The Role of Genetic Background on the Phenotypic Severity of the Osteogenesis Imperfecta Murine (*oim*) COL1A2 Gene Mutation Throughout Postnatal Development

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By

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

THE ROLE OF GENETIC BACKGROUND ON THE PHENOTYPIC SEVERITY OF THE OSTEOGENESIS IMPERFECTA MURINE (*OIM*) COL1A2 GENE MUTATION

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The Role of Genetic Background on the Phenotypic Severity of the Osteogenesis Imperfecta Murine (*oim*) COL1A2 Gene Mutation Throughout Postnatal Development

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ABSTRACT

Osteogenesis imperfecta (OI) is a genetically and clinically diverse disease of type I collagen, the main structural protein in the body. To date, more than 850 distinct OI-causing mutations have been identified representing a broad range of clinical outcomes. It has been well documented that related individuals harboring the same OI-causing mutation can have very different clinical outcomes. But the relationship between genotype and clinical outcome is unclear. The most widely studied mouse model of OI is the osteogenesis imperfecta murine (*oim*) which contains a single base pair deletion in the *col1a2* gene causing a frameshift, thus altering the terminal 48 amino acids. This results in the exclusion of $\alpha 2$ chains from type I collagen molecules and in the production of the homotrimeric collagen [$\alpha 1(I)_3$] isotype instead of the more prevalent heterotrimeric collagen [$\alpha 1(I)_2 \alpha 2(I)$] isotype. The resulting clinical phenotype is similar to that of human OI type III patients, including bone fragility with multiple fractures, bowing of the long bones and cortical thinning.

The goal of this study was to further examine the genotype-phenotype relationship with the long-term goal of identifying possible modifier genes impacting the quality of bone. Modifier genes are those that influence clinical outcome by interacting with genes known to impact bone quality such as the type I collagen genes. As gene expression changes throughout development, we sought to determine if age related changes occurred in our animal model. To this end, we bred the *oim* mutation from the outbred B6C3Fe background to the inbred C57BL/6J (C57) background, known to have low, but phenotypically normal, bone mineral density. Wildtype, *oim*/+ and *oim/oim* animals of each strain were then analyzed at one, two and four months of age to assess quantitative bone parameters such as geometry, strength and stiffness. To determine biochemical parameters, total amounts of type I collagen were determined as well as mineral composition. Pyridinoline crosslinks were also measured to determine if an unusually high level of bone turnover occurs.

This work showed strain related differences at all ages examined, but the presence of the *oim* mutation overrides these differences. Bones from *oim/oim* animals of either strain have altered bone geometry and reduced biomechanical integrity as compared to their wildtype counterparts, although C57-*oim/oim* animals had a more severe phenotype than outbred *oim/oim* animals. *Oim/oim* animals also exhibit an altered mineral composition as well as reduced amounts of type I collagen, which were strain specific. Pyridinoline crosslinks demonstrated strain differences in *oim*/+ and *oim/oim*, but not wildtype, animals.

Taken together, these data indicate a role for genetic background in determining phenotypic severity, although some parameters are more affected by genetic background than others.

CHAPTER 1

Project Summary

Osteogenesis imperfecta (OI) is a genetically and clinically heterogeneous disease characterized by anomalies in type I collagen-containing tissues, such as bone, tendon, skin, and teeth. The disease dates back at least two millennia and effects all ethnic and racial groups equally (Byers 1993). To date, more than 850 distinct OI-causing mutations have been identified (Byers 1993; Dalgleish 1997; Dalgleish 1998; Dalgleish 2006) representing a broad range of clinical outcomes. Clinical manifestations include osteopenia with fractures, skeletal deformities, short stature, dentinogenesis imperfecta and blue-gray sclera (Cole 2002). Most OI-causing mutations are in one of the two type I collagen genes, COL1A1 or COL1A2 (Byers 1993). Patients with OI vary in phenotypic severity, even between related individuals harboring the same mutation. The relationship between genotype and clinical phenotype remains poorly defined.

To better understand the role of genetic background in determining phenotypic severity, the same OI-causing mutation was analyzed on two genetically different strains of mice. The C57BL/6J (C57) inbred strain is phenotypically normal, but is known to have low bone mineral density. The B6C3Fe strain is an outbred hybrid strain composed of two genetic backgrounds, C57BL/6J and C3H/HeJ. The osteogenesis imperfecta murine (*oim*) is a naturally occurring mouse model of OI containing a single nucleotide deletion in the COL1A2 gene, resulting in a non-lethal, recessive OI phenotype. This mutation first arose on the B6C3Fe background and results in exclusion of the proα2(I) collagen chain from the triple helical collagen molecule (Chipman, Sweet et al. 1993). Therefore, these animals produce only homotrimeric type I collagen,

consisting of three α1(I) chains, [α1(I)$_3$] instead of the normal heterotrimeric collagen containing two α1(I) and one α2(I) chains [α1(I)$_2$α2(I)]. This results in a phenotype similar to moderately severe human OI type III, including increased fracture number, bowing of the long bones and decreased body size (Chipman, Sweet et al. 1993).

For this study, the *oim* mutation was bred from the B6C3Fe strain to the C57 background and heterozygote offspring repeatedly backcrossed to wildtype C57 animals. After seven backcross generations, the C57 strain should be 99% genetically identical except for the transferred chromosomal region (Daniel McBride, personal communication). Wildtype, *oim*/+ and *oim/oim* animals of each strain were then analyzed at one, two and four months of age to assess quantitative bone parameters and the impact of the *oim* mutation throughout development. Bone geometry was assessed using μCT analysis before undergoing torsional loading to failure to determine bone strength and stiffness. Bone mineral composition was quantitated using neutron activation analysis while hydroxyproline analysis was used to determine total bone collagen content in the different genotypes and strains. Pyridinoline, a marker of bone resorption, was also measured to determine if the alterations in bone parameters are due to an unusually high level of bone turnover.

We demonstrated that strain related differences in bone parameters do exist at all ages examined. However, the presence of the *oim* mutation had the greatest impact on overall bone quality. Regardless of the strain, bones from *oim/oim* animals have altered geometry and reduced biomechanical properties as compared to their wildtype counterparts. *Oim/oim* animals also exhibit an altered mineral composition as well as reduced amounts of type I collagen. Pyridinoline crosslinks exhibited strain differences in *oim*/+ and *oim/oim* animals.

Taken together, these data indicate that genetic background plays an important role in determining phenotypic severity; however, the presence of the *oim* mutation remains the predominant factor in determining the phenotypic severity regardless of genetic background. These data suggest although the specific collagen mutation has the greatest impact on clinical severity, modifier genes do exist that can enhance bone quality even in the presence of collagen mutations. These modifier genes may offer future therapeutic and pharmacological targets aimed at maximizing bone quality even in the presence of OI-causing mutations.

LITERATURE REVIEW

The Collagen Superfamily and Type I Collagen

The collagen superfamily contains 28 distinct types of collagen encoded by more than 42 genes dispersed across the genome (Jackson; Laboratory; Byers 2001; Myllyharju and Kivirikko 2004; Veit, Kobbe et al. 2006). All collagens are triple helical molecules, although the identity of the chains that make up the molecule are unique to each collagen type (Table I) (Byers 2001). Commonalities among the collagens include the $(Gly\text{-}X\text{-}Y)_n$ sequence, where X is usually proline and Y is usually hydroxyproline (Kielty 1993). The positioning of glycine, the smallest amino acid, at every third position allows for the tight winding of the helix (Byers 2001). The triple helical nature of the collagens allows the molecules to be extremely stable within the extracellular matrix environment. When the α chains are present as single unincorporated chains, they are extremely susceptible to proteolytic degradation, but assembly into the helix protects the chains from degradation by most proteases except for specific collagenases (Byers 2001).

The collagen genes are initially synthesized as precursor molecules that are then modified to make the mature collagen molecule. For the fibrillar collagens, such as types I, II, III and V, these precursor molecules contain a signal sequence, globular carboxy and amino terminal ends, a telopeptide and the triple helical domain (Byers 2001). These individual chains are synthesized on polyribosomes and the growing chains are moved into the rough endoplasmic reticulum and the signal sequence removed. During this time, certain prolyl and lysyl residues are hydroxylated and some of these hydroxylysyl residues are then glycosylated (Byers 2001). The hydroxylation of proline is necessary for the thermal stability of the triple helix while hydroxylation of lysine residues provides

more stable covalent cross-links than the lysyl residues alone (Eyre 1987). The glycosylation of hydroxylysine may have a role in fibril formation and interactions with cells and other macromolecules (Byers 2001). Following synthesis and modification, the procollagen chains are assembled into trimers. The carboxy-terminal region of the proα chains associate with each other and winding proceeds toward the amino-terminus (Cohen, Diegelmann et al. 1992). The triple helical molecule is then moved to the Golgi and packaged into secretory vessels for transport to the extracellular environment (Cohen, Diegelmann et al. 1992). Once there, the globular C- and N-terminal ends are cleaved, leaving only the triple helix (Cohen, Diegelmann et al. 1992). The resulting monomers spontaneously assemble into fibrils via interaction between the telopeptide regions of adjacent monomers and these fibrils are stabilized by intermolecular crosslinks, giving the fibril tensile strength (Cohen, Diegelmann et al. 1992).

Type I collagen is the main structural protein in the body, giving structural integrity to vertebrates and other multicellular organisms (Hulmes 2002). It provides resistance to tensile stress in skin, tendon, bone and cartilage (Hulmes 2002). Type I collagen is a fibrillar collagen, normally found as a heterotrimer, consisting of two α1 chains and one α2 chain [$\alpha1(I)_2\alpha2(I)$]. However, a homotrimeric [$\alpha1(I)_3$] variant has also been identified and is normally found in small quantities in the skin (Moro and Smith 1977; Uitto 1979; Rupard, Dimari et al. 1988), amniotic fluid and embryonically (Jimenez, Bashey et al. 1977; Scriver 1989), in certain tumors and may have a role in wound healing (Haralson, Jacobson et al. 1987; Cohen, Diegelmann et al. 1992). Although homotrimeric type I collagen is compatible with life, evolutionary studies have shown the conservation of the proα2(I) chains

Table I-1: Collagen Superfamily and Associated Diseases

Name	Gene(s)	Chain(s)	Isotypes	Type	Associated Disorders
I§	COL1A1	α1	$\alpha1(I)_2\alpha2(I)$	Fibrillar	OI[a]
	COL1A2	α2	$\alpha1(I)_3$		EDS[b]
II	COL2A1	α1	$\alpha1(II)_3$	Fibrillar	Chondrodysplasias
III§	COL3A1	α1	$\alpha1(III)_3$	Fibrillar	EDS
IV	COL4A1	α1	$\alpha1(IV)_2\alpha2(IV)$	Basement membranes	Alport syndrome
	COL4A2	α2	Others?		
	COL4A3	α3			
	COL4A4	α4			
	COL4A5	α5			
	COL4A6	α6			
V	COL5A1	α1	$\alpha1(V)_2\alpha2(V)$	Fibrillar	EDS
	COL5A2	α2	$\alpha1(V)\alpha2(V)\alpha3(V)$		
	COL5A3	α3			
VI	COL6A1	α1	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Microfibril	Bethlem myopathy
	COL6A2	α2			
	COL6A3	α3			
VII	COL7A1	α1	$\alpha1(VII)_3$	Anchoring	EB[c]
VIII	COL8A1	α1	$\alpha1(VIII)_2\alpha2(VIII)$	Meshwork	?
	COL8A2	α2			
IX	COL9A1	α1	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	FACIT*	Chondrodysplasias
	COL9A2	α2			
	COL9A3	α3			
X	COL10A1	α1	$\alpha1(X)_3$	Meshwork	Chondrodysplasias
XI	COL11A1	α1	$\alpha1(XI)\alpha2(XI)\alpha1(II)$	Fibrillar	Chondrodysplasias
	COL11A2	α2	$\alpha1(XI)\alpha2(V)\alpha1(II)$		
XII	COL12A1	α1	$\alpha1(XII)_3$	FACIT	?
XIII	COL13A1	α1	$\alpha1(XIII)_3$	Transmembrane	?
XIV	COL14A1	α1	$\alpha1(XIV)_3$	FACIT	?
XV	COL15A1	α1	$\alpha1(XV)_3$	Endostatin	?
XVI	COL16A1	α1	$\alpha1\ (XVI)_3$	Other	?
XVII	COL17A1	α1	$\alpha1(XVII)_3$	Transmembrane	EB
XVIII	COL18A1	α1	$\alpha1(XVIII)_3$	Endostatin	?
XIX	COL19A1	α1	$\alpha1(XIX)_3$	Other	?
XX	COL20A1	α1	$\alpha1(XX)_3$	FACIT	?
XXI	COL21A1	α1	$\alpha1(XXI)_3$	FACIT	?
XXII	COL22A1	α1	$\alpha1(XXII)_3$	FACIT	?
XXIII	COL23A1	α1	$\alpha1(XXIII)_3$	Transmembrane	?
XXIV	COL24A1	α1	$\alpha1(XIV)_3$	Fibrillar	?
XV	COL25A1	α1	$\alpha1(XV)_3$	Transmembrane	?
XVI	COL26A1	α1	$\alpha1(XVI)_3$	FACIT	?
XVII	COL27A1	α1	$\alpha1(XVII)_3$	Fibrillar	?
XVIII	COL28A1	α1	$\alpha1(XVIII)_3$	Basement Membranes?	?

§ Indicates presence in bone; * FACIT: fibril-associated collagens with interrupted triple helices; [a] Osteogenesis imperfecta; [b]Ehlers-Danlos syndrome; [c]epidermolysis bullosa

(Bernard, Myers et al. 1983; Deak, van der Rest et al. 1985; Exposito and Garrone 1990; Exposito, D'Alessio et al. 1992) as well a high degree of sequence similarity between the human and mouse α2(I) chains (McBride and Shapiro 1994), indicating an important role for the α2(I) chain in the collagen molecule. The α1(I) chain, as well as homotrimeric type I collagen, may represent an earlier, ancestral form of collagen as fibrillar collagens have been identified in several lower species, including sponges (Exposito and Garrone 1990) and sea urchins (Exposito, D'Alessio et al. 1992). The genes encoding the triple helical portion of the molecule are arranged into exons of 54 bp or multiples of 54 bp, suggesting that these exons may have arisen from an ancestral collagen gene by multiple duplications (Exposito, Cluzel et al. 2002). Gene structure analysis of sponges has shown the same arrangement of exons in multiples of 54 (Exposito, Cluzel et al. 2002). cDNA studies using sponges have shown remarkable similarity between collagen structure of both vertebrates and invertebrates (Exposito and Garrone 1990). The small differences in the gene structure may be due to adaptations made by the animal to best fit into their environment. For example, collagen from worms living at varying depths of the ocean are compared, those living in the deep sea where temperatures are very cold have higher proline content and stabilizing triplets than those found at higher temperatures (Exposito, Cluzel et al. 2002). The addition of the α2(I) chain in higher vertebrates may be a similar adaptation, as the presence of this chain produces a collagen with more biomechanical integrity than homotrimeric collagen (Misof, Landis et al. 1997; McBride, Shapiro et al. 1998).

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a genetically and clinically heterogeneous disease characterized by anomalies in tissues containing type I collagen, such as bone, tendon, skin, teeth and sclera. Clinical manifestations include osteopenia with fractures, skeletal deformities, short stature, skin laxity, dentinogenesis imperfecta and blue-gray sclera (Cole 2002). Most mutations causing OI are in one of the two type I collagen genes, COL1A1 or COL1A2 (Byers 2001), although three new classifications have been identified in which the disease is not associated with defects in type I collagen genes (Roughley, Rauch et al. 2003). OI-causing mutations generally lead to either decreased amounts of normal type I collagen or the production of abnormal $\alpha 1$ or $\alpha 2$ chains (Byers 2001).

Clinically, OI was originally divided into four classifications based on clinical manifestations and severity of the disease (Sillence, Senn et al. 1979), although three additional types have been recently added (Roughley, Rauch et al. 2003). Type I OI is the mildest and most common form characterized by pre-pubertal fractures, normal stature and sclera, and little or no deformation of long bones (Byers 1993; Marini 1998). Type II OI is perinatal lethal often due to pulmonary insufficiency caused by a small abdominal cavity (Byers 1993). Type III is the most severe form compatible with life. Type III patients usually have some long bone deformation at birth that progresses throughout life and can limit ambulation in addition to short stature and blue-gray sclera. The hallmark of type III OI is extreme bone fragility leading to numerous fractures throughout life. Dentinogenesis imperfecta and premature hearing loss are also common (Byers 1993). Type IV OI is variable with severity between that of types I and III (Marini 1998; Cole 2002) and can be characterized by mild to moderate bone deformity

and normal stature and sclera (Byers 1993). Patients with types III and IV may also suffer from scoliosis (Marini 1998). All these forms of OI described are inherited in an autosomal dominant fashion, although autosomal recessive mutations have been documented in types II and III (Byers 1993; Cole 2002).

The three new, and relatively rare, classifications of OI that were recently added describe OI resulting from mutations in non-collagen genes (Roughley, Rauch et al. 2003) and are classified based on bone architecture. These patients would have previously been assigned to OI type IV based solely on clinical presentation (Roughley, Rauch et al. 2003). Patients with type V OI have moderately deforming with hypertrophic callus formation at sites of fracture and calcification of interosseous membranes (Roughley, Rauch et al. 2003). Type VI OI patients have moderate to severe skeletal deformity with osteoid accumulation but without anomalies in calcium, phosphate or vitamin D metabolism indicative of osteomalacia (Roughley, Rauch et al. 2003). Patients with type VII OI are unique as type VII appears to be inherited in an autosomal recessive pattern and is characterized by moderate to severe skeletal deformity and bone fragility as well as a disproportionate shortening of the humerus and femur (Roughley, Rauch et al. 2003).

Ehlers-Danlos Syndrome (EDS): Similarities and Differences to OI

The bone problems that define classical OI are due to mutations in the type I collagen genes. However, other non-bone diseases can also arise from mutations in these genes. Various forms of Ehlers-Danlos syndrome are also caused by mutations in the COL1A1 and COL1A2 genes, but do not produce the bone phenotype characteristic of

OI. Ehlers-Danlos syndrome types VIIA and B (classification: arthrochalasis) are due to mutations in the COL1A1 and COL1A2 genes, but are defined by hyperextensible skin, hypermobile joints and easy bruising with congenital hip dislocation (Burrows 1999). The classical mutations leading to these types of EDS tend to involve mis-splicing of exon 6 in both COL1A1 and COL1A2 genes, although other EDS-causing mutations have been identified. Exon 6 houses the cleavage site for the procollagen I N-terminal peptidase and cross-linking site (Burrows 1999). This mis-splicing results in the retention of the N-propeptide and abnormal fibrillogenesis leading to angular collagen fibril as seen by electron microscopy (Burrows 1999). It is interesting to note that although both EDS and OI are due to mutations in the same genes, the type of mutation can drastically change the affected tissue.

Recently, several patients have been diagnosed with EDS types I/II, characterized by thin, velvety and hyperextensible skin, delayed wound healing, atrophic scarring and easy bruising (Malfait and De Paepe 2005). In these patients, the disease is not caused by mis-splicing of exon 6, but by a lack of α2(I) chains. The first reported patient did not have a history of fractures, but did have slightly blue sclera, a symptom of OI but not EDS, which may be explained by the reduced amount of type I collagen found in both EDS and OI (Hata, Kurata et al. 1988). Upon further examination it was found that this patient carried both genomic copies of the proα2(I) gene and had the same concentration of genomic proα2(I) molecules as controls, but almost a complete lack of α2(I) mRNA was made without any detectable α2 (I) protein (Hata, Kurata et al. 1988). This is in contrast to a human OI type III patient, described below, who most closely resembles the *oim* mouse, the most widely studied mouse model of OI. This patient made both α2(I) mRNA and protein, but was unable to incorporate the mutant proα2(I) chains into the

triple helix, leading to multiple fractures, short stature and bowing of the long bones (Deak, Nicholls et al. 1983; Chu, Rowe et al. 1984; McBride and Shapiro 1994). A second patient was identified who also lacked α2 protein with clinical manifestations of EDS without the skeletal abnormalities seen in OI (Nicholls, Valler et al. 2001). This patient, the child of first cousin parents, also had mild blue sclera as well as ligamentous and joint laxity characteristic of EDS in addition to a history of fracture indicative of OI (Nicholls, Valler et al. 2001). This patient also had the normal gene concentration, but produced an extremely low level of COL1A2 mRNA with a complete lack of α2(I) protein (Nicholls, Valler et al. 2001). Three other patients diagnosed with EDS, but with valvular involvement, were found to have mutations in COL1A2 that lead to the use of cryptic splice sites producing a premature stop codon and an unstable mRNA (Schwarze, Hata et al. 2004). Splicing studies showed these patients removed the same introns as controls, but in a different order, indicating that the order of intron removal was an important factor in determining clinical outcome (Schwarze, Hata et al. 2004). A fourth patient was recently identified in which codon eight of COL1A2 exon 7 contained a cytosine insertion (Malfait, Symoens et al. 2006). This patient did not produce COL1A2 mRNA or protein and was diagnosed with EDS type I/II with mitral valve bulging (Malfait, Symoens et al. 2006). Reduction in the amount of COL1A1 mRNA due to the use of a cryptic splice site can also lead to a mixed EDS/OI phenotype(Symoens, Nuytinck et al. 2004). Taken together these studies indicate a potential role for RNA in determining disease, as seen in repeat expansions causing unstable or altered RNA in diseases such as dystrophic myotonica 1 and 2 (Gatchel and Zoghbi 2005).

The OI patient who lacked α2 chains in his type I collagen still made normal levels of α2 mRNA and was able to make a protein product, but was unable to

incorporate these chains into the triple helix and had classical OI symptoms. The EDS patients presented here do not make any RNA and do not suffer from bone problems, but instead have hyperextensible skin and joints. However, when a patient has a COL1A2 mutation that leads to reduced production of α2 mRNA, the phenotype is less defined with symptoms of both disorders. These studies suggest a potential role for the COL1A2 mRNA in disease progression, as the presence or absence, as well as the amount, of the mRNA may have a role is determining clinical outcome.

Clinical Significance and Current Treatments for OI

Current treatments for patients with OI treat the symptoms, not the underlying cause of the disease. Current standard treatments include bisphosphonates to decrease bone resorption, growth hormone to augment growth and collagen production and bracing to provide support to the bones. Experimental therapies that aim to treat the underlying cause of disease include stem cell transplantation and ribozyme therapy.

Bisphosphonates

Bisphosphonates are a class of molecules currently used to treat OI and osteoporosis. These compounds have three main characteristics, a PCP group and two R groups (Figure I-1). The PCP group is necessary for the binding of the molecule to hydroxyapatite as well as for biological activity (Licata 2005). When the R1 group is an OH- ion, binding to hydroxyapatite is enhanced and the identity of the R2 group determines the potency of the individual bisphosphonate (Licata 2005). Bisphosphonates act on the osteoclast, preventing its resorptive activities by various methods (Licata

2005). It is hypothesized that by reducing the activity of the osteoclast, cortical thickness and bone mineral density will increase and, hopefully, improve the biomechanical integrity of the bone, thereby reducing fracture number.

A study using intravenous pamidronate in patients with OI types I, III and IV found an 88% increase in cortical bone width and a 46% increase in trabecular bone volume, due to increased numbers of trabeculi, after a year of treatment. Markers of bone turnover were reduced 26-75% without any detrimental effects on mineralization (Rauch, Travers et al. 2002). Taken together, these data demonstrate the action of the pamidronate on the osteoclasts without affecting the osteoblasts. By slowing or stopping bone resorption without altering the osteoblasts, the bone volume increased.

Another study compared the efficiency of oral and intravenous bisphosphonate treatment. Patients were divided into groups based on age and severity of OI and then given either intravenous pamidronate or oral alendronate therapy. At the end of the study, all patients had significant increases in bone mineral density regardless of treatment group (Dimeglio, Ford et al. 2005). Patients in both groups showed a linear growth rate with a reduced fracture rate, though not significant. When alkaline phosphatase, a marker of bone formation was examined, both groups showed reduced levels. Both groups also had a reduced level of N-telopeptide, a marker of bone resorption, although the alendronate-treated group had a greater reduction than the pamidronate-treated group (Dimeglio, Ford et al. 2005). This study demonstrates that both oral and intravenous bisphosphonate treatment increase bone mineral density and decrease bone turnover.

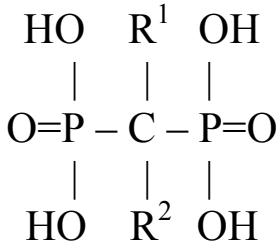

Figure I-1. Functional Domains of the Bisphosphonates. Hydroxyapatite binding is enhanced if R^1 is an OH group. The PCP group is required for biological activity as it acts as a "bone hook" to allow for hydroxyapatite binding. The identity of the R^2 group determines potency of the individual bisphosphonates. Adapted with permission (Licata 2005).

Bisphosphonate therapy has many advantages over other treatment options for patients with OI. Many different types of bisphosphonates are available in both pill and intravenous forms, allowing many treatment options. The pill form is less expensive and does not require hospitalization as the intravenous therapy does, and does not require the puncturing of fragile veins often found in OI patients (Dimeglio, Ford et al. 2005). Positive results have been seen in children and adolescents of all ages with all forms of OI (Rauch, Travers et al. 2002; Dimeglio, Ford et al. 2005).

