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FURTHER CHARACTERIZATION OF THE SKELETAL PHENOTYPE IN A HURLERS SYNDROME MOUSE MODEL AND THE ETHICAL TREATMENT OF CHILDREN IN MEDICINE

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Cell and Molecular Biology

By

Anna Marie McWoods

May 2019

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FURTHER CAHRACTERIZATION OF THE SKELETAL PHENOTYPE IN A HURLERS SYNDROME MOUSE MODEL AND THE ETHICAL TREATMENT OF CHILDREN IN MEDICINE

Biomedical Sciences

Missouri State University, May 2019

Master of Science

Anna Marie McWoods

ABSTRACT

Mucopolysaccharidosis type I (MPS I) is a rare, autosomal recessive disorder caused by the deficiency of the lysosomal enzyme α-L-iduronidase (IDUA). Absence of IDUA results in the accumulation of dermatan and heparin sulfate and ultimately causes multi-system dysfunction. The most severe form of MPS I is Hurlers syndrome, a rapidly progressive disorder that, if left untreated, is fatal. Current treatment options for diagnosed individuals includes hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). These treatments are able to ameliorate the majority of symptoms with the exception of the bone phenotype. This investigation aimed to further characterize the bone phenotype in a knock- in mouse model (IDUA-W392X), containing a nonsense mutation analogous to the IDUA mutation commonly found in human Hurlers syndrome patients. To accomplish this the organic portion of the bone was analyzed. The most abundant bone matrix protein, type I collagen, was indirectly quantified in wild type, heterozygous, and mice without IDUA activity. Findings indicate significantly elevated type I collagen content and bone mass in male IDUA-W392X mice. In order to inspect the extent of IDUA deficiency on bone resorbing osteoclasts, a protocol was establish to examine their activity. Previous investigations have indicated impaired bone remodeling and a decrease in expression of biomarkers for osteoclast differentiation. This study examined protein localization of RANKL, the stimulus responsible for initiating resorption, through immunohistochemical staining of decalcified bone tissue. Finally, this study further defined the rights and protections of children involved clinical research. This was accomplished through developing the discussion of maintaining patient autonomy, retaining open communication between clinicians and patients, and the process of consenting to clinical research.

KEYWORDS: Mucopolysaccharidosis, Hurler syndrome, bone remodeling, type I collagen, hydroxyproline, osteoclast, immunohistochemistry, pediatric bioethics

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May 2019

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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I dedicate this thesis to my father and mother, Emmett Jr. and Rosalyn McWoods. You have played such an integral roles in my life. Thank you for always being there for me and supporting me in my pursuit of a higher education. I love you and appreciate you from the bottom of my heart.

I also dedicate this thesis to my deceased grandparents (Jesse and Vesta Couch and Emmett Sr. and Mary "Chi Chi" McWoods). Thank you for fighting for our civil rights. I would not be able to stand here today without the sacrifices that you made. I wish you could be here to celebrate this accomplishment with us! "U is a part of Us!"

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INTRODUCTION

Hurlers Syndrome

Mucopolysaccaridoses (MPS) encompasses a group of lysosomal storage disorders that arises due to the deficiency of enzymes required to degrade mucopolysaccharides [1,2]. This deficiency results in the accumulation of mucopolysaccharides, or glycosaminoglycans (GAG), within the lysosomes and extracellular matrices of various body tissues [3,4]. The buildup of GAGs ultimately results in abnormal changes in the extracellular matrix that cause cellular dysfunction. There are a large variety of enzymes that degrade GAGs and the deficient enzyme will designate the subset of MPS. In MPS I, α- L- iduronidase (IDUA) is deficient and results in the accumulation the GAGS dermatan and heparin sulfate within lysosomes.

The amount of residual IDUA activity designates the severity of MPS I phenotype (Table 1) [1, 5]. Hurlers syndrome, is the most severe form of MPS I and is a rapidly progressive disorder characterized by multisystem failure [3]. If left untreated, this severe phenotype will result in death at an early age. The intermediate form of MPS I is Hurler-Scheie. Hurler-Scheie is not as progressive but still leads to multisystem organ failure in the late teens or early twenties if untreated. With Scheie, affected individuals experience milder symptoms and if left untreated, a normal life span is expected [1, 6, 7].

Treatment Options

Prior to a formal diagnosis, MPS I individuals commonly undergo corrective surgeries to alleviate disease complications [8]. A wide variety of surgeries are typically performed and include some of the following procedures: ventriculoperitoneal shunts, tendon release, joint

Table 1: Sub-phenotypes of MPS1 Based on Severity Levels and Clinical Indicators

	Severe	Attenuated	
	Hurlers	Hurlers-Scheie	Scheie
Onset and Progression	1 year; rapidly progress	3-4 years	2-12 years; less progressive
Muscle and Skeletal	Course facial features. Spinal deformity Skeletal dysplasia	Skeletal abnormalities and joint stiffness	Joint stiffness and carpel tunnel syndrome
Life Expectancy *(If untreated)*	Death before age of ten.	Death in teens or 20s	Normal life expectancy.

replacement, spinal decompression, and carpal tunnel surgery. These procedures are aimed at improving patient quality of life and are performed primarily during childhood [8]. After receiving a formal diagnosis, patients begin either enzyme replacement therapy or receive a hematopoietic stem cell transplantation.

Hematopoietic stem cell transplantation (HSCT) is the older of the noninvasive therapeutic options. This procedure entails transferring hematopoietic stem cells in either blood or bone marrow from a competent donor into a recipient. The transplanted stem cells should hopefully then result in continuous enzyme expression over a recipient's life time [9]. There are two sub categories for HSCT, bone marrow and umbilical cord blood transplants, each of which comes with their own advantages and disadvantages [4, 10]. With a bone marrow transplant, individuals are at a higher risk for human leukocyte antigen mismatch. Due to the presence of mature cell populations in the bone marrow, the recipient's immune system could reject the transplant [10]. With umbilical cord blood, on the other hand, younger cell populations are present which decreases the likelihood of a transplant rejection. Nevertheless, in order for complete treatment effectiveness, the uncommon practice of fetal diagnosis would be required.

Enzyme replacement therapy (ERT) is the most recently developed therapeutic option for lysosomal storage disorders [11]. This innovative treatment is based on weekly intravenous infusion of recombinant IDUA obtained from human fibroblast or animal cell cultures lines [12]. After ERT, functional IDUA is introduced to body tissues, with the exception of brain and bone tissue. Bio-distribution of the recombinant enzyme within the skeletal system is very restricted, likely due to poor vascular supply resulting in limited enzyme penetration [12]. Conversely, with the central nervous system (CNS), likely the blood brain barrier prevents the recombinant IDUA from reaching the system. If any recombinant IDUA is able to pass the barrier, the minute

amounts are not able to prevent deterioration of the CNS and neurocognitive functions [12]. A humoral immune response can arise due to this treatment and in response, antibodies are produced against the recombinant IDUA. Ultimately, this results in a decrease in GAG clearance levels; however, urinalysis can be utilized to monitor patient urine for increased GAG levels [13].

History of Ethics

Nuremberg Code. Bioethics encompasses the field of ethics involved in biological and medical research. Before guidelines had been set in place, marginalized and incapacitated individuals were commonly subjugated to various experimentations without their consent under the declaration of furthering science and medicine [14]. In the late 1800s, a set of regulations for research involving humans was issued by a Prussian minster. His directive forbad the "treatment" of prisoner without their expressed consent. Several years following this decision, Prussia produced detailed guidelines for non-therapeutic research. This caused political and public debate on the legality of what is consent and prompted the development of guidelines for human experimentation by various countries [14]. Nevertheless, it was not until the conclusion of World War II, when the atrocities experienced by prisoners in Nazi concentration camps was made known to the outside world, that the debate of what is acceptable in biological and medical research became a global discussion. Publicizing the events in Nazi research facilities prompted the formation of universal guidelines for research involving human subjects [15].

During the Nuremberg trials, the Nazi doctors were accused of performing sadistic and torturous human experiments on prisoners of the Germanic State. The judges called upon expert witnesses, three of whom made statements that aided the formation of the Nuremberg Code [19].

The first of these individuals was Leo Alexander, a physician in the US Army Medical Corp. Alexander was charged with gathering evidence and before the trials began, provided the committee with a memorandum title " Ethical and Non-Ethical Experimentation on Human Beings" [15,16]. In this document, Alexander identified three requirements for conduct during human experiments: the rights of the subject, the physician's duty as described in the Hippocratic Oath, and good research practices. As the trials continued, Alexander provided even more specified conditions for what is legal and permissible in experiments with human subjects.

Werner Leibbrand, a German psychiatrist and medical historian, began to openly debate the medical ethics discussed during the Nuremberg trials. He condemned the Nazis doctors and stated that German physicians thought of patients as "mere [objects], like a mail package" [15]. During the cross examination of the evidence, the German defense attorneys countered that the Allied Nations were conducting similar experiments, specifically the malaria experiments previously performed in the United States on prison inmates. The defense lawyers refuted that the concentration camp experiments were a necessary evil and were conducted for the betterment of the Germanic nation [15, 17]. During this debate Leibbrand replied that "the state could order the deadly experiments on human subjects but the physicians remained responsible for [not] carrying them out" [15, 17]. This statement led to physiological experiments becoming the center for the rest of proceedings.

Andrew Ivy, was nominated by the American Medical Association to serve as medical advisor during Nuremberg trials. He presented three principles which were included in a document titled "Principles of Ethics Concerning Experimentation with Human Beings". These principles discussed that experiments should be conducted by qualified individuals on consenting subjects only when evidence from animal experimentation provides substantial data suggesting

that a human model would be the next course of action [15]. The expert testimonies provided by Alexander, Leibbrand, and Ivy helped to confirm that universal principles needed to be established for protecting the rights of human subjects in research. As a result, at the conclusion of the trials, ten principles that came to be known as the Nuremberg Code were established to protect against the horrors observed at the conclusion of World War II (Figure 1) [18].

Declarations of Geneva and Helsinki. Following the conclusion of the Nuremberg trials, a group of physicians met in collaboration to prevent the recurrence of the atrocities of the Nazi experiments. This international team was named the World Medical Association (WMA) and was established in 1945. Together the WMA worked to provide a code of medical ethics that provided guidelines that medical professionals should all hold themselves to [19]. These guidelines became known as the Declaration of Geneva, after the city in which the meetings were held. The Declaration, not only outlined the duties of physicians but also clarified what practices were deemed unethical in the medical profession. At the end of the document, the committee included a pledge, similar to the Hippocratic Oath, that was issued as a charge to their peers.

Though the Declaration of Geneva had good intentions, there was one major drawback. Due to the language used, the entire document could be left up to personal interpretation [19]. To remedy this, the WMA reconvened in 1953. At the conclusion of this meeting, another document was drafted, "Ethical Principles for Medical Research Involving Human Subjects". Similar to its predecessor, the document was renamed after the city where the meeting was held Helsinki. In less than two thousand words, the WMA was able to lay out ethical guidelines for physicians and medical researchers and has been revised 7 times since its conception [20].

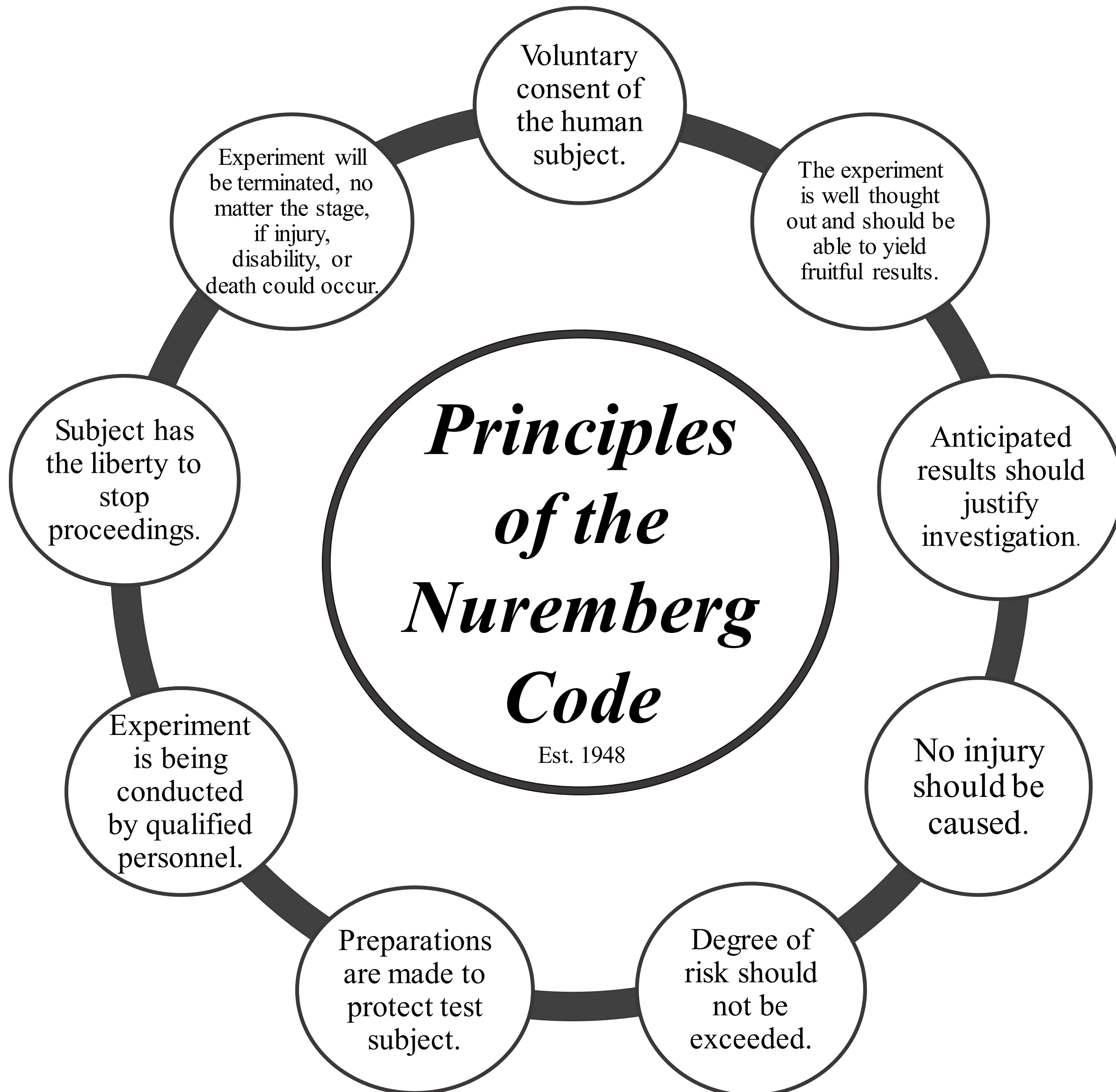

Figure 1: Principles of the Nuremberg Code. The ten principles established at the conclusion of the Nuremberg trials. The Nuremberg code would go on to impact researchers across the globe, who previously thought that this ethical code was already implicit in their work.

