Characterization and Classification of HDACs in Aging Osteoclasts

A THESIS SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY

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Abstract:

Background: As humans age, bone mass is lost due to increased osteoclast and decreased osteoblast function. Bone cell differentiation is regulated by epigenetic changes within histones through acetylation/ de-acetylation. Histone deacetylase removes an acetyl group from a histone, repressing transcription. Several studies have demonstrated that loss of HDAC expression enhances osteoclast activity. There are 18 currently identified HDACs which are divided in 3 classes: I, II, and IV. This study aims to examine expression levels of HDAC class I and II in osteoclasts at 1 and 24-months of age. We hypothesize that osteoclasts from older mice will exhibit lower HDAC expression. This will increase our understanding of how HDAC expression changes in osteoclasts from aging mice. These changes may suggest a possible mechanism by which osteoclast activity is increased in aging osteoclasts.

Methods: Bone marrow cells were flushed from femurs and tibiae of either male or female 1- or 24-month mice. BMMs were harvested and differentiated into osteoclasts at days 0, 2, and 4. They were then lysed to isolate RNA and reverse transcriptase was added to yield cDNA. Samples were subjected to qRT-PCR. Data analysis yielded expression coefficients with standard deviations. True expression was calculated for data sets and examined in graph form showing average with +/- standard deviation. Multiple group comparison ANOVA tests were run with significance set at $p \leq 0.05$.

Results: For HDAC 4, expression at day 4 of differentiation of 24-month females was significantly higher than the 1-month females (p= 0.0273). For HDAC 11, expression at day 4 of differentiation of 1-month males was significantly higher than that of the 24-month males. No other group comparison yielded significance. Overall, expression was similar between age groups and sexes. Expression levels were shown to differ between days of differentiation.

Conclusions: This study functions as a pilot study regarding HDAC classification and expression. To the knowledge of the author, there are no studies to date examining HDAC expression between young and advanced age subjects. The acquired data has numerous outliers which may be disguising areas of significance. The data does not support our hypothesis that expression is lower in advanced age subjects. Limitations of the study include number of test subjects, quality/quantity of cDNA, and accuracy of sample preparation. On a broader scope, the data does not depict distinct expression patterns

for the varying classes of HDACs. It is suggested that each HDAC has varying expression levels at different days of differentiation and might have roles at various stages during expression. This study provides a groundwork for moving forward with more targeted studies based on conditions such as osteoporosis and periodontitis.

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- DNA: deoxyribonucleic acid
- HDAC: histone deacetylase
- HAC: histone acetylase
- BMU: basic multicellular unit
- CTSK: cathepsin k
- IL-1
- IL-6
- TNF-alpha
- Gm-csf: granulocyte macrophage colony stimulating factor
- RANKL: receptor NF-kB ligand
- RANK: receptor NF-kB
- OPG: osteoprotegerin
- SMRT: silencing mediator of retinoic acid and thyroid hormone receptor
- Mef2: myocyte enhancer transcription factor 2
- NAD+: on nicotinamide adenine dinucleotide
- Sirt: sirtuins
- BMM: bone marrow macrophage
- cDNA: complementary deoxyribonucleic acid
- rcf: relative centrifugal force
- rna: ribonucleic acid
- HPRT: hypoxanthine phosphoribosyltransferase 1
- rt-qPCR: reverse transcription quantitative real-time polymerase chain reaction
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Background:

Many major organ systems are involved in the support and protection of the human body, but few are as important as the skeletal system. The main component of the skeletal system is bone, a dynamic organ, which allows for movement and acts as a storage system for many essential minerals to support human life and homeostasis such as calcium. Within bone exist osteoblasts, cells that form bone, osteoclasts, cells that resorb bone, and osteocytes, terminally differentiated osteoblasts that sense mechanical changes. Together these cells create a balance of bone resorption and deposition known as remodeling, to maintain a healthy, strong skeleton. To sustain this balance, precise regulation of each cell's activity must be maintained through coordinated gene expression. Epigenetics is a group of reversible alterations at the DNA (deoxyribonucleic acid) level that regulate gene expression including DNA methylation and the post-translational histone modifications of histone acetylation/de-acetylation and histone methylation/de-methylation.

One core concept of aging is the continued accumulation of genetic damage through life (Lopez-Otin 2013). The continual damage of DNA has widespread effects, particularly on cellular function. As human bodies rely on homeostasis to maintain physiologic health, damage to certain DNA or cells, could, in theory, either decrease physiologic function or allow an advantage to a pathologic function. This notion that progressive cellular DNA damage can lead to weakening of host mechanisms might explain some major age-related links to common conditions such as cancer, atherosclerosis, inflammation, osteoporosis, periodontitis, and heart disease to name a few. Each of these conditions have been shown to have increased prevalence with age. Lopez-Otin et al first described the "9 hallmarks of aging" and laid out a foundation for understanding the mechanism and outlook of aging that is largely cellular based. The authors depict, in no order, altered intercellular communication, genomic stability, telomere attrition, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and epigenetic alterations as the core tenets (Lopez-Otin 2013). This final idea of epigenetics is the central idea of this study.

In today's world, where average life span is continually increasing, we notice a heightened prevalence of bone homeostatic disorders such as osteoporosis, osteopenia, and periodontal disease (Aburto 2020). When lifespan was shorter, these conditions did not pose an appreciable threat (Lane 2006). For example, periodontal disease is an oral condition of bacterial origin which usually caused slow bone loss around teeth as humans age. While seen and acquirable at any stage in life, it is most often detected in adult hood as we age. As there is no cure for this condition, the treatment centers around slowing or halting progression. As people age, it becomes harder and harder to maintain a young adult level of bone.

Osteoporosis is a systemic and pathologic loss and weakening of bone that, while most prevalent in post-menopausal woman, can affect both men and women (Clynes 2020). As the name suggests, osteoporosis leads to a less dense and more porous bone structure. This disease process is considered an aggressive condition as opposed to the accepted gradual decline in bone density in humans as we age. The main sequelae of this diminishing bone density is the significant increase in both fracture risk and fracture occurrence. Osteoporosis presents both medical and personal challenges to society as countless dollars are invested in research as well as treatment of the disease itself and acute situations such as fractures, which costs the USA $17.9 million per year. (Kanis 2019, Clynes 2020). Today, the accepted measurement that depicts overall strength and risk is bone mass. As a person ages, their late life bone mass is determined by their cumulative bone mass growth in their 30s (Clynes 2020). This is of particular importance as beyond the age of 40 bone mass begins to decline. This happens in both males and females, however, women age 50 and older and/or post-menopausal are at risk for osteoporosis which leaves them twice as fracture prone as their male counter parts (Clynes 2020).

To understand the relationship between bone mass and bone loss during aging, it is important to first understand bone metabolism and homeostasis. As previously mentioned, bone is a dynamic organ that ideally undergoes constant remodeling and "refreshing" to accommodate growth, aging, and health. Bone is a critical

tissue in the human body with numerous functions including mechanical support, joint mobility, soft tissue organ protection, erythropoiesis/ hemopoiesis, and electrolyte balance (Rodan 1995). The structure, function, and health of our skeleton is determined by genetics and aided by environmental status. Homeostasis is the body's maintenance of an ideal balance. Bone is no different as it is constantly turning over. This process is regulated by osteoclasts. Bone is continuously resorbed by osteoclasts which is followed by new bone formation and deposition by osteoblasts. The two processes, ideally, are in balance (Rodan 1995). There are 3 cells associated with bone metabolism and regulation: osteoclasts, osteoblasts, and osteocytes. Osteoblasts, derived from mesenchymal stem cells, actively deposit new bone osteoid which will mature into native bone. Osteocytes are terminally differentiated osteoblasts and are able to sense mechanical changes in bone and regulate both osteoblasts and osteoclasts. Osteocytes possess dendritic processes that form a widespread network within bone to allow for communication with osteoblast precursors (Boyce 2012). Osteoclasts, on the other hand, are responsible for bone resorption. Osteoclasts originate from hematopoietic and myeloid precursors similar to monocytes and macrophages (Boyce 2012). Interestingly, bone cells can regulate other bone cells. Bone remodeling begins in bone canals known as basic multicellular units (BMU) formed by osteoclasts. Osteoclasts adhere to bone via a ruffled border rich in actin which creates a "sealed zone" known as a lacuna. Then, they release lysosomal proteases and hydrogen chloride creating an acidic environment. This begins the demineralization and softening of bone which is followed by collagenase, primarily cathepsin K, degrading type I collagen common in bone. The osteoclasts then endocytose the resultant fragments and undergo apoptosis (Boyce 2012). This process is followed by recognition of the defect and deposition of new osteoid by osteoblasts.

Healthy bone remodeling is regulated by many factors such as prostaglandin, parathyroid hormone, and cytokines. These factors exert activity on osteoclasts thereby inducing bone resorption. At the same time, the enhanced osteoclast activity induces osteoblast activity creating a healthy cycle and link (Rodan 1995).

A previous mice model study by Corral et al demonstrated that although osteoblasts are linked to osteoclast function, the same relationship does not necessarily operate in reverse which delineates both bone resorption and deposition as separate processes. This study used an osteocalcin promoter to activate thymidylate kinase and destroy osteoblasts in adult age mice. The result was a total loss of bone formation. On the other side, however, osteoclast volume and function were not affected which led to pathologic bone loss. This mouse model mimicked osteoporosis which has been shown to be a function of osteoclasts hyperactivity. Treatments for osteoporosis are aimed at inhibiting osteoclast function. The result is a class of drugs, bisphosphonates, which function through their high affinity for bone. The result is the apoptosis of osteoclasts thus reducing bone resorption (Drake 2008).

The next step in unlocking knowledge of aging and bone loss is understanding the pathology of osteoporosis. Osteoporosis is characterized by a shift in the bone remodeling homeostasis towards hyperactivity of osteoclasts. It is believed that there are multiple pathologic mechanisms in play for osteoporosis as it is a complex multi-genic condition (Raisz 2005). One mechanism is the structural weakness of bone which may be altered by failure to create healthy amounts of bone. The second mechanism might be excessive resorption. Lastly, the third mechanism might be inadequate bone formation in response to increased osteoclast activity. In bone remodeling, the bone resorption phase is much faster than bone deposition phase (Raisz 2005). Further, in osteoporotic women of post-menopausal age, estrogen deficiency is common. This is associated with increased resorption and decreased osteoclast apoptosis as all bone cells possess estrogen receptors (Riggs 2000). Estrogen plays a key role in downregulating proinflammatory cytokines such as IL-1, IL-6, TNF-alpha, granulocyte macrophage colony-stimulating factor (GM-CSF), and prostaglandins. These cytokines are directly responsible for bone resorption by increasing osteoclast precursors (Riggs 2000). Further, it has been determined that TNF ligand and its receptors are the first step of osteoclast differentiation through activation of the receptor NF-kB ligand (RANKL). RANKL has a high affinity for all cells of the osteoclast lineage

and stimulates function. RANKL is directly responsible for signaling differentiation, resorption activity, and decreasing osteoclast apoptosis (Riggs 2000). Osteoprotegerin (OPG) from osteoblastic lineage cells, which acts a decoy to neutralize RANKL, is increased by estrogen. IL-1 and TNF-alpha both increase RANKL, OPG and GM-CSF. RANKL, OPG, and GM-CSF are each protective against bone loss (Riggs 2000). This mechanism is most directly linked with osteoporosis, but what about the effect of aging on bone loss?

The difference between bone loss and the pathologic bone loss of osteoporosis is the gradual, yet progressive loss of bone mass associated with aging in both men and women. As humans age, the balance of bone resorption and bone formation is altered. Osteoclast activity outpaces osteoblast activity. This loss of balance between osteoblast and osteoclast activity is due to a shift from osteoblastogenesis to adipogenesis within the bone marrow. This has a lipotoxic effect on bone formation and mineralization (Demontiero 2012). The age-related bone decline is also linked with environmental factors like nutrition, level of activity, comorbidities and prescription medications. Systemically, bone loss factors include genetics, peak growth, and hormonal shifts. Peak bone mass is achieved between ages 15-20 in females and later in the 20s in men (Raisz and Seeman 2001). Beyond this age period the body maintains an equal balance between deposition and resorption. Beyond the fourth decade of life, there is a marked decrease in deposition and resorption begins to outpace formation and cortical bone thins leading to porosity increase (Demontiero 2012). This has been confirmed through cross sections studies that indicate a slow decline of bone mineral density beginning at age 40 for both sexes (Khosla and Riggs 2005). As humans approach age 50, when menopause becomes prevalent, females exhibit a more progressive decrease in bone mineral density.

RANK/RANKL and Signaling

There are numerous cytokines and secretory factors that directly affect osteoclast and osteoblast differentiation and function. Besides their role in bone formation, osteoblasts also regulate differentiation of osteoclasts during bone modeling by secreting RANKL. Osteocytes have also been shown to express RANKL.