Although bisphosphonates have the incredible potential to improve the quality for life of OI patients, there are still several unknown factors concerning its safety. While several observatory studies have been done showing improvement in patients, more double-blind placebo studies need to be done. Also, the long-term effects of using bisphosphonates in growing children are not known, especially on the rate of fracture healing and reproduction. One study demonstrated bisphosphonates do not have an adverse effect on fracture healing (Pizones, Plotkin et al. 2005), but this is the only study of its kind to date. There have also been reports of bisphosphonate-induced jaw necrosis (Marx 2003). There is currently no standard method for measuring efficiency of treatment as bone mineral density measurements have inherent variations between measurements and a lack of increase in bone mineral density does not necessarily mean the drug has been ineffective (Licata 2005). Adequate levels of calcium and vitamin D are also required for this therapy (Licata 2005). Additionally, bisphosphonates may not have an effect on the severity of the bone deformation often seen in the more severe cases of OI. The effect of the drugs on scoliosis and dentinogenesis imperfecta is not known.

Very recently, the parathyroid hormone analogue terapeptide was introduced on to the drug market. Unlike the bisphosphonates, terapeptide acts to stimulate osteoblasts in an effort to lay down more bone (Graul 2005). In March 2005 a phase II/III double blind placebo study using 90 OI patients will test the safety and efficiency of the drug. Terapeptide use is contraindicated for use in children and adolescents, as they are at risk of developing osteosarcoma (Graul 2005). Therefore, terapeptides do not currently provide promise for pediatric OI patients.

Growth Hormone Therapy

Growth hormone both stimulates bone metabolism and growth (Antoniazzi, Mottes et al. 2000). It also stimulates collagen metabolism via the action of insulin-like growth factor-1 and insulin-like growth factor binding protein-3 (Antoniazzi, Mottes et al. 2000). One animal study used hGH transgenic mice, previously reported to have increased cortical thickness as well as overall quantity of bone. Biomechanical properties of the vertebrae and femurs from hGH mice were also increased (King, Chase et al. 2005). When the *oim* mouse was crossed with the hGH mice, the caudal vertebrae showed an increased bone volume and cross sectional area (King, Chase et al. 2005). Biomechanical parameters such as maximum load and energy to failure were also improved in *oim*/hGH animals compared to their wildtype counterparts (King, Chase et al. 2005). Stiffness of the vertebrae were not affected in these animals (King, Chase et al. 2005). A related study injected rhGH into *oim* animals. These animals showed increases in mass and femur length as well as bone mineral content. However, bone mineral density was not changed, indicating the rhGH increased bone quantity but not density (King, Jarjoura et al. 2005). Maximum load, energy to failure and ultimate stress were improved in *oim*/rhGH animals compared to wildtype (King, Jarjoura et al. 2005).

Possible mechanisms for the effect of growth hormone include influences on bone mineralization, crystal growth, stimulation of production of bone proteins or alterations in bone metabolism (King, Jarjoura et al. 2005).

One *in vivo* study using growth hormone to treat children with OI types III and IV found that patients with type III OI did not respond to the treatment, but that approximately 66% of type IV patients given the treatment had a marked improvement (Marini, Hopkins et al. 2003). These patients exhibited increased bone mineral density as well as markers of bone formation without changes in fracture rate. Histological examination also showed improvement in bone quality (Marini, Hopkins et al. 2003). Endocrine analyses done before treatment could not predict which patients would respond to the treatment.

While positive effects of growth hormone on bone have been demonstrated in both mice and humans, growth hormone therapy is not a cure all. First, there are few controlled studies in humans and although disease severity plays a role, there currently is no way to predict which patients will respond to the treatment. There have also been reports of positive effects of growth hormone on trabecular, but not cortical, bone (Marini, Hopkins et al. 2003). This corresponds to positive effects on vertebral, but not femoral, bone. Long-term effects are also unknown as studies lasting longer than two years have not been done.

Intermedullary Rodding

Intermedullary rodding is the insertion of a metal rod into a long bone such as the femur or tibia. These rods do not prevent future fracture but instead act as internal splints to stabilize the bone and keep it in alignment. Several rods can be inserted during a

single surgery, although pain and long recovery times may follow. Other risks typical of major surgery, such as bleeding, are also possible. The rod can also move and may need to be replaced as a patient ages of if shifting occurs. The major advantage to rodding surgery is the possibility of increased activity following surgery without casting and long periods of inactivity, which can lead to an increase in fracture rate.

Stem Cell Therapies

Mesenchymal stem cells, also known as marrow stromal cells or MSCs have also been used to treat a type III OI patient. MSCs can differentiate into many cells types in various tissues and are thought to home to sites of injury and repair the tissues by several methods. In a study by Pochampally et al, MSCs from a type III OI patient heterozygote for an IVS 41A+4C mutation in his COL1A1 gene were isolated and expanded in culture. The MSCs were then modified by transfection with a truncated version of wildtype COL1A1 gene and then returned to the patient (2005). The construct contained all the necessary coding sequences while eliminating those sequences with unknown function. This truncation allows for a more efficient transfection while still containing all the regions necessary for proper function. Once transfected, the MSCs from the OI patient overexpressed the COL1A1 gene and produced increased amounts of type I collagen. This allowed the MSC to differentiate into osteoblasts capable of mineralization (Pochampally, Horwitz et al. 2005). And although this system does not remove the abnormal gene product, previous studies have demonstrated that a relatively small amount of wildtype protein is needed to achieve a benefit to the patient. Also, the ability of MSCs to home to areas of injury does not require the direct injection of the MSCs to the bones and are relatively easy to obtain. Additionally, the use of a patient's own cells circumvents typical transplantation issues such as rejection and the need for the patient to

take long-term immunosuppressive drugs. Downfalls of this system include the neomycin cassette which can trigger immune responses and possible mutations due to the construct in the MSCs themselves (Pochampally, Horwitz et al. 2005). How the correction of mineralization achieved in this study will correlate to long-term improvement in lifestyle for the patient is not known.

Another *in vitro* study used MSCs from two OI patients each carrying glycine to serine substitutions in the COL1A1 gene. Adeno-associated virus was used to deliver a COL1A1 construct designed to homologously recombine with the endogenous COL1A1 gene. 31-90% of neomycin-positive clones exhibited gene targeting at one COL1A1 allele (Chamberlain, Schwarze et al. 2004). These positive clones were found to have reduced type I collagen overmodification, a hallmark of OI as well as improved collagen stability as seen by a higher melting temperature (Chamberlain, Schwarze et al. 2004). Fibril diameter was also similar to wildtype in transduced cells while fibrils from untransduced cells were larger than normal (Chamberlain, Schwarze et al. 2004). Transduced MSCs were also able to differentiate into bone as seen by immunohistochemistry and histology (Chamberlain, Schwarze et al. 2004).

The strategy of homologous recombination has the distinct advantage of reducing the possibility of insertional mutations when the construct integrates into the genome of the MSCs. As this system uses homologous recombination of large regions of the COL1A1 gene it will allow for correction of mutations without designing specific constructs for individual mutations. However, delivery via adeno-associated virus may elicit immune responses or inactivate the transduced genes (Pochampally, Horwitz et al. 2005). The presence of the neomycin cassette may also trigger immune responses. This

system also does not differentiate between normal and mutant endogenous genes, leaving open the possibility of potentially detrimental effects on the normal COL1A1 allele.

A third study used osteoprogenitor cells isolated from 8-week old mice transduced with a GFP construct. These cells were then injected into irradiated neonatal heterozygote and homozygote *oim* mice. Osteoprogenitor cells isolated from bone marrow did not express markers of osteoblasts indicating they are not mature bone forming cells (Wang, Li et al. 2006). However, they were able to give rise to GFP-positive cells that were located on surfaces of newly formed bone that did express markers of bone formation such as osteocalcin and osterix (Wang, Li et al. 2006). This study demonstrated the ability of the MSCs to localize to bone and give rise to mature bone forming cells. However, heterotrimers of type I collagen were not detectable in these cells, indicating that a substantial numbers of cells will be required to synthesize bone extracellular matrix (Wang, Li et al. 2006). Variability in the numbers of engrafted cells and the irradiation required to achieve maximum engraftment are other potential problems.

Ribozymes

Therapies aimed at targeting the underlying cause of disease hold a great deal of promise while still in early phases of development. Ribozymes are small catalytic RNA that bind to and cleave RNAs in a very specific manner. Since approximately 25% of mutations causing OI also generate a unique ribozyme cleavage site, the mutation itself provides an opportunity for the suppression of the mutant RNA. Studies were done on fibroblasts from an OI patient with a G907T mutation, a glycine to valine substitution at position 907 in the triple helical domain of the α1(I) chain. The G907T mutation also

resulted in the introduction of a novel ribozyme cleavage site (Dawson and Marini 2000). A mutation-specific ribozyme was designed and transfected into the G907T fibroblasts. This ribozyme was capable of binding to and cleaving approximately 50% of the mutant RNA while leaving the normal RNA undisturbed (Dawson and Marini 2000). Growth rate of the cells was not affected by the ribozyme (Dawson and Marini 2000).

While this study holds promise for the use of ribozymes as a means to treat OI, there are still several obstacles to overcome. The specificity of the ribozymes is both a positive and negative attribute. The biggest benefit to the use of ribozymes is the ability to target only mutant RNA for degradation leaving the normal collagen to carry out normal functions. This degradation will leave the patient with less than the normal amount of type I collagen. However, a reduced amount of phenotypically normal collagen (type I OI) results in a less severe clinical outcome than the presence of abnormal type I collagen (type III OI), which has a dominant negative effect. A unique ribozyme will also need to be designed for each OI-causing mutation. As more than 850 unique OI-causing mutations have been reported, this is not a small problem. Another obstacle to overcome is the amount of mutant allele suppression needed to gain a benefit to the patient. Delivery of the ribozyme as well as toxicity will also need to be tested as studies to date have been done on fibroblasts, not whole animals.

While the various approaches presented here have helped patients with OI, each approach has definite drawbacks. Method of administration, cost, side effects and lack of response from a sub-set of patients are all obstacles that need to be overcome for the medicinal approached. Additionally, molecular approaches aimed at down-regulating specific mutations are labor intensive, requiring mapping of the mutation and generation of specific constructs, ribozymes or oligonucleotides. It is for these reasons that the

identity of modifier genes is necessary. The identity and exploitation of these genes may be the most universal way to improve the clinical outcome for patients with OI and other bone diseases.

Genotype-Phenotype Correlations

Although the clinical manifestations of OI are well characterized, genotype to phenotype correlations are poorly understood. There are two main types of mutations leading to a disease state: included and excluded. Excluded mutations result in haploinsufficiency, in which the patient has half the normal amount of type I collagen. This usually results in a mild phenotype, as the collagen that is produced and incorporated into tissues is normal. Included mutations result in the production of abnormal type I collagen and tend to be more severe. These include substitutions, exon deletions and can result in either incorporation of abnormal collagen into tissues or, in the case of the α2 chain, exclusion of an entire chain. The vast majority of the included mutations are glycine substitutions within the triple helical domain (Gajko-Galicka 2002). For these mutations, the type of substituting amino acid seems to be important in determining clinical outcome (Lund, Astrom et al. 1999). For example, two patients carrying a glycine substitution at position 247 in the α2 chain have been identified. The first patient had a cysteine substitution resulting in OI type III, while the other had a serine substitution causing OI type I, a much milder phenotype (Lund, Astrom et al. 1999). When the substituting amino acids were analyzed for their degree of destabilization of the triple helix, it was determined that certain amino acids were more prevalent in lethal forms of OI than others. For example, alanine substitutions can be found in normal individuals (17 cases) as well as non-lethal (6) and lethal (3) cases of OI. Conversely, asparagine is not found in normal individuals, but is prevalent in lethal OI

with 8 cases versus only one case of non-lethal OI (Persikov, Pillitteri et al. 2004). Although the identity of the substituting amino acid does play a role in determining phenotypic severity, it does not completely explain it, as a broad range of clinical outcomes was seen for each amino acid studied.

This may be further explained by the identity of the chain harboring the mutation as well as the location of the mutation on the chain. Two patients have been identified, each harboring a glycine to serine substitutions at position 247. The mutation in the α1 chain resulted in severe OI type II/III while the α2 chain mutation results in the milder type I phenotype (Lund, Astrom et al. 1999). Each α chain has also been proposed to have a unique distribution of lethal and non-lethal regions. For glycine substitutions in the α1 chain, four non-lethal regions have been identified: 1) serine substitutions at the carboxy-terminal end, 2) cysteine substitutions located mid-chain, 3) 70 amino acid region containing amino acid 352 and 4) a cluster of cysteines located at the amino-terminal end (Marini, Lewis et al. 1993).

The α2 chain regional models proposes three non-lethal regions alternating with two lethal regions, with the only overlap between lethal and non-lethal being at the extreme C-terminal end (Wang, Orrison et al. 1993). Both lethal and non-lethal regions contain single nucleotide substitutions as well as exon deletions (Wang, Orrison et al. 1993). This model proposes that the lethal regions must have important roles in collagen and/or extracellular matrix structure and function. At the fibril level, these lethal regions may have roles in collagen-collagen interactions or interactions with other extracellular matrix components such as osteocalcin (Wang, Orrison et al. 1993).

Recently, a map of ligand-binding sites and mutations of the human type I collagen fibril was constructed. In this map, the α1(I) and α2(I) chains were arranged into overlapping monomers representing the natural periodicity of the collagen molecule (Di Lullo, Sweeney et al. 2002). Ligand-binding sites, as well as known mutations, were then added to the map. This revealed several regions for ligand interaction with type I collagen and that most of these interactions occur near the C-terminal half of the molecule (Di Lullo, Sweeney et al. 2002). It was observed that mutations causing type II OI are evenly distributed throughout the map while mutations causing types III and IV OI have a non-random distribution. These mutations are excluded from several areas within the overlap zones while being randomly dispersed throughout the gap zone. This study also identified 18 type III and IV mutations that localize to residues 226-301 of the α2(I) chain, a region not previously known to contain any OI type II-causing mutations (Di Lullo, Sweeney et al. 2002).

Given that all studies to date have failed to clearly identify a genotype-phenotype map, other factors influencing phenotypic severity must exist. These factors may be differences in splicing at the mRNA level, degradation at the mRNA or protein levels or contribution of modifier genes. Modifier genes are those that influence clinical outcome by interacting with genes known to impact bone quality such as the type I collagen genes. Such genes may be the vitamin D receptor, which facilitates calcium absorption (Heaney 1999) and has been implicated in several bone diseases (Holick 1999), or the estrogen receptors, known to influence bone loss in post-menopausal women (Rosen and Kiel 1999). Parathyroid hormone, a regulator of calcium homeostasis is another possible modifier gene. Parathyroid hormone is known to increase with age (Rosen and Kiel 1999) and with this increase comes a release of calcium from the bone and into the serum

(Juppner, Brown et al. 1999) in addition to a decrease in osteoblast activity and an increase in the number of remodeling sites within the bone (Lukert 1999). Genes impacting intestinal absorption of calcium may be other potential targets. If additional calcium can be absorbed from the diet, this may help strengthen the bone and combat age related bone loss (Eastell 1999). Additionally, genes impacting the rates of bone formation and resorption are obvious targets. If bone formation can be increased while bone resorption is decreased, more bone can be laid down more quickly.

Bone

Bone is a two component composite material made of an organic phase (primarily type I collagen) and a mineral phase (mainly hydroxyapatite crystals). Type I collagen gives the bone toughness, or the ability to of the bone to deflect in response to forces, while the hydroxyapatite crystals give the bone strength and stiffness (Burr 2002). The combination of type I collagen and hydroxyapatite crystals gives bone properties unlike either component alone (Burr 2002). The correct ratio of the two components is also important, as too much mineral leads to brittle bones (Burr 2002), while too little mineral results in weak bones. Hydroxyapatite crystals are normally composed of calcium, phosphate and hydroxy ions $[3Ca_3(PO_4)_2]\bullet(OH)_2$, although several other trace minerals are present and known to impact crystal formation and alignment (Baron 1999; Phillips, Bradley et al. 2000). For example, magnesium is known to inhibit hydroxyapatite crystal formation (Boskey, Rimnac et al. 1992) while fluoride is thought to promote crystal formation (Cheng, Bader et al. 1995). In addition, patients with osteogenesis imperfecta

have been shown to have a reduced Ca/P ratio as compared to normal bones, indicating abnormal mineral (Cassella and Ali 1992).

There are two main types of cells found in bone; osteoblasts are responsible for making matrix components like collagen while osteoclasts are responsible for bone resorption (Favus 1993). Osteoblasts can also become trapped within the matrix they produced and later become calcified deep within the tissue. These trapped osteoblasts are called osteocytes and have long processes that are in contact with processes from other osteocytes or cells lining the bone surface (Favus 1993). These cells provide the bone with an increased surface area, can synthesize new bone matrix which can later calcify and may have a role in local activation of bone turnover (Favus 1993). The balance in activity between these two cell types must be kept in equilibrium in order to maintain proper amounts of bone. Both osteoblasts and osteoclasts produce biochemical molecules that can be used as markers of bone formation and resorption, respectively.

Bone-specific alkaline phosphatase (BSAP) and osteocalcin are non-collagenase proteins produced by the osteoblasts and can be used as markers of bone formation (Khosla and Kleerekoper 1999). BSAP is important in the mineralization process as seen by diseases such as hypophosphatasia, characterized by reduced mineralization of the skeleton and teeth, in which BSAP is lacking, although the exact mechanism is unknown (Khosla and Kleerekoper 1999). Osteocalcin is a molecule of unknown function secreted by the osteoblasts and incorporated into the bone matrix and is often used as an indicator of bone formation. However, osteocalcin can also be released during bone turnover and so potentially can also be considered as a marker of bone turnover (Khosla and Kleerekoper 1999). Osteoblasts also secrete type I collagen and so markers of type I collagen synthesis can also act as markers of bone formation. Carboxy-terminal

propeptide and amino-terminal propeptide of type I collagen are both part of the precursor procollagen molecule and help guide the assembly of the triple helix and are then subsequently cleaved. Measuring these cleavage products allows for determination of the relative amounts of collagen synthesis. However, these cleavage products are not unique to bone, so they really describe collagen synthesis throughout the body (Khosla and Kleerekoper 1999). Conversely, when osteoclasts resorb bone, they break down type I collagen and these breakdown products can be useful in measuring bone turnover. These products include hydroxyproline, an amino acid unique to collagen, pyridinoline cross-links, deoxypyridinoline cross-links, N-telopeptide and C-telopeptide of collagen cross-links (Khosla and Kleerekoper 1999). These breakdown products are also not unique to bone, but instead are a reflection of type I collagen turnover that may be occurring in other areas of the body. The only osteoclast-specific marker of bone turnover is tartrate-resistant acid phosphastase (TRAP), a lysosomal enzyme released when osteoclasts resorb bone. However, several tissues contain acid phosphatases and so serum TRAP may not be bone-specific (Khosla and Kleerekoper 1999).

These bone parameters have been measured in both OI patients and in mice with known differences in bone mineral densities. OI patients have been shown to have a marked increase in bone metabolism as compared to non-affected individuals and that the rate of bone turnover corresponds to the severity of the disease (Cole 2002). It has been demonstrated that strains of mice with higher bone densities have thicker cortexes than those with lower bone densities (Beamer, Donahue et al. 1996). This increase in cortical widths may be due to rates of bone formation and resorption as other studies have shown that strains with higher bone densities have more bone formation and less bone resorption, as well as lower numbers of osteoclasts (Linkhart, Linkhart et al. 1999) when

compared to strains with lower bone densities. Though useful tools, these markers must be kept in context of any mutations when used as a measure of bone quality.

Long bones such as the femur and tibia, are made of two types of bone, cortical and trabecular bone. Cortical bone, also known as the cortex or compact bone, is the dense layer of calcified tissue that comprises the outer portion of the bone. Trabecular bone, also known as cancellous or spongy bone, is the internal space that houses the bone marrow and is found at the epiphysis and toward the growth plates (Favus 1993). The main role of the cortex is to give strength and protection to the bone, while the trabecular bone plays a mainly metabolic role (Baron 1999). Structurally, cortical bone is 80-90% mineralized while only 15-25% of trabecular bone is mineralized (Baron 1999). Histologically, *oim/oim* tibias have a reduced trabecular volume and thinner cortices than wildtype animals (Phillips, Bradley et al. 2000). Although cortical and trabecular bone have different structures and functions, they are comprised of the same matrix components and cell types (Baron 1999). The matrix of the bone is made of type I collagen and other noncollagenous proteins in addition to the hydroxyapatite crystals (Baron 1999). The collagen fibrils align in a stereotypical way to maximize the strength of the tissue and act as a blueprint for mineralization to occur (Schenk, Felix et al. 1993). The toughness of the bone comes from type I collagen while the hydroxyapatite crystals give the bone strength and stiffness (Burr 2002). Therefore, the weaker bones of the *oim/oim* animals may arise from alterations in the collagen, the mineral or a combination of both.

Heritability in Bone Mass

Although the amount of bone a person acquires has a significant environmental component, a large part is also due to genetic factors. Several familial studies indicate that the genetic component may be as much as 70% (McKay, Bailey et al. 1994). As dizygotic twins are like any sib pair, they would be expected to have only 50% of the total genome in common and so would be expected to have a lower heritability than monozygotic twins who have 100% of the genome in common. One female twin study, including both monozygotic and dizygotic twins, measured bone mineral density at several sites including the radius, lumbar vertebrae, femur and trochanter. They found that dizygotic twins had much more variation in their bone mineral density at all sites measured than the monozygotic twins, even when corrected for lifestyle contributions such as caffeine and nicotine intake and number of pregnancies (Slemenda, Christian et al. 1991). However, as the monozygotic twins aged, the effects attributable to environment increased (Slemenda, Christian et al. 1991). In this study, monozygotic twins had a much higher heritability of bone mineral density than dizygotic twins. A twin study using only male twins, both monozygotic and dizygotic, showed that genetic variation in bone mass was due to genes that influence skeletal sites, but does not account for age related bone loss. This bone loss with age is more attributable to environment than genetics (Christian, Yu et al. 1989). Once again it was shown that monozygotic twins had a lower variation and greater heritability in their midshaft radial bone mineral density than the dizygotic twins. As time progresses, the heritability reduces, indicating that genetic factors have a large role in bone acquisition and, therefore, fracture risk, but have a lesser role in determining the rate of bone loss associated with aging (Christian,

Yu et al. 1989). A third study looked at associations in bone mineral densities at the L3 lumbar spine between grandmothers, daughters and granddaughters. This study found a significant positive association between the Z-score values in the mother-daughter and mother-granddaughter pairs (McKay, Bailey et al. 1994). This suggests a familial component to bone mineral density, reinforcing data from twin studies. Twin studies tend to report higher heritability, 80-90%, than familial studies, 50-70% (McKay, Bailey et al. 1994), but this can be explained by the degree of similarity in the genetic material for twins compared to related individuals.

Although these studies are important in demonstrating a genetic component to bone mineral density, they do not speculate on the possible genes that may have a role in determining this density. Linkage studies and quantitative trait loci for bone mineral density are beginning to address the question of which gene or genes may be influencing the acquisition of bone mass. In one study, a family was found to have a high bone density trait that maps to q12-13 on chromosome 11. Following a car accident, the proband, an 18 year old female, was found to have "unusually dense bones," with a Z-score of 5.63 (Johnson, Gong et al. 1997). All other parameters, including bone shape and serum chemistry, were normal. Upon examination of several family members, it was determined that her mother also had a high bone mineral density, as did several other members of her extended family. In this family, the high bone density trait was inherited in an autosomal dominant pattern with Z-scores ranging from 3.42 to 7.80 compared to Z-scores of –0.21 – 1.85 for unaffected individuals. None of the affected individuals had a history of fracture. Linkage analyses demonstrated that the high bone density trait mapped to q12-13, a 30 cM region on chromosome 11 (Johnson, Gong et al. 1997). Further finer mapping and sequence analysis demonstrated a G to T transversion at

position 171 in the LRP5 gene. This results in a glycine to valine substitution, predicted to alter LRP5 interaction with other proteins. A total of 1000 individuals were examined in this study, but only those who were part of the original pedigree with the high bone density had the mutation and all affected individuals carry the mutation (Little 2002).

The LRP5 protein is a member of the low-density lipoprotein-receptors gene family and functions as a co-receptor for Wnt protein (Koller, Ichikawa et al. 2005; Little 2002). The protein has four domains consisting of six YWTD repeats, an epidermal growth factor-like module and an LDLR-like ligand binding domain (Little 2002). The mutation leading to high bone mineral density occurs near the fourth repeat of the first domain, an evolutionarily conserved region. Northern blot analysis demonstrated expression of the LRP5 gene in bone while *in situ* hybridization demonstrated localization to areas involved in bone remodeling, such as the endosteum and trabecular bone within the metaphysis (Little 2002). Further support of the functional importance of the LRP5 gene in determining skeletal strength lies in gain and loss of function mutations. While gain of function mutations lead to high bone density, loss of function mutations have been linked to osteoporosis pseudoglioma syndrome (OPPS) characterized by juvenile osteoporosis, as well as bone deformity and early on-set blindness (Little 2002). Other disorders mapped to this region include autosomal recessive osteopetrosis as well as QTL contributing to normal variation in bone mineral density (Little 2002). Mice deficient in LRP5 have been shown to have low bone mass and body weight as well as eye vascularization, mimicking those symptoms seen in humans. Transgenic mice carrying the high bone density mutation have been shown to have increased trabecular and cortical bone parameters in addition to increased skeletal strength (Little 2002). Additional studies have shown the specific polymorphism as well

as the specific population (men versus women, etc.) being studied are important determinants to the contribution of the LRP5 gene to bone mineral density (Koller, Ichikawa et al. 2005; van Meurs, Rivadeneira et al. 2006).