The American Medical Association (AMA) played an important role in the Nuremberg trials. They were contacted by the American prosecutors and sent one of their members, Andrew Ivy, to serve as medical advisors during proceedings. The conclusion of the Nuremberg trials prompted the refinement and reorganization of the American *Code of Medical Ethics* [22]. After many year of revising, the key principles were distilled down into 10 statements that encompassed the core values and commitments of the AMA. These principles are now considered the standards of conduct for physicians in the United States [21].

IRB and IACUC. Animals and humans play imperative and distinctive roles as research subjects. Without their participation in clinical research, the numerous medical advancements available to modern society would not be feasible. As mentioned previously, historically, the protections surrounding research subjects has not always been forefront for clinical investigations and public awareness was amplified upon the exposure of the atrocities of the Nazi war experiments. In order to protect research subjects, two committees were founded: Institutional Animal Care and Use Committee and the Institutional Review Board.

The Institutional Animal Care and Use Committee (IACUC) was formally established in 1950 and is responsible for the protections of animals in research [22]. As a committee, they review and approve activities that involve animals. IACUC was formed in response to the numerous inconsistences in policies and standard care practices in research labs with animal subjects. Per Public Health Service Policy, an IACUC committee consists of a minimum of 5 members. These members include the following: a veterinarian, a scientist experienced in research involving animals, an individual from a nonscientific area (i.e ethicist, lawyer, clergyman, etc.), and a non-affiliated individual who can represent the interests of the community [22]. At an institutional level, an IACUC committee inspects facilities housing and

working with animals every six months [22]. Additionally they monitor colleges and universities using animals in research. This entails evaluating the researchers' compliance with IACUC requirements. If there are any violations, it is the responsibility of IACUC to report the incidences to federal agencies. When concerns from the public arise regarding the research, IACUC will investigate the issue, address the researchers, and alleviate concerns.

While IACUC oversees research with animal subjects, an Institutional Review Board (IRB) provides the ethical and regulatory oversight for research involving human subjects [23]. They are responsible for reviewing research proposals in studies with humans before research is initiated. Additionally, they have the authority to observe or employ a third party to observe the research being actively conducted and the consenting process for participation [23]. If concerns arise regarding the conduct of the study or how consent was obtained, the committee determines whether an active audit is required and will take appropriate action.

An Institutional Review Board consists of a minimum of 5 members. These members should be both men and women and come from a variety of backgrounds and ethnicities. Together they work to protect the well-being, rights, and welfare of human research participants [24]. It is their responsibility to ensure that the study is in compliance with any relevant local, state, and federal laws. When a study does not meet the necessary criteria, IRB provides a written statement of reasoning to the investigator and the institution they work under [24].

In order to receive IRB approval, the research proposal must meet the ethical standard and provide protections for subjects as outlined in the principles of the Belmont Report [24]. The Belmont Report summarizes the basic ethical principles and guidelines for resolving ethical problems as established by the National Commission for the Protection of Human Subject of Biomedical and Behavioral Studies. The first guideline is in reference to respecting a research

participant's autonomy. This includes acknowledging the autonomy of the subject as well as protecting those with diminished autonomy [25]. Second is beneficence toward the participant. There are two general rules that fall underneath beneficent actions for researchers: do no harm to participants and maximize the benefits of the study rather than the harms [25]. The last guideline established is justice in the sense of the fairness of distribution. This entails that there is a fair selection of study participants and that no person is denied without good reason.

Bone Remodeling Cycle

The skeleton undergoes a continuous remodeling cycle throughout the life span of an individual (Figure 2). This process occurs in five main stages (activation, resorption, reversal, formation, quiescence) and is the result of the actions of osteoclast and osteoblasts [26, 27]. Together these cells collaborate to carry out remodeling and are referred to as a basic multicellular unit (BMU) that spans the bone within a bone remodeling compartment. During activation, osteoclast precursors are recruited to the bone surface. Once there are mature osteoclasts, bone lining cells retract to expose the mineralized matrix [26]. The activated osteoclasts are then able to attach via integrins to dissolve and break down the bone matrix components. During reversal, osteoclast cease resorption of the matrix and bone formation is initiated. This step is primarily stimulated through the release of factors stimulating osteoblast migration and differentiation. Once recruited to the bone remodeling compartment, the osteoblast lay down the matrix and initiates mineralization. At the completion of the bone remodeling cycle, osteoblast remain at the bone surface as inactive bone lining cells [26].

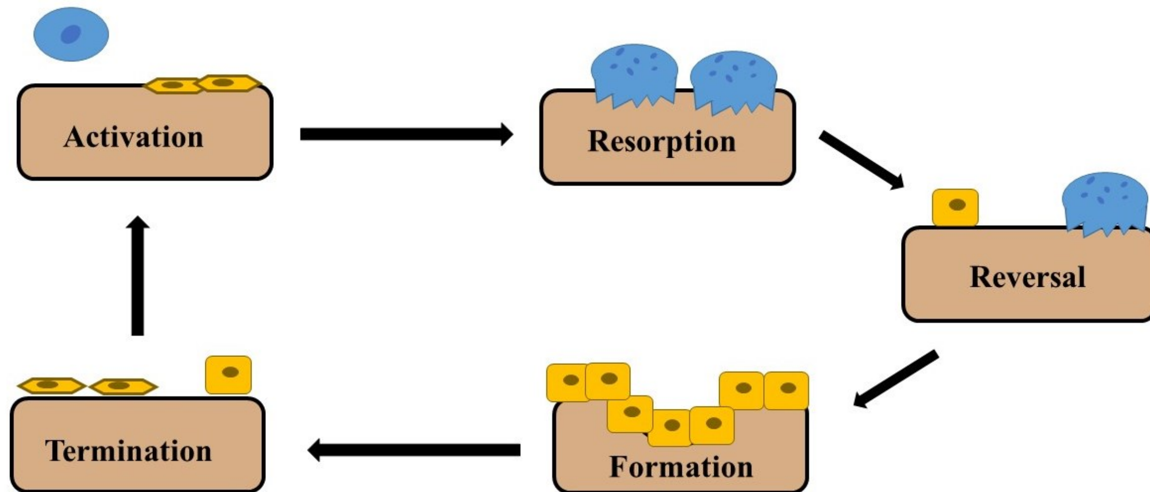

Figure 2: Bone Remodeling Cycle. Five steps of the bone remodeling cycle carried out by the bone cells Together the bone cells collaborate to carry out the remodeling cycle within a bone remodeling compartment. Cells of osteoclast lineage are designated in blue and cells of osteoblastic lineage in yellow.

Bone Cells

Osteoclasts. Osteoclast are the primary bone cells responsible for bone reabsorption and maintaining skeletal integrity. They are derived from hematopoietic monocyte- macrophages [28]. In response to a variety of factors, mononucleated preosteoclast fuse to form the multinucleate osteoclast. These factors include the following: various growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), macrophage colony-stimulating factor (M-CSF), and receptor activator of the NF-κB ligand (RANKL). Upon stimulus from RANKL and M-CSF, osteoclasts differentiate by inducing the expression of the genes encoding for proteins that will characterize the lineage and ultimately the development of a mature osteoclast (Figure 3) [29].

The activation of a mature osteoclast is initiated by RANKL binding to its receptor. Upon RANKL binding, the mature osteoclast is polarized and undergoes internal restructuring. The formation of podosomes, actin- rich structures, aid in the formation of the actin contractile ring within the osteoclast. This actin ring, permits the formation of the sealing zone within which reabsorption can occur (Figure 4). Osteoclast are able to interact with the surface of the bone via integrin attachments, specifically the αvβ3 receptor [30]. This area where the osteoclast is attached to the bone surface is designated by the osteoclast ruffle border (Georgess et al., 2014) .

Located on the basal surface of the osteoclast are V-ATPase proton pumps (Figure 4). These pumps expel protons in the resorption space and along with the chloride imported by the bicarbonate exchanger are able to generate the lower intracellular pH needed to degrade the inorganic minerals of the bone [31]. The organic portion of the bone is degraded by proteases such as TRAP (tartrate-resistant acid phosphatase), cathespin K, and other matrix metalloproteinases. Cathespin K particularly plays an important role in maintaining the sealing

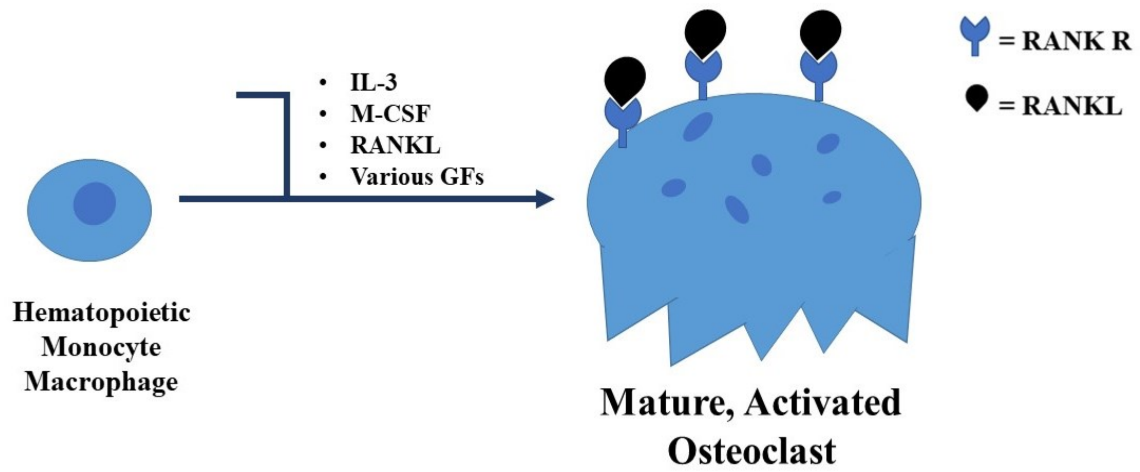

Figure 3: Osteoclast Differentiation. Key steps in the differentiation of hematopoietic monocyte macrophages into mature activated osteoclasts.

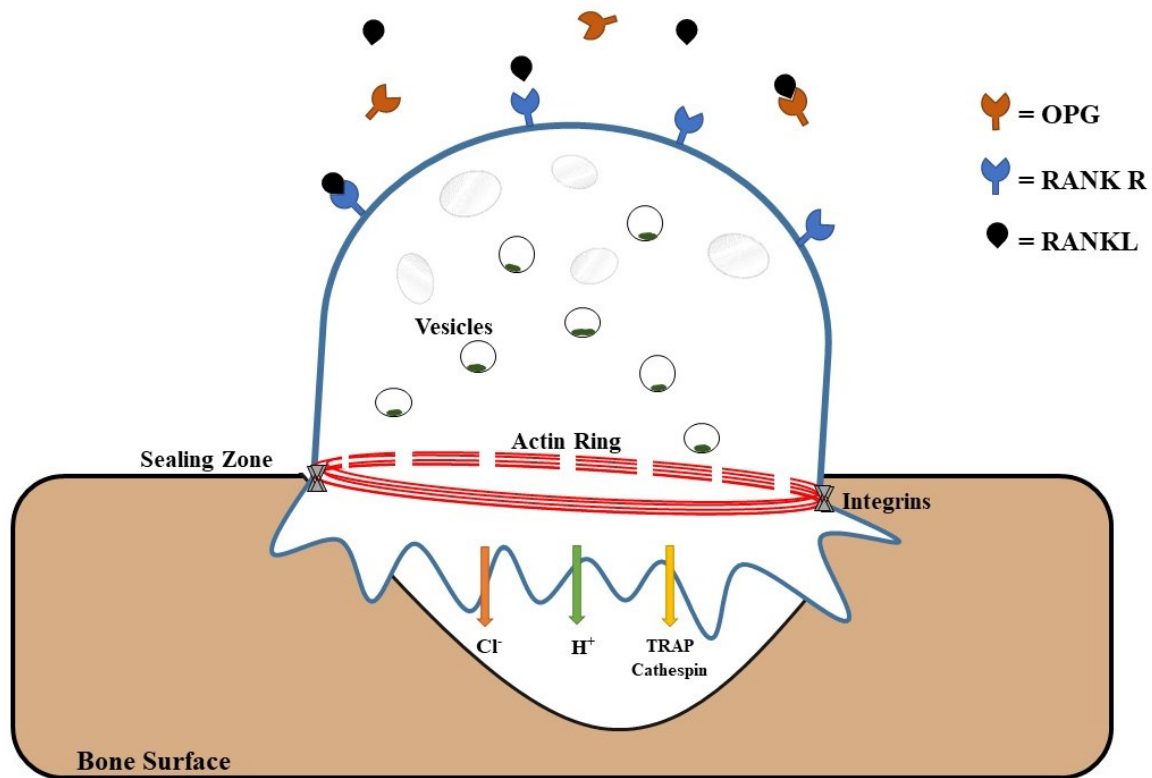

Figure 4: Overview of Osteoclast Activation. Upon RANKL binding its receptor (RANK R) the osteoclast undergoes internal reconstruction. The formation of an actin ring allows for the osteoclast to begin degrading the matrix through proteolysis and acidification. These products are internalized and further degraded in the lysosome or trancytosed. Osteoclast activity is inhibited by the production of the decoy receptor osteoprotegerin (OPG).

zone by cleaving components of the bone matrix (i.e. type I collagen). Upon cathespin K cleavage, the Arg-Gly-Asp (RGD) motif within bone collagen is exposed allowing the αvβ3 receptor is able to attach to [30]. The degradation products produced through the acidification and proteolysis of the bone matrix are then internalized into the osteoclast. Following internalization the products are either further degraded by lysosomes or expelled into the extracellular space by transcytosis [30, 31].

Osteoblasts and Osteocytes. Osteoblasts are mononucleated, cuboidal bone cells that are responsible for generating new bone. These cells are derived from mesenchymal progenitor cells and the differentiation of osteoblastic cell lineage is dependent on the activation of specific transcription factors [28]. The initial differentiation of mesenchymal stem cell into a pre-osteoblast is dependent upon Runx2, a transcription factor [32]. Next these cell undergo three further stages of differentiating with the end result being mature cuboidal osteoblast (Figure 5).