Osteocytes are thought to regulate osteoclast differentiation during bone remodeling. Two cytokines, macrophage colony stimulating factor (M-CSF) and RANKL, are necessary and sufficient for osteoclast differentiation. RANKL binds to RANK receptor expressed on osteoclast surface. Osteoprotegerin (OPG) is a bone protective factor acting as a RANK decoy. Beyond resorption, osteoclasts express coupling factors to regulate osteoblast and osteocyte activity during bone remodeling (Huynh et al. 2017, Novack and Mbalaviele 2016, Park-Min 2017). These coupling factors are expressed independent of osteoclast resorption. It is also suggested that osteoclasts release factors from the bone that act as coupling factors. RANK signaling pathway involves multiple protein kinase cascades resulting in osteoclast differentiation and resorption. When RANK is engaged by RANKL, there are accessibility changes within the nucleosomes and chromatin. This increased accessibility leads to differentiation of osteoclasts through expression of NFATc1 (Boyle 2003, Ghayor & Weber 2016, Novack & Mbalaviele 2016, Park-min 2017). One gene activated by NFATc1 is *cathepsin K (Ctsk).* CTSK is produced and secreted by osteoclasts. Bone resorption occurs locally after an osteoclast attaches to bone and creates a resorption pit. After this step the osteoclast creates an acidic local environment which dissolves the mineral structure. This softened matrix is then removed by metalloproteinases and cathepsin K (Demontiero 2012).

Epigenetics and Histone Acetylation:

Epigenetics refers to the process by which gene expression and phenotype are altered with reversible modifications to DNA. This process does not make changes to the genetic code; however, the modifications are heritable. This potentially heritable status of epigenetic changes plays a strong mediator role in conditions such as cancer (Lawlor 2019). Bone remodeling is controlled through bone cells via epigenetic processes such as histone acetylation/de-acetylation and DNA methylation/de-methylation. Epigenetic regulation occurs in both physiologic and pathologic conditions. Further, epigenetic alteration is one of the hallmark signs of aging. It is poorly understood as to what extent epigenetic changes occur in osteoclasts as men and women age and if those changes contribute to bone loss.

Throughout their lives, osteoclasts are subjected to constant epigenetic changes. As previously stated, histone modifications play an enormous role in gene regulation and expression.

Epigenetic changes primarily involve changes in acetylation and methylation. Nucleosomes which consist of DNA segments wrapped around a histone have an N-terminal tail which is subject to reversible modifications of acetylation and methylation (Bradley 2015, Lawlor 2019). During epigenetic changes, acetyl and methyl groups are either added or removed from DNA or the histones. Acetylation is carried out by histone acetyl transferase (HAT) and de-acetylation is carried out by histone deacetylase (HDAC). Thus, acetylation and deacetylation are epigenetic changes modifying chromatin structure and transcription (Seto 2014). Acetylation is associated with elevated gene expression while deacetylation is associated with gene and transcription repression. Chromatin that has been deacetylated becomes more condensed and more difficult to access for transcription.

Post-translational histone modification can cause functional changes to expression and chromatin structure. This represents a key trait of epigenetic changes. Removal of acetyl from lysine groups allows HDACs to alter transcription and either promote or remove other post-translational modifications such as methylation (Seto 2014). This allows increased binding of the H4 tail to DNA. Contrarily, de-acetylation is proposed to strengthen the histone-DNA interaction. This would make the DNA less accessible, thus repressing transcription (Seto 2014). High levels of acetylation are associated with elevated transcription while low levels of acetylation are associated with gene transcription repression.

According to Lopez-otin et al, aging is the “time-dependent functional decline characterized by loss of physiological integrity, leading to impaired function and increased vulnerability.” There are links between aging and increased epigenetic changes associated with elevated HDAC presence and function, though this remains poorly understood. DNA is subject to aging and may not replicate as readily or as efficiently in advanced age. This knowledge has led to medicinal

targeting of HDACs to treat disorders such these. It is believed there is enormous therapeutic potential here, but the entire area is still poorly understood. Therapeutic targeting has been difficult due to the widespread and varying function of HDACs. Selective targeting is the most ideal route of medicine, though this is difficult. Currently, some HDAC inhibiting drugs appear to be narrowing in on class I HDAC selective therapy (Hull 2016).

HDACS Structure and Function:

Histone deacetylase functions directly by catalyzing hydrolysis of an N6-acetyl-lysine residue within histones. This leads to an acetylated substrate, an acetyl group, and 1 H2O molecule. This makes HDACs an “eraser” of epigenetic information and post-translational modifications of chromatin (Bradley 2015, Hull 2016). The counter of this are histone acetyltransferases that add acetyl groups to chromatin to activate gene expression. However, as typical of our aging bodies, homeostatic measures often become dysregulated and can even be pathologic, which brings up the idea of how aging affects HDAC expression and function. HDACs have been heavily implicated in diseases of bone such as osteoporosis and rheumatoid arthritis (Gillespie 2012)

Today, there are 4 recognized classes of HDACs known simply as class I, II, III, and IV. These classes are based on function and structure primarily. To be clear, however, very little of HDAC presence, levels, functions, and dysregulation is understood. Class III HDACs are generally considered a separate group known as sirtuins and rely on NAD+ for function. Classes I, II, and IV are zinc related (Seto 2014). Basic knowledge of this topic suggests that HDAC1 and HDAC3 are two of the most highly expressed HDACs in bone, though other HDACs have been noted at varying levels (Bradley 2015). Historically, it is accepted that HDACs play an important role in skeletal formation, particularly intramembranous ossification. Intramembranous ossification yields the formation of flat bones in the skull.

The four recognized class I HDACs are HDAC 1, 2, 3, and 8. It is believed that they possess high histone affinity and are thus critical for gene transcription, DNA replication and survival, and cell proliferation in bone (Bradley 2015). While there

is no clear evidence that HDAC over-expression is oncogenic, it has been noted that class I HDAC suppression exhibits anti-tumor effects (Falkenberg 2014). Also critical to this classification is the discovery that class I HDACs are mainly expressed in the nucleus but they can also be found in the cytoplasm (Seto 2014). HDACs 1 and 2 are only active in protein complexes and are highly subject to phosphorylation (de Ruijter 2003). HDAC1 has been found downregulated in osteoblast differentiation (Lawlor 2019). HDAC3 functions differently than HDAC1 and 2 as it involves the SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) pathways and is dependent on phosphorylation. SMRT is a co-repressor protein of nuclear receptors. Essentially, SMRT proteins help bridge the gap of numerous transcription factors and histone modifying enzymes such as HDACs (Watson 2012). HDAC 3 has been shown as the primary enzyme in the repressive function of SMRT. HDAC3 is understood to be crucial in facilitating proper craniofacial development. A study which deleted HDAC3 in a mouse model yielded the phenotype of microcephaly (Singh 2013). HDAC3 is also believed to be integral to other bone tissue formation as its activity is reduced during osteoblast differentiation which increases acetylation and gene expression (Lawlor 2019). However, little is known of its function in life, aging, and bone metabolism. HDAC 8 is also important for skull development, as studies have demonstrated HDAC 8 removal results in impaired calvarial development (Haberland 2019). Contrary to other Class I HDACs, phosphorylation inhibits HDAC 8 enzymatic activity (Seto 2014). HDAC 8 is also known to suppress osteogenic gene expression (Lawlor 2019). Little is known about HDAC8 with regards to its function and role throughout aging. Summarily, class I HDACs are crucial for proper endochonrdral ossification, but their ongoing function during aging is poorly studied and documented.

Class II HDACs are subdivided into classes IIa and IIb and include HDAC 4, 5, 6, 7, 9, and 10. HDACs 4, 5, 7 and 9 belong to the IIa group and HDAC 6 and 10 belong to the IIb group. The main structural difference of class II HDACs from class I is the possession of large NH2- terminal extensions that have binding sites for myocyte enhancer transcription factor 2 (Mef2) and chaperone protein 14-3-3.

When serine residues in the NH2 terminals are phosphorylated, binding of chaperone proteins to class II HDACs is promoted. This is followed by translocation of the HDACs to the cytoplasm. Ultimately, this leads to “de-repression” of genes (Bradley 2015). Curiously, class II HDACs appear to possess much lower affinity levels and enzyme deacetylase activity than their class I counterparts. This means to deacetylate substrates class II HDACs to recruit class I HDACs (Bradley 2015). Like class I HDACS, class II HDACs are also highly implicated in skeletal formation. HDAC 4 was first shown to integrate extracellular signals of bone formation with transcription regulators (Bradley 2015). Bio-engineered mice with HDAC 4 deficiency display early endochondral ossification. Expanding on this, studies have shown that HDAC4 controls chondrocyte hypertrophy during endochondral ossification (Lawlor 2019). Obviously, this will result in skeletal defects (Vega 2004). Interestingly, but not surprisingly, parathyroid hormone is major regulator of HDAC4 function in bone development. Shimizu et al. demonstrated that PTH induces PKA to phosphorylate HDAC4. This results in in lysosomal degradation of HDAC4 (Shimizu 2010). Further, HDAC4 can associate with Mef2c, a transcription factor, inhibiting the normal chondrocyte hypertrophy. This demonstrates a repressive effect of HDAC4 and mef2c (Arnold 2007). HDAC 4, 5, and 7 are subclassified due to their similarities. They have their catalytic domain on the C-terminal as opposed to the other class II HDACs and they share the same co-repressors (de Ruijter 2003). Little is known about HDAC 6, but recent studies suggest a link between HDAC 6 and achondroplasia (Simon 2010, Lawlor 2019). HDAC 7 has been previously shown to act as a negative regulator of osteoclast function, meaning that its presence decreases the activity of osteoclasts (Boyle 2003). HDAC 10 has been found to be very similar to HDAC 6 and both have a unique catalytic domain (Seto 2014). HDAC 9 is shown to interact with Mef2, suggesting its role in muscle metabolism (de Ruijter 2003). Blixt 2017 studied suppression HDACs 6 and 9. It was found that when HDAC 6 is suppressed, osteoclast differentiation is not affected. It has been proposed that HDAC 6 is likely involved with tubulin stability in mature osteoclasts, but the role in

differentiation is yet to be established (Blixt 2017). On the contrary, when HDAC 9 is suppressed, osteoclastogenesis is enhanced (Blixt 2017).

Class III HDACs, sirtuins, are classified as Sirts1-7. It is more appropriate to recognize them as sirtuins rather than HDACs. This is because sirtuins are related to yeast proteins and are dependent on nicotinamide adenine dinucleotide (NAD+) for functionality. They function by moving an acetyl (de-acetylase driven) group from a lysine complex to NAD+. This creates an enzyme feedback inhibitor, which is the basis of sirtuin function (Bradley 2015). Much is poorly understood about sirtuins and they have been seldom studied in depth. As such, there is much debate over their function since studies indicate that different sirtuins function on different proteins. Sirtuins are of interest, however, because there are links to sirtuins controlling metabolism, inflammation, genomic stability, and aging. As related to the topic of interest, bone metabolism and osteoporosis, there are new links that suggest sirt 1 and 6 heavily facilitate endochondral ossification (Bradley 2015).

HDAC 11 is currently the sole member of the class IV HDAC group. If we knew little about the other HDAC groups, we know even less about HDAC 11. Research indicates that HDAC 11 is most related to HDACs 3 and 8 and is present in multitude of tissues, but its function is not known. Results of a sequencing study show that HDAC11 likely has a role in mRNA splicing (Joshi 2013, de Ruijter 2003). Further, there is little understanding about expression levels of HDAC 11 within bone cells and with aging processes.

Role of HDACs in osteoclasts and bone resorption:

During osteoclast differentiation, HDAC 1 expression decreases and HDAC 2 expression increases. HDAC 1 appears to be expressed at the beginning of differentiation and function as a suppressor of transcription. After signaling via RANKL, the expression of HDAC 1 is greatly diminished (Astleford 2020). It is suggested that HDAC 2 is strongly associated with perpetuating osteoclast differentiation. When HDAC 2 is biomechanically suppressed or removed, the result is inhibition of osteoclast differentiation along with diminished fusion during

differentiation (Astleford 2020). Multiple studies have shown that HDAC 7 suppression facilitates osteoclastogenesis and osteoclast hypertrophy and that HDAC 7 directly inhibits osteoclastogenesis (Pham 2011, Jin 2013). This appears connected to the HDAC7's ability to inhibit a transcription factor needed for RANKL related gene expression. A previous study of the Manksy lab demonstrated that when HDAC 7 is removed from mice osteoclasts, the overall bone mass is decreased (Stemig 2015). This suggests that HDAC7 and possibly all class II HDACs provide an osteoprotective function. Studies of HDAC 9 show that there is reduction in bone volume with HDAC9 is knocked out or suppressed (Jin 2015). HDAC 3 has been studied in regard to inflammation and healing. It was determined that HDAC 3 suppression is associated with decreased osteoclast numbers (Molstad 2020). Other studies show that HDAC3 knock out mice display inhibited osteoclast formation in response to RANKL. Thus, it is likely that HDAC 3 and 7 possess opposite roles in osteoclast differentiation with HDAC 3 promoting osteoclast differentiation and HDAC 7 suppresses osteoclast differentiation (Pham 2011). Further, HDAC 3 suppression has been shown to increase leucine-rich repeat phosphatase resulting in decreased matrix creation (Bradley 2013). Expression of HDAC 4 in osteoclasts appears to decrease throughout differentiation, though this significance is not understood (Astleford 2020). Oppositely, HDAC 5 expression rises throughout differentiation upon RANKL signaling and has been suggested to link with NFATc1 as a regulatory mechanism during osteoclast differentiation (Astleford 2020). Currently there are no strong links to the function of HDAC 6 in osteoclast differentiation. These studies might suggest that class I HDACs might function in promoting bone resorption while class II HDACs might have a protective function. They also suggest that HDACs have highly individual roles within osteoclast differentiation.

Summary and hypothesis:

DNA methylation and acetylation are being increasingly implicated in human disease and serve as target for drug development. Further HDACs are clinically relevant today as there are numerous inhibitory molecules for HDACs and are frequently used therapeutically in conditions like arthritis, cancer, diabetes,

epilepsy, heart disease, and of most importance to this study: aging-related disorders (Bradley 2015).

