analysis compared to histology.

With regards to chapter 3 and 4 of this thesis, and the role of PANX3 in OA development. Using more translatable models would be useful. For example,
collaborators of ours have identified PANX3 mutants in humans that is associated with inflammatory OA. We have been working with these mutants in cell culture to characterize how they may influence PANX3 trafficking and alter the function of PANX3 channels. Mice containing this mutant form of PANX3 would be an exciting model to use, and determine if this mutant makes mice more susceptible to various OA inducing interventions compared to WT mice. This is ongoing work in our lab.

As discussed in the limitation section, this thesis is limited in several ways and future experiments can expand these findings and uncover specific mechanisms. To better understand the specific function of PANX3 in adiposity, cell specific KO models should be employed to determine if PANX3 is influencing adipose cell development. For example, adipocyte specific cell KO models will help determine if this phenotype is driven by intrinsic processes in adipocytes. Additionally, inducible KO models may help determine if this is a development phenotype or specific to adulthood.

Future studies should consider the use of a Western diet in place of a high fat diet to study if Panx3 KO mice are protected in an obesogenic environment. The HFD used in this study is not realistic of a human diet, and may have been too high in fat resulting in excessive fat accumulation compared to what would be expected in a Western diet. Additionally, seeing if Panx3 KO mice fed a Western diet and forced to run have improved fat loss would be interesting to investigate.

To my knowledge, there is no evidence that PANX3 is expressed in human fat tissue. To move this project forward in terms of translatability, determining the expression of PANX3 in human fat cells is warranted, and whether these levels change in terms of obesity, sex, and activity levels in humans. Additionally, determining if PANX3 variants are associated with body composition, as has been seen with back pain [42]. Lastly, the current KO models used have provided useful proof of concept evidence for targeting PANX3 in obesity and OA. The generation of pharmacological agents targeting PANX3 function, and whether manipulating its function recapitulates the findings in the KO models is needed. We have filed a Report of Innovation with World Discoveries at Western University entitled ‘Pannexin 3 channels as a new target for obesity and
inflammation.’ Tech ID: W-22-001 to protect the intellectual property of our findings, as these are questions we are currently asking in our research program.

5.12 Overall Conclusion

In conclusion, this thesis shows that the deletion of Panx3 in mice can influence the health and development of a number of tissues. As hypothesized, its deletion resulted in reduced adipose tissue mass, and early OA like changes to joint tissues in adulthood which produces accelerated OA in aging. We also provide evidence that the effect of deleting Panx3 is context dependent – including the sex, age, and exercised state of the animal. Therefore, when investigating the function of Panx3 in obesity and OA, researchers should seek to control for such variables.
References


Confirmation of Publication and Licensing Rights

June 20th, 2023 Science Suite Inc.

Subscription: Agreement number: Journal name:

To whom this may concern,

Student Plan
IG25IFKJZA
Western University Thesis Repository

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Figure 1-1
Figure 2-1A
Figure 5-1
Figure 5-2
Figure 5-3
Figure 5-4
Official Notice of Animal Care Committee (ACC) Approval:

Your new Animal Use Protocol (AUP) 2019-069:1: entitled "The function of pannexins in tissue development, metabolism and obesity" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that: 1) Animals used in this research project will be cared for in alignment with:

- a) Western's Senate MAPPs 7.12, 7.10, and 7.15
  
  [http://www.uwo.ca/univsec/policies_procedures/research.html](http://www.uwo.ca/univsec/policies_procedures/research.html)

- b) University Council on Animal Care Policies and related Animal Care
  
  [http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm](http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm)

2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;

   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;

   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and

   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.

   e) [http://uwo.ca/research/services/animalethics/animal_use_protocols.html](http://uwo.ca/research/services/animalethics/animal_use_protocols.html) 3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will

   a) be made familiar with and have direct access to this AUP;

   b) complete all required CCAC mandatory training (training@uwo.ca); and c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
a) Practice will align with approved AUP elements;

and ACC Leaders; limited to:

b) Unrestricted access to all animal areas will be given to ACVS Veterinarians
c) UCAC policies and related ACC procedures will be followed, including but not

i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to

hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,

http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee

University Council on Animal Care
Ontario Canada N6A 5C1
Fax 519-661-2028 http://www.uwo.ca/research/services/animalethics/index.html

*** THIS IS AN EMAIL NOTIFICATION ONLY. PLEASE DO NOT REPLY ***

Dr. Timothy Regnault, Animal Care Committee Chair
London, 519-661-2111 x 88792
auspc@uwo.ca
The University Animal Care Committee
Curriculum Vitae

EDUCATION

2017 – 2023  Ph.D. Anatomy & Cell Biology  
Specialization in Collaborative Training in Musculoskeletal Health Research  
Western University  
*Thesis:* Pannexin 3 in exercise, obesity, and osteoarthritis.  
*Supervisors:* Silvia Penuela and Frank Beier

2015 – 2017  MSc. Kinesiology  
Brock University  
*Thesis:* Bone structure and bone mineral density in growing male mice is largely unchanged when calcium and vitamin D is fed at levels lower than those present in the AIN93G reference diet.  
*Supervisor:* Wendy Ward