Bone formation, also known as ossification, is canonically classified into two categories: intramembranous and endochondral [33]. Intramembranous ossification is initiated by the differentiation of mesenchymal cells into osteoblasts and does not rely upon a pre-existing cartilage precursor [33]. Mature osteoblasts actively generating un-mineralized bone, or osteoid, through the secretion of a variety proteins (i.e. type I collagen, osteocalcin, osteonectin, alkaline phosphatase, proteoglycans, etc.) [32, 34]. This secretion is stimulated by parathyroid hormone (PTH) and calcitriol (vitamin D). Due to their role, osteoblasts have an enlarged Golgi apparatus and extensive endoplasmic reticulum. Mineralization of the bone matrix is initiated by the high extracellular Ca^{2+} levels, increased PO_4 concentration, and subsequent deposit of crystalline hydroxyapitite (a complex of $Ca^{2+}$and PO_4). Eventually, some of the osteoblasts become embedded in the new matrix being produced. When this occurs, osteoblasts are termed

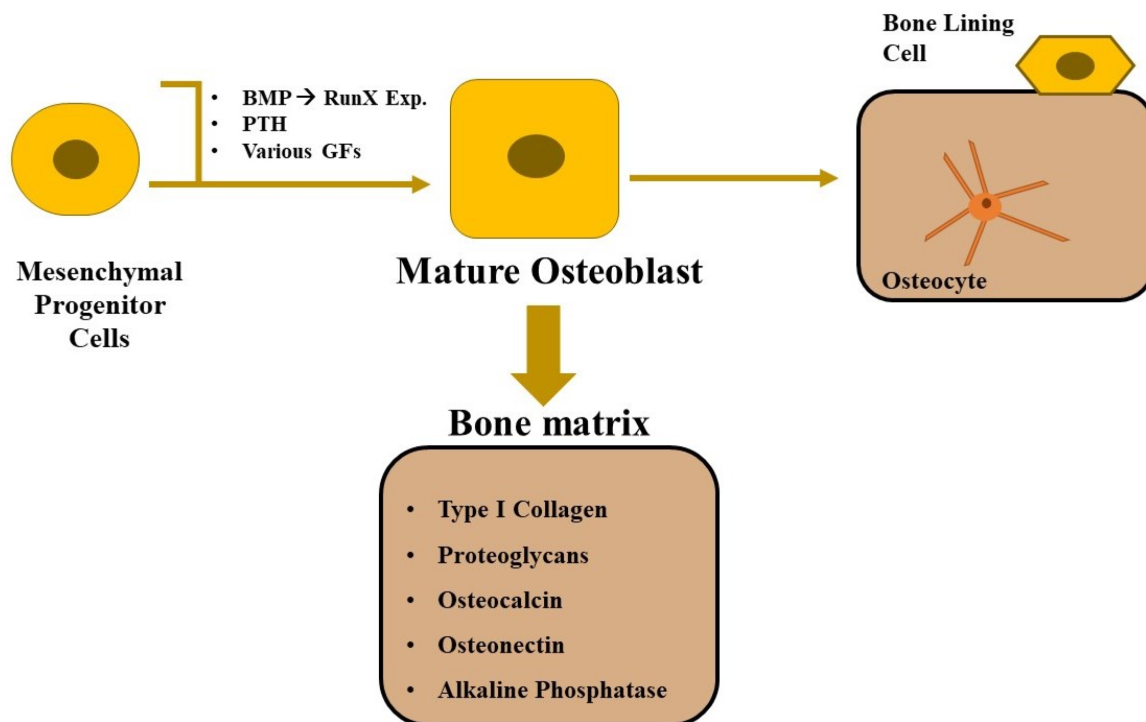

Figure 5: Osteoblast Differentiation. Key steps in the differentiation of mesenchymal cells into osteoblast. Mature osteoblast actively generate osteoid and initiate mineralization through secretion of alkaline phosphatase. Osteoblast that become entrapped within the generated matrix differentiated into osteocytes. Remaining osteoblast that no longer secrete the bone matrix remaining on the surface of the bone as bone lining cells.

osteocytes. The remaining osteoblasts will remain on the surface of the newly generated bone and become bone lining cell [34].

Endochondral ossification, on the other hand, is dependent on a cartilage processor [33]. This form of ossification begins with the aggregation of mesenchymal cells at the cartilage model precursor. After the mesenchymal cells differentiate into osteoblasts, a bone cuff is formed around the cartilage model and calcification is initiated. Connective tissue cells and blood vessels then invade the calcified cartilage allowing endochondral bone to formed [33]. The bones in the extremities and responsible for weight bearing are developed through endochondral ossification. Conversely, the flat bones (i.e. mandible and skull) are formed through intramembranous ossification [33].

Osteocytes the most numerous cells found in bone tissue (Figure 6). As previously described they are former osteoblast that have become entombed within the bone matrix. Osteocytes, in comparison to their osteoblast precursors, contain a higher concentration of proteins related to mineralization and phosphate [28, 35]. These cells predominately are involved in the regulation of the bone cells and are able to detect microalterations to the bone and generate chemical signals to adjust the bone remodeling cycle [28]. These signals are sent via the fingerlike projections, dendrites that are found within the canaliculi, microscopic canals between the osteocyte lacunae in the bone.

Osteoblasts and osteoclasts have a temporary presence in variable locations on the bone; however, osteocytes are present through the entire bone volume and are fairly long-lived cells. This is primarily due to their regulatory roles [28]. Osteocytes are the primary producers of RANKL and OPG. Additionally, they produce PTH needed to activated osteoblasts and stimulate osteoblastic production of RANKL [28, 35]. Bone formation is inhibited by the presence of

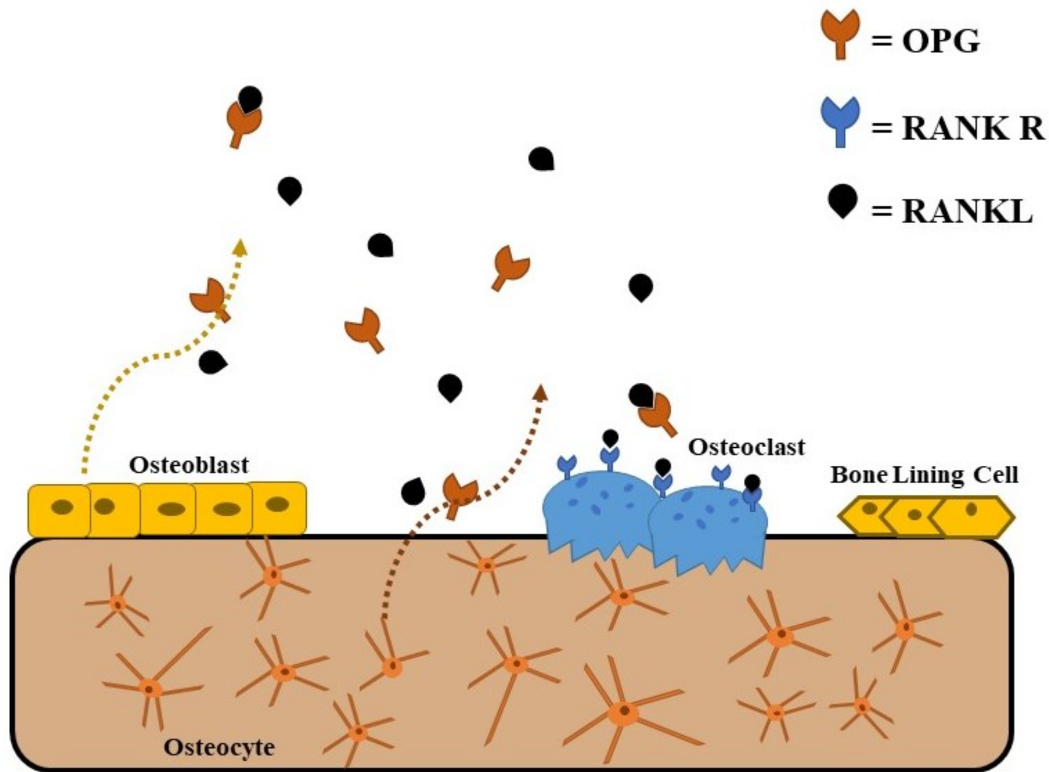

Figure 6: Bone Remodeling Unit. Mature osteoblast, derived from mesenchymal cells, synthesize the bone matrix. Once embedded in the bone matrix osteoblast are termed osteoclast. Together these bone cells generate RANKL to stimulated osteoclasts mediated bone resorption. Osteoclast activity is regulated by the production of osteoprotegerin (OPG), a decoy receptor for the RANK receptor (RANK R). Remaining osteoblasts remain on the surface of the newly generated bone and become bone lining cells.

glucocorticoids and TNF- α. Conversely, these effectors promote osteoclast mediated bone resorption. Resorption activity likewise can be inhibited by calcitonin and bisphosphonates, while also encouraging bone formation. A summary of the effect of certain stimuli on the various bone cells can be found in Table 2.

Components of the Bone Matrix

Type I Collagen. Collagen is the most abundant fibrous protein in mammalian connective tissues. It provide mechanical strength, additional flexibility, and structural support to various tissues [9]. Collagenous proteins typically are categorized based on their function. Host defense collagens aid in the recognition and elimination of pathogens. Transmembrane collagens function as cellular adhesion receptors while the sheet forming and anchoring class connect the basal lamina skin layer to the underlying connective tissue. Similarly, fibril- associates collagens link fibrillar collagens to either the extracellular matrix or each other. These collagenous proteins play a vital role in cellular function, however, they are not as important or abundant as the fibrillar collagens [9, 36]. Fibrillar collagen (types I, II, and III) make up 80-90% of all collagenous proteins within the body.

Type I collagen is the most abundant of the fibrillar collagen and can be in found blood vessels, ligament sclera, tendons, skin, and bone [9]. Within bone tissue, type I collagen makes up approximately 90% of the total proteins present and is secreted by osteoblasts [36]. Type I collagen is a heterotrimer that consists of two α1 chains and one α2 chain (Figure 7). The primary structure of a single chain contains a recurring glycine-proline- hydroxyproline repeat (Gly-X-Y). These repeats aid in the formation of a left- handed helix, which is the equivalent of

Table 2: Functional Effect of Locally Produced Factors on the Bone Cells

Factor	Osteoblast/Osteocytes	Osteoclast
Glucocorticoids	∨ bone formation	∧ bone resorption
Calcitonin	∧ bone formation	∨ bone resorption
Bisphosphonates	∧ bone formation	∨ bone resorption
TNF- α	∨ bone formation	∧ bone resorption

∧**,** increased; ∨**,** decreased

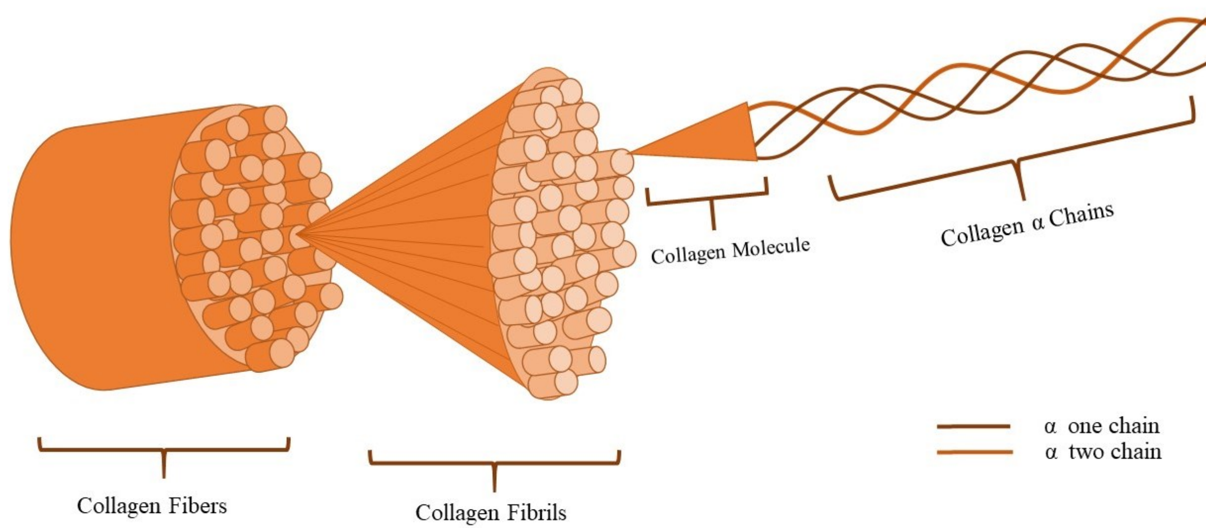

Figure 7: Type I Collagen. Each collagen fiber is made up of numerous fibrils. Collagen fibrils are formed from multiple copies of collagen molecules. A single collagen molecule has a heterotrimeric structure, which consists of two α1 and one α2 chains.

1000 amino acids. The left- handed helices wrap around one another to form a right handed triple helix [9].

The procollagen α chains are synthesized on the ribosomes of the endoplasmic reticulum. To the carboxyl terminus of these α chains, an asparagine-linked oligosaccharide is added. The carboxyl terminus pro-peptides then associate to form trimers covalently linked by di-sulfide binds [9]. Upon linkage, select residues within the Gly- X-Y repeat are covalently modified by the hydroxylation of proline and lysine and the glycosylation of hydroxylysines. These modifications aid in formation of a heterotrimer procollagen molecule. Folded procollagen is then transported to the Golgi apparatus, where it is are laterally associated by disulfide bonds. These chains are secreted from the cell into the extracellular space where the pro-peptides are cleaved from N and C termini. This processing results in the assembly of collagen fibrils which are subsequently stabilized by covalent crossed linkage [37]. These fibrils can then be assembled in larger bundles and are used to attach various connective tissues [9].

Other Matrix Components. The majority of the bone matrix consist of type I collagen, but there are large variety of other non-collagenous proteins (Figure 5). Besides collagen, the two protein classes found in abundance are proteoglycans and glycoproteins [34]. Proteoglycans consist of multiple GAG molecules covalently attached to leucine-rich core protein and are found within the bone matrix [38]. The GAG molecules are long polysaccharides that are made up of disaccharide repeats that are sulfated to different degrees (i.e heparan sulfate, dermatan sulfate, chondroitin sulfate etc.) [39]. Proteins which fall into the proteoglycans are predominantly found within the mineralized matrix and play variety of different roles. They act as co-receptors for certain cytokines in addition to being crucial structural elements. The glycoproteins, also found in relative abundance in the bone are produced in different stages of

osteoblast maturation. Some commonly found glycoproteins are alkaline phosphatase, RGD motif containing proteins, and osteonectin. Overall, this protein class is involved in a large array of activities including bone mineralization and bone cell-matrix interactions [34].