Changes in Bone Quality/Strength with Age

It is well known that the risk of fracture among the elderly population is quite high, although the exact mechanisms of aging and its affects on the skeleton are not well understood. It is known that as the skeleton ages, the bones become porous with an increased number of empty osteocyte lacunae and plugging of the haversian canals and canaliculi, disrupting the normal microarchitecture of the bone (Kloss and Gassner 2006). As the bone ages, collagen content decreases with an increase in concentration of denatured collagen, reducing the bone's ability to resist fracture (Vashishth 2005). Since bone is a composite of collagen and mineral, age-associated changes in collagen are often associated with changes in mineral. As bone ages, there is an increase in high-density mineralized bone that is more susceptible to fractures than younger mineral (Vashishth 2005). There is also an uncoupling of bone remodeling, with a higher rate of bone resorption as compared to bone remodeling (Kloss and Gassner 2006). Studies among the elderly have shown a decrease in osteoblastic activity in the 6^{th} decade, resulting in a reduction in bone formation (Kloss and Gassner 2006). However, osteoclast surface did not change (Kloss and Gassner 2006), resulting in high bone-resorption activity as measured by urinary N-telopeptide, C-telopeptide and deoxypyridinoline levels (Rosen and Kiel 1999). These unusually high levels of bone resorption ultimately lead to bone loss and alteration of skeletal architecture which increases the likelihood of fracture (Rosen and Kiel 1999). There are several biochemical and hormonal contributions that accelerate bone loss seen in the elderly, but environmental factors also have a role.

Lifestyle choices such as smoking and alcohol may contribute to the accelerated bone loss in the aging population (Rosen and Kiel 1999).

The most common factor leading to age-related bone loss is calcium deficiency, due to decreased dietary calcium intake. Secondary to this, is a reduced vitamin D intake, which ultimately leads to reduced calcium absorption (Rosen and Kiel 1999). Studies using dietary supplementation of calcium and vitamin D have shown a preservation of bone mass and a decrease in bone resorption and osteoporotic fractures (Rosen and Kiel 1999).

Another factor contributing to age-related bone loss are hormones. Estrogen deficiency is well known to be a major player in post-menopausal bone loss in women, but men also lose bone mass with age. It may be that absolute estrogen levels are more important than testosterone levels in maintaining bone mass, although exactly how male sex hormones contribute to bone maintenance or loss is unclear (Rosen and Kiel 1999). Changes in growth hormone, and therefore insulin-like growth factor-1, also have a role in bone loss in both elderly men and women. These hormones are known to have a critical role in bone development and maintenance, so that any changes in this pathway may dramatically alter the functioning of the tissue (Kloss and Gassner 2006). Adrenal androgens also decline with age, although exactly what role they play in bone mass maintenance or loss is unclear (Rosen and Kiel 1999). Increases in parathyroid hormone with age also have a role in bone loss in the elderly, although this may be a secondary effect to impaired renal function that often accompanies aging (Rosen and Kiel 1999).

Studies comparing young and old bone have shown that old bone is unable to resist fracture as effectively as young bone. They found that younger bones were able to

compartmentalize damage to the bone to delay the onset of fracture, which older bones were unable to do (George and Vashishth 2006). This compartmentalization may be an attempt to protect other regions of the bone from perpetuation of the fracture. They also found that older bones more readily initiated cracks and that these cracks propagated easier than in young bones (George and Vashishth 2006). Taken together, this study demonstrates that reduced bone quality in old bones increases bone fragility and leads to an increased number of fractures (George and Vashishth 2006).

One possible explanation for this increased propensity to fracture in older bone is a reduction in the mechanical integrity of collagen fibers in the aging bone. A study by Wang, et al showed that following demineralization of the bone, the mechanical integrity of the collagen decreases (Wang, Shen et al. 2002). This corresponds to a 30-50% decrease in the work to fracture measurement, indicating that the bone can take less force before fracturing (Wang, Shen et al. 2002). They also found an 68% increase in pentosidine, an advanced glycation end (AGE) product that forms covalent cross-links between adjacent molecules and, therefore, can be measured (Wang, Shen et al. 2002). AGEs are also known to inhibit bone matrix induced bone formation (Kloss and Gassner 2006), so an increase in AGEs not only effects the quality of the bone, but also the quantity. Differences in elastic modulus and stiffness were also found between old and young bones. Young bones lose stiffness gradually, while old bones lose stiffness much more dramatically, indicating that young bones can control damage initiation better than old bones, thus increasing fatigue life (Diab, Sit et al. 2005). These studies correlate well with the study showing increased bone fragility in older bones, as a decrease in collagen integrity will reduce the toughness of the bone and the increased number of cross-links will increase the brittleness of the bone, allowing fractures to occur more easily.

Although osteoporosis has classically been thought of as a disease of elderly women, a new perspective is beginning to emerge. Osteoporosis is now being considered a pediatric disease. Exercise studies have shown that the earlier an exercise program is initiated, the greater the accretion of bone (Branca 2006). This gain in bone may prevent the onset of osteoporosis or, at least, delay the age of onset. During the normal growth process, bone grows in both length and diameter (Specker 2006). Both of these increases alter the geometry of the bone and, therefore, biomechanical integrity. Longer and wider bones are better able to withstand torsional loads and, therefore, will not break as easily as shorter, narrower bones (Turner 2006). During puberty, the porosity of the bone increases due to increased bone turnover, contributing to the increased number of fractures often seen in adolescents during puberty (Specker 2006). During puberty, body and muscle mass both increase, putting added stresses on the bone. These added stresses are theorized to improve biomechanical integrity by the mechanostat theory, a mechanism by which the bone responds to changes in loading with alterations to bone architecture (Skerry 2006). These changes in loading can be positive, as in the case of chronic exercise, or negative, as seen with patients on long-term bed rest. If the force is moderate and sustained over a long period of time, it can induce changes in geometry that will better allow the bone to resist forces, ultimately resulting in a stronger bone (Skerry 2006). Post-puberty, the rate of remodeling slows and porosity decreases, but the now larger bone continues to mineralize with a concomitant decrease in fracture rate (Specker 2006).

Studies showing changes in bone strength throughout development as well as during the aging process further contribute to our understanding of modifier genes impacting bone strength. Studying how the gene expression profile changes with age, we

can better understand which genes may have a role in determining bone biomechanical integrity.

Bone Properties in Inbred Mouse Strains

To try to better understand the role of genetic background in determining bone mineral density, animal models are essential. However, phenotypically normal mice can have a wide range of bone mineral densities. Outbred mice have a mixed genetic background and can have a broad range of phenotypes, thus mimicking the human population. Inbred mouse strains represent genetically identical animals with each strain being genetically unique from every other inbred strain, thus adding to their appeal for controlled studies. In a study of 11 inbred strains, the highest femoral bone mineral density and thickest cortices were seen in C3H/HeJ (C3) animals, with the lowest bone density and thinnest cortices being in C57BL/6J (C57) animals (Beamer, Donahue et al. 1996). These particular strains serve as progenitors for inbred strains designed specifically for the study of inherited traits and so are the most studied (Beamer, Donahue et al. 1996). C3 animals had thicker cortices with a smaller cross-sectional area as compared to C57 animals (Akhter, Iwaniec et al. 2000). C57 animals also had approximately half the bone volume as compared to C3 animals (Akhter, Iwaniec et al. 2000). They also have a greater osteoclast surface with reduced osteoid surface as compared to C3 (Akhter, Iwaniec et al. 2000), indicating a difference in bone turnover and formation may have a role in the overall differences seen in the two strains. This is further supported by a recent study in which a mutation was identified in the alkaline phosphatase gene in C57 animals known to have reduced bone mineral density (Klein, Carlos et al. 2005). Biomechanical studies are consistent with the bone mineral density and geometry analyses, with C3 animals having a higher ultimate and yield load, ultimate

and yield stress, and modulus, in addition to being stiffer than their C57 counterparts (Akhter, Iwaniec et al. 2000). Taken together, these data indicate that the C3 animals have increased biomechanical properties, in part due to the thick cortex coupled with a small cross-sectional area and reduced regions of bone turnover and larger areas of bone formation.

To further characterize the observed differences in bone strength, QTL studies have been done in which chromosomes of the hybrid offspring of C3 and C57 matings were mapped. In these studies, the time of peak bone mass was determined to be four months of age, confirming the previously held dogma (Beamer, Shultz et al. 2001). Bone mineral density was measured using pQCT and heritability of bone density in the femur was estimated to be 83%. When the distribution of bone densities were plotted, the C57 progenitors had the lowest density, the C3 progenitors had the highest and the F1 hybrid offspring had intermediate values (Beamer, Shultz et al. 2001). QTL mapping revealed several loci distributed throughout the genome that contributed to the variation seen in the bone mineral densities. 10 of these loci had some impact on femoral mineral density, with QTLs on chromosomes 1, 4 and 18 having the greatest effect, while five others were unique to the femur (2, 6, 12, 13 and 16), accounting for 8.36% of the variation in bone density observed (Beamer, Shultz et al. 2001). The QTLs do not appear to work independently, but rather have an additive effect on bone density (Beamer, Shultz et al. 2001).

This concept was further proven in another genome-wide QTL analysis. In this study, the femurs of 999 female mice were analyzed for single bone traits such as bone mineral density and bone strength as well as pleiotropic or multiple gene, effects (Koller, Schriefer et al. 2003). This study found QTLs on chromosomes 10 and 12 that were

linked specifically to variations in biomechanical properties, while chromosome 17 harbors a QTL unique to bone structural properties (Koller, Schriefer et al. 2003). Pleiotropic QTLs affecting all bone parameters studied were found on chromosomes 4 and 14, while QTLs affecting either bone density or bone structure/stiffness were found on chromosomes 1, 8, 13 and 17 (Koller, Schriefer et al. 2003). All QTL associated with changes in biomechanical properties were also associated with changes in either bone mineral density, cross-sectional moment of inertia or both (Turner, Sun et al.). Several of these loci have been shown to be highly preserved across species, including humans, indicating an important role in bone health (Koller, Schriefer et al. 2003).

Once candidate QTLs were identified, five congenic mouse strains were developed in order to try to understand the effect of each individual QTL on bone mineral density. Congenic strains are genetically identical (inbred) mice except for a single transferred chromosomal segment. These strains were made by transferring genomic DNA from the QTL region of the chromosome from a C3 animal to a C57 host animal (Turner, Sun et al. 2003). In this manner, the recipients are C57 at all their loci expect for the transferred region allowing for study of the effect of the QTL and the role of genetic background in determining bone mineral density. These studies showed that an individual QTL had an impact on the structure and function of the C57 femora, but did not bring the C57 femora to the normal C3 level in either shape or strength (Turner, Sun et al. 2003). This may be due to the isolation of a single QTL, as it has been shown that the action of the QTLs are additive (Beamer, Shultz et al. 2001).

Taken together, these studies demonstrate the presence of genes throughout the genome that act together to impact the quality and strength of bone. These QTLs are

potential modifier genes that may represent possible therapeutic targets for the treatment of OI.

Current Mouse Models of OI

There are currently several mouse models of OI being used to study the disease and the effect of the various OI-causing mutation on the clinical outcome. These models represent a wide variety of mutations including glycine substitutions, deletions, transcriptional inhibitors and mini-gene insertions, and represent the broad range of clinical severity seen in the human OI population. Some carry their mutations in the α1(I) chain while others affect the α2(I) chain. All have unique advantages while some have distinct disadvantages.

Mov-13

The *Mov-13* mouse is a transgenic model carrying a murine retrovirus in the first intron of the COL1A1 gene resulting in blocked transcription (Bonadio, Saunders et al. 1990) and, ultimately, a null α1 allele. Homozygote animals fail to produce any α1 chains and, therefore, cannot make type I collagen and die mid-gestation (Bonadio, Jepsen et al. 1993). Heterozygotes produce 50% of the normal amount of type I collagen, although the collagen they have is normal in structure, mimicking human OI type I (Bonadio, Jepsen et al. 1993). Heterozygotes were found to have reduced long bone strength and increased brittleness that were somewhat improved by the skeletal adaptations the animals made (Bonadio, Jepsen et al. 1993). This animal is maintained on the inbred C57 background allowing for genotype-phenotype studies as well as gene dosage effects as overexpression of an α1(I) gene rescued the phenotype (Bonadio, Jepsen et al. 1993). Disadvantages of this model include the transgenic nature, unnatural

cause of disease and retrovirally induced leukemia as well as the lethality of the homozygotes. The penetration of the mutation is also incomplete, as seen by the ability of odontoblasts to efficiently transcribe and translate the *Mov-13* allele into functional proα1 chains (Bonadio, Saunders et al. 1990). Studies undertaken on this model include geometric and biomechanical studies (Bonadio, Saunders et al. 1990; Bonadio, Jepsen et al. 1993), collagen content analyses (Bonadio, Saunders et al. 1990), histological examination (Bonadio, Saunders et al. 1990; Bonadio, Jepsen et al. 1993) and mineralization studies (Bonadio, Jepsen et al. 1993).

Human COL1A1 Minigene Construct Mouse

An older transgenic mouse model of OI utilizes two constructs containing portions of the human COL1A1 gene. The first is a minigene containing an 11 Kb portion of gene including the promoter, first five exons and introns and the last six exons and introns (Sokolov, Mays et al. 1993). The second construct was the same as the first but containing a deletion removing most of the regulatory sequence of the first intron (Sokolov, Mays et al. 1993). The transgenic animals were made by injecting one of the two constructs into fertilized embryos. These two constructs allow for testing of the regulatory sequences within the normal COL1A1 sequence as well as a mean to quantitatively assay the expression of the minigenes relative to the endogenous gene at both the RNA and protein levels (Sokolov, Mays et al. 1993). Another advantage of this system is that antisense oligonucleotides can be generated in an attempt to correct the mutant gene. One major disadvantage of this system is the low yield of transgenic mice following injection. Only about 10% of the injected embryos survive to sexual maturity (Khillan, Li et al. 1994). This can be explained by the high rate of lethality of mutations affecting the α1(I) chain. This system is also not ideal for studying genotype-phenotype

correlations because of the wide range of copy number, anywhere from 1-50, seen in transgenic animals, with the degree of phenotypic severity correlating with the copy number (Sokolov, Mays et al. 1993). The presence of both mutant and endogenous α1(I) chains also makes dissection of the contribution of the mutant protein difficult. The unusual nature of the cause of disease, as well as the mix of human and mouse protein, also makes translation to humans complicated. Studies done on this model include phenotypic rescue studies (Khillan, Li et al. 1994) as well as age and tissue-specific expression studies (Sokolov, Mays et al. 1993).

Brittle IV (Brtl IV)

The Brtl IV mouse model is a relatively new transgenic mouse model of OI created using the Cre/Lox system and first described in 1999. This model carries a glycine to cysteine substitution at position 349 of the COL1A1 gene, leading to the production of mutant α1 chains (Forlino, Porter et al. 1999). The heterozygote animals have a variable phenotype similar to human OI type IV patients and are the result of a glycine substitution, the most common molecular cause of OI in the human population. Another advantage of this model is the ribozyme site designed in the original construct that will allow for study of the correction of the mutant allele (Forlino, Porter et al. 1999). The transgenic nature of this animal is a potential disadvantage, as the addition of exogenous genetic material may have as yet unknown effects on the clinical outcome. For example, some of the heterozygote animals died in the perinatal period due to respiratory insufficiency for unknown reasons (Forlino, Porter et al. 1999). Additionally, animals carrying the stop cassette produced an alternative splice site of the mutant transcript, designated as BrtlII, were perinatal lethal due to the partial expression of an α1

chain carrying a non-collagenous insertion (Forlino, Porter et al. 1999). The maintenance of this mutation on a mixed genetic background also makes genotype-phenotype correlations difficult. Data available on this model include structure and stability studies on the mutant collagen (Kuznetsova, Forlino et al. 2004) as well as biomechanical studies of bones from these animals at varying time points (Kozloff, Carden et al. 2004). More information on this model can be found in appendix I.

Osteogenesis imperfecta murine (*oim*)

The most widely used model is the osteogenesis imperfecta murine or *oim*, described below. This model was first described in 1993 as lacking the α2 chain of type I collagen and, therefore, producing only homotrimeric type I collagen (Chipman, Sweet et al. 1993) and is unique because the disease causing mutation is naturally occurring, free of any genetic manipulation. Homozygous animals mimic human OI type III and is the most severely affected of any of the viable mouse models. Heterozygote animals mimic the milder OI type I phenotype, with bone biomechanical integrity intermediate to wildtype and homozygotes (Saban, Zussman et al. 1996). Extensive studies have been done using this model to study the role of the α2 chain in collagen structure and function (Misof, Landis et al. 1997; Kuznetsova, McBride et al. 2003), the consequence of producing only homotrimeric type I collagen on non-mineralized tissues (Phillips, Pfeiffer et al. 2002; Pfeiffer, Franklin et al. 2005), the role of collagen in mineralization (Cassella and Ali 1992; Camacho, Landis et al. 1996; Fratzl, Paris et al. 1996; Misof, Landis et al. 1997; Phillips, Bradley et al. 2000) and the biomechanical properties of bone and tendon (McBride, Shapiro et al. 1998; Camacho, Hou et al. 1999). Additionally, attempts at correcting the defect have been done using bone marrow cells from these animals (Oyama, Tatlock et al. 1999; Niyibizi, Smith et al. 2001). The biggest

disadvantage of this model is that although the phenotype mimics that seen in humans, the type of mutation and mode of inheritance are extremely rare. Most OI-causing mutations are glycine substitutions, not deletions causing homotrimeric type I collagen production. The *oim* mutation also has an autosomal recessive mode of inheritance, which is unusual in the human OI population where most mutations are dominant. The maintenance of the *oim* mutation on an outbred mouse background also limits genotype-phenotype studies. Transfer of the mutation to inbred mouse strains is an easy solution to this problem and will be discussed further.

G610C

Another new mouse model of OI is the G610C mouse. This is also a transgenic model of OI created using the Cre/Lox system. This is the first mouse model of OI that mimics the human condition in both genotype and phenotype. This model is based on an Amish population in which 64 individuals in 37 nuclear families are all heterozygote for the same glycine to cysteine substitution at position 610 of the $\alpha 2$ chain causing OI type I/IV. This population is the first to have such a large number of people all carrying the same mutation. In appendix I we demonstrate reduced biomechanical integrity as seen by a reduction in the force needed to break the bone as well as tensile strength without changes in geometry. This model has several unique advantages. First, this model will allow for direct mouse-human comparisons, as fibroblast lines from the human patients exist. It also presents a unique opportunity to study the genetic variation seen among people with the same mutation but different clinical outcomes. The mutation is currently maintained on two inbred backgrounds, C57 and DBA/2J, which are known to have low and high, but phenotypically normal, bone mineral densities, respectively. This will allow for study of genotype-phenotype correlations, as well as gene dosage effects since

both heterozygote and homozygote offspring are viable. The biggest disadvantage of this model is the transgenic nature of the animal and the possibility of unknown effects of the genetic manipulation.

COL1A1 Hammerhead Ribozyme Mouse

Another very new mouse model of OI contains a hammerhead ribozyme against a human COL1A1 minigene. This minigene is different from the previously described minigene in that it contains only 130 bp of the human gene (Toudjarska, Kilpatrick et al. 2001). Preliminary cell culture studies in calvarial osteoblasts have shown that transfection of the ribozymes into cells carrying the minigene are able to specifically cleave mutant transcript and downregulates the expression of the mutant RNA (Toudjarska, Kilpatrick et al. 2001). It was later determined that a multimeric version of the ribozyme was much more potent in downregulating the mutant transcript and protein, ablating nearly 100% of both mRNA and protein (Peace, Florer et al. 2005). Mice carrying either the ribozyme, minigene or both have been created and are currently being evaluated [Peace, in preparation]. This system holds the possibility to study downregulation of the mutation as a means to correct the mutation as well as the degree of phenotypic reversion following these treatments. A disadvantage of this system is the transgenic nature of the construct and unknown effects having such a large exogenous gene will have on other tissues and systems.

All of the models described above are viable models in which to study OI. Each model has a unique set of characteristics making it the model of choice for various studies. It is important to note that the model used should be carefully chosen to best address the question being asked as there is not a perfect model.

Table I.2: Summary of Animal Models of OI

Name	OI Type	Gene	Mutation	Protein Consequence	Human Patient(s)	References
Mov-13	I	Col1a1	Retroviral insert	Haploinsufficiency of α1 chains	No	Bonadio, 1990; Bonadio, 1993
Minigene	Mild	Col1a1	11 Kb human minigene insert	Abnormal α1 chains	No	Sokolov, 1993, Khillan, 1994
Brtl IV	IV	Col1a1	Glycine substitution	Abnormal α1 chains	Yes	Forlino, 1999; Kozloff, 2004; Kuznetsova, 2004
Oim	III	Col1a2	Deletion	Homotrimer	Yes	Chipman, 1993; See text for others.
G610C	I/IV	Col1a2	Glycine substitution	Abnormal α2 chains	Yes (many)	Personal communication with Dr. Daniel McBride
Ribozyme	Mild	Col1a1	130 bp human minigene insert	Abnormal α1 chains	No	Toudjarska, 2001; Peace, 2005

Osteogenesis Imperfecta Murine (*oim*) Model

The *osteogenesis imperfecta* murine (*oim/oim*) is a naturally occurring mouse model of OI originally identified at Jackson Laboratory due to small size and bone deformity. This mouse contains a spontaneous G deletion at position 3983 in the proα2(I) collagen (COL1A2) gene causing a frameshift during translation of the mutant transcript resulting in an alteration of the terminal 48 amino acids. The abnormal C- terminal end of the α2(I) collagen propeptide chain prevents it from associating with the proα1(I) collagen chains (Chipman, Sweet et al. 1993; McBride and Shapiro 1994). Therefore, these animals produce homotrimeric type I collagen, consisting of three α1 chains [$\alpha1(I)_3$] instead of the normal heterotrimeric collagen containing two α1 chains and one α2 chain [$\alpha1(I)_2\alpha2(I)$], causing a non-lethal, recessive OI type III phenotype (Chipman, Sweet et al. 1993). The *oim/oim* mutation results in a clinical phenotype including reduced body mass and reduced bone mineral content and density (Phillips, Bradley et al. 2000), cortical thinning, bowing of the long bones with fractures and callus formation, similar to that of a human OI type III patient (Pihlajaniemi, Dickson et al. 1984; Chipman, Sweet et al. 1993), who also produced only homotrimeric type I collagen. This patient, the child of consanguineous parents, has a 4 base pair deletion in the coding region for the carboxypropeptide of the proα2 chain which also causes a frameshift and prevents incorporation of the α2 chain into the triple helix (Nicholls, Pope et al. 1979). Chu et al demonstrated that fibroblasts from this patient were able to produce normal levels of α2(I) mRNA which were translated into protein (Deak, Nicholls et al. 1983), that was not able to associate to form the triple helix. Clinical features of this patient included numerous spontaneous fractures, above normal IQ

and slowed motor development (Nicholls, Osse et al. 1984). Gross examination at five years of age showed shortening of the limbs as compared to the rest of the body, severe deformities and bowing of the limbs and hypermobility of the right upper arm (Nicholls, Osse et al. 1984). X-rays showed severe osteoporosis and multiple fractures in the limbs in addition to osteoporosis of the lumbar spine. Dentinogenesis imperfecta was not seen (Nicholls, Osse et al. 1984). Biochemical analysis demonstrated an unusually high level of alkaline phosphatase while transverse sections of skin show a normal collagen fibril banding pattern but localized regions of fibrils of abnormal size and shape (Nicholls, Osse et al. 1984), characteristics shared by the *oim/oim* mouse.

Differences Between Heterotrimeric and Homotrimeric Type I Collagen

To test the differences in the strength of the collagen between wildtype, heterozygous (*oim*/+) and homozygous (*oim/oim*) animals, tail tendon was isolated and subjected to stress/strain experiments. When tail tendon from *oim/oim* animals is subjected to stretching, it was only able to be stretched 6-7% before breaking, while wildtype tendon was stretched 12-16%, a statistically significant two-fold difference (Misof, Landis et al. 1997). *Oim*/+ tendon had intermediate, but still statistically significant, values, stretching 8%. When the collagen was visualized, *oim/oim* collagen fibrils had a much smaller diameter than either wildtype or *oim*/+ animals (McBride, Choe et al. 1997; Misof, Landis et al. 1997). Homotrimeric type I collagen also has a higher melting temperature, but slower rate of unfolding than wildtype type I collagen, possibly affecting the rate of tissue growth and response to insult (Kuznetsova, McBride et al. 2003). In addition to the altered

properties of homotrimeric type I collagen, measurements of hydroxyproline, an amino acid unique to type I collagen, demonstrated a reduction in the overall amount of collagen in the bones (Camacho, Hou et al. 1999), aorta (Pfeiffer, Franklin et al. 2005) and left ventricle (Weis, Emery et al. 2000) of *oim/oim* animals.

Since homozygous *oim* animals produce only homotrimeric type I collagen, an isotype not normally expressed post-embryonically except in small quantities in the skin (Uitto 1979) as well as in disease and injury states, it is not surprising that the mineralization of *oim* bone and teeth is also altered. Small-angle x-ray scattering has revealed alterations in the mineral phase of bones from *oim/oim* animals as compared to their wildtype and heterozygous counterparts. Femurs and tibias from *oim/oim* animals have thinner crystals that tended to be more random in their orientation than heterozygote littermates (Fratzl, Paris et al. 1996). When neutron activation analysis was used to identify and quantitate specific minerals in incisors and femurs, *oim/oim* mice were found to have a very different mineral composition than their wildtype and heterozygous littermates, with the most dramatic differences seen in the femur (Phillips, Bradley et al. 2000). *Oim/oim* femurs have increased magnesium and fluoride with reduced sodium, while *oim/oim* incisors have reduced magnesium as compared to wildtype and reduced fluoride as compared to heterozygotes (Phillips, Bradley et al. 2000). These differences may reflect a tissue-specific mineral composition as well as reflect the impact of mutations in type I collagen on the mineral phase of bones and teeth.