This study is focused on the expression of histone deacetylases (HDACs) which are negative regulators of gene expression. HDACs remove acetyl groups which results in more compact chromatin. There are 18 HDACs which are divided into four classes based on structure, function and subcellular localization. Class I HDACs include HDAC1, 2, 3 and 8 while class II include HDAC 4, 5, 6, 7, 9 and 10. Using this information, this study aims to further determine if expression of HDACs change in osteoclasts from young and old animals. The information will form the basis of future studies involving understanding how epigenetic changes regulate bone loss during aging. HDAC expression will be tested and calculated using cDNA created from osteoclasts harvested from both male and female mice of ages 1 month and 24 months. This most accurately reflects the status of human young adults and elderly respectively.

This study is intended to act as a pilot study focused on class I, II, and IV HDACs within varying age groups of mice. This information is hypothesized to be critical in understanding pathology of bone conditions like osteoporosis and periodontitis. Information gained may have therapeutic value or warrant more specific studies. We hypothesize that osteoclasts from 24-month-old mice will have lower expression of HDACs compared to 1 month old mice. We further hypothesize that females in both the 1-month-old and 24-month old groups will have high HDAC expression compared to that of their male counterparts because many HDACs have been linked to promotion of osteoclast function. We believe there is an age-related relationship with higher HDAC prevalence in females, which may explain the higher amounts of bone loss in older females vs males.

Aim:

1. Examine expression levels in osteoclasts of class I, II and IV HDACs in osteoclasts from 1- and 24-month-old mice in both males and females at days 0, 2, and 4.

2. Compare HDAC expression relationships with regards to aging and among established classes of HDACs

Methods

Conducted in the Mansky lab, this study consists of 12 C57BI/6 mice test subjects. 1 month old mice were selected due to their human age comparison of young adulthood while 24-month-old mice were selected to replicate a more advanced human age. The four test groups are 1 month old males, 1 month old females, 24month old males, and 24-month-old females. To obtain an average and a standard deviation, there are 3 test sets within each testing group. Mice were obtained and aged before being euthanized to harvest bone marrow. RNA was collected from osteoclasts, cDNA was reverse transcribed from RNA, and qRT-qPCR was performed to obtain HDAC expression levels.

Culturing of mouse osteoclasts:

All mice care and use was approved and reviewed by the University of Minnesota Institutional Animal Care and Use Committee, IACUC protocol number 2104-39006A. All euthanasia was performed by CO_2 inhalation. Bone marrow was harvested from femora and tibiae of the above outlined mice groups. Bone marrow macrophages (BMMs) were then differentiated into osteoclasts. Beginning two days after re-plating BMMs (referred to as day 0), cells were provided 1% CMG 14-12 supernatant (Dr. Sunao Takeshita, Nagoya City University, Nagoya, Japan) containing macrophage-stimulating colony factor (M-CSF) and 10 ng/ml RANKL (R&D Systems) every 48 hours (on days 0, 2, and 4) until the desired experimental end point.

RNA extraction and analysis:

Each osteoclast sample was combined with 500 uL of TRIzol (Ambion, life Technologies) and repeatedly pipetted to lyse osteoclasts. Samples were removed from freezer and allowed to thaw at room temperature for 5 minutes. Each sample then received 100uL of chloroform and was vortexed for 10 seconds and incubated for 3 minutes. Samples then subjected to centrifugation at 12 rcf (relative centrifugal force) for 15 minutes at 4 Celsius. The aqueous phase was separated

into a new tube and combined with 250 uL isopropanol. After 10 minutes of incubation at room temperature, samples were centrifuged at 12 rcf (relative centrifugal force) for 10 minutes at 4 Celsius. The liquid was decanted from the tubes with special care not to disturb the residual pellet. Each pellet then received 500 uL of 75% ethanol and was again vortexed. The tubes were then all centrifuged at 7.5 rcf for 5 minutes at 4 Celsius. After completion, the pellet was then allowed to dry in an upright position. RNA synthesis was complete by resuspending the pellet in 20 uL of RNase-free water and then stored in -80 Celsius freezer.

cDNA synthesis After thawing, RNA samples, the concentration of each sample was then tested by adding 2 uL RNA into 98 uL of TE-Buffer. These tubes were quantified using a UV spectrometer. Each sample was combined with 4 uL iScript Reaction Mix, 1 uL iScript Reverse Transcriptase, and 15 uL of RNase-free/ DEPC treated water for a total volume of 20 uL per sample. The original samples and reaction mix were then placed into PCR tubes and placed into thermal cycler and run through PCR function iScript. The PCR conditions were 95 Celsius for 30 seconds, 58 C for 30 seconds and 72C for 30 seconds. This was followed by melting curve analysis at 95 C for 5 sec, 56 C for 5 sec and 65 C increased to 95 C at 0.5 C increments every 5 seconds. The result was cDNA.

Real Time PCR Protocol:

To begin, master mixes were prepared for each HDAC (1-11) using SyBR Green. In each master mix tube, the following were combined: HDAC # forward primer 0.1 uL per sample, HDAC # reverse primer 0.1 uL per sample, 10 uL per sample SyBR Green, and 8.8 uL DEPC-treated water per sample. Simultaneously, a master tube was made using HPRT forward and reverse as opposed to HDAC primers with the same proportions. Next, rt-qPCR tubes were laid out and labeled on a master plate for days 0, 2, and 4 for each HDAC of a given test set along with one for HPRT days 0, 2, and 4. 19 uL of master mix was pipetted into each rt-qPCR tubule according to labelled HDAC. Lastly, 1 uL of cDNA corresponding to the sample set

and days 0, 2, and 4 were added to the appropriate rt-qPCR tubes. Tubes were sealed and centrifuged to ensure mixing. Tubes were loaded into rt-qPCR machine (Bio-rad) and samples were subjected to a pre-established real time protocol at 58 Celsius.

Data Analysis:

All cq values were individually labelled according to master plate templates. To calculate adjusted expression, cq value of the HDAC was subtracted from the corresponding HPRT differentiation day of the matching set. The resultant value was then used to calculate true expression with a power function. The formula used was expression= (1/power (2,adjusted cq)) * 100. For example, the number 2 was raised by the power of adjusted cq. The resultant value was treated as the expression relative to HPRT expression. After calculating all expressions, a mean with standard deviation was calculated and displayed in graph form for each data set, i.e 1-month male set 1/ HDAC 1, 1-month male set 2/ HDAC1, 1-month male set 3/ HDAC1, 1-month female set 1/HDAC 1, and so on. Calculations were made according to manufacturer specifications (Bio-Rad). The level of significance was set to $p<0.05$.

Results

We searched for significance between the days of expression as well as between age groups and between sexes of matching age groups. Thorough analysis and calculation yielded only two comparisons displaying significance. These comparisons will be discussed in the below sections. The remainder of comparisons did not show significance and numerical *p* values are not displayed for consideration. Post-hoc analysis was performed via a Tukey test comparing means of all treatments to all other treatment means.

HDAC 1

To determine if HDAC expression changes with sex and/or age, we cultured osteoclasts from bone marrow of 1 and 24-month-old male and female mice. Expression levels of HDAC1 (Fig 1) in the females measure at a much higher level compared to males at the 1-month time point. It is also clear that both males and females in the 1-month group follow the same pattern with expression peaking at day 2 (Fig 2). Looking at the 24-month groups, both males and females display similar levels of expression.

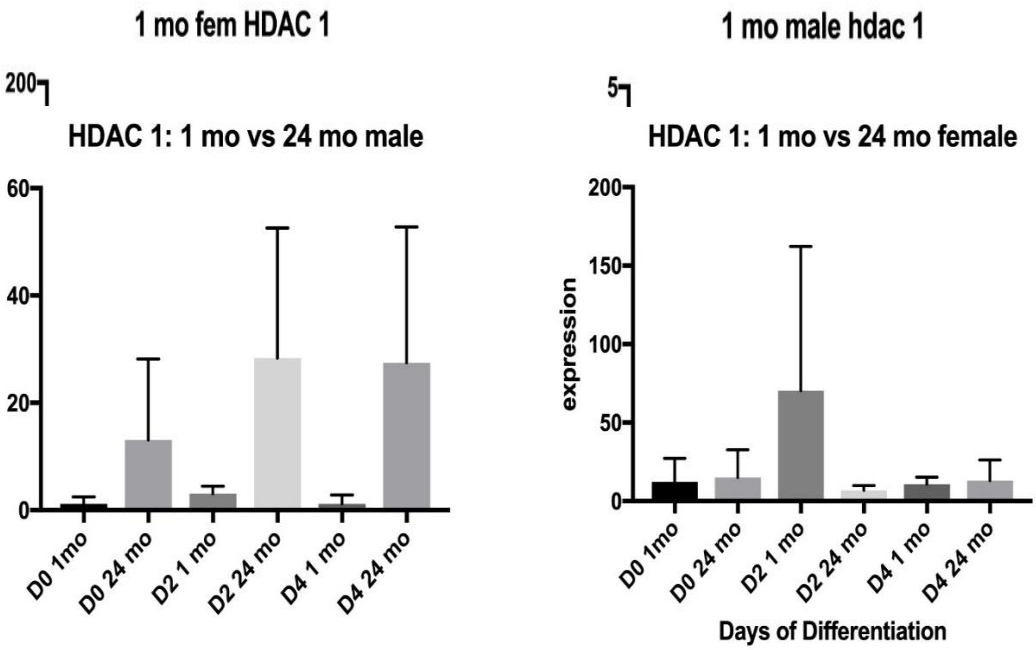

Fig 2: Bar graphs depicting comparison among males and females between age groups of 1-month and 24-month. Average is displayed +/- standard deviation

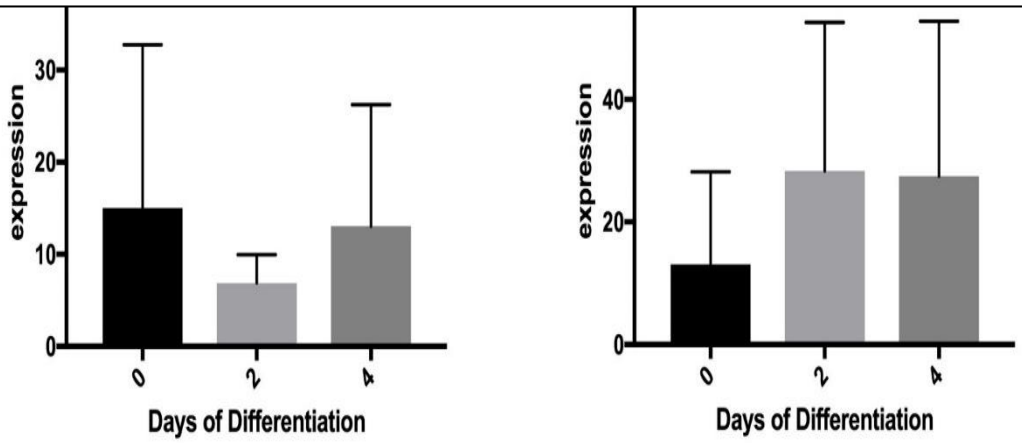

Fig 1: HDAC 1 expression of 1-month females, 1 month males, 24-month females, 24 month males. Average +/- standard deviation

HDAC 2

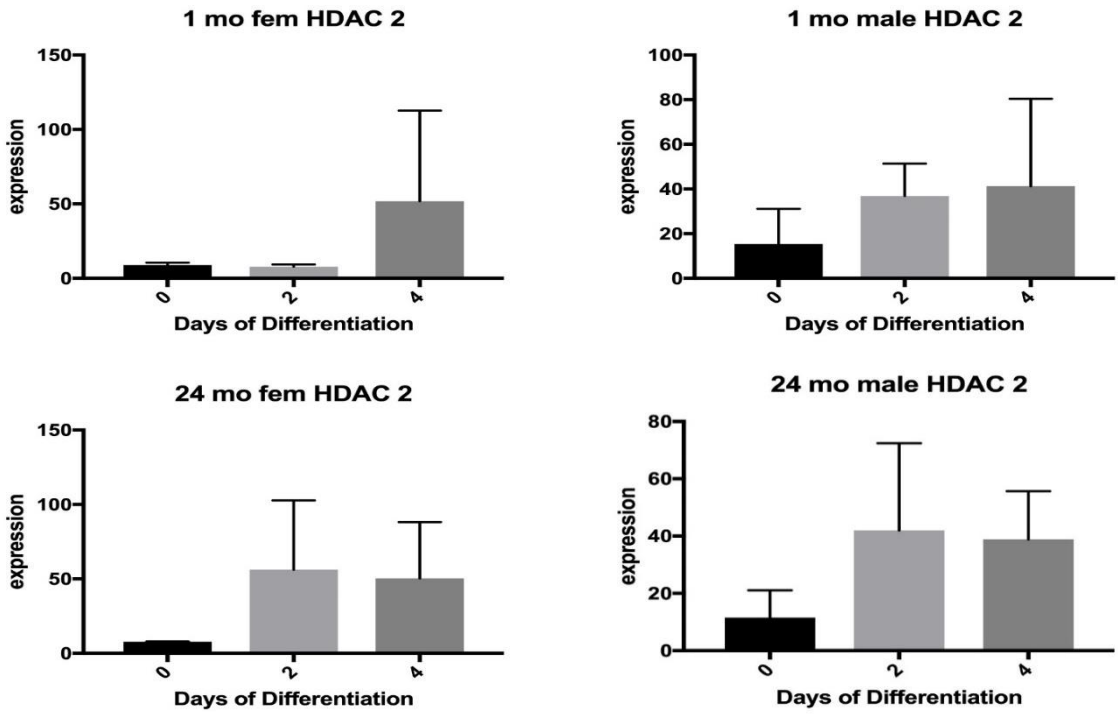

Fig 3: HDAC 2 expression of 1-month females, 1-month males, 24-month

In 1-month females, expression is very similar on days 0 and 2, then increases on day 4. In the 1-month male group, expression is lowest on day 0 and increases in day 2 and day 4 (Fig 3). The 24-month female group has relatively low initial expression and increases on day 2. The male 24-month counterparts display a