IDUA-W392X Mouse Model

Development. There are a wide range of animal models employed in the characterization of MPS I; however, these were not useful in the evaluation and development of innovate therapies [5]. Often these animals did not carry mutations identified in the clinically severe form of MPS I, thus limiting prospective investigations. To gain a better understanding of the bone abnormalities associated with MPS I, Wang et. al generated a mouse model that would reflected the most severe phenotype, Hurlers syndrome. The generated mice have a W392X nonsense point mutation (TGG→TAG) within exon nine of the IDUA gene. This mutation corresponds to the W402X mutation commonly found individuals diagnosed with Hurlers syndrome.

The W392X mouse line was generated by transfecting embryonic murine stem cells with linearized constructs of IDUA-W392X. Integration was verified by an *EcoR*I digest and gel electrophoresis (Figure 8). *Cre*-mediated recombination was used to remove the neomycin resistance cassette, which yielded 34 bp fragment difference between the wild-type and mutant alleles (Figure 8B) [5]. This size difference was used to distinguish between genotypes of mice generated for the study. The IDUA-W392X mouse line was produced from transfected H4 embryotic stem cell clones that was crossed against C57BL/6J mice. When the lysosomal enzyme activity in the IDUA mice was assessed, the IDUA homozygous mice had 30-50% of the projected IDUA mRNA levels and low levels of enzymatic activity. The deficiency in enzymatic

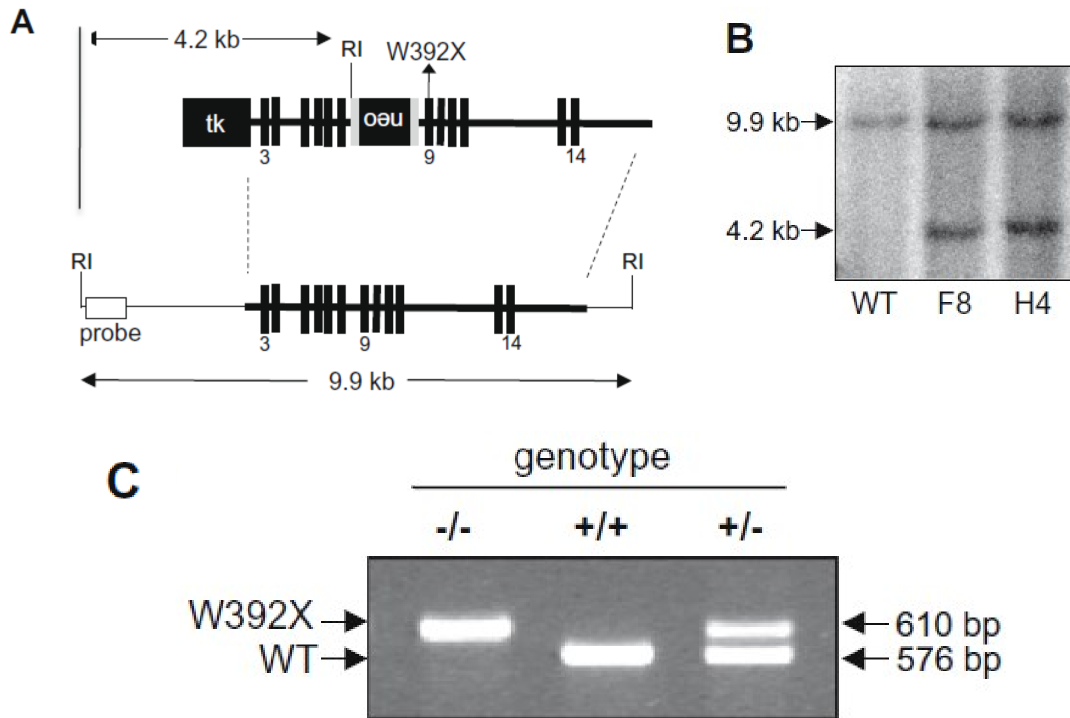

Figure 8: Generation of the IDUA-W392X knock-in mouse. (**A**) The IDUA-W392X targeting construct (upper) was integrated into the mouse IDUA locus (lower) by homologous recombination (dashed lines). The bold horizontal lines indicate the targeted region. The bold vertical lines indicate exons (not shown to scale). Exons 3, 9, and 14 are numbered. The W392X mutation was introduced into exon 9. The sizes of the genomic DNA fragments after *EcoRI* digestion in both wild-type and targeted IDUA loci are indicated as well as the region of probe binding used in Southern blotting. tk, thymidine kinase gene; neo, neomycin-resistance gene (flanked by loxP sites shown as gray bars). (**B**) Southern blot of wild-type and two targeted ES cell clones (denoted as F8 and H4) resulted in a 9.9 kb fragment from the wild-type IDUA locus and a 4.2 kb fragment from the targeted IDUA locus. (**C**) PCR of the IDUA allele using genomic DNA derived from tail snips (following Cre recombinase-mediated excision of the neo cassette) results in a 576 bp product from the wild-type IDUA allele and a 610 bp product from the W392X allele [5]. Copied from Wang et al. 2010 with permission.

activity caused a significant increase of GAG tissue storage [5]. As the animals aged, the elevated GAG levels were maintained and prominent morphological changes were observed. These observations were likened to an individual diagnosed with Hurlers syndrome.

Skeletal Abnormalities. The generation of the IDUA-W392X mouse line provided a way to assess the skeletal abnormalities and defects associated with Hurler syndrome. At birth the affected mice were indistinguishable from wild-type littermates; however as the IDUA mice aged, morphological changes became very apparent. The zygomatic arch in the mice noticeably thickened by 15 weeks of age and became even more pronounced by 35 weeks (Figure 9A) [5]. At 5 weeks of age, the IDUA mice showed a 15% decrease in femur length when compared to age-matched wild type controls. This deference in femur length became less evident as the mice continued to age. By 35 weeks, the IDUA mice developed increased thickness in femur diaphysis as has been described in humans (Figure 9B) [5].

The skeletal abnormalities in the IDUA-W392X mice were further characterized by Oestreich et. al in 2015. They found that the IDUA mice weighed more compared to their littermates at 16 weeks of age. When the skeletal microarchitecture of tibias was analyzed, the IDUA mice displayed elevated trabecular number and connectivity. The mice also had a decrease in trabecular separation which suggests that the deficiency of IDUA causes an increase in bone material, thus causing a changes in microarchitecture [1]. When the bone mineral density was analyzed in tibias, the IDUA homozygous mice displayed a 50% increase porosity while the heterozygous mice displayed a 12.2% increase (Figure 10). The increased porosity observed in the IDUA homozygous mice could indicate there is an increase in osteocyte lacunae. This would also point toward an increase in osteoblasts which, likely are functionally compromised.

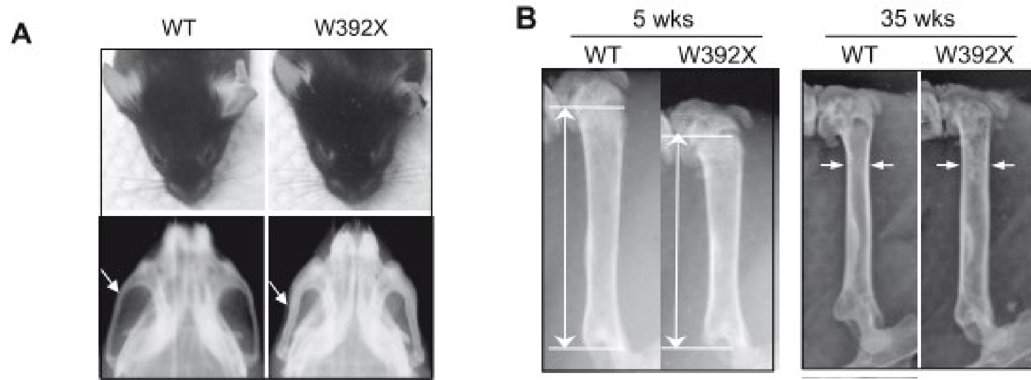

Figure 9: **Morphological Assessment of the IDUA-W392X Mice**. (**A**) Photographs and radiographs of the heads of 35 week old WT and IDUA-*W392X* mouse (**B**) Radiographs of the femurs of a wild type and IDUA-W392X mouse at 5 weeks and 35 weeks of age. Arrows indicate which region of the bone was measured respectively [5]. Copied from Wang et al. 2010 with permission.

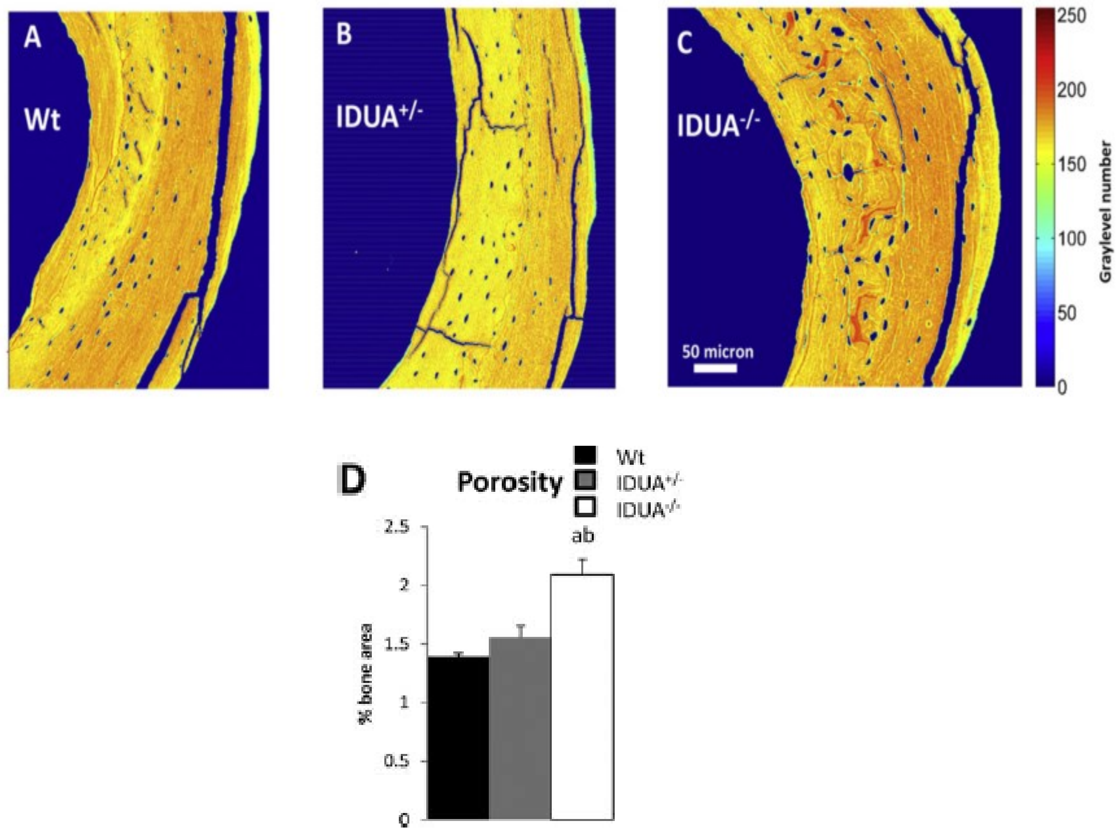

Figure 10: **Bone Mineral Density Distribution (BMDD) and Porosity Analysis as determined by Scanning Electron Microscopy.** Representative segments of female **A**) WT, **B**) $IDUA^{+/-}$, and **C**) $IDUA^{-/-}$ cross-sectional BMDD images of the medial section of the mid-diaphysis of the tibia (range of 256 gray level numbers, with red representing the greatest density of calcium and blue the least). For each sample the whole cross-sectional image was used to determine the BMDD histogram. **D**) Porosity quantification of Wt (black bar), $IDUA^{+/-}$ (gray bar) and $IDUA^{-/-}$ (white bar) showing an increase in porosity in the $IDUA^{-/-}$ tibiae. Values are means ± SE. $p \leq 0.05$ compared to sex-matched Wt, b$p \leq 0.05$ compared to sex-matched $IDUA^{+/-}$. WT (n = 5), $IDUA^{+/-}$ (n = 5), and $IDUA^{-/-}$ (n = 4) [1]. Copied from Oestrich et al. 2010 with permission.

The effect of IDUA deficiency has been investigated in other mouse models. These studies found that in the absence of IDUA activity, a pathological enlargement of the lysosomes within cells of osteoblastic lineage results (Figure 11) [40]. In these mice the osteoclast contained numerous vacuoles which contained products unable to be degraded. Additionally, osteoclasts from the IDUA deficient mice had a less well-defined ruffled border, indicating impaired bone resorption. Reduced numbers of both bone forming units and osteoclasts were found in the IDUA deficient mice. These finding indicated that bone remodeling is impaired in the absence of IDUA activity.

When the cortical bone was analyzed in the IDUA-W392X mouse model, the material composition for the mice was altered. The homozygous females displayed an increased carbonate/phosphate ratio, decreased phosphate/collagen, and decrease phosphate/matrix ratios when compared to their wild type and heterozygous littermates (Figure 12) [1]. Conversely, the IDUA homozygous males displayed slightly elevated carbonate/phosphate and phosphate/matrix ratios, with a normalized phosphate to collagen ratio. When the bone strength was assessed in the IDUA mice, both homozygous males and females displayed a 75% increase in bone strength [1]. When the strength of the bone materials was assessed (tensile strength), both males and females showed reduce levels when compared to littermates (Figure 13). Females also had an additional decrease in elasticity. These findings suggest that the increased bone strength associated with the IDUA deficiency results in changes in bone material properties due to dysfunctional osteoblasts, thus affecting bone biomechanics and overall causing an increase in torsional strength [1]

MPS I is characterized by a thickening of bone. As discussed previously, type I collagen makes up the majority of the bone matrix. The ratio in which the type I collagen mRNAs are transcribed is two α1 chains and one α2 chains (Furth, Worth, & Ackerman, 1991) . In order to

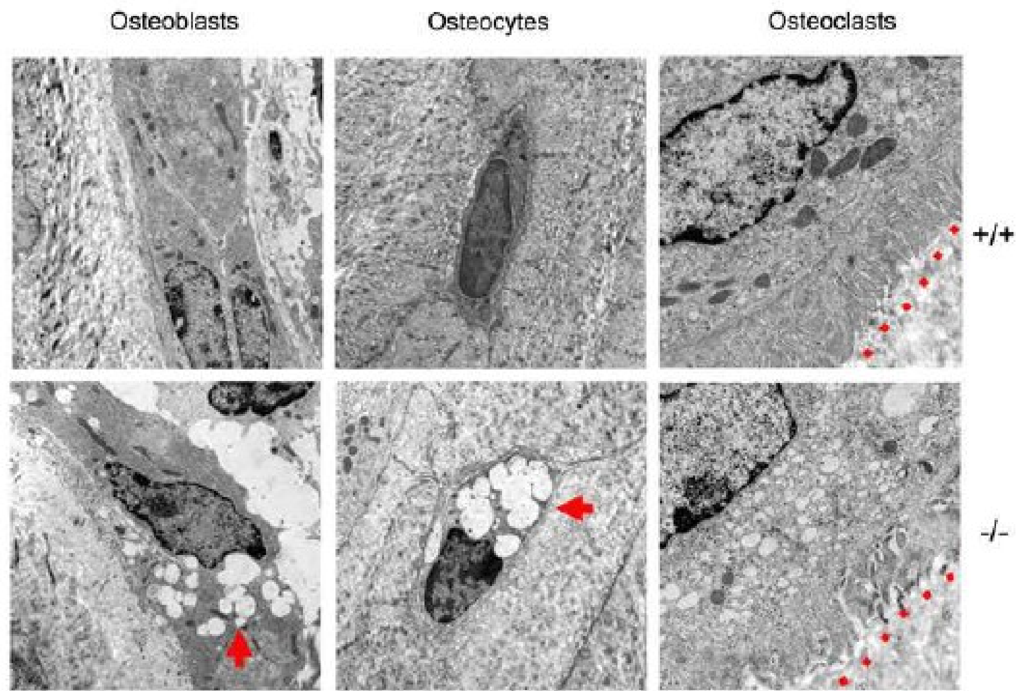

Figure 11: **Electron Microscopy of Bone Cells**. EMs from 24 week old wild type and IDUA deficient mice reveals pathological lysosomal storage (indicated by arrows) in osteoblasts and osteocytes from IDUA deficient mice. IDUA deficient mice osteoclasts contain numerous small vacuoles and their ruffled border (indicated by dotted red lines) was less developed compared with wild type sections [40]. Copied from Kuehn et al. 2015 with permission.