Several studies have been aimed at further understanding the biochemical and structural differences between heterotrimeric and homotrimeric type I collagen. Although homotrimeric collagen can self-assemble into fibrils, the critical concentration for fibril

formation *in vitro* is 40-fold higher than for heterotrimeric collagen, making homotrimeric fibrillogenesis less efficient (McBride, Kadler et al. 1992). This is further supported by osmotic stress force measurements indicating that the α2 chain enhances intermolecular attraction between collagen molecules as seen by lower hydration of heterotrimer fibrils as well as interactions with non-collagenous molecules (Kuznetsova, McBride et al. 2001). These non-collagenous interactions may be particularly important in bone, where non-collagenous proteins interact with collagen to form the composite material (Kuznetsova, McBride et al. 2001). Homotrimeric collagen also possesses an enzyme cleavage site approximately 100 residues from the C-terminus that is susceptible to enzymatic digestion below its melting temperature that heterotrimeric collagen does not have (McBride, Choe et al. 1997). X-ray diffraction studies indicate a loss of axial order and crystalline lateral packing for homotrimeric as compared to heterotrimeric collagen, although transmission electron microscopy shows a relatively normal axial banding pattern (McBride, Choe et al. 1997). Homotrimeric collagen fibers contain more water than heterotrimeric collagen, prohibiting the close packing of the fibers seen in heterotrimeric collagen (Miles, Sims et al. 2002). Homotrimeric collagen also has an increased number of cross-links, possibly as a compensatory mechanism for reduced biomechanical integrity (Weis, Emery et al. 2000; Pfeiffer, Franklin et al. 2005). These studies of homotrimeric type I collagen suggest that the α2 chain has an important role in 1) rates of triple helix formation, 2) structural stability of the triple helix and 3) normal collagen fibril packing.

In summary, these studies all contribute to the understanding of the weaker bones seen in the *oim/oim* mouse. In addition to reduced amounts of type I collagen, the homotrimeric collagen is weaker possibly due to misalignment of the fibrils, reduced fibril

diameter or both. The homotrimeric type I collagen also has an increased melting temperature and degree of cross-linking as well as a reduced rate of unfolding, perhaps influencing tissue remodeling or response to injury. As expected, the alterations in type I collagen have an impact on the mineral phase of the bone. *Oim/oim* animals have less mineral as seen by reduced bone mineral content and density measurements. The mineral they have is made of thinner and less well aligned crystals than wildtype animals. Changes are also seen in the composition of the mineral between *oim/oim* and wildtype animals. Taken together, these studies provide some of the answers to the reduced biomechanical integrity seen in *oim/oim* bones.

Goals

The goal of this study was to determine the role of genetic background and age on the phenotypic severity of the *oim* mutation. The *oim* model provides a unique opportunity to study the role of genetic background in determining clinical outcome as many aspects of the mutation have been well studied in both mineralized and non-mineralized tissues. However, the maintenance of the mutation on an outbred genetic background makes study of modifier genes difficult. To this end, the *oim* mutation was transferred to the C57BL/6J mouse strain, described above. The mutation was then analyzed in the context of both backgrounds at each of three ages representing childhood, adolescence and post-puberty or peak bone mass. At all ages examined, strain differences did occur, but the oim mutation overrode these differences. The *oim* mutation generally caused smaller body size, altered femoral geometry, reduced biomechanical integrity, altered tibial bone mineral composition and reduced hydroxyproline content. However, C57 *oim/oim* animals generally had the most severe phenotype as determined by biomechanical integrity.

CHAPTER II

THE *OIM* MUTATION: ROLE OF GENETIC BACKGROUND IN DETERMINING PHENOTYPIC SEVERITY AT PEAK BONE MASS

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MULTIELEMENT ANALYSIS OF BONE FROM THE OSTEOGENESIS IMPERFECTA MODEL (*OIM*) MOUSE USING THERMAL AND FAST NEUTRON ACTIVATION ANALYSIS

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ABSTRACT

To investigate the role of genetic background in determining clinical outcome in osteogenesis imperfecta (OI), the *oim* mutation was evaluated on two separate backgrounds, the outbred B6C3Fe and the inbred C57BL/6J backgrounds. The osteogenesis imperfecta murine (*oim*) model produces a phenotype similar to human OI type III, characterized by bowing of long bones and reduced biomechanical integrity. How genetic background impacts the severity of an OI phenotype has not been characterized. Femurs and tibias from four month-old wildtype, *oim*/+ and *oim/oim* animals from each strain were analyzed along with a serum marker of bone turnover. *Oim/oim* mice of both strains exhibited reduced body mass regardless of strain. μCT analysis showed altered femoral geometry between strains and genotypes. Torsional ultimate and tensile strengths were significantly reduced in *oim/oim* femurs from both strains, but did not exhibit strain differences. Torsional stiffness showed genotype differences within the C57 strain, which were not seen in outbred animals. Energy to failure exhibited significant strain differences for all genotypes as well as being reduced in *oim/oim* femurs from both strains. Collagen content of femurs was significantly reduced in *oim/oim* mice of both strains as well as exhibiting significant strain specific differences in wildtype animals. Neutron activation analysis demonstrated mineral-specific differences in tibial mineral composition between genotypes and strains. Pyridinoline crosslinks quantitation showed strain differences in *oim*/+ and *oim/oim* animals. Taken together, these studies indicate an important role for genetic background in determining clinical outcome by impacting bone geometry, reducing biomechanical integrity and collagen content as well as altering bone mineral composition. However, presence of the *oim* collagen mutation had the greatest impact on the pathogenic outcome of bone quality and strength.

INTRODUCTION

Bone is a two component composite material made of an organic phase, primarily type I collagen, and a mineral phase, mainly hydroxyapatite crystals. Type I collagen is normally found as a heterotrimeric molecule, comprised of two α1 chains and a single α2 chain [$\alpha1(I)_2\alpha2(I)$], however a homotrimeric variant containing only α1 chains [$\alpha1(I)_3$] has also been identified embryonically (Jimenez, Bashey et al. 1977; Scriver 1989), in some tumors (Moro and Smith 1977; Uitto 1979; Rupard, Dimari et al. 1988) and may play a role during the wound healing process (Haralson, Jacobson et al. 1987; Cohen, Diegelmann et al. 1992). Within the bone, the collagen fibrils align in a stereotypical way to maximize the strength of the tissue and act as a blueprint for mineralization to occur (Schenk, Felix et al. 1993). The toughness of the bone comes from type I collagen while the hydroxyapatite crystals give the bone strength and stiffness (Burr 2002). The combination of type I collagen and hydroxyapatite crystals gives bone properties unlike either component alone (Burr 2002). The correct ratio of the two components is also important, as too much mineral leads to brittle bones (Burr 2002), while too little mineral weakens bones. Long bones such as the femur and tibia, are made of two types of bone, cortical and trabecular bone. Cortical bone, also known as the cortex or compact bone, is the dense layer of calcified tissue that comprises the outer portion of the bone. Trabecular bone, also known as cancellous or spongy bone, is the internal space that houses the bone marrow and is found at the epiphysis and toward the growth plates (Favus 1993). The main role of the cortex is to give strength and protection to the bone, while the trabecular bone plays a metabolic role (Baron 1999).

Osteogenesis imperfecta (OI) is a genetically and clinically heterogeneous disease characterized by anomalies in type I collagen-containing tissues, such as bone, tendon, skin, and teeth. OI is most commonly caused by mutations in one of the two type I collagen genes, COL1A1 or COL1A2 (Byers 1993). The disease can be subdivided into four categories based on clinical manifestation (Sillence, Senn et al. 1979). Type I OI is the mildest form, usually due to haploinsufficiency, characterized by normal stature with an increased number of pre-pubertal fractures (Byers 1993; Marini 1998). Type II OI is perinatal lethal often due to pulmonary insufficiency (Byers 1993). Type III OI is the most severe form compatible with life. Type III OI patients are generally wheelchair bound and have progressive bowing of the long bones, short stature, blue-grey sclera and reduced biomechanical integrity manifested by numerous fractures throughout life (Byers 1993). Type IV is variable exhibiting clinical severities intermediate to OI types I and III. These patients have mild to moderate bone deformity with normal stature (Byers 1993).

The osteogenesis imperfecta murine (*oim*) model mouse is the most widely studied mouse model of OI. The *oim* mouse was originally identified at Jackson Laboratory when a spontaneous mutation arose, producing small animals with skeletal deformities. It was later determined that the phenotype arose due to a single base deletion in the COL1A2 gene leading to the exclusion of the $\alpha 2$ chain from the mature type I collagen molecule, producing solely homotrimeric type I collagen (Chipman, Sweet et al. 1993). This mouse model has been shown to have reduced biomechanical integrity (McBride, Shapiro et al. 1998; Camacho, Hou et al. 1999), altered bone mineral composition (Phillips, Bradley et al. 2000) and reduced amounts of type I collagen in

bones (Camacho, Hou et al. 1999), tendon (McBride, Choe et al. 1997), left ventricle (Weis, Emery et al. 2000) and aorta (Pfeiffer, Franklin et al. 2005).

Although the disease-causing genes for OI are known, understanding how genotype causes phenotype is still poorly understood. More than 850 distinct OI-causing mutations have been mapped (Dalgleish 1997), with most common type of mutation being glycine substitutions. Most OI-causing mutations are limited to single individuals or families, making the study of genotype-phenotype correlations difficult. Several attempts have been made to associate a given genotype with a specific phenotype with minimal success (Nuytinck, Wettinck et al. 1997; Lund, Astrom et al. 1999; Di Lullo, Sweeney et al. 2002). The identity of the chain harboring the mutation, the location of the mutation within the chain, and the substituting amino acids are also important determinants of clinical outcome. Given that all studies to date have failed to clearly define a genotype-phenotype map, other factors influencing phenotypic severity must exist.

Differences in contribution of genetic background to bone geometry and strength have been widely studied in inbred strains of mice, especially C57BL/6J (C57) and C3H/HeJ (C3) mice (Beamer, Donahue et al. 1996; Akhter, Iwaniec et al. 2000). C57 mice have been found to have decreased biomechanical integrity compared to C3 mice (Akhter, Iwaniec et al. 2000). The differences in bone strength seen between C3 and C57 animals is, in part, due to differences in bone geometry and turnover (Beamer, Donahue et al. 1996; Akhter, Iwaniec et al. 2000). Taken together, these data indicate that the C57 animals have decreased biomechanical properties, in part due to the altered geometry and differences in bone turnover.

Although much data is available on specific collagen mutations in mouse and humans as well as differences between inbred strains of mice, studies to evaluate the role of genetic background and modifier genes on specific collagen mutations have not been done. The *oim* mouse, which has been extensively characterized at the molecular, biochemical and physiological levels, is normally maintained on the outbred B6C3Fe background (with genetic contributions from both C57BL/6J and C3H/HeJ backgrounds) and was recently bred onto the inbred C57BL/6J background.

To determine the role of genetic background and potential modifier genes on clinical outcome, wildtype, heterozygous (*oim*/+) and homozygous (*oim/oim*) mouse femurs and tibias of each strain (outbred and C57) were evaluated at four months of age, the time of peak bone mass in mice (McBride, Shapiro et al. 1998). Femoral geometry was analyzed by μCT analysis while biomechanical integrity was determined by torsional loading to failure. Mineral composition was determined using neutron activation analysis and collagen content was quantitated using the hydroxyproline assay. A serum marker of bone turnover, pyridinoline crosslinks, was also quantitated to determine if alterations in bone turnover occur. Our results demonstrate an important role for genetic background in determining the clinical outcome in *oim*/+ and *oim/oim* animals. However, the presence of the *oim* mutation ultimately had the greatest impact on phenotypic severity.

METHODS

Animals

Heterozygote B6C3Fe *a/a-Col1a2*$^{oim/J}$ animals were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in an AAALAC accredited facility at the

University of Missouri-Columbia. To produce the C57-*oim* line, the *oim* mutation was bred from the outbred B6C3Fe background to the inbred C57BL/6J (C57) background. *Oim*/+ heterozygote offspring were repeatedly backcrossed to wildtype C57 animals so that after seven backcross generations, the C57-*oim/oim* animals are 99% genetically identical except for the transferred region. C57-*oim/oim* animals, as well as their wildtype and heterozygote (*oim*/+) littermates, used in experiments had been backcrossed at least 11 generations. The C57 congenic background was verified using approximately 65 microsatellite markers with an average of three markers per chromosome with a mean genetic distance of 20 centiMorgans between markers (Research Animals Diagnostic Laboratory, Columbia, MO). Animals had ad libitum access to food and water (Purina 5008 Formulab Diet; Purina Mills Inc., Richmond, IN) and cared for in compliance with an approved University of Missouri Animal Care and Use Protocol. Genotypes (wildtype, *oim*/+, *oim/oim*) were determined by polymerase chain reaction and restriction fragment length polymorphism assay (Phillips, Bradley et al. 2000).

Experimental Design

Animals were sacrificed at four months of age via CO_2 asphyxiation, weighed, blood harvested by cardiac exsanguination, serum isolated and stored at –80^{o} C until use. Tibias were removed, cleaned of soft tissue attachments and snap frozen in liquid nitrogen prior to long-term storage at –80^{o} C. Femurs were removed, cleaned of soft tissue attachments, wrapped in strips of sterile gauze and stored at –80^{o} C in sterile 1X phosphate buffered saline (PBS).

μCT Analysis

Geometric parameters were defined from right femurs by microCT (μCT) scan analysis (MicroCAT II, ImTek, Inc., Knoxville, TN) prior to *ex vivo* torsional loading to failure. The μCT image slices were reconstructed in 3D using the Amira 3.1 software package (Mercury Computer Systems/TGS, Hudson, NH) to measure total femur length and to locate the mid-shaft slice. The mid-shaft slice was modeled as a hollow elliptical cross-section and was used to determine periosteal ($_p$) and endosteal ($_e$) anterior-posterior (d_p; minor diameter of the bone cross section) and medio-lateral (Dp and De; major diameters) dimensions (Figure 1; Camacho, et al, 1999). The Amira software allows points to be plotted on either side of the section in the desired direction and a histogram to be plotted across the given cross section. From the histogram, the edges of the cortical bone were reproducibly identified by using a bone density threshold of

$$P_{max} + (P_{max}-P_{min})/3 \qquad 1)$$

The image analysis software was then used to draw mutually perpendicular major and minor diameter lines to the edges of the cortical bone, and to determine numerical values of the diameters.

To calculate marrow cavity diameter, the average of the endosteal diameters in both the anterior-posterior and medio-lateral directions were taken. Cortical bone widths were determined by averaging the two widths taken in the anterior-posterior direction and the two taken in the medio-lateral direction. Polar moment of area (K) was calculated using

$$=\pi[(D_p\text{^}3)(d_p\text{^}3)(1-q\text{^}4)/((D_p\text{^}2)+(d_p\text{^}2))] \qquad 2)$$

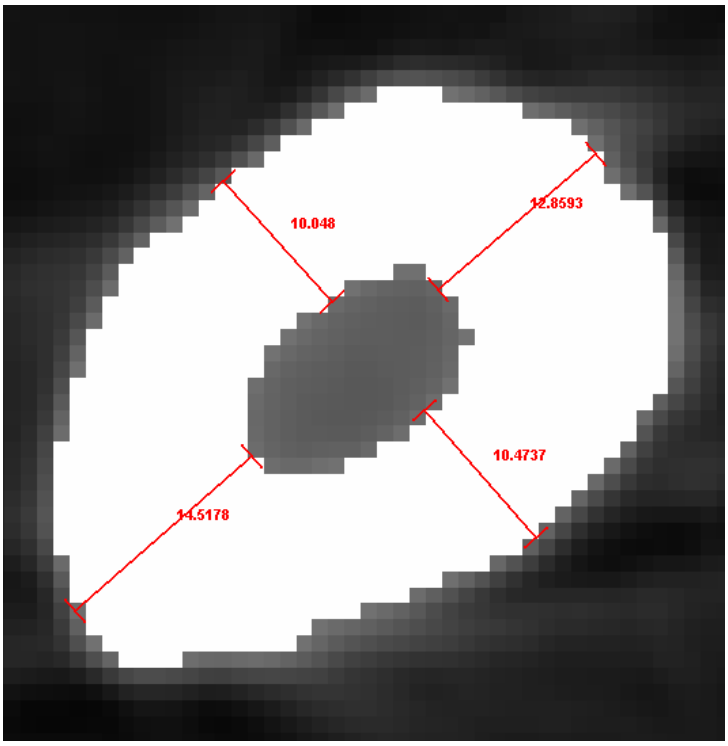


Figure II-1. CT Scan Analysis. Cross-section of the mid-shaft slice of a femur. Lines show procedure for measuring cortical bone width in both the major (medo-lateral; D_p) and minor (anterior-posterior; d_p) directions. Dimensions for the marrow cavity (D_e) were obtained in a similar manner. Image provided by Dr. Chris Winkelmann.

Torsional Loading to Failure

A torsional holder was machined for each femur to be tested from 0.035" wall ½ inch outer diameter steel tubing (McMaster-Carr, Chicago, IL) (Figure 2). The long axis of the femur was visually aligned with and centered on the axis of the holder using the holder side struts as guides. The bone was threaded through a paper washer to hold the bone in place and provide a surface onto which 5 minute epoxy (Devon, Riviera Beach, FL) could be poured, thus securing the femur ends in the holder. The holder was then placed in a test fixture mounted on a TA-HDi testing machine (Stable Micro Systems, Surrey, UK). A cross bar was inserted, preventing the upper end of the holder from rotating about its long axis (Figure 3a). A Dremel tool cut-off wheel was then used to sever the two steel struts (Figure 3b). The lower end of the holder was rotated at a constant speed (0.75 radians/sec) by raising the test machine cross head at 10 mm/sec to pull on the 0.045" diameter aircraft cable attached to the 5 Kg load cell in the cross head with other end wrapped around the 1.00" diameter shaft of the test fixture (Figure 3c).

The computer software controlling the test machine acquired and stored the crosshead position, cable force (F), and sample time at a rate of 200 samples/sec. A digital camera (Sony DSC-S75, Oradell, NJ) was used to take pre- and post-test photos (Figure 4) of each specimen, as well as a video-audio clip of each test to obtain a record of the mode of femur failure. Applied torque T was calculated from F using

$$T = (F - F_{\text{friction in fixture}})*0.13006 \quad \text{(Nmm)} \qquad 3)$$

Torque T was plotted as a function of relative angular displacement θ (rad) between the ends of the L=7 mm long exposed section of femur shaft (L=4.76 mm for 1 month specimens; Figure 5). Torsional stiffness Ks (Nmm/rad) was determined as the slope of a

Figure II-2. Torsional Loading to Failure Specimen Holder. Window length: 7mm for femurs from 2 and 4 month animals and 4.76 mm for femurs from 1 month animals.

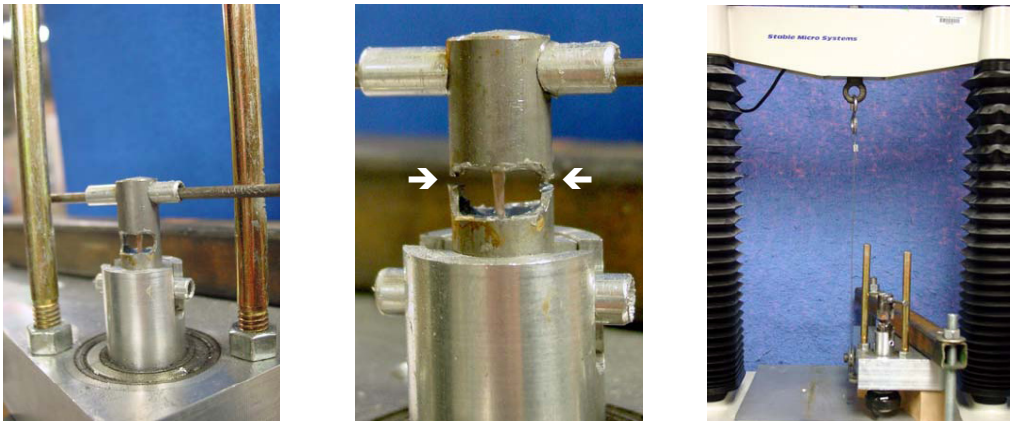


Figure II-3. Torsional Loading to Failure Experimental Set-Up

A. Cross-bar prevents upper portion of femur from rotating.

B. Dremel tool cuts struts (arrows), allowing femur to rotate.

C. Cable attached to load cell wraps around the bottom of the test fixture.

line fit by linear regression to the initial linear region of this plot (between 5<T<10 Nmm for 2 and 4 month samples; between 2<T<5 for 1 month samples; Figure 5). Ultimate tensile strength Su (N/mm^2) of the femur's cortical bone was determined by substituting T_{max} and the mid-shaft cross section diameters into

$$Su = \text{shear stress } \tau_{max} = T_{max}/R \qquad (N/mm^2)$$

$$\text{where } R = [\pi D_p d_p^2(1 - q^4)]/16 \qquad (mm^3) \qquad 4)$$

The mid-cross section diameters were used to determine its polar moment of area parameter K (mm^4) for a hollow ellipse (Roarck and Young 1975).

$$K = [(\pi D_p^3 d_p^3)(1-q^4)]/[16(D_p^2+d_p^2)] \qquad (mm^4)$$

$$\text{where } q=D_e/D_p \qquad 5)$$

The femur's shear modulus of elasticity G (Nmm^2) was determined using

$$G = KsL/K \qquad (Nmm^2) \qquad 6)$$

where Ks is torsional stiffness, L is the exposed length of femur and K is the mid-shaft cross section polar moment of area determined by μCT analysis.

The strain energy absorbed in the femur to fracture U (Nmm) was calculated as the area under the T versus θ curve until failure from T=2 to T_{max} (see Figure 5).

Hydroxyproline Assay

Left femurs were lyophilized in a Freezemobile 12SL (Virtis, Gardiner, NY) for three hours prior to being cut in half. Each half was then weighed (Model AG245, Mettler Toledo, Columbus, OH) and measured. The proximal (hip) end of the femur was used for further analysis (Stegemann and Stalder 1967). Each sample was crushed using a mortar and pestle in 500 μl 6 N HCl and transferred to a glass screw-top test tube. An

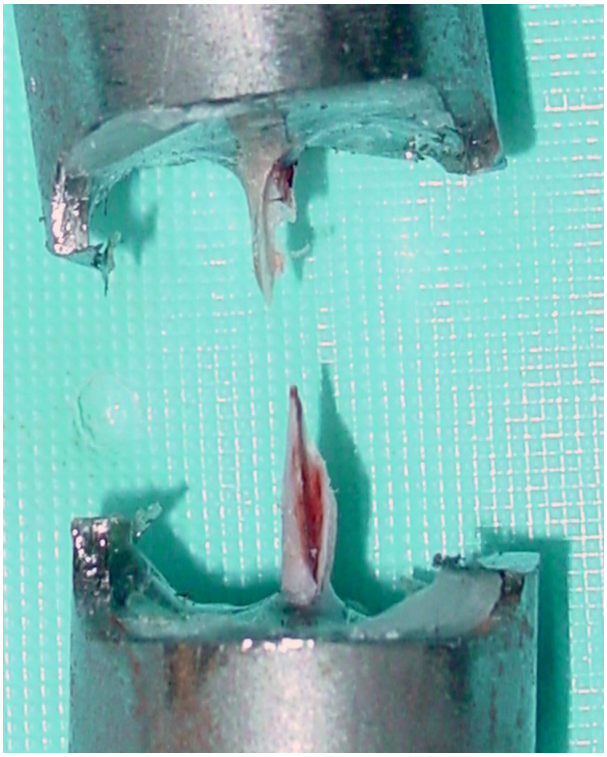


Figure II-4. Torsional Loading to Failure Post-Test Photo. Note the 45 degree spiral fracture typical of torsional loading to failure.