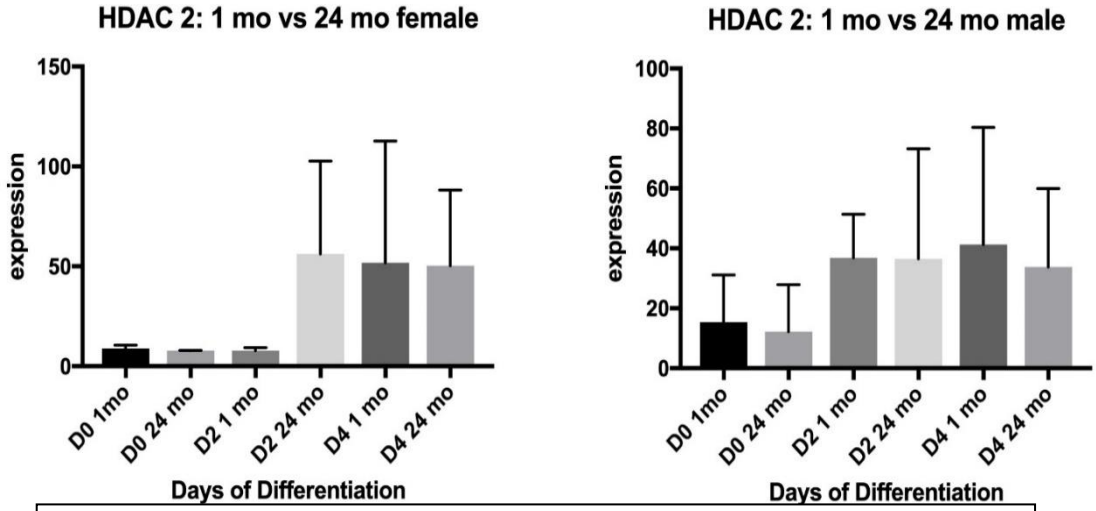

Fig 4: Comparison of 1 and 24-month females and males of HDAC 2 with average +/- standard deviation

similar progression. On the other hand, 1-month males and females appear to have similar average expression levels. A direct comparison between ages depicts a similar range of expression between the 1-month and 24-month groups (Fig 4). This is different than the pattern noted in HDAC 1.

HDAC 3

Within HDAC 3 groups (Fig 5), again, the 1-month females display the pattern of low expression at day 0, increasing at day 2. 1-month males appear to have similar expression levels throughout differentiation. Comparing 1-month males and

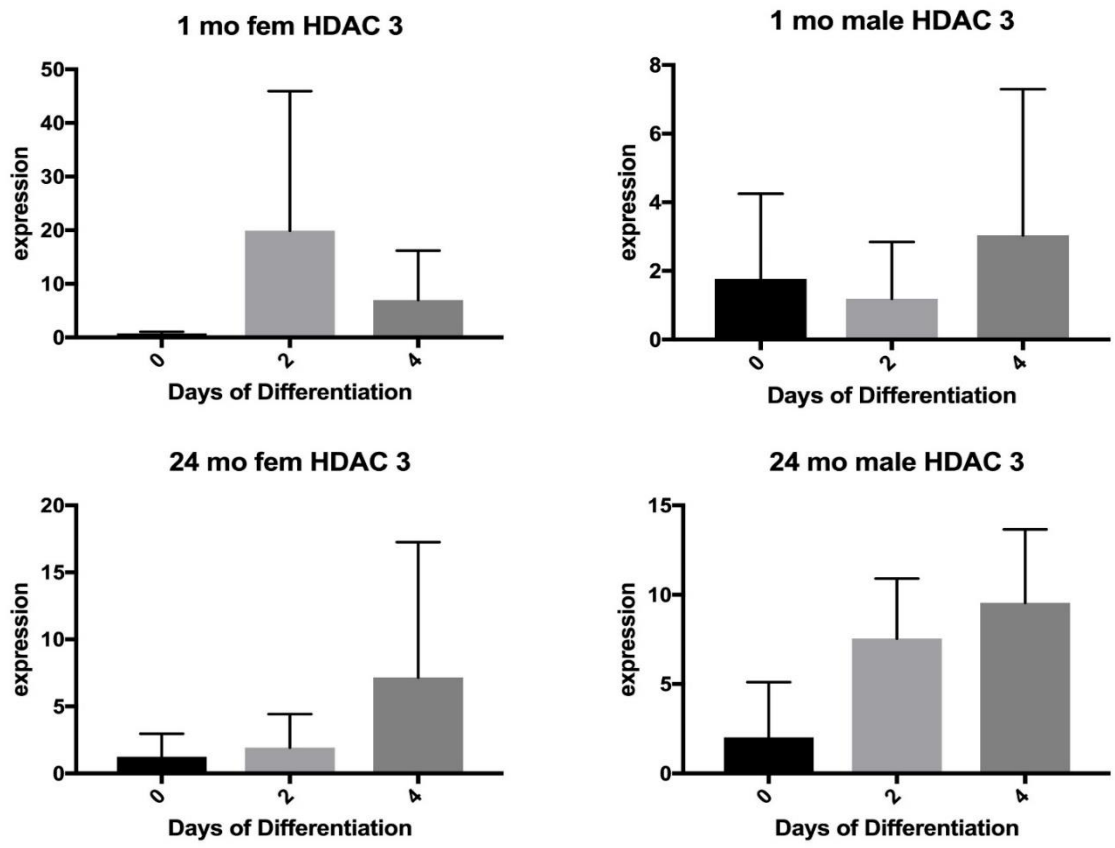

Fig 5: HDAC 3 expression of 1 month females, 1 month males, 24 month females, 24 month males. Data displayed in bar graph form with average depicted by bar +/- standard deviation

females,there is generally much higher levels of expression in the 1-month females. 24-month females display relatively similar expression levels at days 0 and 2, which is followed by an increase on day 4. 1-month females overall have higher expression of HDAC3 compared to 24-month females. 24-month males display a progressive increase in expression from day 0 to day 4. There are also

generally similar levels of expression when comparing 24-month males and females.

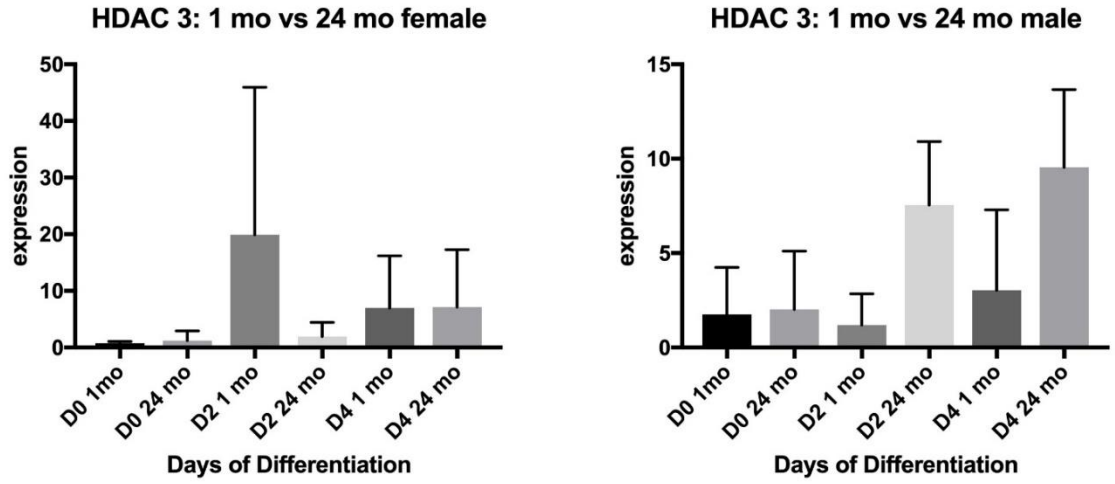

Fig 6: Comparison of 1 and 24-month females and males of HDAC 3. Average displayed +/- standard deviation.

HDAC 4:

1-month females display the expected expression curve with lowest expression day 0, and an increase at day 2. Analyzing the 24-month females we see expression is highest day 0 and 4 and actually drops at day 2 (Fig 7). 24-month males display a gradual, growth in expression from day 0 to day 4. One thing that is clear, the 24-month females have significantly more HDAC 4 expression than the 1-month subjects with a *p* value of 0.0273 (Fig 8). Comparison of the 1-month and 24-month males, however, shows there is only slightly higher expression in the older group.

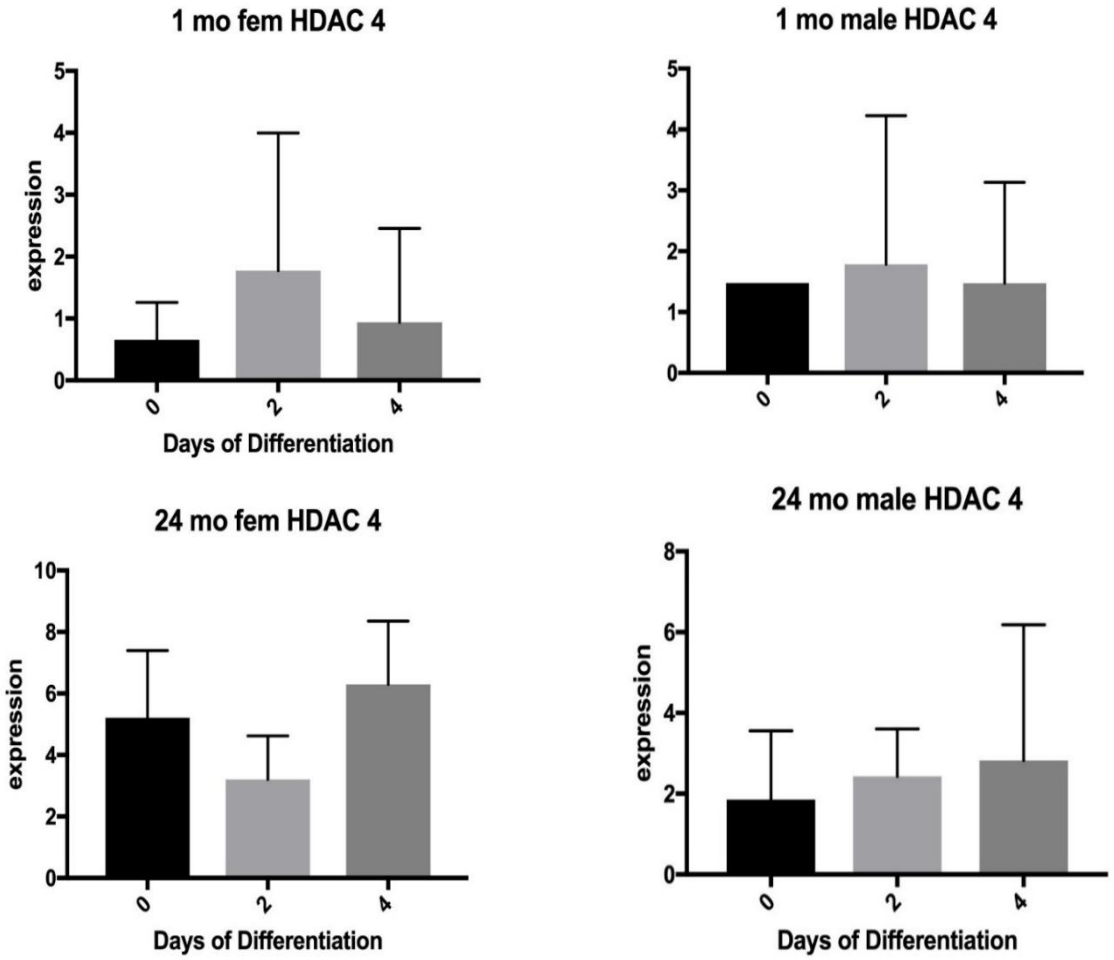

Fig 7: HDAC 4 expression of 1-month females, 1-month males, 24-month females, 24-month males displayed with average +/- standard deviation.