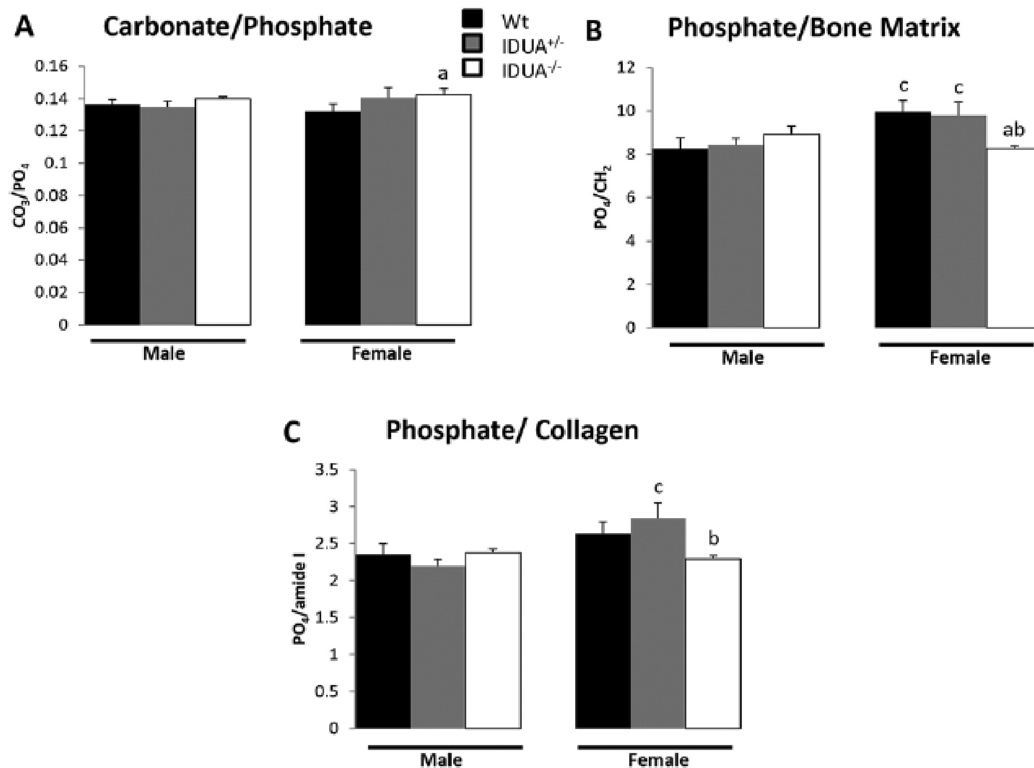

Figure 12: **Physiochemical Composition of the Tibial Cortical Bone.** Composition was Composition was determined using Raman spectroscopy. **A**) Carbonate/phosphate ratios [(CO_3^{2-} /PO_4^{3-}); indication of carbonate substitution of phosphate in the crystal lattice] were increased in tibiae from IDUA$^{-/-}$ females compared to WT. **B**) Phosphate to bone matrix ratios [(PO_4^{3-} /CH_2); indication of the relative amount of mineral phosphate to organic matrix] was decreased in tibiae from IDUA$^{-/-}$ females compared to WT and IDUA$^{+/-}$ littermates of the same sex. **C**) Phosphate to collagen ratios [(PO_4^{3-} /amide I); indication of the relative amount of phosphate mineral to collagen] was decreased in tibiae from female IDUA$^{-/-}$ compared to IDUA$^{+/-}$ littermates and had a decreased trend compared to sex-matched WT littermates ($p = 0.06$). Values are means ± SE. $^{A}p \leq 0.05$ compared to sex-matched WT, $^{B}p \leq 0.05$ compared to sex-matched IDUA$^{+/-}$, $^{C}p \leq 0.05$ compared to genotype-matched male. Male WT (n = 5), IDUA$^{+/-}$ (n = 5), and IDUA$^{-/-}$ (n= 4); Female WT (n = 5), IDUA$^{+/-}$ (n = 4), and IDUA$^{-/-}$ (n = 5) [1]. Copied from Oestreich et al. 2010 with permission.

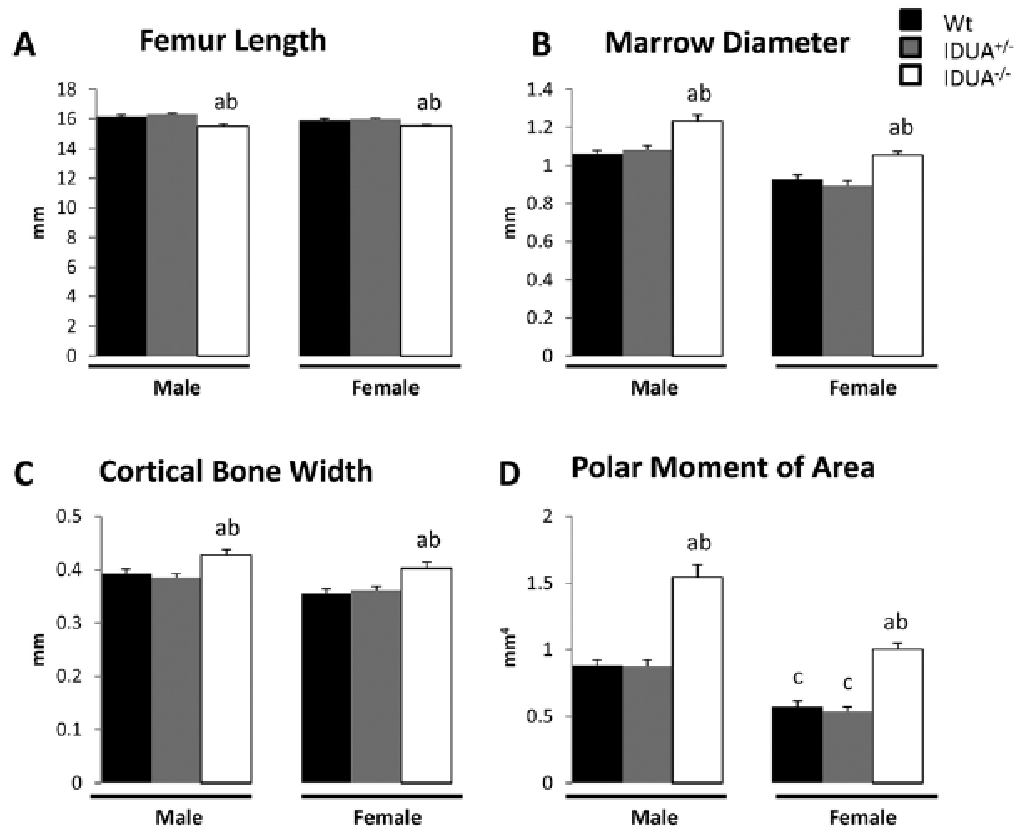

Figure 13: **Geometric Parameters**. Parameters and polar moment of area of male and female WT, IDUA$^{+/-}$, and IDUA$^{-/-}$ femora as determined by μCT. **A**) Femoral length was decreased, and **B**) marrow diameter, **C**) cortical bone width, and **D**) polar moment of area were increased in IDUA$^{-/-}$ femurs (white bar) compared to sex-matched WT and IDUA$^{+/-}$ femora. Values are means ± SE. $^{A}p \leq 0.05$ compared to sex-matched WT, $^{B}p \leq 0.05$ compared to sex-matched IDUA$^{+/-}$, $^{C}p \leq 0.05$ compared to genotype-matched male. Male WT (n=10), IDUA$^{+/-}$ (n=9), and IDUA$^{-/-}$ (n=11); Female WT (n=7), IDUA$^{+/-}$ (n=7), and IDUA$^{-/-}$ (n=10) [1]. Copied from Oestreich et al. 2010 with permission.

determine if bone thickening is caused by an increase expression of type I collagen, the expression levels of the chains was analyzed in wild type, heterozygous, and mice without IDUA activity. Results showed no significant changes in the α1 and α2 expression levels in the IDUA-W392X mice (Figure 14A) [41]. When osteoclastogenesis expression markers were assessed, a decrease in RANK and OPG receptors were found in mice carrying the mutated IDUA allele (Figure 14B) [41]. The RANK receptor especially is key in the differentiation of the mature, multinucleated osteoclast. A decrease in RANK receptor expression could be indicative of decrease in mature osteoclast able to resorb bone tissue.

This investigation intends to further characterize the skeletal abnormalities associated with MPS I and to address bioethical aspects of experimental medicine in pediatric cases. To accomplish this goal, the type I collagen content in the tibias and femurs of wild type, heterozygous, and mice without IDUA activity will be assessed. Additionally, this study will establish a protocol for examining osteoclast activity. This will be assessed through immunohistochemistry of RANKL, the stimulus initiating osteoclast resorption, in the tibias of wild type males and males without IDUA activity. Furthermore, the rights and protections of children in research will be defined through the discussion of patient autonomy, the importance of doctor and patient relationships, and the consenting process for clinical research.

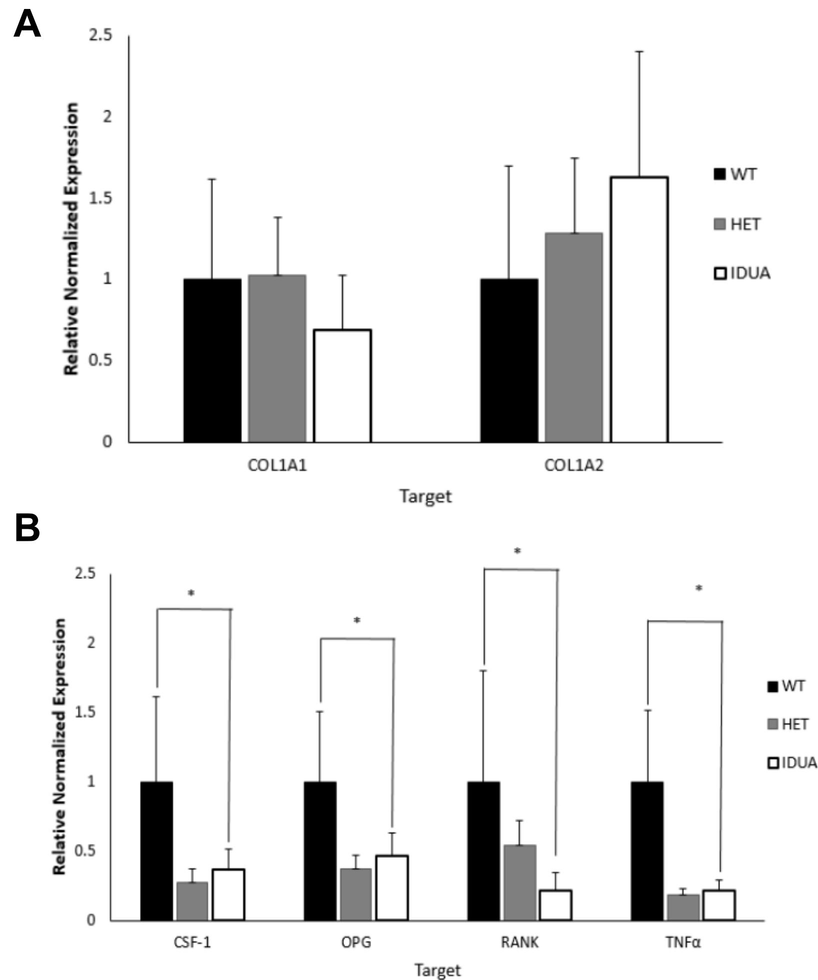

Figure 14: **Expression Analysis.** (**A**) Type I Collagen Expression levels as defined by relative quantification PCR. Total RNA was isolated from tibiae of wild-type (n=4), heterozygous (n=4), and IDUA(-/-) (n=4) mice, transcribed into cDNA and measured for relative expression levels of targets by KAPA PROBE qPCR. Targets included Type I collagen Alpha chain 1 (COlA1) and Type I collagen alpha Chain 2 (COL1A2). (**B**)Expression levels for Biomarkers Involved in Osteoclastogenesis as defined by relative quantification PCR. Total RNA was isolated from tibiae of wild-type (n=4), heterozygous (n=4), and IDUA($^{-/-}$) (n=4) mice, transcribed into cDNA and measured for relative expression levels of targets by KAPA PROBE qPCR. Targets include, Colony Stimulating Factor-1 (CSF-1), osteoprotegerin (OPG), Receptor Activator of Nuclear Factor κ B (RANK) and Tumor Necrosis Factor α (TNFα). Significance was determine if $p < 0.05$ based on one-way student t- test (*CFX Manager Verizon 3.0*). Asterisks denotes significance difference between heterozygous to wild- type (OPG) or significance difference between heterozygous and IDUA ($^{-/-}$) as compared to wild-type (CSF-1), (RANK) and (TNFα) [41].