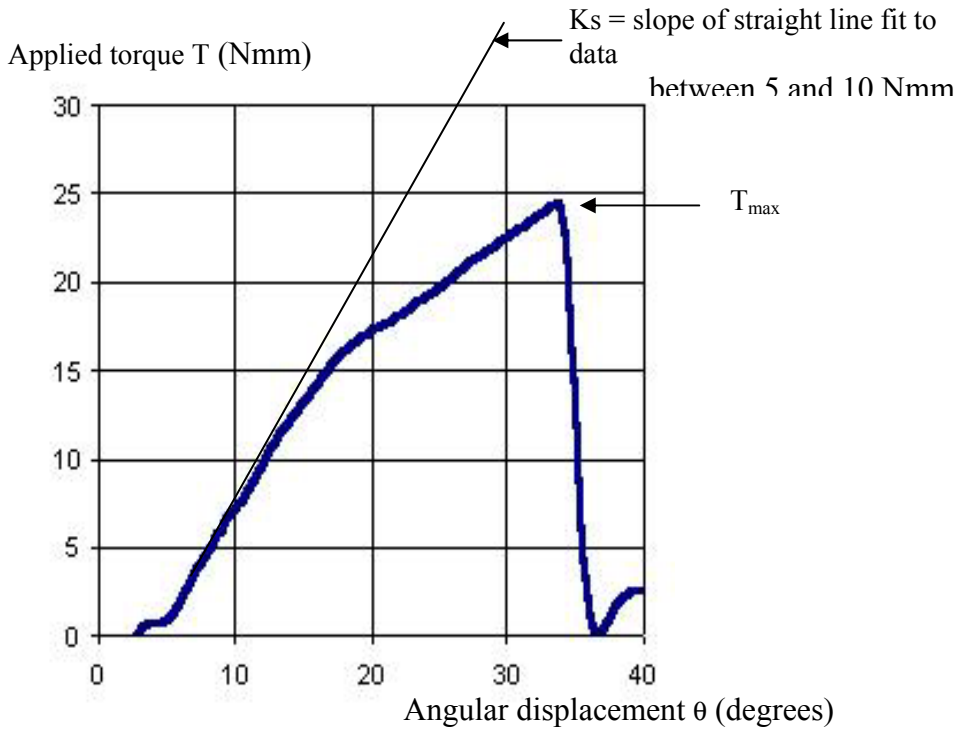


Figure II-5. Typical Torque vs. Angular Displacement Graph

additional 500 µl 6N HCl was used to wash the mortar; this was added to the screw-top test tube. The samples were then hydrolyzed at 124^{o} C for three hours. The samples were allowed to cool to room temperature and the caps removed. The HCl was then neutralized under vacuum in a desiccator at 70^{o} C overnight or until the sample was dried. The samples were then resuspended in 0.001 N HCl and allowed to sit for at least 30 minutes at room temperature. 10 µl of sample was removed and transferred to a 1.5 ml microcentrifuge tube and brought to 100 µl with 0.001 N HCl. Aliquots of hydroxyproline at 4, 2, 1, 0.5 and 0.25 µg/100 µl were used as standards to generate a standard curve. 100 µl freshly made Chloramine T solution was added to all samples, standards and the blank (100 µl 0.0001 N HCl) and allowed to stand at room temperature for 9-10 minutes. 1.2 ml Erlich's reagent was then added and the tubes incubated at 55^{o} C for 30 minutes (Stegemann and Stalder 1967). Absorbances were then read at 558 nm on a Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY).

Neutron Activation Analysis

Before analyses, the epiphyses were removed from the snap frozen left tibias from each genotype and strain (n=8) and the shaft demarrowed. The cortical shaft samples were then delipified for 10 minutes via acetone sonication (Model G-80-80-1, Laboratory Supplies Company, Hicksville, NY), washed with two water sonications (ten minutes each) and lyophilized in a Freezemobile 12SL (Virtis, Gardiner, NY) for two and a half hours. The samples were weighed (Model AT261, Mettler Toledo, Columbus, OH) and loaded in high-density polyethylene vials. A foam spacer was placed on top of the specimen to ensure a similar sample geometry and placement for all tests.

Standards and samples were activated by a thermal neutron flux of $5x10^{13}$ n/cm^2/s for specific times (see Table II-1) to activate F, Na, Mg, Ca, K and Zn and a 5 x 10^{12} n/cm^2/s flux to activate P. Milk powder, non-fat milk powder and bone ash were used as standards for all elements (NIST SRMs 8435,1549 and 1400, respectively, Bethesda, MD). The samples and standards were counted using a high-resolution gamma-ray spectrometer with a HPGe detector face (EG&G Ortec, Oak Ridge, TN). The spectrometer was connected to a spectroscopy amplifier (Tenelee 244, Oak Ridge, TN) and a loss-free counting module (Model 599, Canberra-Nuclear Data, Meriden, CT). Data acquisition and processing was performed using an ADC (Model 581, Canberra-Nuclear Data) and computer (Digital MicroVax, Maynard, MA).

Pyridinoline Assay

Determination of serum pyridinoline, a marker of bone turnover, was done using the Metra Serum PYD EIA Kit according to the manufacturer's protocol (Quidel, San Diego, CA). Blood was stored with 5µl heparin on ice prior to being centrifuged at 6000 rpm to separate serum from red blood cells. Serum was then stored at –80^o C until use. Briefly, 200 µl serum was added to the provided spin column and spun at maximum speed for 30 minutes or until eluate was colorless. 50 µl Reagent 1 and 25 µl diluted standard, control or undiluted filtered sample was added to a 96-well plate. 75 µl of cold pyridinoline antibody was then added to each well and the plate incubated overnight at 4^o C in the dark. The plate was then washed three times using 250 µl 1x wash buffer. 150 µl enzyme conjugate was then added and the plate incubated at room temperature for 1 hour and then washed three times with 250 µl 1x wash buffer. 150 µl working solution

Table II-1. Instrumental NAA Parameters

Ion	Nuclear Reaction	Photopeak (keV)	Irradiation Time	Decay Time	Count Time	Count Position
F	$F^{19}(n,\gamma)F^{20}$	1633	7 sec	15 sec	30 sec	Face
P	$P^{31}(n,\alpha)Al^{28}$	1779	15 sec	1 min	3 min	Face
Na	$Na^{23}(n,\gamma)Na^{24}$	1368	7 sec	1 min	10 min	1 spin[b]
Mg	$Mg^{26}(n,\gamma)Mg^{27}$	844, 1014	7 sec	1 min	10 min	1 spin
Ca	$Ca^{48}(n,\gamma)Ca^{49}$	3085	7 sec	1 min	10 min	1 spin
K	$K^{41}(n, \gamma)K^{42}$	1524	95 sec	EOI[a]	30 min	1[c]
Zn	$Zn^{68}(n, \gamma)Zn^{69m}$	438	95 sec	EOI[a]	30 min	1[c]

[a] EOI: End of Irradiation Time is the specific time the sample exited the reactor. All samples and standards were decay corrected back to this time.

[b] Count position was in a rotating position 2.5 cm from the detector face.

[c] Count position was stationary 2.5 cm from the detector face.

was then added and the plate allowed to sit at room temperature for 40 minutes before adding 100 µl stop solution. The plate was then read at 405 nm using four-parameter calibration KC Junior (BioTek Instruments, Winooski, VT) software capable of analyzing y=(A-D)/(1+(x/C)^B)+D on a µQuant plate reader (BioTek Instruments).

Statistical Analyses

All statistical analyses were done using SAS (SAS Institute Inc., Cary, NC). Data collected from 4-month old animals were analyzed using a general linear model. When heterogeneous variations made it necessary, a log transformation was used to stabilize the variation. If this log transformation failed to stabilize the variation, a ranking procedure was used (Conover and Iman 1982). Mean differences were determined using Fisher's Least Significant Difference (LSD). Means and standard deviations presented are the actual numbers, although p values reflect the transformed or ranked data. All results are presented as mean ± standard error. Differences were considered to be statistically significant at a p value < 0.05.

RESULTS

C57-*oim* line is >99% pure C57BL/6J

DNA, isolated from tail tips from C57 breeding pairs underwent microsatellite analysis using approximately 65 microsatellite markers. 3 markers per autosome were analyzed with an average genetic distance of 20 centiMorgans between markers (Goto, Ebukuro et al. 2005). All samples analyzed were shown to be >99% C57.

Oim/oim mice have reduced body mass

Oim/oim mice have reduced whole body mass (14-16%) at four months of age as compared to their wildtype counterparts, regardless of strain (Figure II-6). C57-*oim/oim* animals were also significantly smaller (14%) than their *oim*/+ littermates. When like genotypes of different strains were compared, C57 animals were 18-25% smaller than outbred animals.

Determination of Bone Geometry

To determine if the *oim* mutation or genetic background caused alterations in femoral geometry, femurs from four month-old animals underwent μCT analysis. Measurements included total femur length, cortical bone width, marrow cavity diameter and polar moment of area. *Oim/oim* femur lengths were 3-4% shorter than wildtype femurs of the same strain (Figure II-7a). *Oim*/+ femurs from the C57 strain were intermediate in length, but not statistically significant, to wildtype and *oim/oim*; however outbred *oim*/+ were 0.90 $\pm$ 0.09 mm and 0.47 $\pm$ 0.03 mm shorter than wildtype or *oim/oim*, respectively. When like genotypes of different strains were compared, C57 wildtype and *oim/oim* were 3 and 4 % shorter than their outbred counterparts, respectively, while *oim*/+ were not different between strains. Marrow cavity diameter also showed both genotype and strain differences. C57-*oim/oim* femurs had a 14% smaller marrow cavity diameter than C57-wildtype (Figure II-7b), while outbred *oim/oim* femurs had a 17% smaller diameter. Outbred *oim/oim* animals also showed a significantly smaller (18%) marrow cavity than outbred *oim*/+. C57 *oim*/+ had marrow

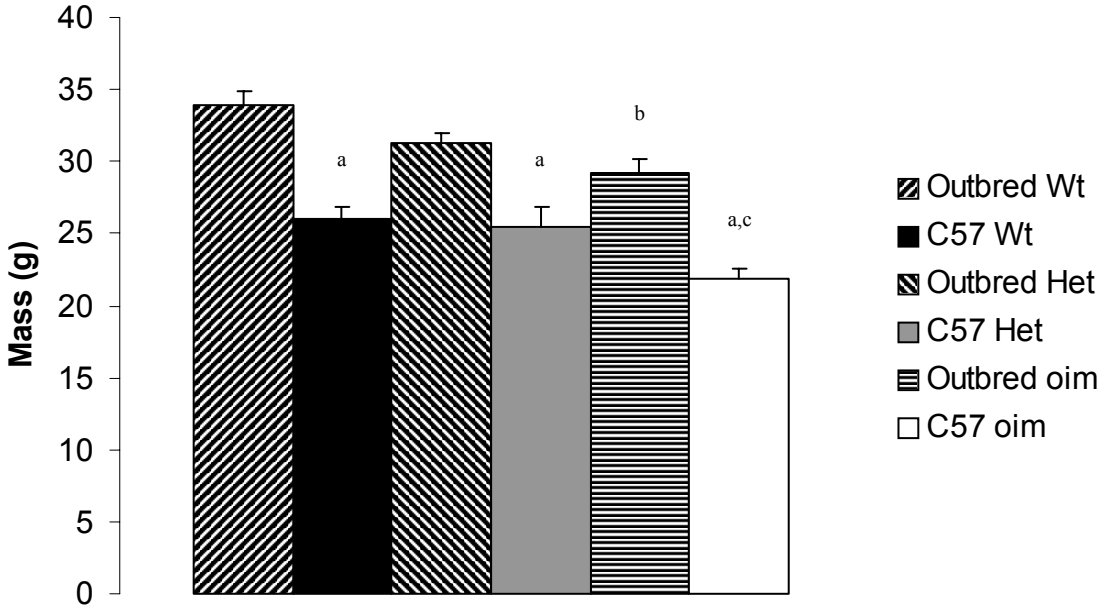


Figure II-6. *Oim* mice exhibit reduced whole body mass as compared to wildtype. C57 mice are smaller than outbred mice of like genotype. [a] p value <0.0001 compared to outbred; [b] p value < 0.05 compared to outbred Wt; [c] p value <0.05 compared to C57 Wt and Het. [outbred Wt, n=12; C57 Wt, n=21; outbred Het, n=33; C57 Het, n=11; outbred *oim*, n=11; C57 *oim*, n=15]

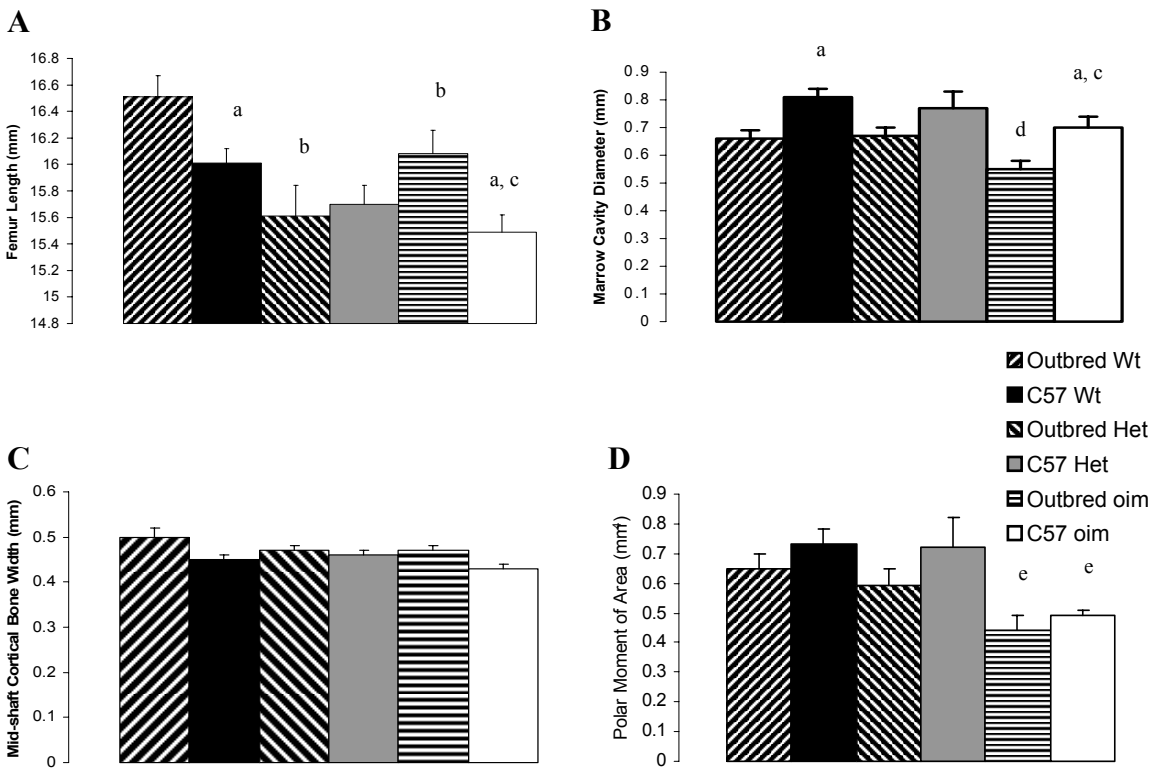


Figure II-7. Femoral geometry as determined by µCT analysis. [a] p value <0.05 compared to outbred; [b] p value <0.05 compared to outbred Wt; [c] p value <0.05 compared to C57 Wt; [d] p value <0.0001 compared to outbred Wt and <0.05 compared to outbred Het; [e] p value <0.05 compared to Wt of the same strain. [Wt, n=13; Het, n=8; outbred *oim*, n=12; C57 *oim*, n=11]

cavity diameters intermediate to wildtype and *oim/oim*, while outbred wildtype and *oim*/+ had almost identical marrow cavity diameters. C57 animals had 13-21% larger marrow cavities than like genotypes of the outbred strain.

Cortical bone width was not significantly different between genotypes or strains. C57 animals did exhibit a linear trend in which wildtype animals had the largest cortical bone width and *oim/oim* animals had the smallest with heterozygotes being intermediate (Figure II-7c). For the outbred strain, *oim/oim* animals had smaller cortical bone widths, while wildtype and *oim*/+ were virtually identical.

Polar moment of area, a measure of the bone's ability to resist torsion, was also measured using μCT analysis. The larger the polar moment, the less the bone will twist. *Oim/oim* animals of both strains exhibited a significant reduction (~32%) in their polar moment of area compared to wildtype animals of the same strain (Figure II-7d). Like in other geometrical parameters, C57 heterozygotes had intermediate values compared to wildtype and *oim/oim*, though not statistically significant, while the polar moment of area in outbred heterozgyotes were almost identical to wildtype. There were no strain differences in polar moment of area between like genotypes.

Torsional Loading to Failure: A Measure of Bone Biomechanical Integrity

To determine biomechanical integrity, the same femurs analyzed via μCT scans were then subjected to torsional loading to failure. The combination of μCT analysis and torsional loading allows for the separation of macroscopic whole bone properties from microscopic material properties. Macroscopic properties describe the bone as a whole, including any geometrical adaptations the animal may have made. Microscopic

properties subtract out the geometry, describing only the material the bone is made of.

Torsional ultimate strength (T_{max}) is a macroscopic property that measures the ultimate torsional force needed to break the bone. The corresponding microscopic material property is tensile strength (Su), which measures the ability of the material to resist forces without breaking. Torsional stiffness (Ks) is the macroscopic property that describes the stiffness, or ability of the bone to resist torsion without breaking. Shear modulus of elasticity (G) represents the corresponding material property, which describes the elasticity of the bone material. The final macroscopic property, energy until failure (U), describes the amount of energy a bone is able to absorb until it fractures.

T_{max} was significantly reduced in *oim/oim* femurs from both C57 and outbred strains as compared to their corresponding wildtype femurs (52 and 56% of +/+ values) and *oim*/+ (19%), respectively (Figure II-8a). While not significant, the T_{max} of *oim*/+ from both strains was intermediate to wildtype and *oim/oim*. No significant strain differences were evident in T_{max}. Tensile strength (Su), the corresponding material property, exhibited similar results. *Oim/oim* animals from both strains had a 38% significant reduction in their tensile strength as compared to wildtype (Figure II-8b). However, outbred *oim/oim* animals were also significantly reduced compared to their heterozygote counterparts as well. When strains were compared, wildtype C57 femurs had a significantly reduced tensile strength compared to wildtype outbred femurs (24% reduction). *Oim*/+ femurs from both strains exhibit intermediate tensile strength as compared to wildtype and *oim/oim*, though not statistically significant.

Energy until failure, another macroscopic property, showed that outbred wildtype femurs were able to absorb the most energy prior to breaking (Figure II-8c). Strain

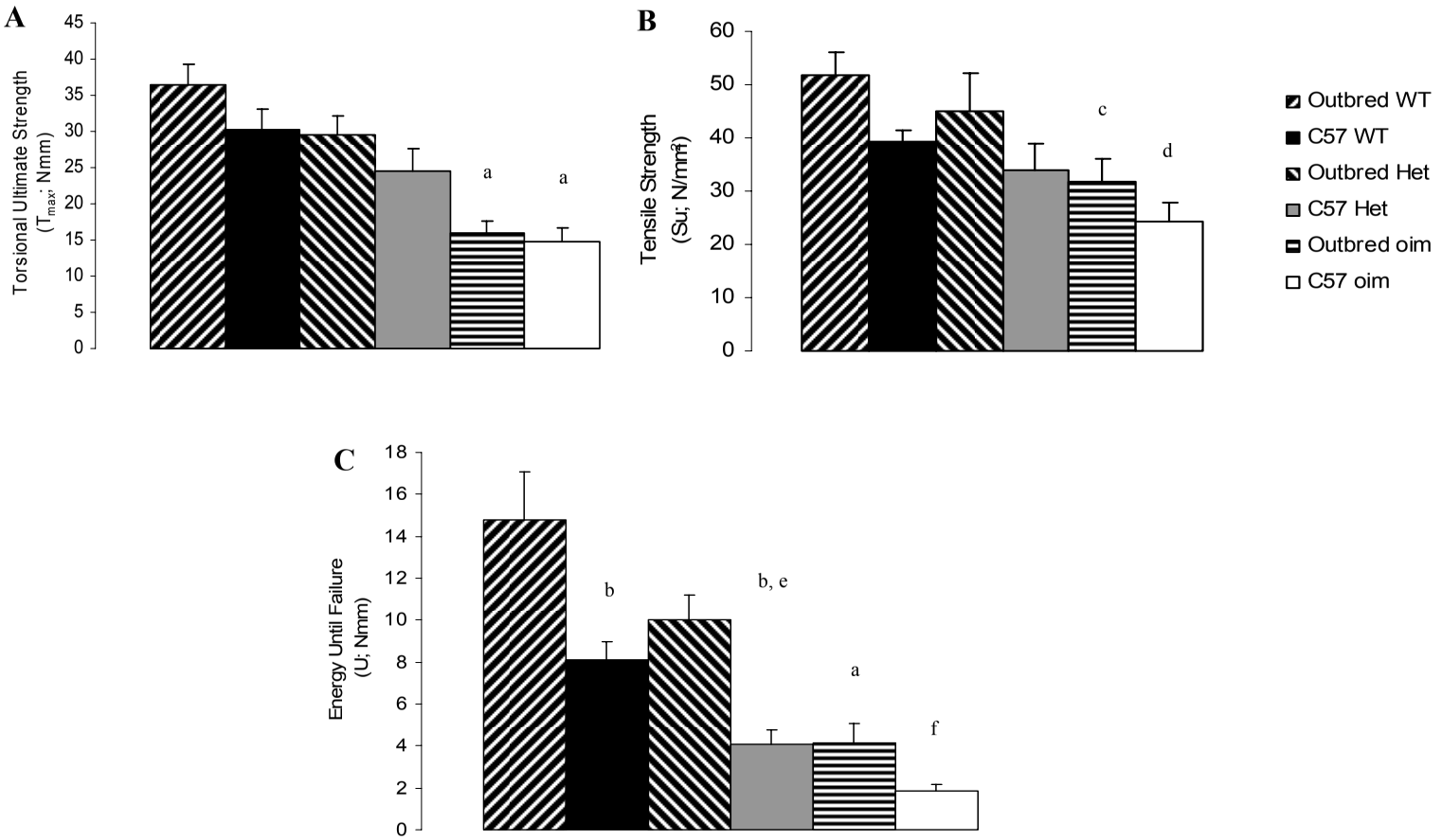


Figure II-8. Biomechanical integrity as determined by torsional loading to failure. [a] p value <0.0001 compared to Wt and <0.05 compared to Het of the same strain; [b] p value <0.05 compared to outbred; [c] p value <0.05 compared to outbred Wt and Het; [d] p value <0.05 compared to C57 Wt; [e] p value <0.05 compared to Wt; [f] p value <0.0001 compared to Wt. [outbred Wt, n=11; C57 Wt, n=12; outbred Het, n=6; C57 Het, n=8; outbred *oim*, n=12; C57 *oim*, n=8]

differences were seen between wildtype and heterozygote animals with outbred femurs able to absorb more energy than C57 femurs of the same genotype. Outbred wildtype and *oim*/+ femurs were able to absorb 45% and 60% more energy than C57 wildtypes and *oim*/+, respectively. Strain differences were not seen with *oim/oim* animals, although the p values were close to significance (p=0.0658) and the use of ranked data reduces the power of the numbers.

Stiffness had a very different profile than T_{max} or Su. Torsional stiffness (Ks) was only different between strains for *oim*/+ mice, but not wildtype or *oim/oim*, animals (Figure II-9a). There were no genotype differences in stiffness in either strain. When the microscopic counterpart, shear modulus of elasticity (G), was examined, there were no strain or genotype differences found (Figure II-9b).

Bone Mineral Composition as Determined by Neutron Activation Analysis

To determine if strain differences and/or the *oim* mutation have an impact on the mineral component of bone, the bone mineral composition was determined for tibias of each genotype and strain by neutron activation analysis. Both genotype-specific and strain-specific mineral changes were seen. Fluorine was significantly different between strains for all genotypes, with C57 animals having higher fluorine levels than their outbred counterparts (Figure II-10). Within the C57 strain, *oim/oim* tibias had significantly more fluorine than either C57 wildtype or C57 *oim*/+ tibias. Outbred *oim/oim* tibias also had significantly more fluorine than their wildtype and heterozygote counterparts. For both strains, wildtype tibias contained the least amount of fluorine with *oim/oim* having the most.

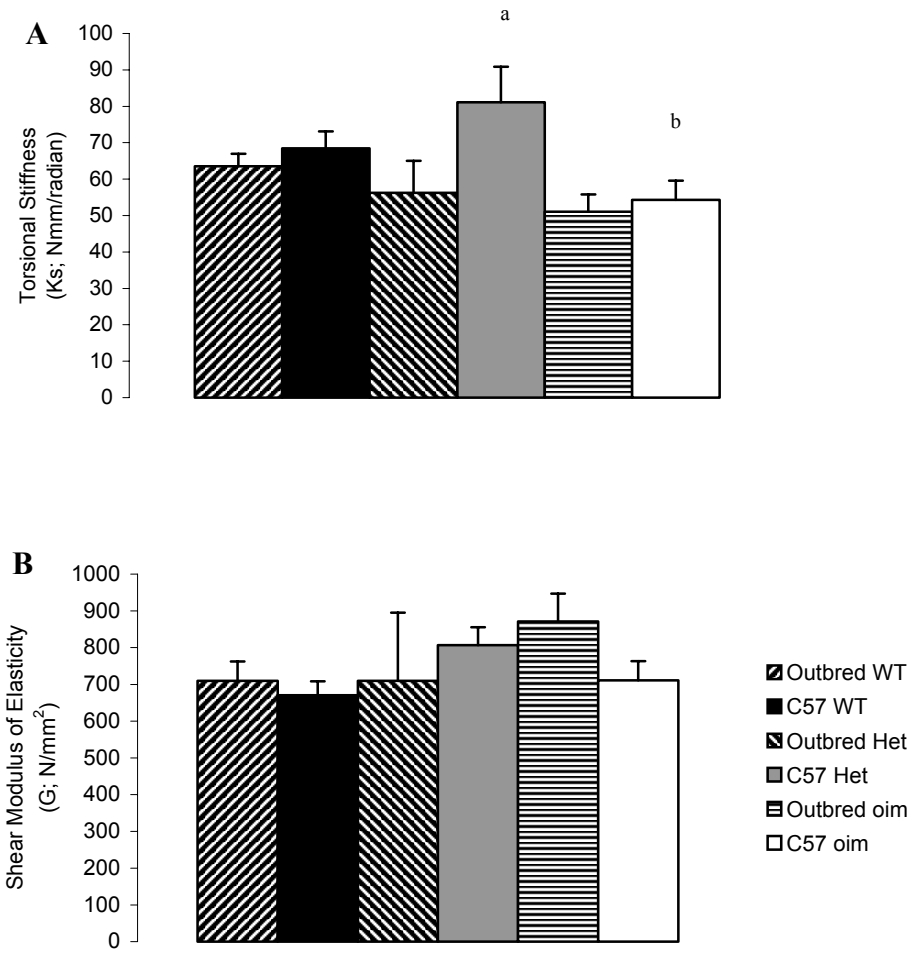


Figure II-9. Femoral stiffness as determined by torsional loading to failure. [a] p value <0.05 compared to outbred Het; [b] p value <0.05 compared to C57 Het. [outbred Wt, n=11; C57 Wt, n=12; outbred Het, n=6; C57 Het, n=8; outbred *oim*, n=12; C57 *oim*, n=7]

Phosphorus levels were also altered, both between strains and genotypes. Strain differences were seen between heterozygotes, but not wildtype or *oim/oim* tibias (Figure II-10), although outbred animals always had more phosphorus than C57 animals regardless of genotype. Genotype differences were also seen within the C57 strain. C57-*oim/oim* tibias had more phosphorus compared to wildtype and heterozygote C57. Within the outbred strain, both *oim*/+ and *oim/oim* animals had more phosphorus than wildtype animals.