2008 – 2012  BAHSc. Exercise Science & Health Promotion (High Honours)  
Sheridan College  
*Thesis:* Changes in hip flexor passive compliance do not account for improvement in vertical jump performance following hip flexor static stretching.  
*Supervisor:* Trevor Cottrell

SCHOLARSHIPS & AWARDS

2023  Bone & Joint Institute Transdisciplinary Postdoctoral Fellowship Award, Western University
2023  Western University Postdoctoral Fellowship Award, Faculty of Health Science
2023  Suzanne M. Bernier Best Publication Award, Anatomy and Cell Biology, Western University
2022  Best Poster, International Gap Junction Conference, Spain
2021  Honorary Mention, Canadian Student Health Research Forum, Poster Presentation
2021  Ontario Graduate Scholarship, Western University
2020  Ontario Graduate Scholarship, Western University
2019  Ontario Graduate Scholarship, Western University
2018  Nominee, Vanier Canada Graduate Scholarship, Western University
2017  Ontario Graduate Scholarship, Western University
2017  Nominee, Vanier Canada Graduate Scholarship, Western University
2016  Frederick Banting & Charles Best Canadian Graduate Scholarship (CIHR), Brock University
2015  Green Shield Canada Graduate Fellowship in Health Science, Brock University
2012  Top Presenters Award, Ontario Kinesiology Association, Graduate Poster Presentation
2012  Silver Medal Award (graduating top of the class), Sheridan College
2012  Applied Research Leadership Award, Sheridan College Applied Research and Innovation
2012  Exercise Science & Health Promotion Research Award, Sheridan College
2011  Top Presenters Award, Ontario Kinesiology Association Graduate Poster Presentation
2009  Exercise Science & Health Promotion Award

**RESEARCH PUBLICATIONS (n = 14)**


TEACHING EXPERIENCE

2022 Lecturer
Western University, Bone & Joint Institute
MSK 9000A: Biomedical & Bioengineering Concepts

2022 Lecturer
Western University, School of Kinesiology
KINESIOL 2992A: Fitness Assessment and Strength Training

2022 Professor
Fanshawe College, School of Applied Science and Technology
BIOL 7012: Microbiology 3 – Immunology & Virology

2019 – 2022 Graduate Teaching Assistant
Western University, Faculty of Medicine & Dentistry
9501 Anatomy for Physical Therapy
9524 Anatomy for Occupational Therapy

2015 – 2017 Graduate Teaching Assistant
Brock University, Faculty of Applied Health Science

2013 – 2015  **Sessional Instructor**  
Sheridan College, Faculty of Applied Health & Community Studies

2012 – 2015  **Instructor**  
Bryan College, Faculty of Massage Therapy and Faculty of Advanced Health & Fitness Trainer

**UNDERGRADUATE STUDENT SUPERVISION**

<table>
<thead>
<tr>
<th>Year</th>
<th>Student Name</th>
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<tbody>
<tr>
<td>2022 – 2023</td>
<td>Geneva Harold</td>
</tr>
<tr>
<td>2021 – 2023</td>
<td>Rehanna Kanji</td>
</tr>
<tr>
<td>2021 – 2022</td>
<td>Justin Tang</td>
</tr>
<tr>
<td>2020 – 2021</td>
<td>Christopher Zhang</td>
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**PROFESSIONAL EXPERIENCE**

<table>
<thead>
<tr>
<th>Year</th>
<th>Position Details</th>
</tr>
</thead>
</table>
| 2015 - Present | Kinesiologist (13935)  
EXERCISE SCIENCE  
Brent Wakefield (Sol-proprietor) |
| 2015 | Research Assistant  
Brock University  
St. Catharines, ON |
| 2012 - 2013 | Head Kinesiologist/Team Leader  
pt Health Seniors Wellness Division  
Sudbury & North Region |
| 2012 | Kinesiologist  
Centric Health Management  
Milton, ON |
| 2011 | Exercise Physiologist Intern  
Cleveland Clinic of Canada  
Toronto, ON |

**PRESENTATIONS/ABSTRACTS**

“Pannexin 3 deletion in mice results in knee osteoarthritis and intervertebral disc degeneration after forced treadmill running.” [Poster], London Health Research Day, London ON 2023

“Pannexin 3 deletion in mice results in knee osteoarthritis and intervertebral disc degeneration after forced treadmill running.” [Poster], Gordon Research Conference: Cartilage Biology, Tuscany, Italy 2023
“Sex, diet, and exercise affect the significance of deleting Panx3 in mouse models of obesity and osteoarthritis.” [Poster], International Gap Junction Conference, La Coruna, Spain 2022

“Pannexin 3 channels regulate tissue architecture, barrier function, keratinocyte adhesion, and inflammatory responses during skin gaining.” [Abstract], International Gap Junction Conference, La Coruna, Spain, 2022

“Channel-independent function of golgi-localized pannexin 3.” [Abstract], International Gap Junction Conference, La Coruna, Spain, 2022

“Pannexin 3 regulates fat accumulation and inflammation in response to forced exercise and high fat diet in male mice.” [poster], Canadian Student Health Research Forum, 2021