QUANTIFICATION OF BONE COLLAGEN LEVELS

Materials and Methods

The type I collagen content of tibias and femurs from wild type (WT), heterozygous ($IDUA^{+/-}$), and mice without IDUA activity ($IDUA^{-/-}$) was indirectly quantified with a hydroxyproline assay. Bone samples obtained from age- matched mice were received from a collaborator, Dr. Charlotte Phillip's lab at the University of Missouri. All samples were stored in 1x PBS at -20ºC until used in the assay. Soft tissue was manually removed from the bone exterior. Subsequently, heads of the long bones were removed and samples were lyophilized in a ThermoFischer USV400® to extract any remaining bone marrow. Following lyophilization, samples were weighed and then dissolved in 0.5 mL of 6 N HCl and ground into a fine powder. Samples were transferred into glass tubes with an additional 0.5 mL of 6 N HCl and dried at 124ºC for three hours. After drying, samples were cooled to room temperature, placed in a NaOH pellet lined desiccator within an 85ºC water bath, and dried under a vacuum for approximately eighteen hours.

After cooling, the bone powder was reconstituted in 0.001 N HCl and incubated at room temperature for thirty minutes. During incubation, samples were vortexed occasionally. Reconstituted samples were used in a colorimetric assay using Chloramine T (Sigma Aldrich) and Ehrlich's reagent (4-dimethylaminobenzaldehye; Sigma Aldrich). Reagents were made fresh for each run. Chloramine T, distilled water, and 0.001 N HCl were added to 1.5 µL of each sample and incubated at room temperature for ten minutes. Following incubation, Ehrlich's reagent was added to the samples and then incubated in a 55 ºC water bath for twenty minutes. Samples were removed and cooled at room temperature for five minutes. One hundred

microliters of each sample was plated on a 96 well plate along with hydroxyproline standards and absorbances were measured on the SpectraMax® Paradigm® Multi-Mode Detection Platform at 558nm. Hydroxyproline standard concentrations used are as follows: 3µg/100 µL, 2 µg/100 µL, 1 µg/100 µL, 0.5 µg/100 µL, 0.25 µg/100 µL, and 0.125 µg/100 µL. Samples were read in duplicate and made relative to a 0.001 N HCl blank. The hydroxyproline concentration was determined by a standard curve equation and an one-way *ANOVA* statistical test was used to evaluate the different concentrations across genotypes and sexes.

Results

Hydroxyproline is an amino acid found only in collagenous tissues. It is synthesized by the post-translation hydroxylation of select proline residue in collagen fibers and makes up the Y position of the repeating tripeptide Gly-X-Y, unique to collagen. Due to the exclusivity of hydroxyproline, bone collagen levels can be indirectly quantified with a hydroxyproline assay (Figure 15). The type I collagen content was assessed in wild type (WT), heterozygous (HET), and mice lacking IDUA activity (IDUA) in both male and female mice. (Figures 16 and 17).

Wild type males contained an average hydroxyproline concentration of 0.007 µg/µL ($SD = 0.0036$) and heterozygous mice 0.006 µg/µL ($SD = 0.0032$) (Table 3). Mice without IDUA activity contained on average 0.015 µg/µL ($SD = 0.005$) of hydroxyproline, which was found to be significant when compared to both the WT and HET mice ($p=0.0005$). Additionally, the IDUA male bones weighed significantly more (Figure 13B; $p=0.0019$). Females of the same genotype had hydroxyproline concentration as follows: WT 0.005 µg/µL ($SD=0.0025$), HET 0.006 µg/µL ($SD = 0.0022$), and IDUA 0.006 µg/µL ($SD = 0.0024$). No significance was found on either the hydroxyproline content or bone weight in the female mice (Figure 17B).

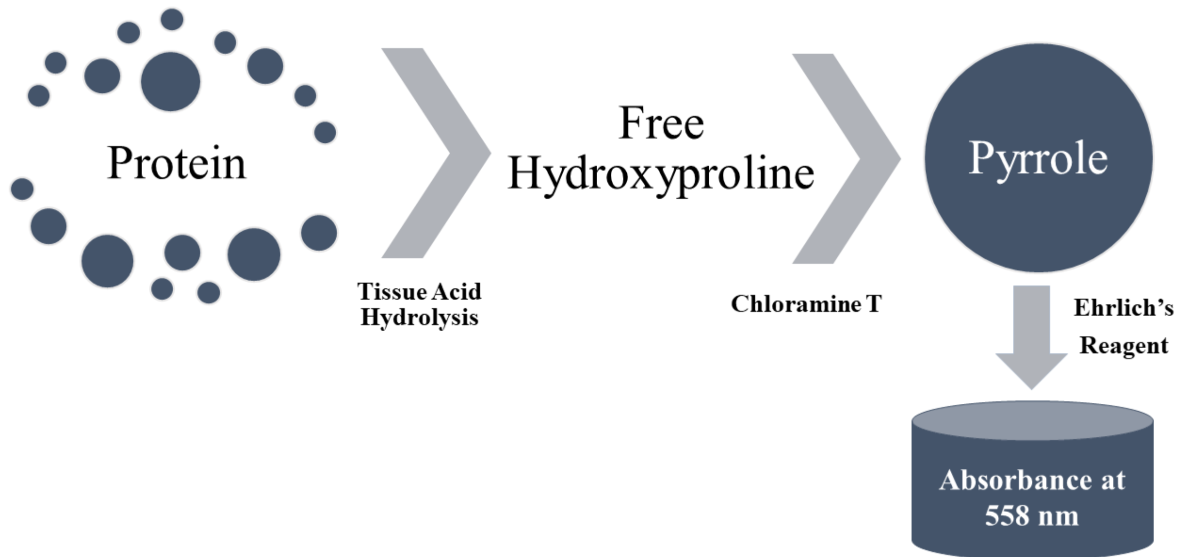

Figure 15: Methodology for the Hydroxyproline Assay. Acid hydrolysis breaks the peptide bonds of the type I collagen within the bone powder, thus freeing up hydroxyproline. Chloramine T converts the freed hydroxyproline into pyrrole. Ehrlich's reagent reacts with pyrrole to produce a chromophore. The absorbance can be read at 558 nm.

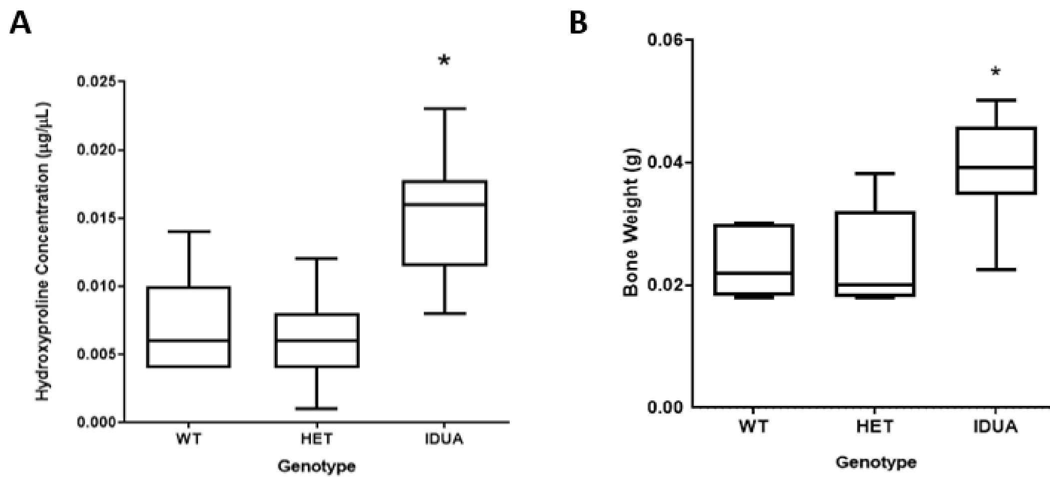

Figure 16: Comparison of the hydroxyproline concentration (A) and bone weight (B) in male mice. Total sample size includes 22 mice. Bone weights were taken after lyophilization and excludes one outlier. The absence of α-L- iduronidase activity designated by IDUA (n=8), heterozygous genotype designated by HET (n=7), and wild-type designated by WT (n=7). Bars designate the spread of sample concentrations. One-Way *ANOVA* used to determine statistical difference across genotypes *(* = p<0.05*).

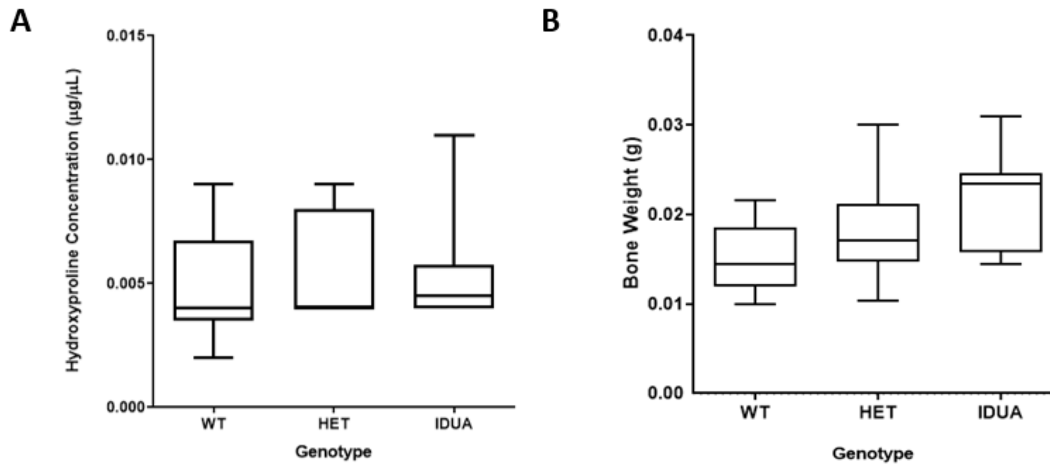

Figure 17: Comparison of the hydroxyproline concentration (A) and bone weight (B) in female mice. Total sample size includes 23 mice. Bone weights were taken after lyophilization and excludes three outlier. The absence of α-L- iduronidase activity designated by IDUA (n=8), heterozygous genotype designated by HET (n=9), and wild-type designated by WT (n=6). Bars designate the spread of sample concentrations One-Way *ANOVA* determine no statistical difference across genotypes.

Table 3: Hydroxyproline Statistical Comparison

Sex	Genotype	Average Concentration (μg/μL)	Concentration Range	Standard Deviation	Standard Error of Means (SEM)	n
Male	WT	0.007	0.004-0.010	0.0036	0.0014	7
	HET (+/-)	0.006	0.001-0.012	0.0032	0.0012	7
	IDUA (-/-)	0.015	0.008-0.023	0.0045	0.0016	8
Female	WT	0.005	0.002-0.009	0.0025	0.0010	6
	HET (+/-)	0.006	0.004-0.009	0.0022	0.0007	9
	IDUA (-/-)	0.006	0.004-0.011	0.0024	0.0009	8

Previously, it has been reported that there is an increase in cortical bone width and whole body mass in both male and female mice without IDUA activity. To assess the effect of increased collagen content in relationship to bone weight, the hydroxyproline concentration for each mouse was examined relative to their bone weight (Figure 18 and 19). IDUA males on average displayed increased type I collagen content based on bone weight when compared to the males of other genotypes ($p=0.0142$). IDUA females showed no significant decrease in type I collagen in comparison to other genotypes.

Conclusion

Mucopolysaccharidosis (MPS) is a group of lysosomal storage disorders that arise due to the deficiency of enzymes needed to degrade glycosaminoglycans (GAGs). With MPS type I (MPS I), the enzyme α-L-Iduronidase (IDUA) is deficient. The absence of IDUA results in the accumulation of GAGs within various tissue and ultimately causes multisystem dysfunction. In diagnosed individuals, the level of functional IDUA determines the severity of the disease. The majority of symptoms caused by IDUA deficiency are ameliorated with current therapeutic options with the exception of the skeletal abnormalities.

Skeletal abnormalities characteristic of MPSI include decreased femur length in addition to increase cortical bone thickness. Bone tissue is composed of both organic and inorganic materials and the ratio between the two will affect the over structural integrity of the bone. Previous investigations have shown that in the W392X mouse model that females have an altered CO_3 to PO_4, PO_4 to bone matrix, and PO_4 to bone matrix ratio [1]. IDUA males did not display as drastic alteration in comparison to the other genotypes; however, males did show an increase in torsional stiffness on biomechanical testing. The majority of the bone matrix consists of type I

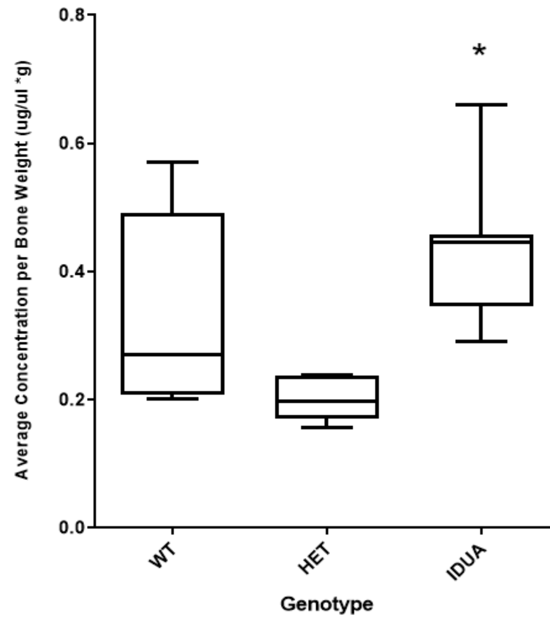

Figure 18: Comparison of the average hydroxyproline concentration in relationship to bone weight in male mice. Bone weights were taken following lyophilization. Total sample size includes 22 mice, four outliers excluded. The absence of α-L- iduronidase activity designated by IDUA (n=8), heterozygous genotype designated by HET (n=7), and wild- type designated by WT (n=7). Bars designate the spread of sample concentrations One-Way *ANOVA* used to determine statistical difference across genotypes (* = $p<0.05$).