When sodium was analyzed, strain differences were apparent for wildtype and heterozygote tibias with outbred animals having more sodium than C57 animals (Figure II-10). The same trend held true for o*im/oim* tibias, although not significantly different ($p=0.0617$). C57-*oim/oim* tibias showed significantly reduced levels of sodium compared to both C57 wildtype and C57 *oim*/+ tibias. Outbred *oim/oim* animals showed significantly reduced levels of sodium compared to outbred wildtype and *oim*/+ tibias. Outbred *oim*/+ tibias also had statistically reduced sodium levels compared to outbred wildtype tibias.

The next mineral to be analyzed was magnesium. Strain differences were only seen between *oim/oim* animals, with C57-*oim/oim* tibias having elevated magnesium compared to outbred *oim/oim* tibias (Figure II-10). Within the C57 strain, genotype differences were seen between wildtype and *oim*/+, wildtype and *oim/oim* as well as *oim*/+ and *oim/oim*. C57 wildtype tibias had the lowest levels of magnesium and C57-*oim/oim* tibias had the highest levels with C57 *oim*/+ being intermediate. Outbred *oim/oim* tibias had more magnesium than outbred wildtype tibias. Outbred *oim*/+ were

not significantly different from either wildtype or *oim/oim*, although p values were close to significance (p=0.06).

Calcium, known to be an important mineral in bone tissue, was also analyzed. Strain differences were only seen in heterozygote animals, with C57 animals having reduced amounts of calcium (Figure II-10). Within the C57 strain, *oim/oim* tibias had an increased amount of calcium compared to wildtype with *oim*/+ being intermediate. The same was true of the outbred strain, although within this strain, *oim*/+ were also significantly different from wildtype with values similar to *oim/oim*.

Strain and genotype differences in the calcium-phosphate ratio were not seen. Within the outbred strain, *oim/oim* animals had the lowest ratio with *oim*/+ being intermediate to wildtype and *oim/oim*. Within the C57 strain, wildtype tibias had the lowest ratio with *oim/oim* intermediate to *oim*/+ and wildtype tibias.

Potassium was also analyzed. Strain differences were only seen between *oim*/+ animals, with outbred *oim*/+ having higher levels of potassium than C57 *oim*/+ (Figure II-10). The only genotype differences were seen between outbred *oim*/+ and *oim/oim*, with *oim/oim* having lower levels than *oim*/+.

The final element to be analyzed was zinc. Strain differences were not observed (Figure II-10). The only genotype differences were within the outbred strain between wildtype and *oim*/+ as well as wildtype and *oim/oim*, with wildtype having the lowest amount of zinc and *oim/oim* having the highest.

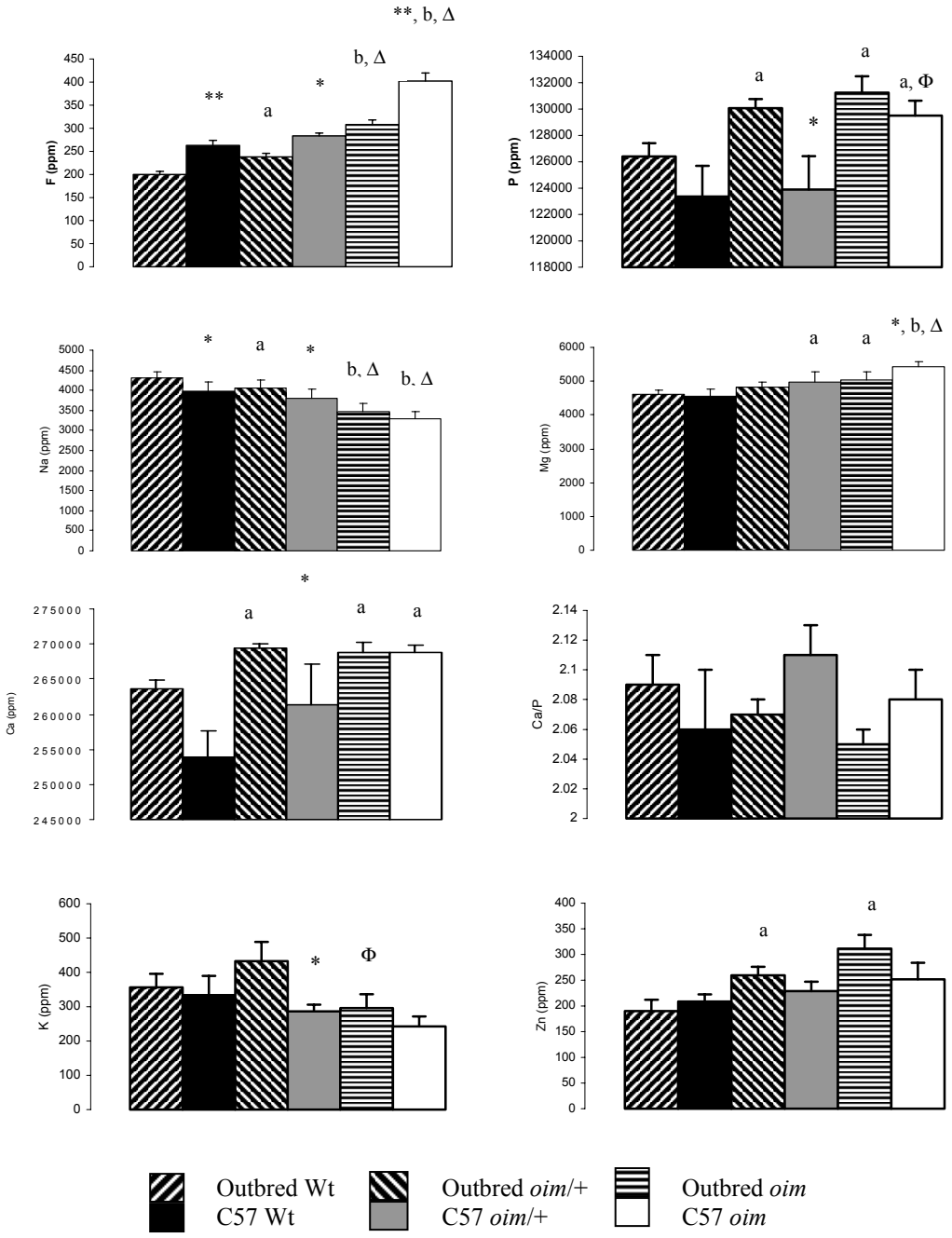


Figure II-10. Mineral composition of tibias. * p value ≤0.05 compared to outbred; ** p value ≤0.0001 compared to outbred; [a] p value ≤0.05 compared to Wt; [b] p value ≤0.0001 compared to Wt; [Φ] p value ≤0.05 compared to Het; [Δ] p value ≤0.0001 compared to Het. [Wt, n=8; Het, n=8; *oim*, n=8]

Determination of Total Collagen Content Using the Hydroxyproline Assay

Hydroxyproline is an amino acid unique to collagen and, therefore, is used as an indirect measure of the amount of collagen present. Strain differences were only seen between wildtype femurs (Figure II-11), with outbred wildtype animals having 25% less collagen than wildtype C57 femurs. Within the C57 strain, significant differences were seen between wildtype and *oim/oim* as well as between *oim*/+ and *oim/oim*. *Oim/oim* femurs contained 42% less collagen than wildtype femurs and 31% less than *oim*/+. A similar, but less dramatic, trend was seen within the outbred strain, with *oim/oim* femurs having 33% less collagen than wildtype and 25% less than *oim*/+.

Pyridinoline Crosslink Quantitation: A Marker of Bone Turnover

Pyridinoline is a type of non-reducible crosslink found within the collagen molecule. When bone is resorbed by osteoclasts, collagen is broken down. As this breakdown happens, these crosslinks remain intact and, therefore, can be used to measure the breakdown of collagen (Cohen, Diegelmann et al. 1992; Dogan and Posaci 2002). Strain differences in the quantity of pyridinoline were not evident in wildtype animals but were seen between *oim*/+ and *oim/oim*, with C57 animals having lower pyridinoline content than outbred (Figure II-12). Genotype differences were not seen within either strain.

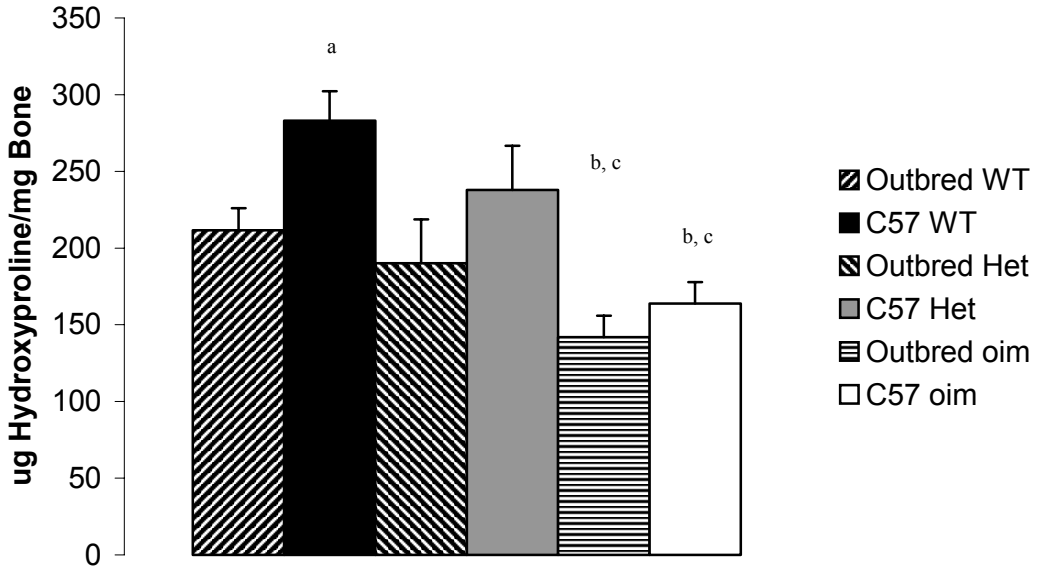


Figure II-11. Hydroxyproline assay as a measure of collagen content. [a] p value <0.05 compared to outbred; [b] p value <0.05 compared to Wt; [c] p value <0.05 compared to Het. [outbred Wt, n=13; C57 Wt, n=7; outbred Het, n=8; C57 Het, n=8; outbred *oim*, n=13; C57 *oim*, n=7]

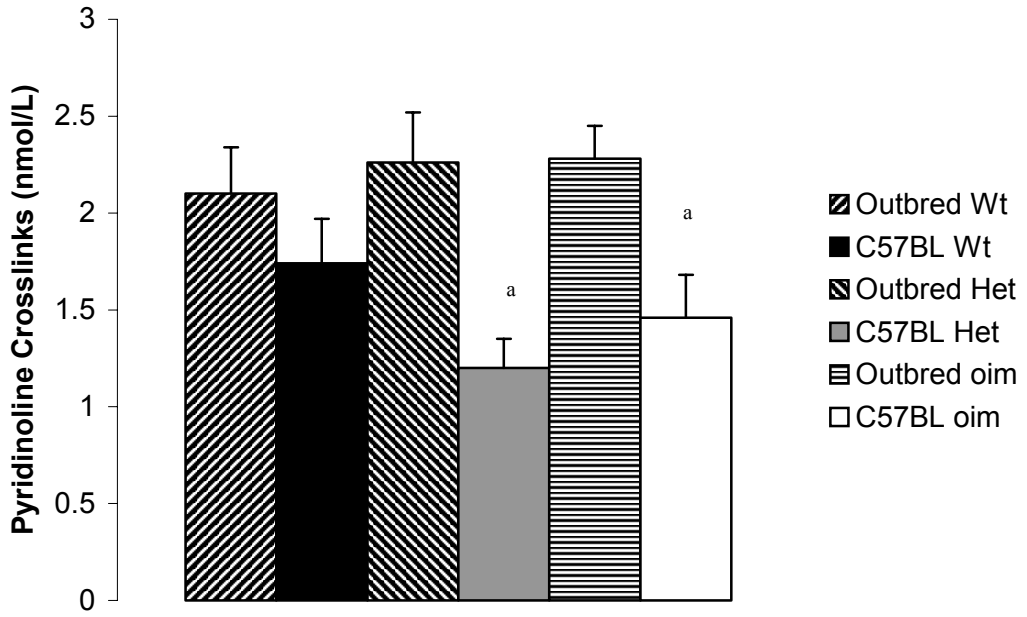


Figure II-12. Pyridinoline content: a measure of bone turnover. [a] p value <0.05 compared to outbred. [outbred Wt, n=7; C57 Wt, n=9; outbred Het, n=8; C57 Het, n=9; *oim*, n=8]

DISCUSSION

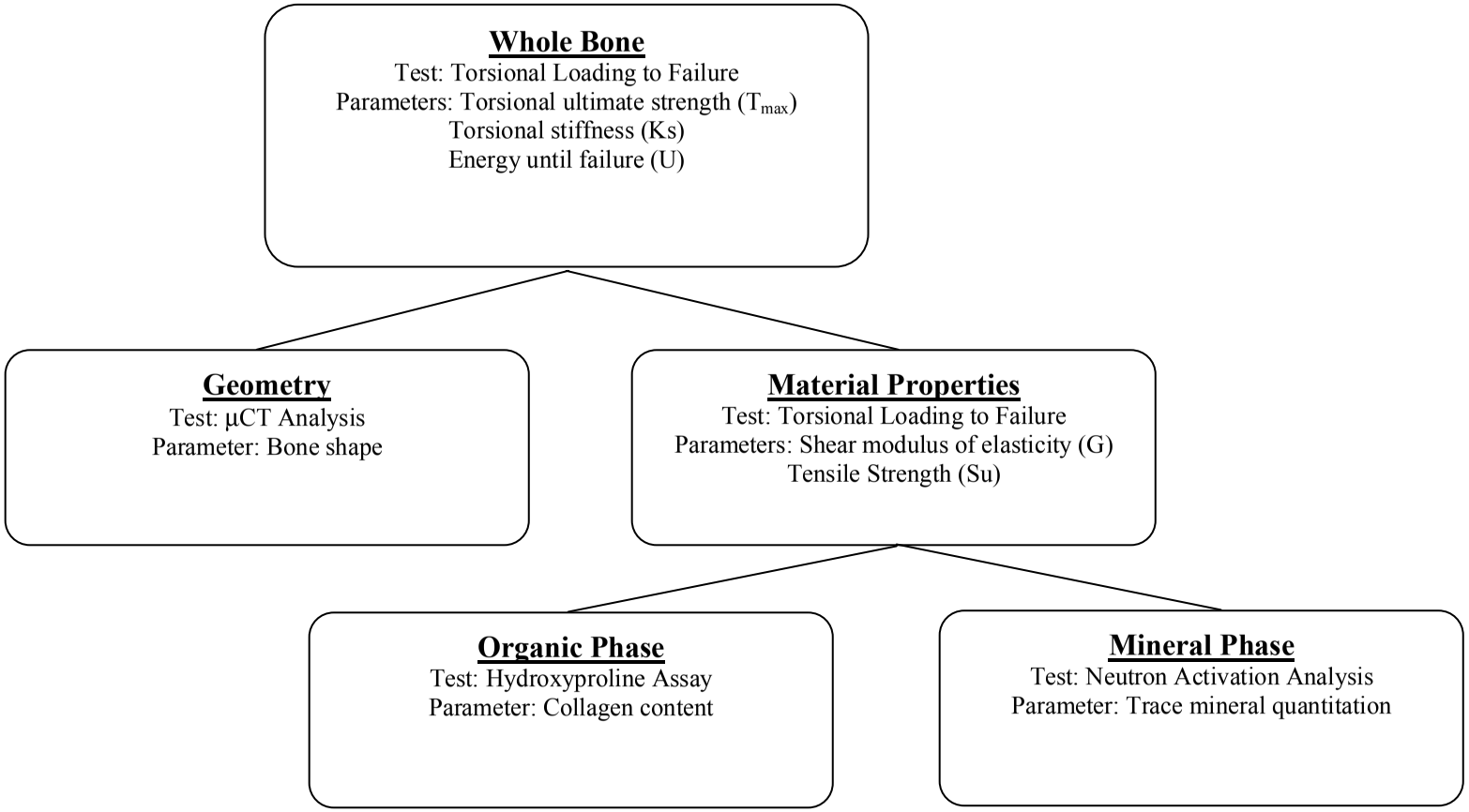
The goals of the study presented here were twofold. The first goal was to find those bone parameters that were altered by the presence of the *oim* mutation and associated with changes in the quality and integrity of bone. The second goal was to determine which of these parameters were also impacted by the strain, and the modifier genes contained therein. In order to achieve these goals, we aimed to 1) compare two separate mouse strains and 2) compare the impact on bone quality and integrity of the *oim* mutation on these two genetic backgrounds, outbred and congenic.

With so many OI-causing mutations known, it is difficult to determine why a specific genotype causes a given phenotype. It is known that the identity of the substituting amino acid plays a role in clinical outcome and certain amino acids, such as asparagines, are more devastating than others, due to the destabilizing effects on the triple helix (Persikov, Pillitteri et al. 2004). The identity of the chain harboring the mutation is also important, as each chain is proposed to have a unique distribution of lethal and non-lethal regions (Marini, Lewis et al. 1993; Wang, Orrison et al. 1993). However, this does not fully explain the broad range of clinical outcomes seen in OI patients, suggesting that modifier genes may affect the clinical outcome of the OI-causing mutation. Modifier genes are those that may influence the collagen genes to impact the clinical outcome of an OI-causing mutation.

The approach we took was to examine bone quality by several parameters (Figure II-13). Torsional loading to failure was used to determine biomechanical properties of the whole bone, including any geometrical adaptations the animal may have made. The combination of the μCT analyses and torsional loading to failure permitted separation of

whole bone macroscopic properties from microscopic material properties. The whole bone properties can be further subdivided into two categories: bone geometry and material properties. Bone geometry was assessed using μCT analysis while material properties were determined using a combination of μCT analysis and torsional loading to failure. Material bone properties describe only the material the bone is made of, essentially subtracting out any geometrical adaptations. Both subtle changes in bone geometry and weaker material properties can have a large impact on the biomechanical integrity of the bone. Material properties can then be further subdivided into the organic and mineral phases, which make up the two-component system of bone. The organic phase is primarily type I collagen while the mineral phase is made of mainly hydroxyapatite crystals. This combination of type I collagen and hydroxyapatite gives the bone properties unlike either component alone (Burr 2002). Collagen content was determined using the hydroxyproline assay. Hydroxyproline, an amino acid unique to collagen, is used as an indirect measure of the amount of collagen in a tissue. Since the main type of collagen in bone is type I, this assay was used as a relative measure of the impact of the strain and the *oim* mutation on the amount of type I collagen in the bone. The hydroxyapatite crystals are normally composed of calcium, phosphate and hydroxy ions $[3Ca_3(PO_4)_2]\bullet(OH)_2$, although several other trace minerals are present and known to impact crystal formation and alignment (Baron 1999; Phillips, Bradley et al. 2000). Neutron activation analysis is a unique method that allows quantitation of individual trace minerals within the bone. Using these multiple levels of analyses (3 dimensional structure, biomechanical integrity and mineralization), we were able to characterize the C57-*oim* mouse line and show that

Figure II-13. Experimental Strategy to Determine Bone Quality



C57-*oim/oim* animals typically have a more severe phenotype than outbred *oim/oim* animals.

The two mouse strains used in this study were the inbred C57BL/6J and the outbred B6C3Fe. Inbred mouse strains have long been used to examine the contribution of genetic background to a given trait. Studies comparing the bone shape and quality of C3H/HeJ and C57BL/6J mine found several key differences. C3 animals were found to have the higher femoral bone mineral density and thicker cortices compared to C57 animals, although both strains fall within the normal range of bone mineral densities for mice (Beamer, Donahue et al. 1996). C3 animals also had a smaller cross-sectional area and twice the bone volume compared to C57 (Akhter, Iwaniec et al. 2000). The small cross-sectional area with thicker cortices of the C3 animals would imply a stronger bone, which is consistent with the biomechanical studies (Akhter, Iwaniec et al. 2000). Consistent with this, C57 animals were found to have a greater osteoclast surface with reduced osteoid surface as compared to C3 (Akhter, Iwaniec et al. 2000), indicating that C57 animals have increased bone resorption with a reduction in the area of bone formation. This is further supported by a recent study in which a mutation was identified in the alkaline phosphatase, a marker of bone formation, gene in normal C57 animals (Klein, Carlos et al. 2005).

The outbred B3C3Fe strain is a genetic mix of these two inbred strains C57BL/6J and C3H/HeJ. The combination of the two genetic backgrounds which have very different bone mineral densities gives the outbred strain a great deal of variation in phenotypic severity. Each F2 offspring will receive a unique combination of genes from each of the two genetic backgrounds, with some receiving more genes from the stronger C3H background and others receiving more from the weaker C57 background. Jackson

Laboratory currently maintains the *oim* mutation on this outbred genetic background by repeated backcrossing *oim/oim* to B6C3Fe heterozygotes to produce outbred mice heterozygous for the *oim* mutation. Because of the contribution of the C57 genetic background to the outbred genetic background, we predicted that there would be differences between the two strains because of the contribution of the C3H strain to the outbred strain, but that these differences would not be as drastic as comparisons between two very dissimilar strains, such as C57BL/6J and C3H/H3J.

Total Body Mass

Outbred *oim/oim* mice have previously been shown to have a reduced body size compared to wildtype animals (Phillips, Bradley et al. 2000) and this finding was confirmed in the current study. The finding that C57-*oim/oim* animals showed a reduced body size compared to wildtype is not surprising as they carry the same mutation as outbred *oim/oim* animals. This is also consistent with what is seen in human OI type III, which the *oim* mutation mimics, which causes patients to have short stature (Byers 1993). The finding that C57-*oim/oim* animals had a greater reduction in body size than outbred *oim/oim* animals may be explained by hybrid vigor, since wildtype C57 animals have been shown to have comparable body weights to other inbred strains (Akhter, Iwaniec et al. 2000). The intermediate body mass of *oim*/+ of both strains is consistent with findings that these animals mimic the milder phenotype of OI type I (Saban, Zussman et al. 1996).

Femoral Geometry

Bone shape (geometry) has a large role in determining biomechanical integrity and a subtle change in cross-sectional geometry, in particular, can have a large impact on the ability of the bone to resist forces without breaking (Bonadio, Jepsen et al. 1993; Rubin and Rubin 1999; Hamrick 2003; Turner 2006). In order to assess bone geometry,

	Geometry	
	Strain	Genotype
Femur Length	Yes (wildtype only)	Yes
Marrow Cavity Diameter	Yes (wildtype and *oim*)	Yes
Cortical Bone Width	No	No
Polar Moment of Area	No	Yes
	Biomechanics	
	Strain	Genotype
T_{max}	No	Yes
Su	Yes (wildtype only)	Yes
Ks	Yes (heterozygotes only)	Yes
G	No	No
U	Yes (wildtype only)	Yes
	Organic and Mineral Phases; Bone Turnover	
Collagen Content	Yes (wildtype only)	Yes
Mineral Composition	Yes*	Yes
Pyridinoline Crosslinks	Yes (*oim* only)	No

Table II-2. Summary of strain and genotype differences. Strain differences were seen in geometry and Su, but the *oim* mutation overrides these differences. Collagen content was reduced in *oim* femurs, but strain differences also exist as C57-*oim* femurs showed the greatest reduction in collagen content. Mineral composition showed mineral specific strain and genotype differences. * Mineral-specific differences.

several parameters were examined. Homozygosity for the *oim* mutation had the same general effect on femoral geometry, regardless of strain, producing shorter femurs with smaller marrow cavity diameters and polar moment of area with unchanged cortical bone widths compared to wildtype femurs of the same strain. However, the impact of the genetic background became apparent when femoral geometry of outbred *oim/oim* and C57- *oim/oim* femurs were compared. C57-*oim/oim* femurs were shorter with larger marrow cavity diameters with the same cortical bone width as outbred *oim/oim* femurs. The increased size of the marrow cavity seen in C57-*oim/oim* femurs may be a compensatory mechanism to distribute stresses over a larger cross-sectional area (Akhter, Iwaniec et al. 2000). The shortness of the C57-*oim/oim* femurs may allow the animal to compact the potentially weaker cortical bone, which provides strength to the bone, in an attempt to prevent fracture. The polar moment of area (K) is a geometrical description of the bone cross-section. The larger the value, the less the bone will twist (http://www.efunda.com/math). When femurs from *oim/oim* animals are compared to wildtype femurs, the polar moment of area is smaller for *oim/oim* animals. The larger polar moment of area of wildtype femurs indicates that they will twist less in response to torsional loads. Strain differences were not seen, indicating the two strains have similar abilities to resist twisting based solely on this parameter. This correlates with macroscopic stiffness data (Ks) which also shows reduced stiffness in *oim/oim* animals, indicating that wildtype femurs can twist more (are less brittle) than *oim/oim* femurs, indicating it can take more of a twisting force before it breaks.