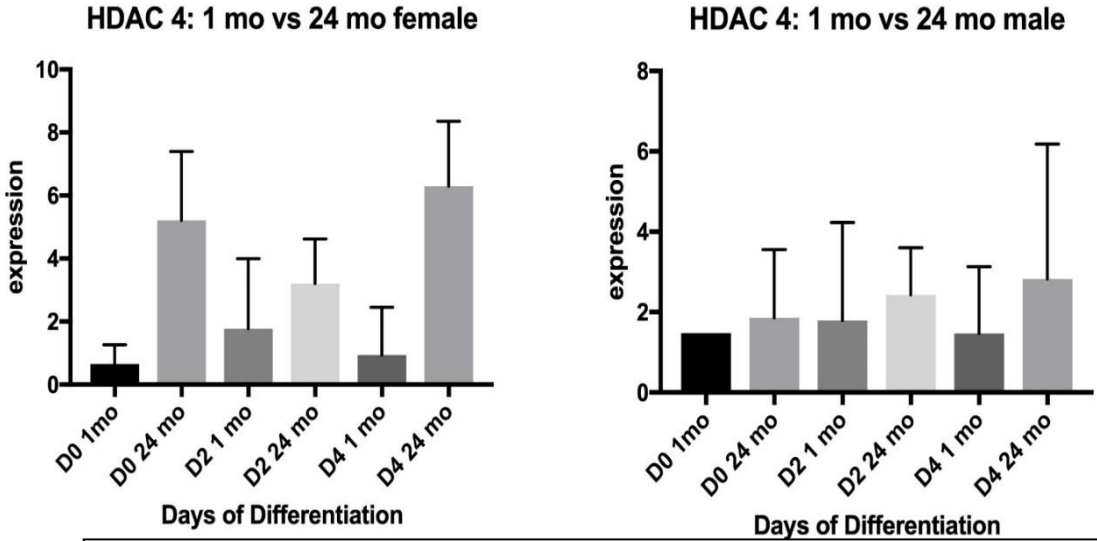

Fig 8: Comparison of 1 and 24-month females and males of HDAC 4 displaying average +/- standard deviation. P=0.0273 comparing D4 24mo fem vs D4 1mo fem

HDAC 5:

In the HDAC 5 test groups (Fig 9), the 1-month female displays lowest expression at day 0, increases significantly at day 2, then drops near initial value at day 4. The 1-month males begin with almost no expression, increase slightly at day 2, and then increase for day 4. The 24-month females display a similar pattern to that of the HDAC 4 24-month females where the day 4 expression is the highest. Expression levels are similar between 1-month males and females. On the other hand, the 24-month females show significantly higher HDAC 5 expression than their 24-month male counterparts. Viewing fig 10, the expression levels between 1-month and 24-month females are similar aside from day 4 which is higher in the

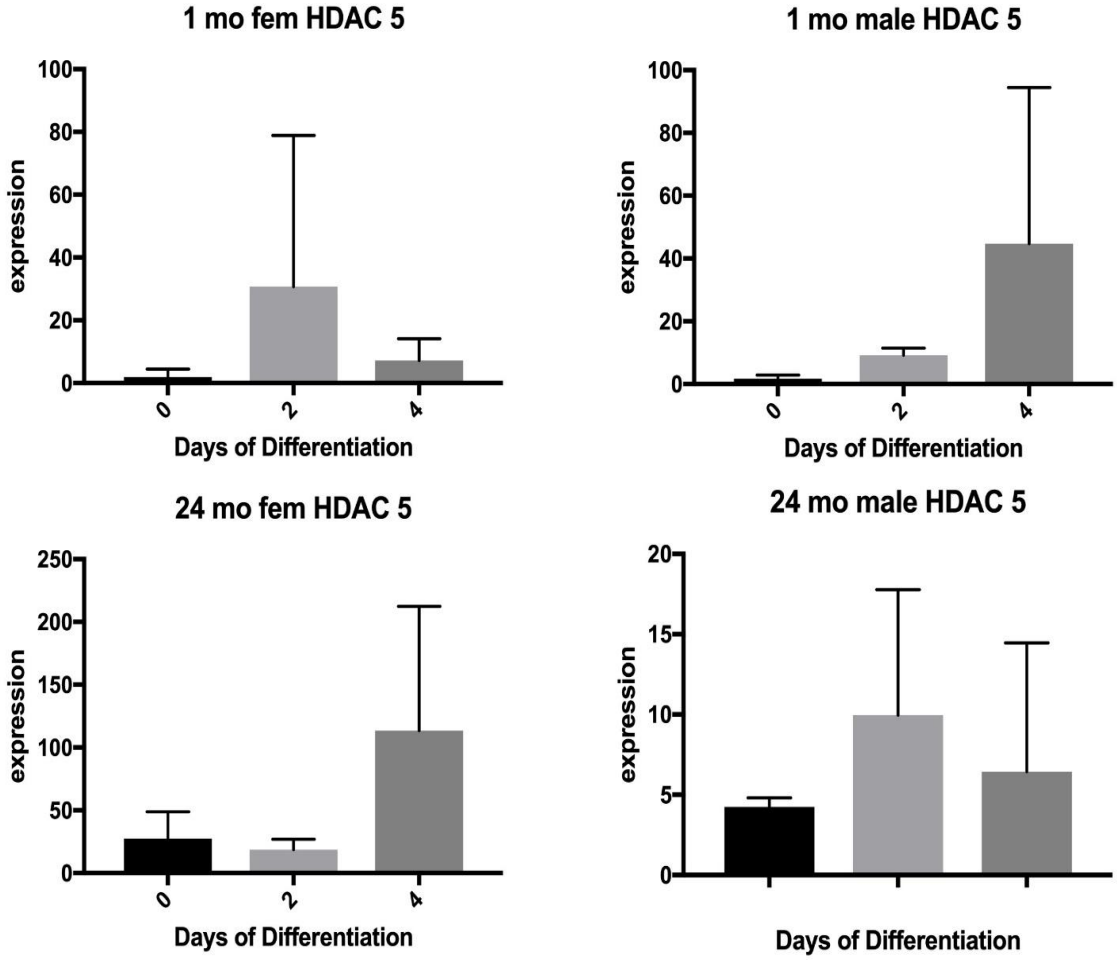

Fig 9: HDAC 5 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average depicted by bar +/- standard deviation.

24-month samples. In the 1-month males, there is significantly higher day 4 expression than that of the 24-month males.

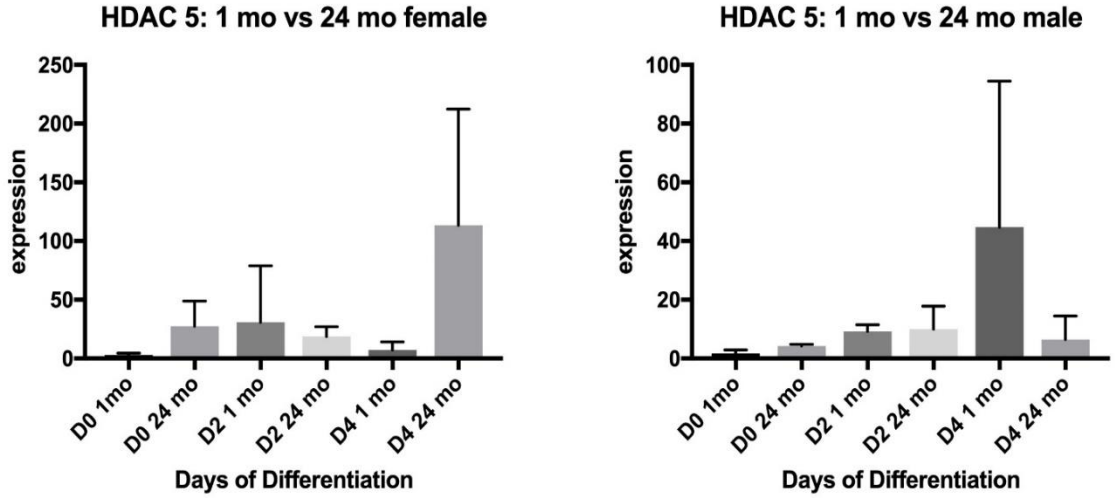

Fig 10: Comparison of 1 and 24-month females and males of HDAC 5. Average +/- standard deviation

HDAC 6:

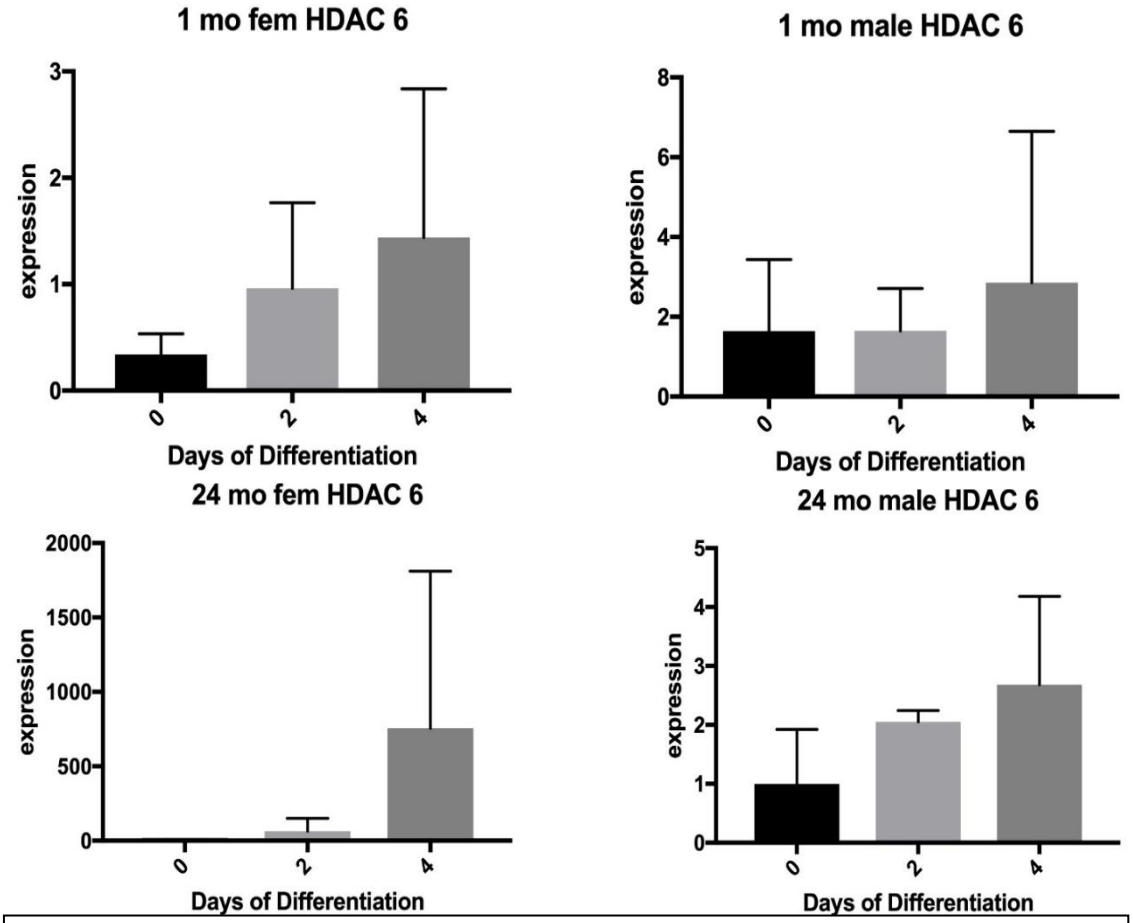

Fig 11: HDAC 6 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average +/- standard

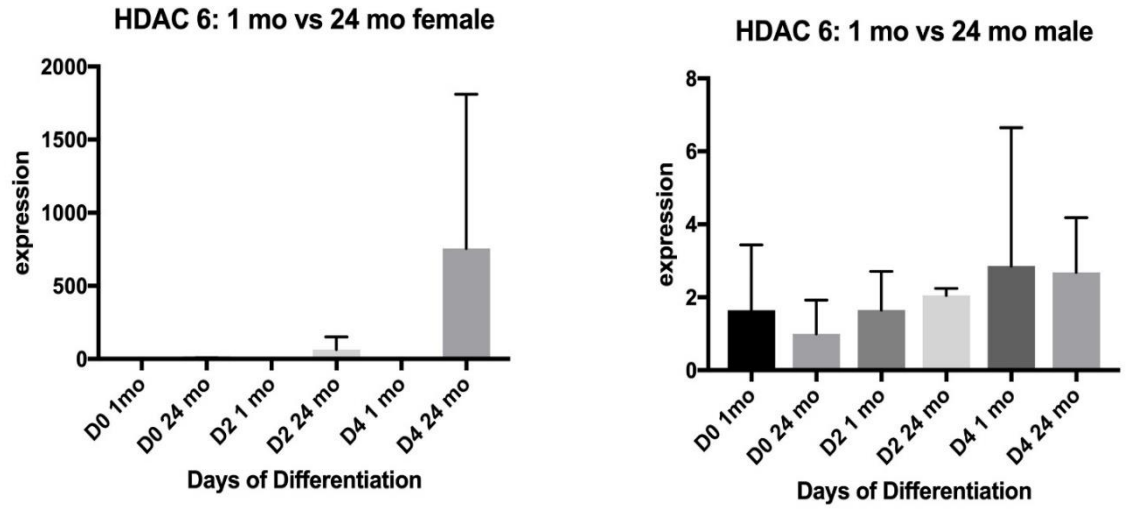

Fig 12: Comparison of 1 and 24-month females and males of HDAC 6. Average +/- standard deviation

In our HDAC6 expression levels, the 1-month females have overall less expression than their male counterparts (fig 11). The 1-month males also had very little change in expression between day 0, 2 and 4. When comparing the age groups (Fig 12), it is apparent that HDAC 6 is expressed significantly higher in 24-month females than 1-month females. On the male side, expression values are relatively similar to each other.