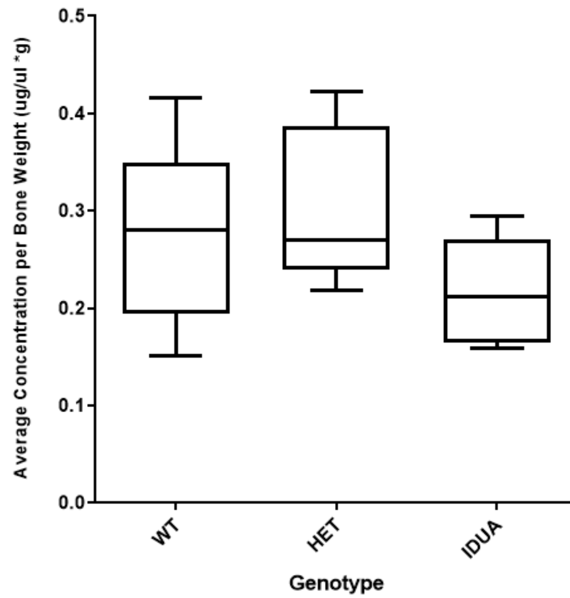

Figure 19: Comparison of the average hydroxyproline concentration in relationship to bone weight in female mice. Bone weights were taken following lyophilization. Total sample size includes 23 mice, two outliers excluded. The absence of α-L- iduronidase activity designated by IDUA (n=8), heterozygous genotype designated by HET (n=9), and wild- type designated by WT (n=6). Bars designate the spread of sample concentrations. One-Way *ANOVA* determine no statistical difference across genotypes.

collagen and the expression levels of the collagen chains in this model were analyzed and determine to be similar to what is found in the wild type mice [41]. Due to the absence of over expression of the collagen chains in these mice, the decision to quantify the bone collagen content was made to further characterize the MPSI skeletal abnormalities.

In order to quantify the type I collagen content in the IDUA-W392X mice, a hydroxyproline assay was performed. Bone collagen levels were assessed in wild type, heterozygous, and mice lacking IDUA activity (Figure 20). The male IDUA mice on average displayed double the amount of hydroxyproline in comparison to WT mice. When the collagen levels were normalized to the bone weight (Figure 18), IDUA males again showed a trend of significance. It can be inferred from these findings that the increase in collagen content in the IDUA males results in the increase bone mass. Additionally the increased collagen levels would also cause the increase torsional stiffness as previously described. The bones of IDUA females weighed more than opposing genotypes but unexpectedly did not display elevated collagen levels. When the collagen levels were normalized to the bone weight (Figure 19), the IDUA females showed lower concentration than WT or HET mice. This indicates that the increased bone mass is not caused by elevated bone collagen levels and could be due to altered physiochemical composition of the bone.

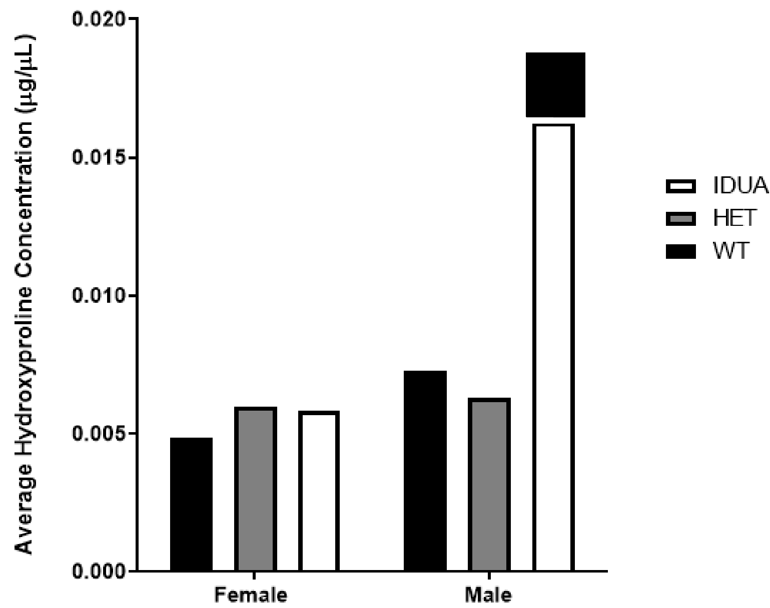

Figure 20: **Comparison of the average hydroxyproline concentration.** Total sample size includes 22 male and 23 females, outliers excluded. The absence of α-L- iduronidase activity designated by IDUA (males n=8; females n=8), heterozygous genotype designated by HET (males n=7; females n=9), and wild- type designated by WT (males n=7, females n= 6). One-Way *ANOVA* used to determine statistical difference across genotypes in male mice (* =$p<0.05$).

IMMUNOHISTOCHEMICAL STAINING OF RANKL IN TIBIAS

Materials and Methods

Decalcification and Paraffin Embedding of Tibias. Bone samples obtained from age-matched mice were received from a collaborator, Dr. Charlotte Phillip's lab at the University of Missouri. All samples were stored in 1X PBS at -20ºC until used in the assay. The left tibias of wild type males (WT) and male mice without IDUA activity (IDUA $^{-/-}$) were thawed and rinsed in deionized water. After bone weights were recorded, specimens were transferred to glass vials, completely submerged in Cal-Ex™ II Fixative/Decalcifier (Thermofischer), and incubated at room temperature for 36 hours. After decalcification, samples were rinsed in deionized water and placed in embedding cassettes.

Samples were processed in a Leica ASP300S in a twelve hour overnight cycle. Following two formalin washes, samples were bathed in increasing concentrations of ethanol. The ethanol washes were succeeded by four xylene, an absolute ethanol, and deionized water wash before samples were submerged in paraffin. The tibias were then removed from the tissue processer, mounted in paraffin, and stored at 4 ºC until sectioning. Five to seven micrometer thick sagittal sections of the embedded samples were cut on a Leica RM2315 microtome and mounted on *Superfrost Plus* (Fischer Scientific) microscope slides. Mounted samples were stored in a slide box at room temperature.

Immunohistochemical Staining of RANKL. Samples were dewaxed in a procedure adapted from AbCam's protocol for immunohistochemistry (IHC) of paraffin embedded sections. Modifications were made due to sample loss in various stages of the procedure. Slides were submerged in two xylenes baths for three minutes and followed by decreasing ethanol

washes (two absolute, 95%, and 70%). After dewaxing, slides were rinsed in cold deionized water for three minutes. Heat induced antigen retrieval steps were excluded from IHC procedure due to loss of tissue samples. The bone tissue was permeabilized in two 5 minute washes in 1X TBS with 0.025% Triton. The tissues was then block for two hours in 10% rabbit serum (AbCam) with bovine serum albumin (BSA) at room temperature. After blocking, excess rabbit serum was drained and samples were cover with 5 µg/mL of anti-RANKL antibody (ab45039) and incubated at 4 ^{o}C overnight. The following day, slides were washed twice for five minutes in 1X TBS with 0.025% Triton. Samples were then covered in 5 µg/mL of goat anti- rabbit IgG antibody (ab205718) and incubated at room temperature for one hour. Incubation was succeeded by covering sample in 3,3'-diamniobenzidine (DAB, Sigma Aldrich) for ten minutes. Samples were then rinsed in deionized water, dehydrated, and mounted with Permount® (Fisher Scientific).

Results

The activation of an osteoclast is dependent on RANKL binding to its receptor. This ligand is produced by cells of osteoblastic lineage to initiate bone resorption. As previously discussed, the expression levels of the RANK receptor is significantly decreased in mice without IDUA activity (Figure 14). Due to this decrease, the quantity of RANKL was assessed in WT and IDUA male mice (Figure 21). Preliminary staining with DAB indicants decreased amounts of RANKL in IDUA males in comparison to the WT; however due to histological artifacts no conclusions can be made.

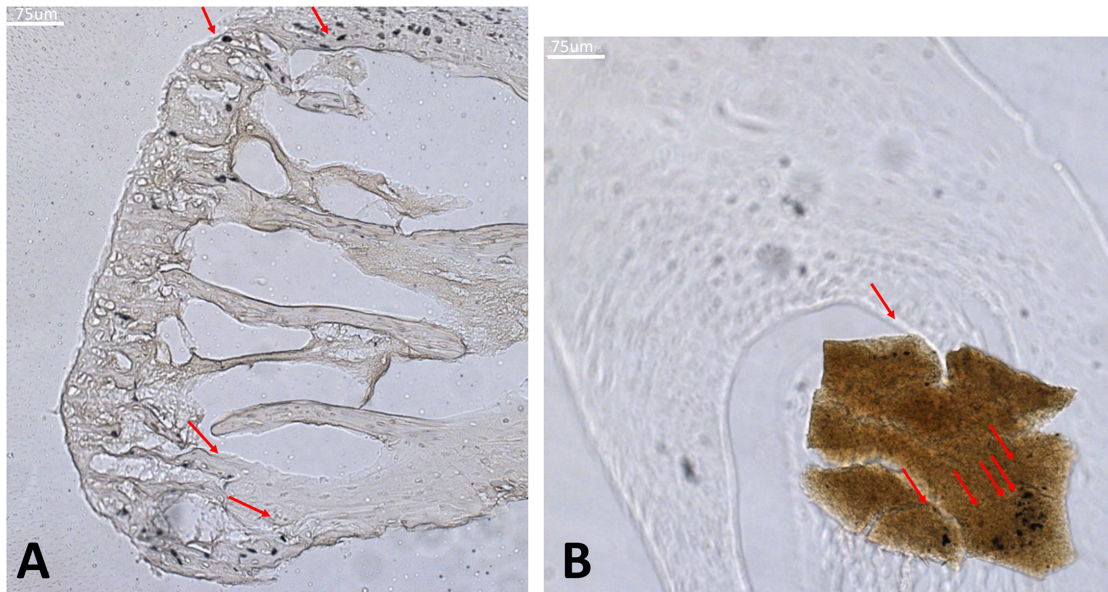

Figure 21: Immunohistochemical Staining. Representative images of IHC decalcified tibias. The left tibias of IDUA (**A**) and WT (**B**) males were sectioned in the sagittal plane and stained with DAB. Red arrows indicated areas of high RANKL concentration. Bright field image taken at 10X magnification on a Confocal Microscope.

Conclusion

Throughout the life span of an individual, the skeleton is continuously undergoing remodeling. Bone remodeling is initiated by activation of the bone cells responsible for resorption, osteoclasts. Upon stimulus from receptor activator of the NF-κB ligand (RANKL), preosteoclasts begin to differentiate into mature osteoclasts capable of resorption. Preliminary histological analysis indicates lower RANKL quantities in the bone cell population of IDUA mice; however due histological artifacts, no conclusions can be made. Further repetitions are required to elucidate differences in RANKL levels across genotypes.

There were several limitations to this investigation. The initial 24 hour incubation insufficiently decalcified the tibias causing the samples to fall out of the wax mount during sectioning. Additionally, some samples were not able to be completely sectioned due to the presence calcified tissue pockets. To remedy this, the bone decalcification period was increased to 36 hours. The majority of problems were elevated, however, imaging reveled multiple layers within the sectioned tissue after immunohistochemical staining. The multiple layers were more pronounced in the IDUA males (data not shown). Due to the increased bone mass in the IDUA males, an increase incubation time in the decalcification solution will be required moving forward.

The delicate nature of the sectioned bone initially resulted in the loss of samples during heat induced antigen retrieval steps. To preserve samples for use in immunohistochemcial staining, the antigen steps were removed from the procedure which could indirectly affect the amount of the target antigen, RANKL. Certain fixatives result in the obscuring of the epitope and usually these antigens are unmasked through antigen retrieval. The removal of this step could have resulted in an even further decrease of detectable antigen in all samples. Other antigen

retrieval methods, such as enzymatic retrieval, could prevent the loss of sample. Additionally, due to the relative thinness of the sections, there was difficulty in preventing the samples from folding in on itself during mounting on microscope slides (data not shown). This could be alleviated by increasing the hot water bath temperature during the mounting.

THE IMPORTANCE OF BIOETHICS IN RESEARCH

Ethics plays a critical role in research involving living subjects; however, it was not until the conclusion of World War II that a global discussion began about what is and is not permissible in research. Historically, marginalized and incapacitated individuals (i.e. minorities, women, and children) were commonly subjugated to various experimentations without their consent under the declaration of furthering science and medicine. After decades of discussion, a consensus was met and produced a code of ethics to which clinical researches are expected adhere to. Additionally, Institutional Review Boards (IRB) were established at various institutions in order insure researcher compliancy in the protection of the rights of human test subjects.

Before initiating any investigations with humans as test subjects, an IRB board reviews the proposal for the conduction of experiments. In the examination of the research proposal, an IRB board makes sure that ethical standards are met as outlined in the Belmont Report [24]. These standards included the following: maintaining patient autonomy, beneficent and non-maleficent actions, and justice in participant selection. When the subjects of interest fall into the category of the historically marginalized, even more consideration is taken during proposal review. In cases dealing with terminal patients, prioritization in placed upon ensuring that the subject's rights and protections are considered throughout duration of the experiment despite their health status. This section aims to define the rights and protections of children in research through the discussion of patient autonomy, the importance of doctor and patient relationships, and consenting to clinical research.

Autonomy

Patient autonomy is fundamental to the field of medical ethics. It is defined as the right of a rational individual to make their own decision and is the moral justification for the informed consent process [42]. The importance of autonomy in medical research and in a clinical setting have been realized within the past century due to guidelines being set in place for medical practitioners. As mentioned previously, it was the social norm for marginalized and incapacitated individuals to be subjugated to various experimentations without their consent under the declaration of furthering science and medicine [14]. Upon the conclusion of the Nuremberg trials, the world saw medical research in a new light and revealed various short comings. It can be attributed to the code of medical ethics that the importance of maintaining and nurturing patient autonomy was realized.

It is established that individual autonomy consists of the following requirements: liberty and agency. Liberty encompasses the freedom from controlling influences [43]. Agency, on the other hand, refers to the capacity for intentional action or self- governance and begins to develop in early adolescence [42]. The ability to exercise self- governance also includes the concept of respect for others [44]. The freedom from external incentives and capacity to exercise free-will play a pivotal role in giving informed consent. These facets of autonomy require balancing multiple interests and practical wisdom. Additionally, early adolescences is the time frame where building relationships and respecting another individual's autonomy starts to develop. It is for these reasons that in cases involving children, proxies are designated.

In healthcare, the norm is to respect a patient's autonomy and approach it so that the patients have a say in their care. However, there are some situations in which physicians diagnose and treat their patients without their consent [45, 46]. These situations can best be

described as selective medical paternalism, in which a course of action in the best interest of the patient is chosen without obtaining consent. In medicine, this is in reference to a physician distributing resources to their patients and directing their patient's care [46]. This being said, medical paternalism should not be the endpoint or solution before moving forward with a treatment plan. The physician in such cases should encourage active participation in the decision-making process. In cases where the individual is incapacitated or a minor, the medical professional should work with the appointed surrogate decision-maker.