Femoral Biomechanical Strength and Stiffness

The use of torsional loading to failure, in conjunction with μCT analysis, allowed separation of whole bone properties from material bone properties. Whole bone properties describe the bone as a whole while material properties describe only the material the bone is made of. When wildtype animals from the two genetic backgrounds are compared, there were no strain differences in T_{max} between C57 wildtype and outbred wildtype; however, the tensile strength of the bone material in C57 wildtype was inherently weaker than outbred bone material suggesting that the changes in geometry are able to compensate for the material differences. However, in *oim/oim* femurs, this geometrical compensation does not appear to happen. *Oim/oim* femurs exhibit reductions in both T_{max} and Su, indicating the material the bone is made of is weaker than in wildtype, and that the changes in geometry or strain cannot compensate for this weakness. The ability of the femur to absorb energy before breaking is a parameter not previously reported in the *oim* literature. The findings that measures of the strength of the femur are significantly reduced in *oim/oim* animals of both strains is consistent with previous studies reporting the weaker biomechanical integrity of *oim/oim* bone (McBride, Shapiro et al. 1998; Camacho, Hou et al. 1999) and is also consistent with human OI type III patients who have numerous fractures throughout their lifetime (Byers 1993). *Oim*/+ animals of both strains were intermediate to wildtype and *oim/oim*, consistent with earlier reports (Saban, Zussman et al. 1996). Outbred femurs of any genotype were able to absorb a great deal more energy before fracturing than the corresponding C57 femurs. This is consistent with studies examining various strains of inbred mice that found femurs from C57 animals were the weakest strain in every parameter examined, although energy

until failure was not tested (Akhter, Iwaniec et al. 2000). Taken together, these findings suggest that both whole bone and material properties are altered in these animals in such a way that *oim/oim* femurs are not able to resist biomechanical stress to the same degree as wildtype femurs.

When the stiffness of the femurs was analyzed, differences in the stiffness of the material were unchanged between strains and genotypes. However, whole bone stiffness showed differences between heterozygotes of the two strains as well as between C57-*oim/oim* and C57 *oim*/+, although the significance of these differences is unclear. The possibility exists that the mix of heterotrimeric and homotrimeric type I collagen, in conjunction with alterations in bone geometry, has an impact on the ability of the bone to resist twisting forces. Previous studies showed a significant reduction in torsional stiffness in outbred *oim/oim* femurs compared to both wildtype and *oim*/+ femurs (Camacho, Hou et al. 1999). However, these animals were 11-13 months of age at the time of sacrifice, while the animals tested in this study were only four months of age. Studies of aging bone have shown that older bones were unable to compartmentalize damage to the bone and more readily initiated cracks that propagated easier than in young bone (George and Vashishth 2006). Therefore the older *oim/oim* bones of the previous study may have shown a greater deviation from wildtype both because of the mutation and the bone changes associated with age.

Type I Collagen Content

The reduction in the amount of type I collagen seen in *oim/oim* animals, along with alterations in bone mineral composition, also contribute to the weaker bones seen in these animals. Previous studies on outbred *oim/oim* animals demonstrated a 20%

reduction in collagen content of humerii (Camacho, Hou et al. 1999), and 35 % reduction in the left ventricle (Weis, Emery et al. 2000) and aorta (Pfeiffer, Franklin et al. 2005). The present study demonstrates a 33% decrease in femora. The finding that wildtype outbred femurs had 25% less collagen than wildtype C57 femurs was surprising, as the stronger bones of the outbred line were predicted to have more collagen than the weaker bones of the C57 line. However, collagen contributes to the toughness of the bone, or ability of the bone to deflect in response to forces, while the hydroxyapatite crystals give the bone strength and stiffness (Burr 2002). Therefore, wildtype outbred bones may have something in the mineral composition that is able to compensate for the reduction in collagen content to allow the bone to better withstand forces. The finding that C57-*oim/oim* femurs had the greatest reduction in collagen content is consistent with the observation that they have the weaker genetic background.

Trace Mineral Composition

While collagen in an important contributor to the material properties of the bone, the mineral phase cannot be ignored. While hydroxyapatite crystals are normally made of calcium, phosphate and hydroxy ions [$Ca_{10}(PO_4)_6(OH)_2$], many impurities exist that impact the crystallinity, stability and morphology of the crystals (Grynpas 1990; Boskey, Rimnac et al. 1992; Cheng, Bader et al. 1995). Instrumental neutron activation analysis (NAA) provides an exceptionally sensitive method by which to measure trace minerals. NAA is a non-destructive analysis in which a sample is irradiated to produce a radioactive nuclide, which will decay by emitting γ rays. The number of γ rays emitted indicates the quantity of a particular element, while the energy of the γ rays indicates the identity of the element (Website). NAA has several unique properties making it a

superior method by which to study trace mineral composition. The non-destructive nature of NAA allows for the samples to be used for other downstream applications and the sensitivity of the system allows for analysis of small samples in which other methods have failed. NAA can easily quantifying as little as 0.1-1000 ng of an element, although this is element specific (www.missouri.edu/~murrwww). When necessary, sensitivity of the system can be improved by moving the sample closer to the detector, counting for a longer period of time, increasing the irradiation time or by decreasing the decay time of the sample (www.missouri.edu/~murrwww).

Because of the instrumental approach to this analysis, there is little opportunity for sample contamination. NAA is also free of matrix interference because most of the samples are transparent to both the neutron (the probe) and the γ ray (analytical signal) (www.missouri.edu/~murrwww). Complications can arise when different elements in the sample produce γ rays of similar energy, although this problem can be overcome by choosing alternative energies at which to quantitate the element of choice or by waiting for the shorter-lived of the two nuclides to decay before counting.

Minerals thought to impact bone quality include fluoride, phosphorus and magnesium. In the current study, fluoride was elevated in C57 tibias compared to outbred tibias. *Oim/oim* tibias also had elevated fluoride levels compared to wildtype, regardless of strain. Fluoride is known to have biphasic effects, being beneficial at low doses but detrimental at high doses (Cheng, Bader et al. 1995). Riggs, et al showed that fluoride therapy in postmenopausal osteoporotic women caused thickening of the trabeculi, but induced loss of cortical bone, putting the bone at risk for fracture (Riggs, O'Fallon et al. 1994). Consistent with this, association of relatively high levels of

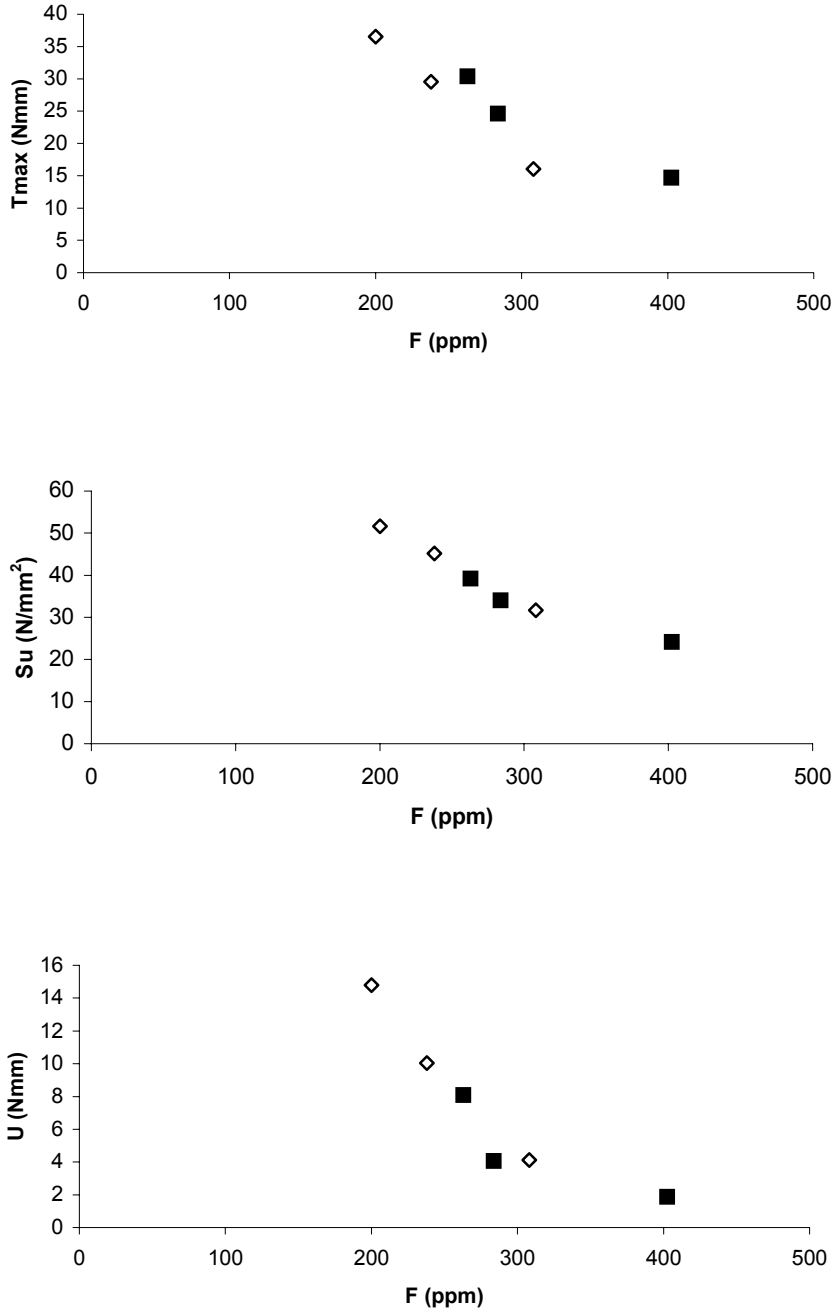


Figure II-14. Association of fluoride levels with biomechanical parameters. Fluroide negatively correlates with Tmax, Su and U. The higher the level of fluoride, the weaker the bone, regardless of strain. Squares: outbred; diamonds: C57.

fluoride in *oim/oim* tibias corresponds with weaker bones as seen by reductions in T_{max}, Su and U (Figure II-14). This association is consistent for wildtype C57 tibias, which have higher levels of fluoride and are weaker than outbred wildtype tibias, and heterozygote tibias of both strains, which are intermediate to wildtype and *oim/oim* in both fluoride levels and biomechanical integrity.

Phosphorous is one of the main components of hydroxyapatite crystals and plays a role in bone mineralization. *Oim/oim* tibias exhibit elevated levels of phosphorous compared to wildtype. This finding is different from Phillips, et al who did not find differences in phosphorus levels between outbred genotypes (2000). However, this may be a reflection of the specific bone used in each study; Phillips, et al used femora while the current study used tibias. Additionally, Phillips, et al used femurs from 3-12 month old animals, not a single age group as was done in the present study. Different bones types also exhibit differences in Ca/P ratios (Tzaphlidou and Zaichick 2003; Tzaphlidou, Speller et al. 2005) and subtle differences in the Ca/P ratio may be more apparent in larger bones such as femurs. No association between phosphorus levels and biomechanical integrity was found. The elevated levels of phosphorus may be a compensatory mechanism by the bone to increase mineralization and, therefore, strength the bone, as long term hyperphosphatemia is known to cause soft-tissue calcification (Hruska and Lederer 1999).

Magnesium also showed significant differences between genotypes and strains at four months of age. *Oim/oim* tibias of both strains had significantly more magnesium than wildtype, with C57-*oim/oim* having significantly more magnesium than outbred *oim/oim* tibias. Magnesium level was also associated with decreased biomechanical

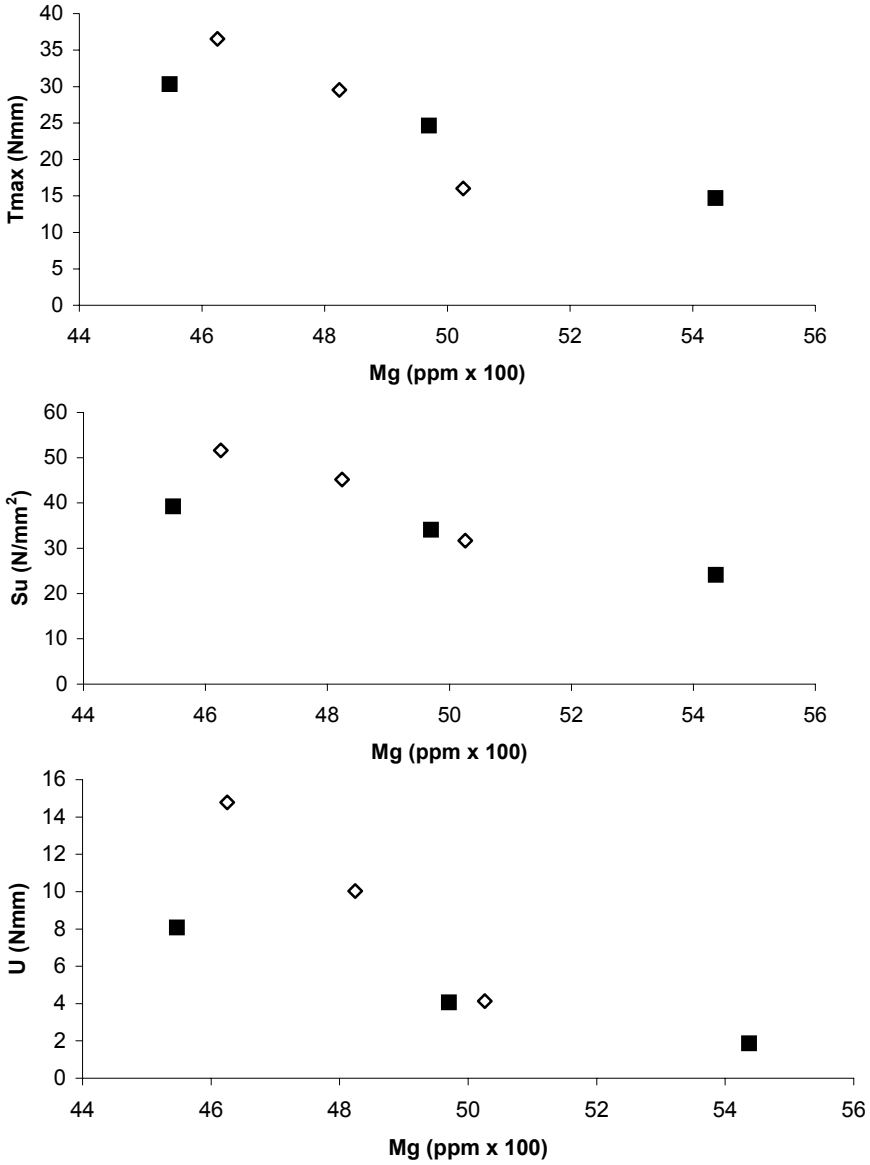


Figure II-15. Association of magnesium levels with biomechanical parameters. Magnesium negatively correlates with T_{max}, Su and U. The higher the level of magnesium, the weaker the bone, regardless of strain. Squares: outbred; diamonds: C57.

parameters, with C57 animals exhibiting a stronger association than outbred (Figure II-15). Fratzl, et al demonstrated that *oim/oim* cortical bone has thinner and less well aligned crystals, contributing to the reduced strength of the bone (1996). As reduced magnesium is known to increase crystal size and perfection (Boskey, Rimnac et al. 1992), an increase in magnesium would decrease crystal size and, therefore, decrease the biomechanical integrity of the bone.

The lack of difference in the Ca/P ratio between wildtype and *oim/oim* tibias, as well as a lack of correlation between Ca/P and biomechanical parameters, may be explained by the type of bone used in this study. The Ca/P ratio has been used in humans as an indicator of the severity of the disease. The lower the ratio, the worse the clinical severity (Cassella, Garrington et al. 1995). However, four months of age is the time of peak bone mass in mice (McBride, Shapiro et al. 1998), corresponding to post-puberty in humans. OI patients have been shown to have an improvement in fracture number after puberty (Kozloff, Carden et al. 2004) and this may be reflected in an increased Ca/P ratio. It has also been observed that differences in the Ca/P ratio exist between different bone types (Tzaphlidou and Zaichick 2003; Tzaphlidou, Speller et al. 2005) and subtle differences in the Ca/P ratio may be more apparent in a larger bone such as the femur.

Potassium was reduced in *oim/oim* tibias regardless of strain, but did not demonstrate an association with biomechanical parameters. Potassium is thought to contribute to an alkaline environment that may be protective to the bone (Tucker 2003). The theory that an acidic environment may lead to bone loss has been supported by several short term studies in both men and women (Frassetto, Todd et al. 2000; Buclin, Cosma et al. 2001; Sellmeyer, Stone et al. 2001). Diets high in phosphorus, chloride and

several amino acids, but low in fruits and vegetables and vitamin C are thought to lead to reduced bone mineral density and increased fracture risk (Tucker 2003) while alkalizing nutrients such as potassium inhibit bone resorption (Buclin, Cosma et al. 2001).

Sodium content was reduced in *oim/oim* tibias of both strains. Within both the outbred and C57 strains, there is a positive association between sodium content and biomechanical strength (Figure II-16). Regardless of strain, the *oim/oim* tibias have the lowest sodium content and the weakest bone as determined by reductions in T_{max}, Su and U as compared to wildtype.

Zinc also shows an association between mineral level and biomechanical parameters. Increased levels of zinc are positively associated with T_{max}, Su and U (Figure II-17). The increased levels of zinc seen in *oim/oim* tibias may be an attempt by the animal to reduce bone turnover. Zinc has been shown to have positive effects on bone growth and can stimulate proliferation and differentiation of osteoblasts as well as inhibit osteoclast formation (Igarashi and Yamaguchi 2001). By increasing the amount of zinc in the bone, the animal may be trying to exploit these positive bone effects. When serum pyridinoline crosslinks were quantitated, strain differences were only seen between *oim*/+ and *oim/oim* animals, with C57 animals having lower pyridinoline than outbred animals. Since there were no strain differences between wildtype animals, this implies that the weaker bones of C57 animals are not associated with increased bone turnover. Hybrid *oim*/+ and *oim/oim* animals have higher PYD levels than C57 *oim*/+ and *oim/oim* animals possibly due to mixed genetic background overlaid by bad collagen.

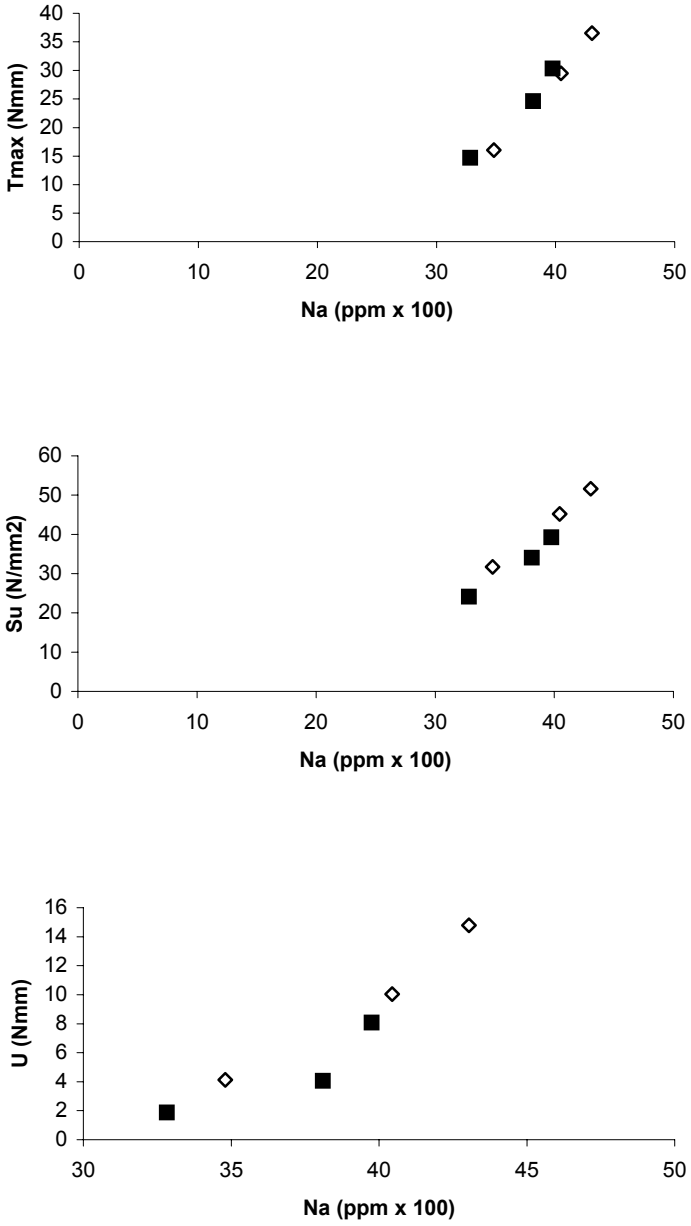


Figure II-16. Association of sodium levels with biomechanical parameters. Sodium positively correlates with T_{max}, Su and U. Squares: outbred; diamonds: C57.

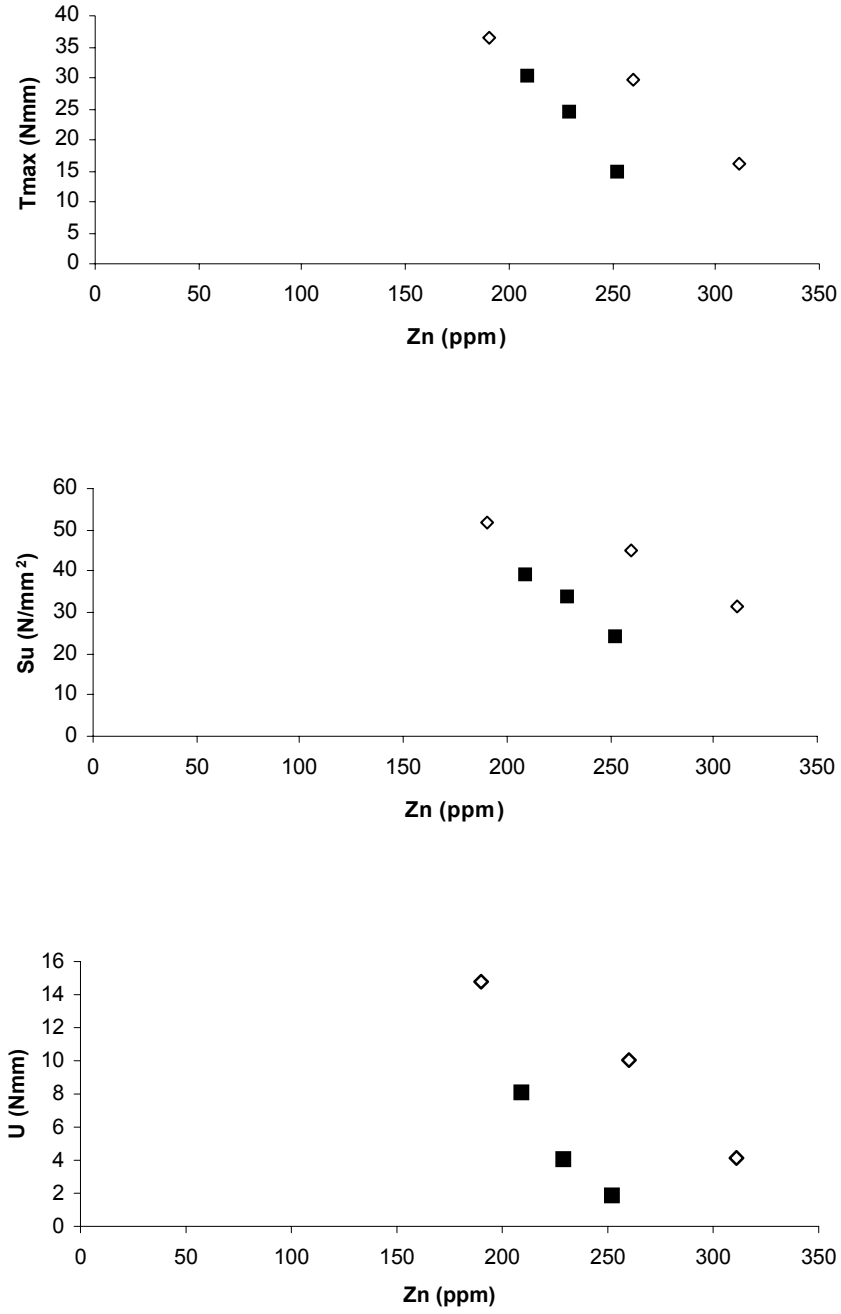


Figure II-17. Association of zinc levels with biomechanical parameters. Zinc negatively correlates with T_{max}, Su and U in a strain specific manner. Squares: outbred; diamonds: C57.

The finding that many parameters were unchanged between wildtype animals of the two strains is not surprising given that the outbred line is partly comprised of C57 genes. The outbred line is comprised of two distinct genetic backgrounds, C57BL/6J and C3H/HeJ and each offspring will contain a unique mix of genes from each of the two backgrounds, which is indicated by the variability in many parameters. The finding that hybrid heterozygotes were the most variable is not surprising given that they have a mixed genetic background as well as a mixed pool of type I collagen.

In conclusion, several strain and genotype differences exist relating to bone geometry and strength as well as collagen content, mineral composition and pyridinoline crosslinks (Table II-4). These data demonstrate that genetic background, and the modifier genes contained therein, have an important role in determining bone biomechanical integrity. However, the presence of the *oim* mutation overrides strain differences, ultimately proving to be the predominant determinant of bone strength.