HDAC 7:

Overall, there was very little expression of HDAC 7 in the 1-month female group (Fig 13). Expression was negligible on day 0, then increased on day 2 and day 4. This expression follows a similar pattern to most 1-month females discussed thus far. The 1-month group has similar expression levels on days 0 and 2, and 4. The 24-month females have significantly higher expression that their 1-month

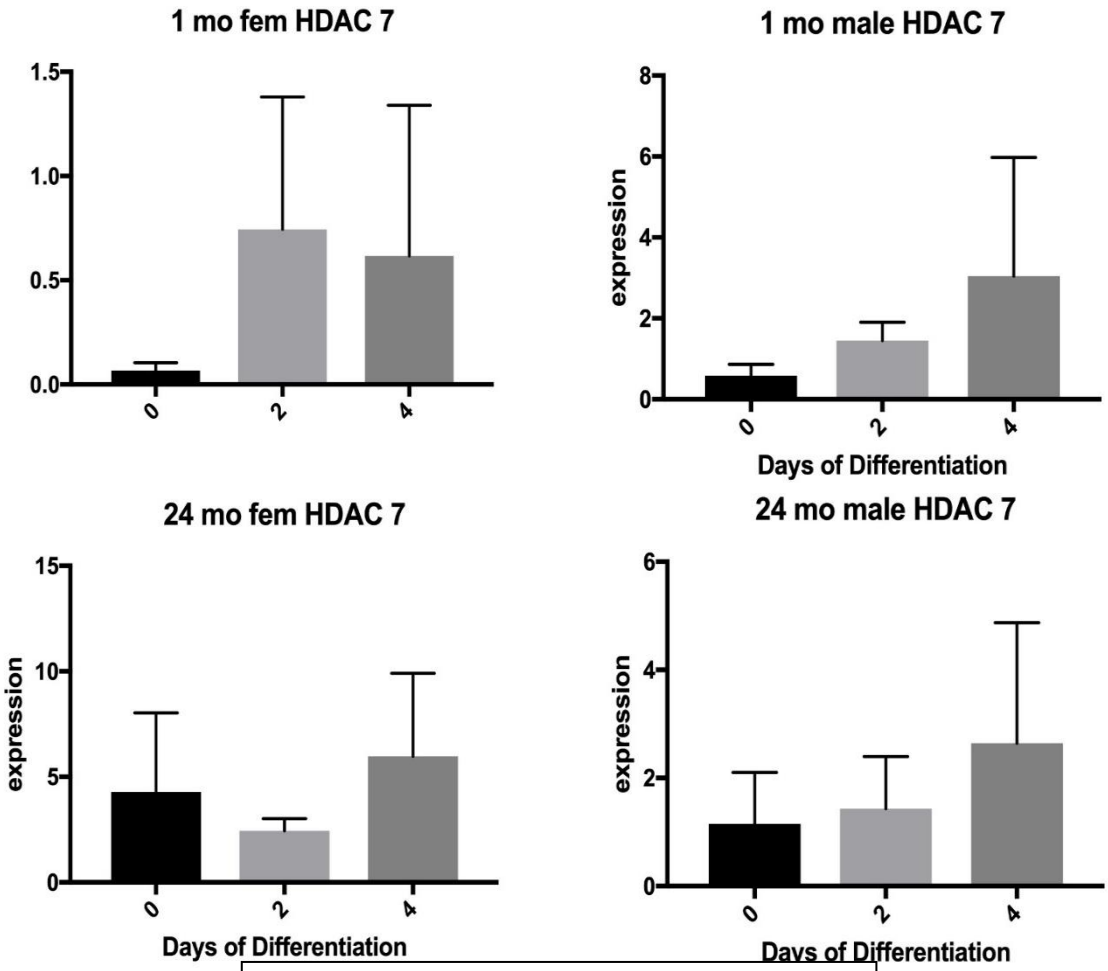

Fig 13: HDAC 7 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average +/- standard deviation

counterparts (Fig 14). The 24-month males have a gradual increase in expression from day 0-4 and have expression values similar to that of the 1-month males (Fig 14).

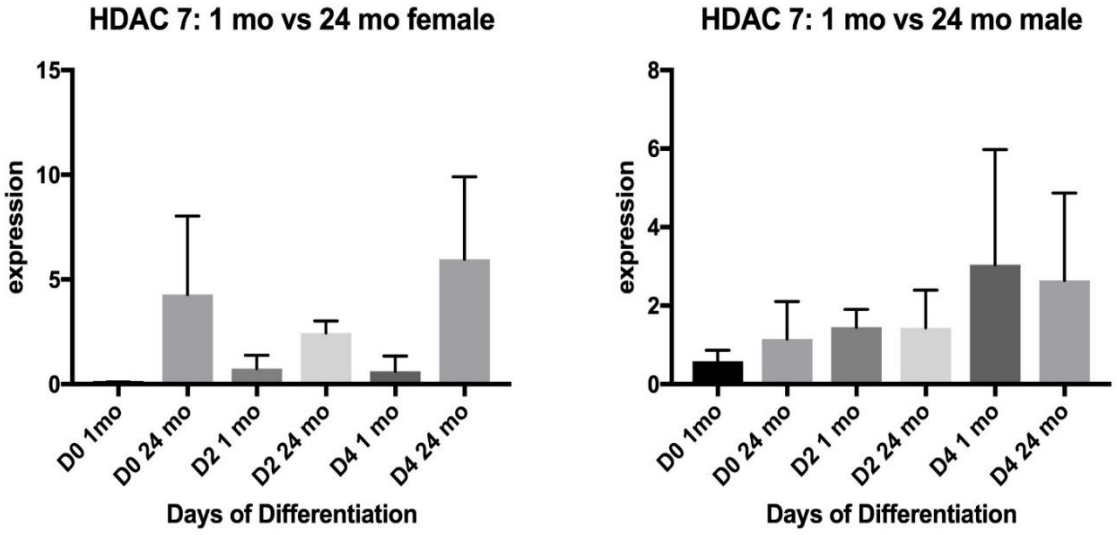

Fig 14: Comparison of 1 and 24-month females and males of HDAC 7 displaying average +/- standard deviation

HDAC 8:

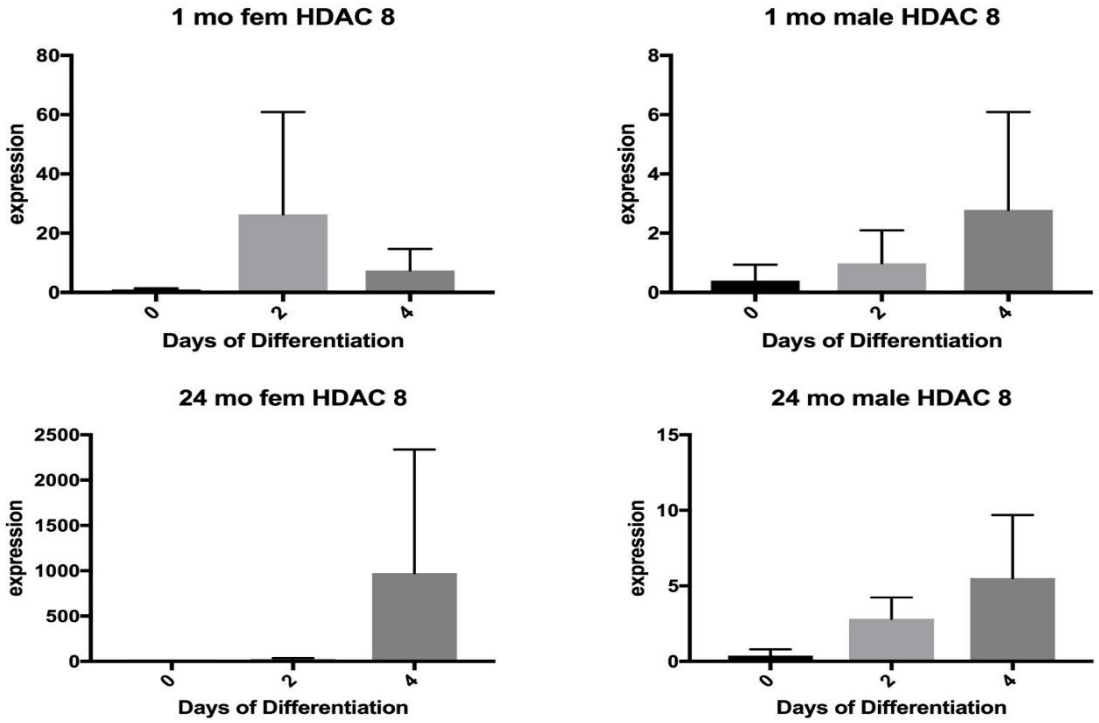

Fig 15: HDAC 8 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average depicted +/- standard deviation

Within the 1-month female group there was very little expression at day 0 while the average expression of day 2 was higher. This followed the same pattern as most HDACs within 1-month female groupings. The HDAC 8 expression in 1-month

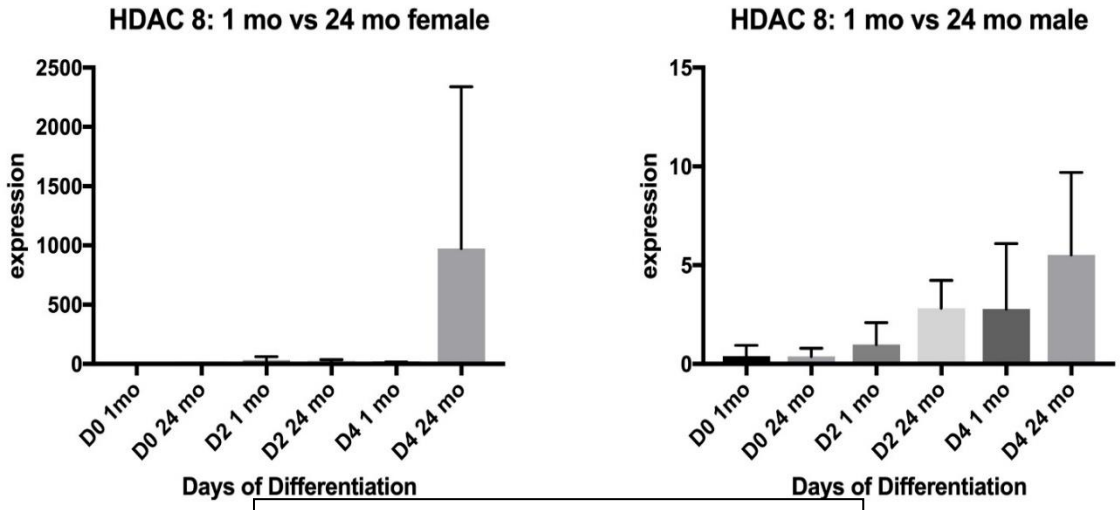

Fig 16: Comparison of 1 and 24-month females and males of HDAC 8 with average +/- standard deviation

males had steady increase from day 0 to day 4, again following the pattern displayed by most HDACs to this point. There is similar expression at day 0

between 1-month and 24-month males, but there is higher expression at days 2 and 4 in the 24-month male group than the 1-month group (Fig 16).

HDAC 9:

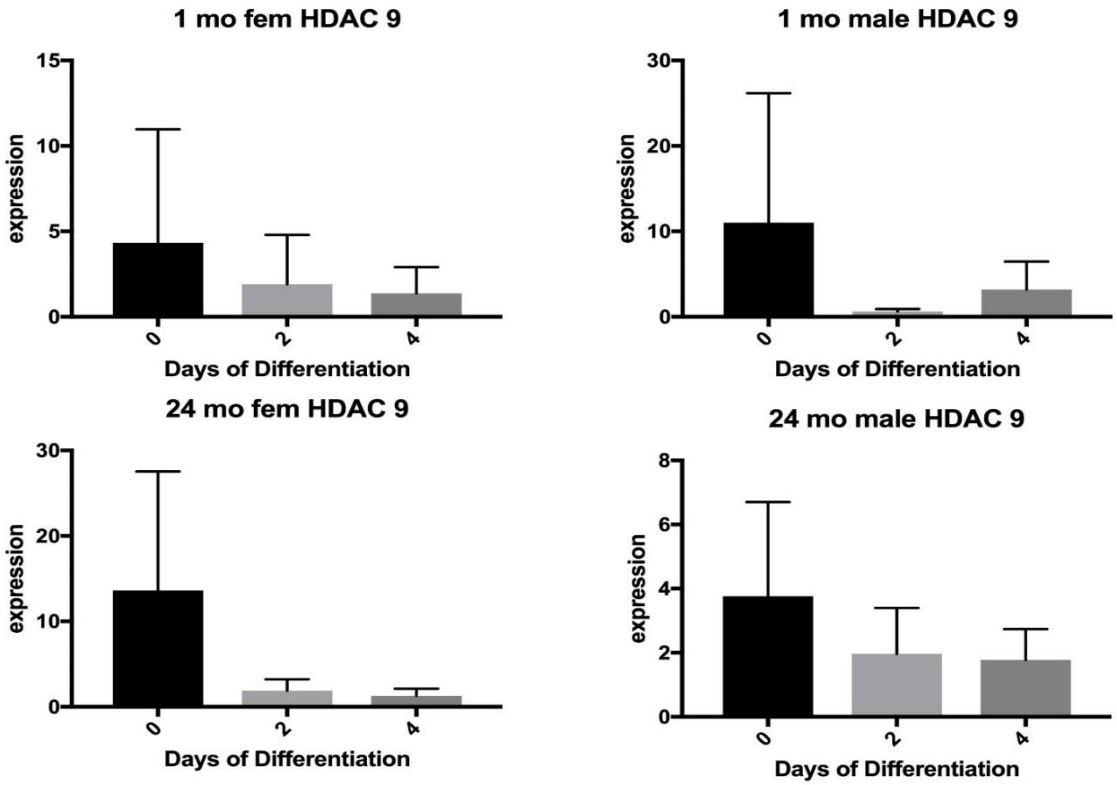

Fig 17: HDAC 9 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average +/- standard deviation