The concept of autonomy can be very difficult to traverse in clinical ethics. As has previously been described, autonomy is the right of a rational individual to make their own decision. This being said, in certain cases, autonomy has been abused. A common example, would be the anti-vaccination movement. Though the scientific foundation for this movement has been disproven, the anti-vaccination movement still disseminates misinformation. They argue that as an autonomous individual, their right to not vaccinate themselves or their children should be respected. Due the public health risk, unvaccinated individuals are barred from certain public locations and activities. The anti-vaccination movement argues that their civil rights should not be denied. They as Americans, have the constitutional right to deny vaccination of themselves and their children based off their personal beliefs. However, due to the life threatening risks caused by unvaccinated individuals in modern society, this group is often ostracized.

A major argument against the anti-vaccination movement is the consistent circulation of pseudo-science. Countless experts have come forward to provide evidence supporting vaccinations, but their voice falls upon deaf ears. This is further exacerbated by the abundance of information available on the internet and often results in the refusal to listen to expert opinions. It

is this perversion of scientific knowledge by the uneducated that has resulted in the re-emergence of illnesses in the 21st century. At this point, the question of autonomy then becomes: How wrong does an autonomous individual get to be, before they are denied civil rights? For many, this is the point at which society as a whole will be harmed by the actions of one.

Doctor and Patient Relationships

The foundation for doctor and patient relationship is the establishment of trust [47, 48]. Through the establishment of trust, high-quality healthcare is able to be delivered, which is the objective of each patient visit. In the absence of trust, there is a negative effect which has been expressed by both physicians and patients [49]. Historically, the most effective method for fostering trust is building rapport. For this to occur successfully, there must be effective communication between both parties and is determined by the physician's bedside manner [48]. Bedside manner entails the development of interpersonal skills and appropriate communication levels. Often, these are the indicators that patients will use to evaluate the proficiency of their physician.

The ability to relay information in a way that is comprehensible to the general public is key to bedside manner. This must be executed in a way that does not make the patient feel incompetent or belittled. Conveying complex medical concepts is a daunting task; however, it is important in building rapport and traversing the informed consent process. From the patient's point of view, a medical professional who is unable to relay complex concepts is intimidating. Depending on the patient, they may seek another opinion or may decide to defer directly to the physician's opinion though they are not sure what is actually happening. It can be argued then

that the informed consent process has not been completed. Medical professionals should work on sharpening these skills and be able to detect and overcome verbal barriers.

In clinical trials, the aforementioned communication gap can be more difficult to transverse. Due to the pioneering experiments being conducted, an in-depth knowledge base may not be established. In such situations the physician should use discretion in their explanations and make it apparent that there is no definitive answer. They may provide their rational for the experiment but should not get tied up in using technical jargon, as at that point they would lose patient involvement. In cases with children, the ability to relay information to the child for assent and to the surrogate for consent may prove even more difficult.

Once the information is delivered to the patient, there are a variety of factors that will affect how they decide to move forward. In modern society, many patients recognize that they are not to be passive in the informed consent process [48]. They will draw upon their own perspective, knowledge on the subject, societal or cultural norms, and personal experiences when evaluating the expert's opinion [48]. For instance, say an individual who is a chronic smoker goes in for their yearly physical. After completing the physical exam, the doctor recommends that the patient should quit smoking due to a high risk of lung cancer. Though this is an expert's recommendation, the patient ultimately will decide what they think is best. In today's society it is frowned upon to smoke as proven by numerous, publically available studies and has been circulated to the public through service announcements. If however, it was common to see both young and old community members smoke growing up, the individual's views on the habit may differ. Some argue that the current approach of promoting public disapproval of smoking is coercive to some degree. A narrative riddled frequently with brash imagery is implement, which

could be perceived as threatening the free will of citizens. One could argue that this methodology is unjustifiably coercive, however, a decision is still being made after the message is delivered.

Concepts of Informed Consent and Assent

Informed Consent is the process in which an adult freely agrees to a treatment upon receiving information on treatment outcomes [42, 51]. In a clinical setting, this process is initiated by the physician and begins with explanation of the diagnosis. During this conversation, the physician explains the benefits and risks associated with the potential treatments and is followed by their professional opinion and recommendation. At the conclusion of the informed consent process, the patients should have an understanding of all items of concern, be able to assess alternatives, and decide if they agree to the set treatment plan [51]. Usually, before moving forward with treatment, a waiver is signed by the consenting individual stating that they have been made aware of any side effects.

In cases with minors, consent is obtained from their guardian. Legal adults are able to consent based on the standard of autonomy; however, with assent, the main focus is on the developmental capacity of the minor. In pediatric cases, surrogate decision makers, parents, or guardians have the legal authority to make medical decisions on behalf of minors [42, 51]. These surrogates must be competent and possess the knowledge needed to make decisions regarding someone else's health. After obtaining consent from the legal guardian, the child's opinion is taken into account. During the assent process, the minor is approached as an individual with emerging autonomy and practitioners seeks their agreement to participate in the treatment plan. In the course of obtaining a child's assent, health professionals assess their patient's respective

awareness of their condition, what is expected by their diagnosis, and projected outcomes of their treatment.

Conflicts that Arise in the Consent Assent Process

There are variety of conflicts that may arise during the consent assent process. Non-maleficence refers to doing no harm to the patient and in the context of medicine and science is used in reference to physical pain [16, 52]. Clinicians seek to do no harm in treating their patients; however what they perceive to be for the patient's best interest may be seen in a different light. From the patient's point of view, harm may go beyond the physical and could encompass emotional and spiritual harm. Depending on the individual, the threat of emotional or spiritual harm could far out weight the risk of physical harm. Such differences commonly arise due to cultural or religious beliefs. Certain faiths steadfastly believe that any form of modifications done to one's physical form (including blood transfusions, surgeries, etc.) will place their eternal soul at risk. Medical professionals should be well versed and practiced in cultural competency so that the customs and beliefs of their patients are taken into account while obtaining consent. This may draw out the process but will aid in establishing rapport with the patient and their family.

The already sensitive nature of the aforementioned cases can become further exacerbated in pediatric medicine. The best course of action in situations where the guardian refuses treatment for religious reasons is to involve the government. As ruled by the US Supreme Court in case of Prince vs Massachusetts, surrogates cannot make martyrs out of their guardians. It is the policy of the America Academy of Pediatrics that " all legal interventions apply whenever [minors] are endangered or harmed, without exemption based on [their guardians] religious

beliefs" [53,54]. As the ruling authority, it is necessary for the government to remain neutral on topics of worldview and religion; however, from the states' point of view, the physical well-being of its citizens will always out -weigh liberty. If there is any doubt in cases dealing with cultural and religious beliefs, legal advice and a second opinion is encouraged. Ideally, through open conversation with the aforementioned parties, a resolution can be met that will benefit the child.

Adolescent cases also have proven to be difficult cases to maneuver with clinical investigations. When it comes down to a final decision in pediatric cases, if the case involves young children the wishes of their parents or guardian commonly prevail; however, in adolescent cases, a minor's refusal of treatment is approached differently [42]. Due to developing autonomy, adolescents are involved with the decision regarding their well –being, to a certain degree [44, 51]. The capacity to make a decision, matures with both the time and experience of an individual. The developmental stage of the minor should be considered by clinicians and their preferences accordingly incorporated into any treatment plan [44]. In some circumstances, the surrogate's decision may put the minor's wellbeing at risk by refusing diagnostic procedures or a treatment. Though it may be impossible to convince the legal guardian in every situation, the clinician should make an effort to resolve the situation and discuss pros and cons at a level that is comprehendible to all parties [42]. In cases where the guardian refuses to provide the necessary care that the minor needs, medical professionals should contact State officials and not interfere. Only in emergencies when the minor's life is at risk should the clinician move forward without completing the consent process [42].

Navigating the assent process can be further exacerbated in cases where the child is affected by an uncommon or terminal condition. Clinical researchers are able to characterize the

physical manifestations but until a knowledge base surrounding the molecular mechanism have been established, they are unable to effectively treat all phenotypic symptoms. Additionally, through the collection of empirical evidence, the full extent of the emotional and physical toll is not apparent. A prime example is Hurlers syndrome patients. As discussed previously, Hurlers is the most severe form of a rare autosomal recessive disorder and affects mainly children. The physical abnormalities that arise due to this disorder have been characterized extensively through a range of animal models and has helped in developing treatment that ameliorates the majority of symptoms, with the exception of bone phenotype. Without experiencing the disease first hand, researchers are unable to comprehend the physical and emotional toll placed on the child. Due to these circumstances, it can be argued that the assent of such individuals should hold more weight. The affected child is able to provide a unique perspective and their position results in a greater understanding of the implications regarding their treatment. For these reasons, in such cases if the child is affected by an uncommon condition, their assent to treatment should hold more sway during the consent-assent process than others in their age group.

There are a range of factors that could affect the capacity of adolescents to give their voluntary assent. These factor include but are not limited to: the knowledge base of the individual, their mental and physical health status, and experiences with making decisions. In cases where a minor has dealt with the side effects of treatment from an early age, their opinion during the consent-assent process holds more influence than a young child [42, 44]. For instance, say there is a sixteen year old who was chronically ill and after years of treatment and dosage changes, they are finally on a treatment plan that has made their symptoms manageable. Their guardian is ecstatic and requests that the dosage be increase. The physician sees no harm in increasing the dosage to the previous amount, but when the question is posed to the patient, they

ask that the current treatment plan continue. They reveal that their side effects were much more difficult to manage at the higher dosage and their quality of life decreased. Due to a developing sense of autonomy and familiarity with treatment and illness, the adolescent's opinion will play a larger role moving forward. If there is further disagreement, the physician should act as the mediator to develop a plan of action that is desirable to both parties.

Due to financial, emotional, and physical dependency upon their caregivers, minors (both children and adolescent) may defer automatically to their guardian's decision without regard for their own feelings. This is a common behavior, however to complete the consent-assent process, the minor's assent must be given completely voluntarily. In such cases, the physician has the responsibility to introduce the topic of the child participating in decisions regarding their care and including them in conversations by asking their opinion. This being said, medical professionals cannot assume that the legal guardian will provide an impartial decision and have not previously persuade the child to agree or refuse a treatment [51]. To minimize occurrences and ensure that all parties are well informed, it is recommended that physicians collaboration with a Child Life Specialist. In general, the court is hesitant to interfere with a guardian's authority but they also understand that one of their roles is to protect the interest of those unable to protect themselves [55]. The choice to remove child from their guardian should be done only when a high threshold of evidence has been reached.

Conclusions

Within the past century, there has been an influx of new diagnostic and therapeutic options in the medical field. The development of innovative techniques has a complex history and a significant portion of these experiments were carried out at the expense of moral

ambiguity. Guidelines to combat these inconsistencies have been put in place and are updated with the expansion of the knowledge base. These guideline have also aided in establishing the roles in clinical investigations. Typically, research now begins in an animal model and is transitioned to humans once substantial data has been provided. Additionally, committees have been instituted to protect the rights and welfare of research participants, whether human or mammalian.

Clinical investigations involving human subjects have established that autonomy be maintained. Autonomy consists of a self-governing individual agreeing to participate outside of additional influences. In order to participate in clinical studies, the individual goes through the process in which informed consent is obtained. This practice entails the establishment of a doctor-patient relationship and relay of information. When the consenting individual is a minor, this process is altered. A surrogate decision maker ultimately makes the decision regarding their charge but the child is treated as an individual with developing autonomy. Especially in cases with rare disease, the affect individual is able to provide a unique perspective that transcends age groups. Therefore, when the affected individual is a child, such as in Hurlers syndrome, their assent to treatment should have more influence.

At its fundamental level, research is pursuit of knowledge and as scientists and medical professionals we have a responsibility to expand the knowledge base. We are given the responsibility to relay what has been discovered to the general population. This can significantly be impeded by an inability to communicate findings without being dismissive. It is our responsibility to cultivate the necessary communication skills to deliver information that can benefit others. Additionally, as researchers and medical professionals, it is imperative to maintain patient autonomy, especially due to the current rate of advancements being made in

science and technology. We are striving to benefit humanity in investigations being conducted; however, one can very quickly lose their humanity through compromise. Those compromises may start out small, but could eventually lead to large scale concessions. At that point, the likelihood of rationalizing unethical behaviors is probable and one could argue they are no better than the Nazi scientists. As scientist, we should not only hold ourselves but also our peers to a high ethical standard. If we allow compromises to be made, no longer will we be able to hold the trust of those we seek to assist.

FUTURE DIRECTIONS

Mucopolysaccharidosis type I is a lysosomal disorder which arises due to deficiency of α-L-Iduronidase (IDUA). The absence of IDUA activity results in the progressive accumulation of the glycosaminoglycans, dermatan and heparin sulfate. Ultimately, the pathological accumulation of dermatan and heparin sulfate results in multi-organ system failure. For affected individuals there are limited treatment option which are only able to improve quality of life and have no effect upon the skeletal abnormities. The findings in this investigation will aid in further characterization of the skeletal abnormalities in the IDUA-W392X mouse model. It is anticipated that continued investigations with this mouse model could expound on the molecular mechanisms causing the MPS I skeletal phenotype. This study also aimed to address bioethical aspects of experimental medicine in pediatric cases. These aspects were addressed through discussion of the rights and protections of children in research.

The type I collagen content in the tibias and femurs of wild type, heterozygous, and mice without IDUA activity was assessed through a hydroxyproline assay. This assay determined that male IDUA mice had significantly elevated collagen levels in comparison to other genotypes. Furthermore, it was determined that in IDUA males that the increased collagen content resulted increased bone mass. Additional investigations regarding the bone matrix composition are required. It is likely that the IDUA deficiency also affects the accumulation of proteoglycans within the matrix. Proteoglycans consist of multiple GAG molecules covalently attached to leucine-rich core protein and are found within the bone matrix. This can further assessed through histological analysis of bone proteoglycan content or through quantification of sulfated glycosaminoglycans.

In order to evaluate osteoclast activity, immunohistochemical staining for the osteoclast activator RANKL was performed. Preliminary results suggest lower RANKL levels in mice without IDUA activity. Further analysis in both male a female mice should clarify if the decrease is significant across genotypes. Moreover, immunohistochemical staining of other osteoclastogenesis markers could elucidate the extent of impaired bone remodeling in IDUA mice. Additionally, it has been reported in other MPS I models that the osteoclast are not able to degrade bone in the shortage of functional IDUA. It was determine in this study that the accumulation of GAGs prevents cathespin K activity [30]. Cathespin K is the first protease that is secreted by activated osteoclast. It is primarily involved in the degradation of the collagen matrix and is essential in forming the sealing zone in which bone resorption occurs. If indeed cathespin K activity is impeded, this could be indicative of the decrease in active osteoclast in MPS I models. If the hydroxyproline assay in this study were modified to treat the bone powder with cathespin K before tissue hydrolysis, the amount of collagen degraded by the protease could be determined across genotypes.

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