“Pannexin 3 regulates fat accumulation and inflammation in response to forced exercise and high fat diet in male mice.” [oral], Anatomy & Cell Biology Research Day, Western University, 2020

“Pannexin 3 regulates adipose deposition in response to forced exercise and high fat diet in a sex specific manner.” [poster], London Health Research Day, 2019

“Pannexin 1 and Pannexin 3 regulate body fat accumulation in mouse models of diet-induced obesity.” [poster], Experimental Biology, Orlando Florida, 2019

“Bone Structure and Bone Mineral Density in Growing Male Mice is Largely Unchanged when Calcium and Vitamin D is Fed at Levels Lower Than Those Present in the AIN93G Reference Diet” [poster and tour], ASBMR Annual Meeting, Denver, Colorado, 2017

“Bone Structure and Bone Mineral Density in Growing Male Mice is Largely Unchanged when Calcium and Vitamin D is Fed at Levels Lower Than Those Present in the AIN93G Reference Diet” [poster], ASBMR Symposium: Current Concepts in Bone Fragility – From Cells to Surrogates, Denver, Colorado, 2017

‘Joint Healthy Functional Exercise Techniques Workshop’ [oral & practical], Musculoskeletal Health Education Forum, Brock University, St. Catharines, ON, 2017

‘Olympic Weightlifting for Sport Performance’ [2 day oral & practical], Peterborough, ON, 2016

‘Metabolic Conditioning’ [oral], Sheridan College, Brampton, ON, 2013

‘The relationship between hip flexor extensibility and vertical jump performance in active college-aged male’ [poster], Ontario Kinesiology Association, Annual Graduate Poster Presentation, Niagara Falls, ON, 2012

‘Reliability of goniometric and trigonometric techniques for measuring hip extension flexibility using the modified Thomas test’ [poster], Sheridan Applied Research Showcase, Oakville, ON, 2012
‘Reliability of goniometric and trigonometric techniques for measuring hip extension flexibility using the modified Thomas test’ [poster], Ontario Kinesiology Association, Annual Graduate Poster Presentation, Markham, ON, 2011

‘Squat exercise and the structural integrity of the knee joint’ [oral], HEAL - 30229, Introduction to Resistance Training. Sheridan College, Brampton, ON, 2011

‘Olympic weightlifting techniques with dumbbells for fitness and strength’ [oral], Peel Regional Secondary Schools, Mississauga, ON, 2010

VOLUNTEER ACTIVITIES, OUTREACH & MEDIA

<table>
<thead>
<tr>
<th>Year</th>
<th>Activity</th>
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<tbody>
<tr>
<td>2019 – Present</td>
<td><strong>Science Communication</strong>  @exercisescience</td>
</tr>
<tr>
<td></td>
<td>• Creating exercise science-related posts on social media (Instagram)</td>
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<tr>
<td></td>
<td>• Providing evidence-based fitness and exercise technique information.</td>
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<tr>
<td>2020 – Present</td>
<td><strong>Western Strength Club</strong></td>
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<tr>
<td></td>
<td>Creator &amp; Strength Coach</td>
</tr>
<tr>
<td></td>
<td>• Created a virtual workout group (3-4 days a week) for the Western community (consisting of 10-15 participants) to overcome barriers to health and fitness during the COVID19 pandemic.</td>
</tr>
<tr>
<td>2019 – 2020</td>
<td><strong>Ghanaian Association of London Middlesex</strong></td>
</tr>
<tr>
<td></td>
<td>Mathematics Tutor</td>
</tr>
<tr>
<td></td>
<td>• Helped develop program. Tutoring math to students in London’s Ghanaian community, assisting them with understanding lessons and concepts, solving practice problems and homework, and preparing for examinations.</td>
</tr>
<tr>
<td>2008 – 2012</td>
<td><strong>Exercise Science and Health Promotion Applied Degree Committee</strong></td>
</tr>
<tr>
<td></td>
<td>Student Rep</td>
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<tr>
<td>2012</td>
<td><strong>Pearson Airport Fire Department</strong></td>
</tr>
<tr>
<td></td>
<td>Health Promoter</td>
</tr>
<tr>
<td>2008 – 2012</td>
<td><strong>Sheridan Strength Club</strong></td>
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<tr>
<td></td>
<td>Co-Founder &amp; Strength Coach</td>
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CURRENT/PAST MEMBERSHIPS

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<tr>
<th>Year</th>
<th>Membership</th>
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<tbody>
<tr>
<td>2016 – Present</td>
<td>Registered Kinesiologist, College of Kinesiologists of Ontario (13935)</td>
</tr>
<tr>
<td>2012 – 2014</td>
<td>Ontario Kinesiology Association</td>
</tr>
</tbody>
</table>
2009 - 2016  Certified Personal Trainer, Canadian Personal Trainers Network
2012  CancerSmart Rehab Certification, Wellsprings

INTELLECTUAL PROPERTY

Report of Innovation (ROI). World Discoveries. Western University
‘Pannexin 3 channels as new targets for obesity and inflammation’
Disclosed
Filing Date: 2021/08/27