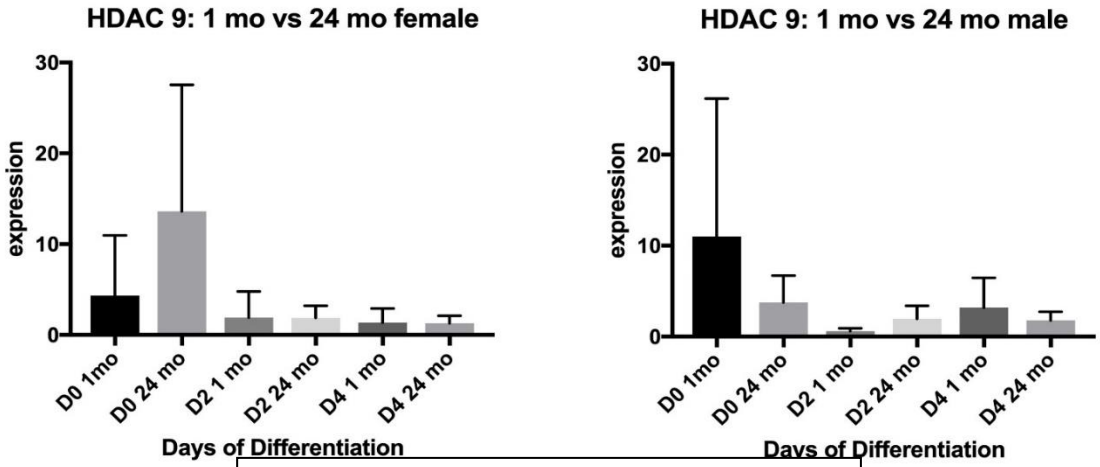

Fig 18: Comparison of 1 and 24-month females and males of HDAC 9. Average +/- standard deviation

HDAC 9 expression in 1-month females is highest at 1 then decreases for days 2 and 4. In 1-month males, expression is very high on day 0, it then drops at day 2 and day 4. The expression of HDAC 9 in the 24-month females is highest at day 0, then drops to low levels of expression at days 2 and 4. Expression of HDAC 9 is also highest at day 0 for the 24-month males, though it is significantly less than that of the 24-month females (Fig 17). Figure 18 shows that, aside from day 0, both 1-month and 24-month females have similar expression.

HDAC 10:

HDAC 10 (Fig 19) is most highly expressed at day 2 in the 1-month females and follows similar pattern to most 1-month females. In the 1-month males, HDAC 10 is most highly expressed at day 4 and lowest at day 0, which is consistent with the majority of HDACs. Overall, there is higher expression in 1-month females than 1-month males. HDAC 9 expression within the 24-month males follows the same

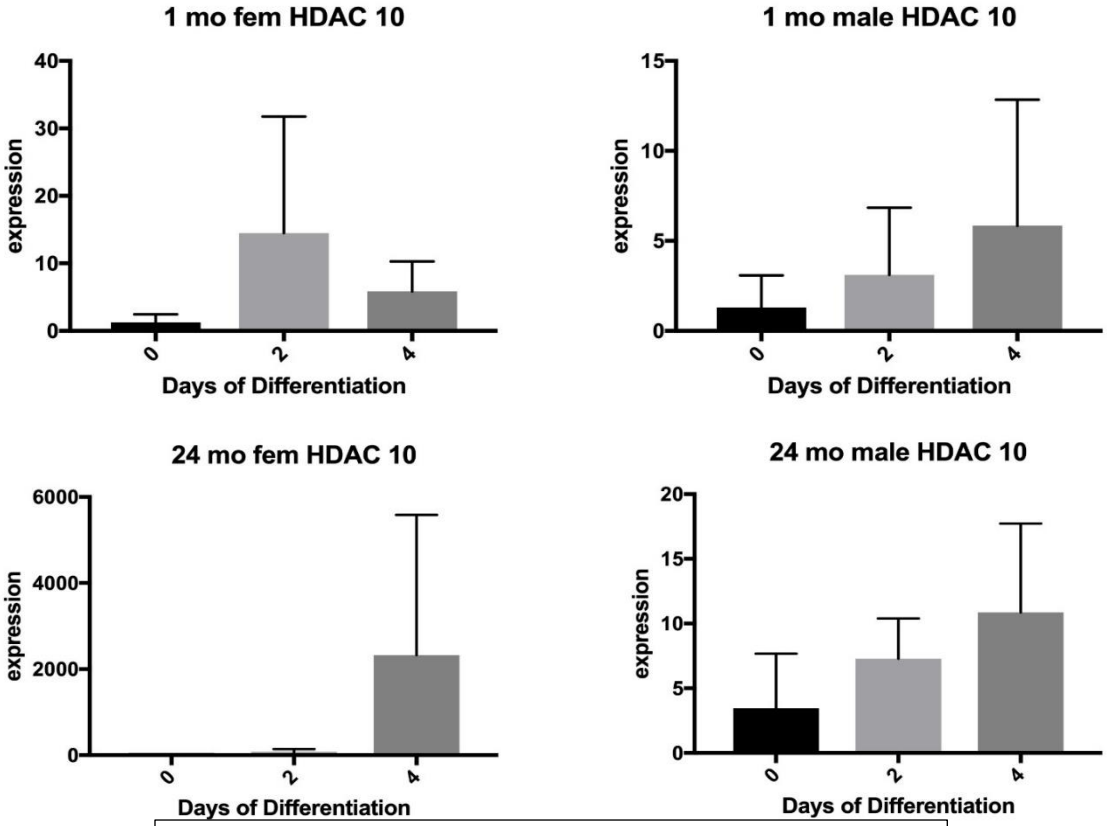

Fig 19: HDAC 10 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average +/- standard deviation

pattern as the 1-month group displaying lowest expression at day 0 and highest expression at day 4. Figure 20 demonstrates that expression is higher in both 24-month females and 24-month males than their 1-month counterparts.

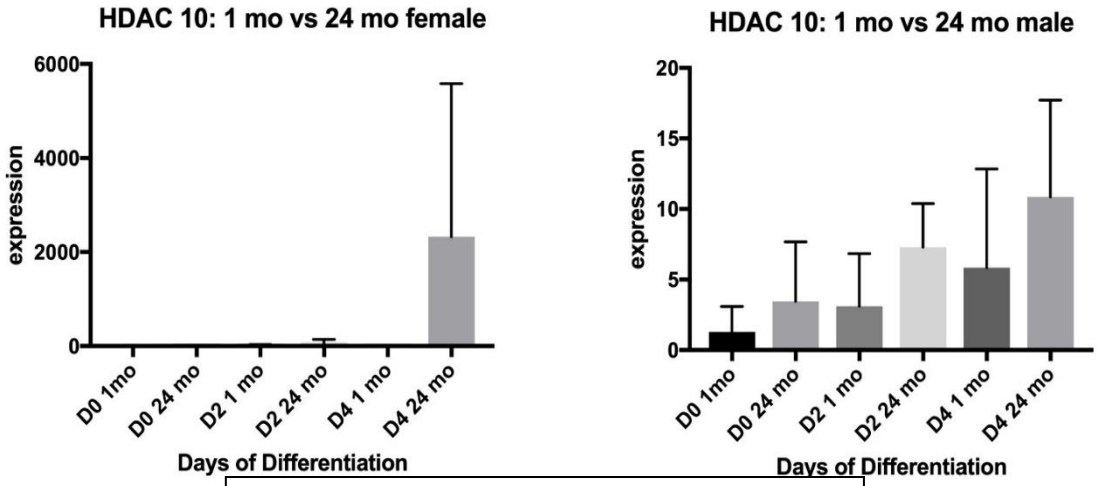

Fig 20: Comparison of 1 and 24-month females and males of HDAC 10 with average +/- standard deviation

HDAC 11:

Overall, HDAC 11 is negligibly expressed at days 0 and 2 but has higher expression at day 4 (Fig21). There Is significantly more expression in the 1-month males than the 24-month males at day 4 of differentiation (P=.0187) (Fig 22).

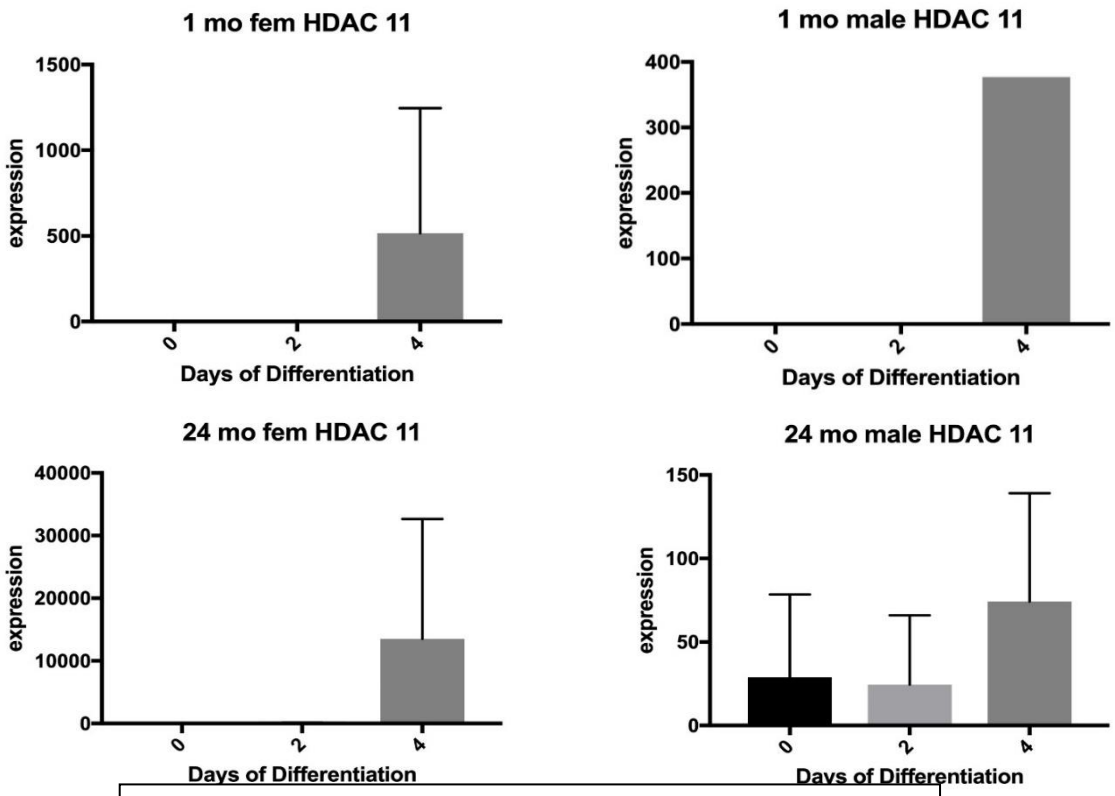

Fig 21: HDAC 11 expression of 1-month females, 1-month males, 24-month females, 24-month males. Data displayed in bar graph form with average +/- standard deviation

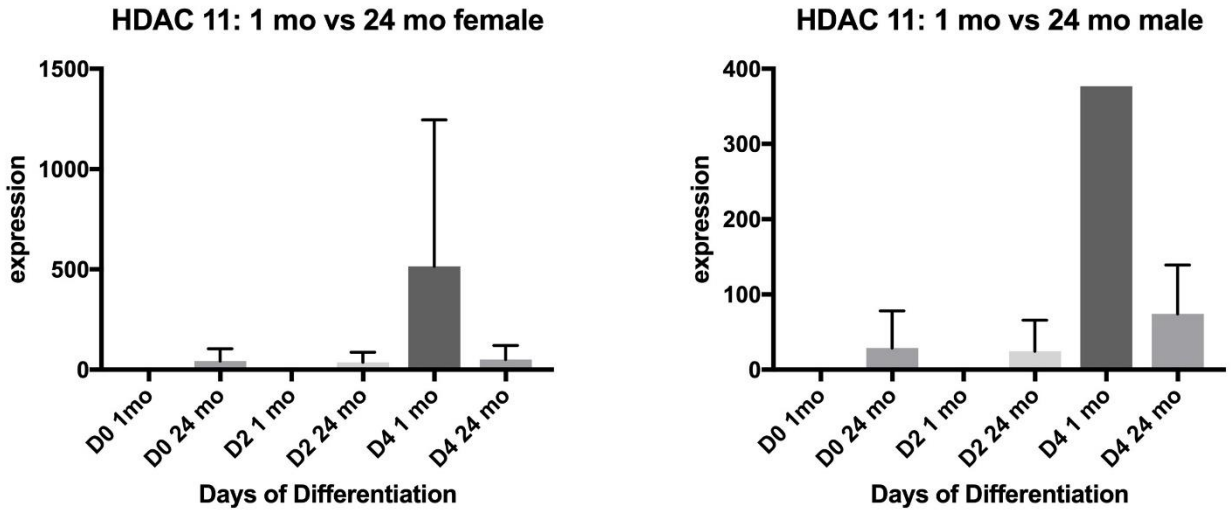

Fig 22: Comparison of 1 and 24-month females and males of HDAC 11 with average +/- standard deviation

Discussion

This study serves as a pilot project aimed at analyzing expression of HDACs from young and old mice. To the knowledge of the author, there are no studies comparing HDAC expression within osteoclasts at different ages. There were 3 main aims of this study. First, we aimed to examine expression levels of class I, II, and IV HDACs in male and female subjects representing young and old. Second, using the resultant expression levels, we aimed to provide a direct comparison of HDAC expression of young age and advanced age. The third aim was to establish a database for reference in future studies.

As previously discussed, HDACs have been linked to bone metabolism as well as many other functions (Boyle 2003). HDACs are currently being explored as a target in diseases such as cancer (Bradley 2015). They have also been hypothesized to have regulatory roles that may be involved in age-related bone loss and/or osteoporosis.

Given the limited understanding of HDAC function and its role in aging, the information gained from this study can provide a baseline for many future studies regarding aging and bone metabolism. The aim is that this data can lead to future studies on the role of HDACs in osteoporosis. Both osteoporosis and periodontitis

are chronic, degenerative osseous conditions. Osteoporosis is marked by pathologic decrease in bone mass. Though this can occur in both males and females as well as young and elderly subjects, it is most closely associated with post-menopausal women. It is also important to realize that, even in the absence of pathologic osteoporosis, bone mass is lost in both sexes throughout aging. Though not considered a lethal condition, osteoporosis is highly prevalent, accounts for large medical care costs, and can negatively impact quality of life (Kanis 2019). As such, osteoporosis is major point of research in the pharmaceutical industry and medications such as bisphosphonates have become mainstream for treatment of osteoporosis. These medications are imperfect, however, as they come with side effects and increased risk of osteonecrosis with trauma. It is important to continue searching for new therapy targets for this condition. On the other hand, periodontitis is highly prevalent throughout the world population and shares similar pathology to osteoporosis. It is characterized by loss in the supporting bone of the mandibular and maxillary alveolus. Periodontitis is a bacterial induced inflammatory condition that leads to progressive bone loss, while osteoporosis is believed to be a product of hormone alteration and aging (Cantley 2016). What they both have in common is the strong association with age related severity if not diagnosed and managed early.

As I discussed in the introduction, bone metabolism is a product of osteoclast function and regulation of resorption/deposition and osteoblast regulation of bone formation. Differentiation of osteoclasts is controlled by the RANK/RANKL stimulating function. RANK is a receptor expressed on the surfaces of hematopoietic stem cells and osteoclasts/osteoclast precursors. RANKL is the effector ligand and is typically produced and expressed by osteoblasts and osteocytes. When RANKL binds to RANK, the result is osteoclast differentiation through targeting of NFATc1 (Park-Min 2017). HDACs and HATs are responsible for gene accessibility, allowing for the uncoiling and recoiling of DNA on histones, thus facilitating transcription. HDACs remove an acetyl group, causing the DNA to “coil” while HATs add an acetyl group leading to “un-coiling”. We hypothesized HDAC expression would be lower in 24-month mice than 1-month mice.

When viewing the expression levels of HDAC 1 during differentiation, the 1-month subjects display an opposite pattern of expression than the 24-month subjects. There is also much higher expression of HDAC 1 in advanced age males. This is in line with previous studies which have suggest that HDAC 1 promotes osteoclast function (Faulkner 2019). Further, there is similar expression between males and females overall, which does not support my hypothesis.

HDAC 2 is universally expressed the lowest at day 0, which might suggest it has little role in the induction of differentiation. Though the elevated levels at days 2 and 4 suggest it might have a role in regulating osteoclast fusion. This appears in line with findings of Faulkner 2019 and supports the idea that HDAC 2 has a role inhibiting osteoclast differentiation.

HDAC 3 is most highly expressed in 1-month females at day 2, though there might be an outlier in the data, and is negligibly expressed at day 0. The 24-month females have highest expression at day 4. This suggests that HDAC 3 may be important for facilitating differentiation (1-month samples) and fusion (24-month samples). There is also less overall production of HDAC 3 in the 24-month samples which fits with my hypothesis that expression is lower in advanced age.

HDAC 4 expression is similar between 1-month males and females and displays the same pattern with highest expression at day 2. On the other hand, the 24-month females have more expression of HDAC 4 than their male counterparts, which supports my hypothesis. Higher expression of HDAC 4 at day 0 in 24-month females suggests that HDAC 4 might play a role in induction of osteoclast differentiation. Comparison of differentiation day 4 in the 1-month females and 24-month females yielded a significant difference and a p value of 0.0273. However, it important to point out that there are what appear to be extreme outliers skewing the calculated average. As such, I don't believe this is a true representation of the relationship. There is higher expression in 24-month samples that 1-mo samples, which does not support my hypothesis.

In HDAC 5, there is higher expression in females than males, which supports my second hypothesis, however, expression is higher in the 24-month samples than 1-month samples which does not support my first hypothesis.

Again, HDAC 7 is more highly expressed in 1-month males than 1-month females. There is also much higher HDAC 7 expression in 24-month females than 1-month females, which does not support my hypothesis. Also, there does not appear to be a real difference in expression between males and females.

HDAC 8 expression values reveal an outlier for day 4 for the 24-month females which skews the data. In the 1-month samples, expression is lowest at day 0 and is highest in the females at day 2 and highest in the males at day 4. There is generally, thought mildly higher expression in the 24-month samples than the 1-month samples which does not support my hypothesis. However, the 1-month females have higher expression than the 1-month males, which does support my second hypothesis.

Day 0 expression of HDAC 9 is higher in 24-month females than that of the 1-month, while the remaining days' expression is similar. It is interesting that is significant expression on day 0 for all test groups for HDAC 9. This suggest that HDAC 9 might have a role in initiation of osteoclast differentiation. The difference between 24-month females and 1-month females does not support my hypothesis, but it supported by the males, which have lower expression in the 24-month group.

There is higher expression of HDAC 10 in 24-month males than 1-month males, which does not support my first hypothesis. Due to outliers and large scales, viewing differences between data sets is difficult and inconclusive. Further testing and possibly elimination of the outliers would be required for more detailed understanding of these results.

HDAC 11 presented very little day 0 and 2 expression in the 1-month females, 1-month males, and 24-month females. However, each of these groups was characterized by a massive jump on day 4, though the extreme levels presented in the data suggest an outlier. Previous studies have suggested that HDAC 11 has

little activity at the first stages of differentiation, and this data supports this. It is possible that HDAC 11 plays a role in fusion during differentiation. It is curious, however, that the 24-month male group appears to have fairly high expression of HDAC 11 at all stages of differentiation. Comparison of differentiation day 4 between 1-month and 24-month males yielded a significant difference with a *p* value of 0.0187. However, this significant difference is likely the product of an inaccurate average of the 1-month sample because we could only successfully obtain one data point. This makes the calculation of a true average impossible. It is possible that the one value we obtained is an outlier seeing how high it is. With this in mind, I do not think this significant value is a true indication of the relationship.

The first goal of this study was to assess HDAC expression in males and females of young and advanced age. Further, we aimed to create a framework database for classification of Class I, II, and IV HDACs and . It appears that the presented data is a good start, but due to areas of missing data and outliers, the *n* of each test group is too small to provide a truly representative data set. To make this data more reliable, I would confer with a biostatistician in the future to determine an appropriate *n* to yield an accurate depiction. A major limitation with increasing the *n* is the cost associated with 24-month mice. To truly establish an age relationship regarding HDACs and epigenetics, more advanced age mice will be required. Waiting 24 months to age mice delays testing and has a high cost. To this point, it is much easier and cost friendly to test 1-month samples. It would also be valuable in the future to run each test set 3 times. This would allow us to create a truly representative average expression of each individual. I think this is important because there can be major variations and fluctuations in testing samples. This may be caused by pipetting technique or even contamination. The main limitation to running each test set 3 times is the amount of cDNA required. There is a limit to how much usable cDNA can be fabricated from a single harvest of RNA; I must also acknowledge there can be great error and inconsistency in this test model. Pipettes can vary in accuracy and reproducibility, particularly at the low increments used in this study. There is also potential for contamination and user error in the

model. Another consideration is the quality of cDNA samples. There can be variance in the strength and quality of cDNA. This can lead to non-representative expression levels. As seen in the collected data, there are numerous extreme outliers. These can be attributed to user error, poor accuracy of equipment, or contamination. Despite the outliers that are clearly impacting the calculated averages, I believe that if the outliers are removed and the same calculations are made, the result will show more areas of significance. It is also possible that without the outliers, the data is more representative of the target sample pool.

We also wanted to compare expression levels in males and females. The collected data has such high ranges and variations, it is impossible to make any clear conclusions. However, the data present does suggest that the initial hypothesis that females would have higher expression than their male counterparts appear to be false. The same is true for my other hypothesis which stated that there would be decreased HDAC expression in 24-month samples than 1-month samples. The current data in fact suggests that there is elevated HDAC expression in the advanced age group. This is important as our basic understanding of HDACs says that when highly expressed they play an inhibitory role by preventing transcription. We wrongly assumed that if levels of HDAC decreased, then osteoclast differentiation would increase in advanced age. It has become clearer that is not this straight forward or general of a relationship. This data suggests that HDAC expression varies highly between HDAC types. Some HDACs increase in expression through age, while others appear to decrease in expression. Still, other HDACs do not appear to be significantly affected by age. At this point, it is likely necessary to reclassify HDACs individually. It might also be misguided to speak to the inhibitory or promotor function of HDACs regarding overall osteoclast function and even the number of osteoclasts present. Based on previous studies and the data presented here, it is more likely that each individual HDAC functions in a different stage of differentiation (Bradley 2015). We know that HDACs are gene repressors or “erasers”. However, that is not always a negative function. For example, repressing of one gene could cause an increase in osteoclast

differentiation, while repressing of another gene could decrease the overall osteoclast function.

We aimed to create a base understanding of HDAC expression, which may be used in further studies. HDACs are currently being targeted in therapy for numerous conditions. We are hoping that this data can be used to focus in on diseases of bone metabolism such as osteoporosis and periodontitis. No current pharmaceuticals are targeting HDACs for these conditions and we believe that with further studying, HDACs may provide a feasible therapeutic target for these conditions. There is a major limitation within this study, however, which should be addressed in further studies to truly identify the link between HDACs and osteoporosis. Mice have a very limited life span and a different reproductive age cycle than humans. Ultimately, mice do not experience menopause the way human females do. This means that female mice maintain relatively normal hormonal levels through their lifespans. Keeping this in mind, the data in this study is only applicable to normal age-related bone mass loss. This is supported by the presented data because there is generally little difference between males and females. A further study needs to use cDNA created from OVX (ovariectomy) mice to again test HDAC expression. Adding this component, I would expect to see appreciable differences in expression between males and females of advanced age. This study could hold the key to unlocking some understanding and a potential drug target for osteoporosis. I would also recommend progressing to a human model as well. There are a few studies of osteoclast precursor cells harvested from human blood samples that suggest this may be a viable study medium to gain some understanding of HDAC expression in humans.

Another future study should center around the concept of periodontitis. This study utilized cells harvested from the long bones of mice legs. Long bones possess strong hematopoietic capacity and have different regulatory actions than flat bones such as the jaw. I would like to test HDAC expression of cDNA harvested from mice jaws of mice both affected and not affected by periodontitis. The data presented in this study is relevant to periodontitis because, much like normal bone

loss with aging, periodontitis is subject to an age-related equation of bone loss (Cantley 2016). Knowledge about how aging affects functions of osteoclasts could provide further insight into the way periodontitis is treated and diagnosed. A recent study observing HDAC expression in gingival tissues found that HDACs 1, 5, 8, and 9 have elevated expression in tissue samples taken from patients diagnosed with chronic periodontitis (Cantley 2016). Meanwhile, a more recent study linking HDACs to periodontitis was able to demonstrate the regulatory role of HDAC 3 in the inflammatory process of gingival fibroblasts (Lagosz 2020). Both of these studies have centered around HDAC presence and function in gingival tissues, but there appears to be very limited information regarding HDAC functions in jaw osteoclasts.

I came into this study believing that HDACs were more black and white, either playing a positive or negative role in osteoclast differentiation, but I now believe this is false. It is more likely that each class plays a separate role. Boyle 2003 suggested that class II and IV HDACs are osteoprotective, preventing pathologic resorption. Pham 2011 indicated that class I HDACs may promote osteoclast activity. If we take these to be true, we expect that 24-month mice to have decreased levels of class II and IV HDACs compared to 1-month mice. It would also be understood that class I HDACs would be more highly expressed in 24-month mice compared to 1-month mice. Instead, it is very clear that it relates to the specific HDAC, and more importantly, which gene is being repressed.

In conclusion, this study serves as a pilot study and classification database for class I, II, and IV HDAC expression in osteoclasts harvested from mice long bone. The first aim of this study was to assess the overall HDAC expression in osteoclasts to identify potential targets of future studies. This aim was met successfully by the study. I do believe the data presented is not strong enough with the current test subject number. To key in on specific HDACs, a greater *n* is recommended. This data does suggest, however, that each HDAC may a different role within osteoclasts, and some might not play a significant role at all. The varied expression within established HDAC classification suggests that a classification

system based on molecular structure is inadequate for understanding function. The second aim was to assess the effect of aging on HDAC expression to establish a link between aging and bone loss. This study can pave the way for examining mechanisms for age-related bone degradation conditions such as osteoporosis and periodontitis. I hypothesized that HDAC expression would be higher in the advanced age group. This hypothesis was neither proven, nor disproven as some HDACs exhibited this pattern, while others did not. I further hypothesized that female subjects would display greater HDAC expression than their male counter parts. Again, this was neither proven nor disproven. Some HDACs did display greater expression in females than males, while others had very similar expression levels. This topic requires further testing. This data can be used to begin in-depth studies of individual HDACs which appear to have roles in the differentiation of osteoclasts.

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