CHAPTER III

THE *OIM* MUTATION: ROLE OF GENETIC BACKGROUND IN DETERMINING PHENOTYPIC SEVERITY THROUGHOUT POSTNATAL DEVELOPMENT

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ROLE OF GENETIC BACKGROUND IN DETERMINING PHENOTYPIC SEVERITY THROUGHOUT POSTNATAL DEVELOPMENT AND AT PEAK BONE MASS IN COL1A2 DEFICIENT MICE (*OIM*)

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ABSTRACT

It has been documented that OI patients, especially those with types I/IV, tend to have an age-associated decline in fracture number post-puberty. In order to study the impact of genetic background on the biomechanical integrity of the *oim* mutation through development, the *oim* mutation was evaluated on both the outbred C3B6He and inbred C57BL/6J strains at one, two and four months of age. These ages correspond to childhood, puberty and post-puberty in humans. We also wanted to determine if strain or genotype differences seen at four months of age were more severe at the younger ages. Femurs and tibias from wildtype, *oim*/+ and *oim/oim* animals of each strain at one, two and four months of age were analyzed for bone parameters along with a serum marker of bone turnover. μCT analysis demonstrated altered femoral geometry between strains and genotypes as well as between the different ages. *Oim/oim* animals had reduced bone strength as compared to wildtype at all ages, although bone strength in both wildtype and *oim/oim* animals did improve with age. Mineral composition showed association of fluoride, magnesium, sodium and zinc levels with biomechanical integrity in both strains and all genotypes at all ages. *Oim/oim* animals also had reduced collagen content as compared to wildtype at all ages. Pyridinoline crosslinks were highest at two months of age, regardless of strain or genotype. These studies indicate that genetic background plays a role in the severity of the *oim* phenotype at all ages and that the severity of the disease does lessen with age. However, the presence of the *oim* mutation is the major determinant of disease severity, as *oim* animals remain weaker than wildtype animals throughout development.

INTRODUCTION

It is well known that the risk of fracture increases as the skeleton ages, although the exact mechanisms of aging and its affects on the skeleton are not well understood. Several features of aged bone lead to an increased fracture susceptibility. Total collagen amounts decrease while the quantity of denatured collagen increases (Vashishth 2005). Changes in the mineral component do not allow the bone to resist fracture as well as in younger bone (Vashishth 2005). Unusually high levels of bone resorption coupled to reduced osteoblast function ultimately lead to bone loss and alteration of skeletal architecture which increases the likelihood of fracture (Rosen and Kiel 1999; Kloss and Gassner 2006). These changes may be due to several factors, including biochemical and hormonal contributions as well as environmental factors and lifestyle choices.

Estrogen deficiency is well known to be a major player in post-menopausal bone loss in women, but men also loose bone mass with age, although exactly how male sex hormones contribute to bone maintenance or loss is unclear (Rosen and Kiel 1999). Other hormones known to have a role in normal bone function and maintenance include growth hormone and, therefore, insulin-like growth factor-1. Both are known to have a critical role in bone development and maintenance as well as a role in bone loss in both elderly men and women (Kloss and Gassner 2006). Adrenal androgens also decline with age, although exactly what role they play in bone mass maintenance or loss is unclear (Rosen and Kiel 1999). Increases in parathyroid hormone with age also have a role in bone loss in the elderly, although this may be a secondary effect to impaired renal function that often accompanies aging (Rosen and Kiel 1999).

Studies comparing young and old bone in the absence of any disease causing mutations have shown that old bone is unable to resist fracture as effectively as young bone. Younger bones did not initiate cracks as easily as older bones and these cracks did not propagate as easily as in older bones (George and Vashishth 2006). Other studies have shown a reduction in the mechanical integrity of collagen fibers as well as an increase in advanced glycation end products (AGEs), a type of collagen crosslink, in the aging bone (Wang, Shen et al. 2002). This increase in AGEs will not only affect the quality of the bone, but also the quantity. These two studies correlate well with each other as a decrease in collagen integrity will reduce the toughness of the bone and the increased number of cross-links will increase the brittleness of the bone, allowing fractures to occur more easily.

Studies have also been done on the aging skeleton of the *oim* mouse. These studies demonstrated that *oim/oim* bone remains weaker than wildtype bone from three to $\geq$18 months of age (McBride, Shapiro et al. 1998). Although *oim/oim* animals did increase their torsional rigidity with age, they never reached wildtype values. This is consistent with observations that fracture number in OI patients tends to decrease post-puberty (Paterson, McAllion et al. 1984) and is consistent with the theory that bone from OI patients plays "catch-up" with increasing age to attempt to correct for the reduction in the amount and quality of the bone (Ramser, Villanueva et al. 1966).

Studies on other mouse models of OI have also shown post-pubertal improvements in bone strength. Femurs from Brtl mice were unable to resist the same forces as wildtype animals at three months of age, but by 12 months, Brtl and wildtype mice were able to resist the same loads (Kozloff, Carden et al. 2004). Mov-13 animals

also demonstrated age-associated improvements in phenotypic severity. Mov-13 animals showed similar bending moments as wildtype animals at 15-weeks, whereas at 8 weeks, Mov-13 animals were weaker (Bonadio, Jepsen et al. 1993). Taken together, these studies demonstrate an age-associated improvement in bone strength, despite diverse OI-causing mutations.

Although osteoporosis has classically been thought of as a disease of elderly women, a new perspective is beginning to emerge in which osteoporosis is considered a pediatric disease. Exercise studies have shown that the earlier an exercise program is initiated, the greater the accretion of bone. This gain in bone may prevent the onset of osteoporosis or, at least, delay the age of onset. During the normal growth process, bone grows in both length and diameter (Specker 2006). Both of these increases alter the geometry of the bone and, therefore, biomechanical integrity. Longer and wider bones are better able to withstand torsional loads and, therefore, will not break as easily as shorter, narrower bones (Turner 2006). During puberty, the porosity of the bone increases due to increased bone turnover, contributing to the increased number of fractures often seen in adolescents during puberty (Specker 2006). During puberty, body and muscle mass both increase, putting added stresses on the bone. These added stresses are theorized to improve biomechanical integrity by the mechanostat theory, a mechanism by which the bone responds to changes in loading with alterations to bone architecture (Skerry 2006). These changes in loading can be positive, as in the case of chronic exercise, or negative, as seen with patients on long-term bed rest. If the force is moderate and sustained over a long period of time, it can induce changes in geometry that will better allow the bone to resist forces, ultimately resulting in a stronger bone (Skerry

2006). Post-puberty, the rate of remodeling slows and porosity decreases, but the now larger bone continues to mineralize with a concomitant decrease in fracture rate (Specker 2006).

Because no literature exists on the effect of the *oim* mutation prior to three months of age, wildtype, heterozygous (*oim*/+) and homozygous (*oim/oim*) mouse femurs and tibias of each strain were evaluated at each of three ages during development. One month of age in mice corresponds to childhood in humans, while two and four months of age in mice are equivalent to puberty and post-puberty, respectively, in humans (Kozloff, Carden et al. 2004). We sought to determine if the more severe phenotype seen in C57 animals is seen at all developmental stages as well as to determine if C57 animals ever reach the same level of phenotypic severity seen in outbred animals. We also sought to determine if strain or genotype differences were exaggerated at a younger age.

Femoral geometry was analyzed by μCT analysis while biomechanical integrity was determined by torsional loading to failure as described in Chapter II. Mineral composition was determined using neutron activation analysis and collagen content was quantitated using the hydroxyproline assay. Pyridinoline crosslinks were also quantitated to determine if changes in bone turnover occur. Our results demonstrate an important role for genetic background in determining the clinical outcome in *oim*/+ and *oim/oim* animals. However, presence of the *oim* mutation ultimately had the greatest impact on phenotypic severity. These studies indicate that genetic background plays a role in the severity of the *oim* phenotype at all ages and that the severity of the disease does improve with age. However, presence of the *oim* mutation is the primary determinant of disease

severity, regardless of age, as the *oim* animals remain weaker than wildtype animals throughout development.

METHODS

Experimental Design

Animals were sacrificed at one, two or four months of age via CO_2 asphyxiation, weighed, blood harvested by exsanguination, serum isolated and stored at –20^{o} C. Tibias and femurw were removed and cleaned of soft tissue attachments prior to being snap frozen in liquid nitrogen (tibias) or wrapped in strips of sterile gauze soaked in sterile 1X phosphate buffered saline (PBS) (femurs) and stored at –80^{o} C.

μCT Analysis and Torsional Loading to Failure

Geometric parameters were defined from right femurs by microCT (μCT) scan analysis prior to *ex vivo* torsional loading to failure as described in on page 58.

Hydroxyproline Assay

Left femurs from one, two and four month-old animals were used for quantitation of hydroxyproline content as described on page 63.

Neutron Activation Analysis

Left tibias to be used for neutron activation analysis were prepared as described on page 66. Standards and samples (tibias from two and four month animals) were activated by a thermal neutron flux of $5x10^{13}$ n/cm^2/s for specific times (see Table III-1)

Table III-1. Instrumental NAA Parameters

Ion	Nuclear Reaction	Photopeak (keV)	Irradiation Time[a]	Irradiation Time[b]	Decay Time	Count Time	Count Position
F	$F^{19}(n,\gamma)F^{20}$	1633	7 sec	15 sec	15 sec	30 sec	Face
P	$P^{31}(n,\alpha)Al^{28}$	1779	15 sec	15 sec	1 min	3 min	Face
Na	$Na^{23}(n,\gamma)Na^{24}$	1368	7 sec	15 sec	1 min	10 min	1 spin[d]
Mg	$Mg^{26}(n,\gamma)Mg^{27}$	844, 1014	7 sec	15 sec	1 min	10 min	1 spin
Ca	$Ca^{48}(n,\gamma)Ca^{49}$	3085	7 sec	15 sec	1 min	10 min	1 spin
K	$K^{41}(n,\gamma)K^{42}$	1524	95 sec	360 sec	EOI[c]	30 min	1[e]
Zn	$Zn^{68}(n,\gamma)Zn^{69m}$	438	95 sec	360 sec	EOI[c]	30 min	1[e]

[a]Irradiation time for tibias from two and four month animals.

[b]Irradiation time for tibias from one month animals.

[c] EOI: End of Irradiation Time is the specific time the sample exited the reactor. All samples and standards were decay corrected back to this time.

[d] Count position was in a rotating position 2.5 cm from the detector face.

[e] Count position was stationary 2.5 cm from the detector face.

to activate F, Na, Mg, Ca, K and Zn and a 5 x 10^{12} n/cm^2/s flux to activate P. Tibias from one month-old animals were activated by the same neutron flux but the irradiation time was doubled for all elements, except P (Table III-1). A correction factor of $1^{-exp(-L*IT2)}/1^{-exp(-L*IT1)}$ where IT is irradiation time and L=LN(2)/half life was applied to the data from one month-old tibias to allow for quantitation using the standard comparator method as for two and four month-old tibias.

Pyridinoline Assay

Determination of serum pyridinoline, a marker of bone turnover, was done using the Metra Serum PYD EIA Kit according to manufacturer's protocol and as described on page 67.

Statistical Analysis

All statistical analyses were done using SAS (SAS Institute Inc., Cary, NC). Data from the three groups were analyzed as a 3 x 2 x 3 factorial (3 genotypes, 2 strains and 3 ages). All other analyses were performed as described on page 69.

RESULTS

The *oim* mutation and genetic background both impact acquisition of body mass throughout development

When total body mass was determined, outbred animals were found to have increased body mass as compared to C57 animals, regardless of genotype (Figure III-1; compare squares to triangles). Consistent with data seen in four month-old animals,

oim/oim animals had reduced body mass as compared to their wildtype littermates, regardless of strain or age. C57 *oim/oim* animals were also significantly smaller than their heterozygote littermates at all ages while outbred *oim/oim* animals were significantly smaller than their heterozygote littermates at one month of age only. Within a given genotype and strain, significant age differences were seen, with four month animals having more body mass than one month animals.

Determination of Bone Geometry

Femur length (FL) increased with age in both strains and in all genotypes, except in outbred wildtype between two and four months of age (Figure III-2a). C57 and outbred wildtype femurs exhibited strain differences in femur length at all ages with outbred animals having longer femurs (Figure III-2a, compare squares and triangles). Heterozygotes were only different at one month of age with outbred having longer femurs. Outbred *oim/oim* had longer femurs than C57 *oim/oim* femurs at both one and four months of age, but not two months. Genotype differences were seen at all ages in outbred animals, but only at four months of age in C57 animals. At one and two months of age, the only genotype differences were between outbred wildtype and *oim/oim* animals with wildtype animals having longer femurs than *oim/oim* littermates (Figure III-2a). At four months of age, C57 wildtype animals had longer femurs than *oim/oim* animals, while outbred wildtype animals had longer femurs than both *oim*/+ and *oim/oim* animals. *Oim*/+ femurs were also longer than *oim/oim* a femurs.

Marrow cavity diameter (MCD) also changed with age, although in a strain and genotype specific manner. Within the C57 strain, all genotypes increased their MCD

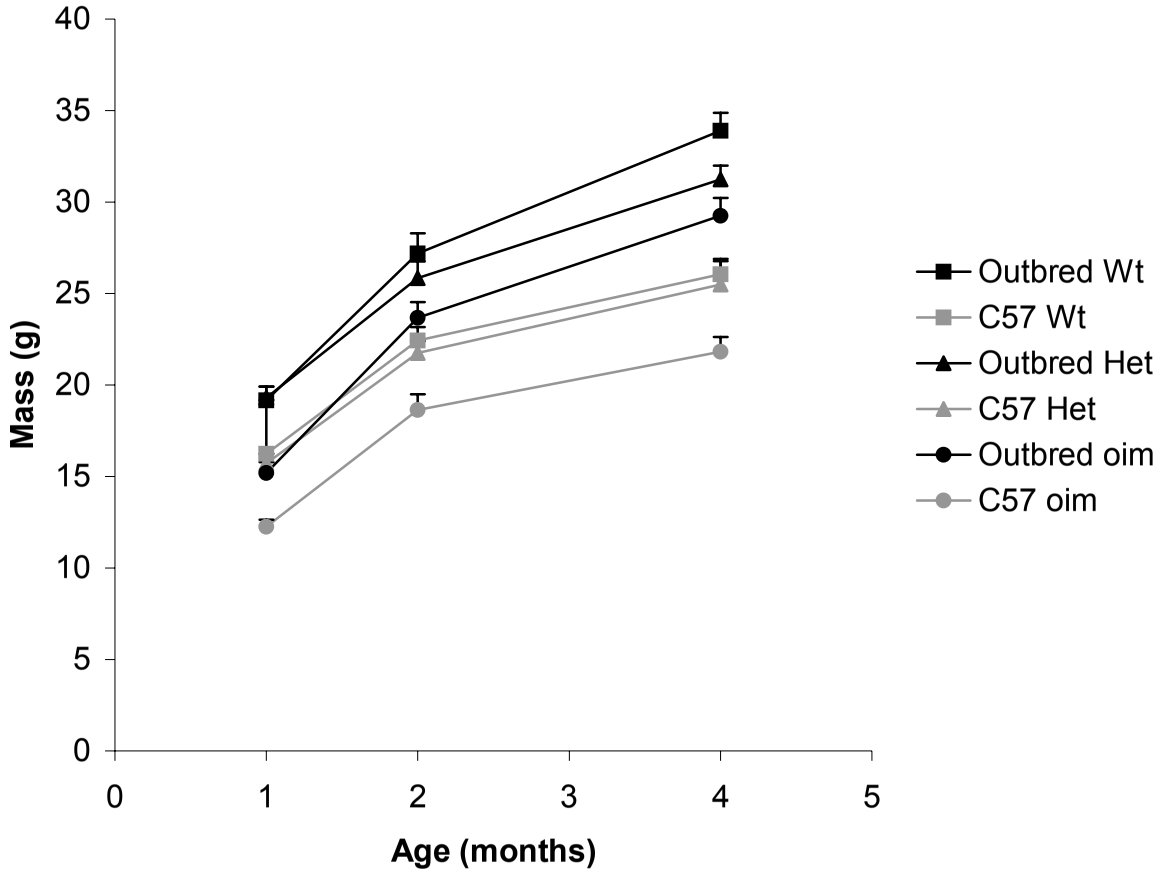

Figure III-1. Genotype and strain both impact total body mass at one, two and four months of age. C57 *oim/oim* animals had reduced body mass as compared to C57 *oim*/+ and wildtype animals at all ages (p values <0.05). Outbred *oim/oim* animals had reduced body mass as compared to outbred wildtype at all ages (p value <0.05). C57 animals had reduced body mass as compared to age and genotype matched outbred animals (p values <0.05). [per age class: Wt, n=12-21; Het, n=11-36; *oim*, n= 7-19]

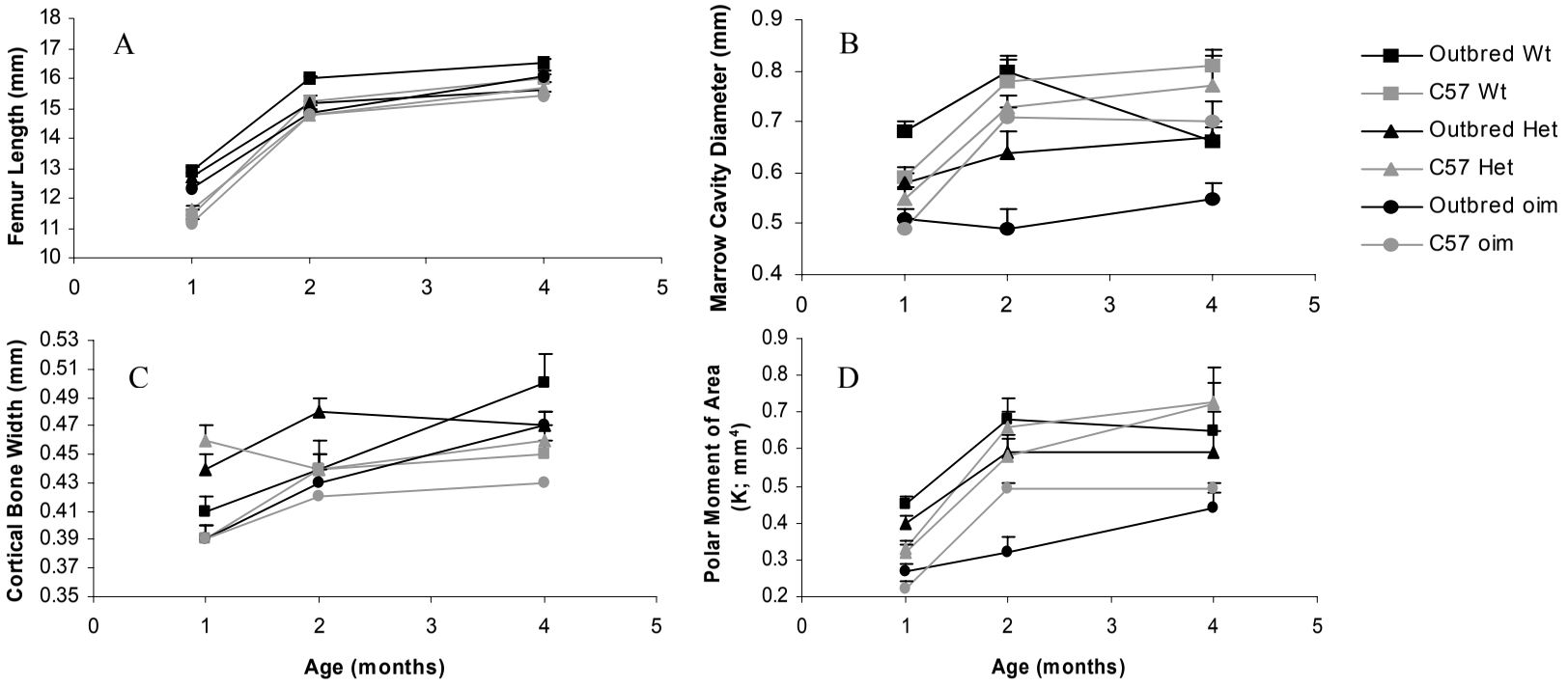

Figure III-2. Femoral geometry of C57 and outbred Wt, *oim*/+ and *oim/oim* mice at 1, 2 and 4 months of age. FL had Strain differences in Wt (all ages) and *oim/oim* femurs (1 and 4 months). Outbred Wt femurs were longer than *oim/oim* femurs (all ages); C57 Wt femurs were longer than *oim/oim* femurs (4 months). MCD showed strain differences in Wt animals (1 and 4 months) and in *oim/oim* animals (2 and 4 months). Wt femurs of both strains had larger MCDs than *oim/oim* femurs (1 and 4 months); outbred Wt femurs were also larger at 2 months. CBW had strain differences in Wt femurs (1 month) and in *oim/oim* femurs (4 months). Outbred Wt femurs had larger CBWs than *oim/oim* femurs (1 month). K showed strain differences in Wt femurs (1 month) and in *oim/oim* femurs (2 months). Wt femurs had a larger K than *oim/oim* femurs in both strains at all ages. p values <0.05. [per age class Wt, n=8-18; Het, n=8-15; *oim*, n=8-14]

between one and two as well as one and four months of age (Figure III-2b). Within the outbred strain, *oim*/+ animals increased their MCD between one and four months of age while outbred wildtype animals increased their MCD between one and two months of age, but decreased between two and four months of age. Strain differences were seen between wildtype animals of the two strains at one and four months of age, with outbred diameters being larger at one month, but smaller than C57 at four months. C57 *Oim*/+ animals had larger MCD than outbred *oim*/+ animals at two months only. C57 *oim/oim* femurs had larger MCD than outbred *oim/oim* femurs at two and four months of age.

Genotype differences in marrow cavity diameter were also seen. At one month of age, C57 wildtype and *oim*/+ femurs had larger MCD than *oim/oim* femurs. Within the outbred strain, wildtype femurs had larger MCD than either *oim*/+ or *oim/oim* femurs. Outbred *oim*/+ femurs also had a larger MCD than *oim/oim* femurs. At two months of age, the only genotype differences were seen within the outbred strain. Wildtype femurs had larger MCD than either *oim*/+ or *oim/oim* femurs; *oim*/+ were also larger than *oim/oim*. At four months of age, wildtype femurs had larger MCD than *oim/oim*, regardless of strain; *oim*/+ also had larger MCD than *oim/oim* femurs.

Cortical bone width (CBW) increased with age, with the exception of C57 *oim*/+ femurs, which decreased at two months of age (Figure III-2c). Outbred wildtype and *oim/oim* femurs demonstrated age-associated differences in CBW between one and two, two and four and one and four months of age. Both outbred and C57 *oim*/+ femurs were significantly different between one and two and one and four months of age. C57 wildtype and *oim/oim* femurs had increased CBW at two and four months of age as compared to femurs at one month of age. Strain differences were seen between wildtype

animals at one month of age, with C57 animals having smaller CBW widths than outbred animals. C57 *oim*/+ femurs had a larger CBW than outbred *oim*/+ femurs at one month of age, but smaller CBW at two months of age and similar CBW at four months of age. Finally, femurs from *oim/oim* outbred animals had a larger CBW than C57 *oim/oim* animals at four months of age.

Polar moment of area (K) also increased with age, regardless of strain or genotype. Wildtype and *oim*/+ animals of both strains showed age-associated increases between one and two and one and four months of age (Figure III-2d). *Oim/oim* animals of both strains also showed a similar increase between one and four months of age. C57 *oim/oim* femurs also had an increase between one and two months of age, while outbred *oim/oim* femurs increased between two and four months of age. Strain differences were seen between wildtype femurs of the two strains at one month of age, with outbred wildtype femurs having a larger polar moment than C57 wildtype femurs. *Oim*/+ femurs from the outbred strain also had a larger polar moment than *oim*/+ femurs from the C57 strain at one month of age. Conversely, *oim/oim* femurs from the C57 strain had a larger polar moment than *oim/oim* femurs from the outbred strain at two months of age.

Genotype differences were also seen at each age point, with wildtype animals always having a larger polar moment of area than *oim/oim* femurs, regardless of strain. At one and four months of age, both C57 and outbred wildtype and *oim*/+ femurs had larger polar moments than *oim/oim* femurs. At two months of age the same held true, except C57 *oim*/+ were not significantly different from *oim/oim* femurs for this parameter.

Torsional Loading to Failure: A Measure of Bone Biomechanical Integrity

Bone biomechanical integrity was measured by three parameters: torsional ultimate strength (T_{max}), tensile strength (Su) and energy until failure (U). T_{max}, or the maximum strength required to break the bone, is a macroscopic bone property describing the bone as a whole, including any geometrical adaptations the bone may have made. This parameter increased in all genotypes with age, although *oim/oim* femurs remained weaker than both wildtype and *oim*/+ femurs at all ages. T_{max} increased with age in wildtype femurs between one and two as well as two and four months of age, regardless of strain (Figure III-3a). *Oim*/+ femurs of both strain increased in torsional strength between one and two as well as one and four months of age. Additionally, *oim*/+ outbred femurs increased between two and four months of age. *Oim/oim* femurs increase in strength between one and four and two and four months of age in both strains. *Oim/oim* femurs from the C57 strain also increased between one and two months of age. Strain differences were only seen at one month of age in wildtype and *oim*/+ femurs, with outbred wildtype being stronger than C57 wildtype, but C57 *oim*/+ femurs stronger than outbred *oim*/+ femurs.

Genotype differences in T_{max} were also seen at each age point, although the difference in strength between the genotypes increased with age. At one month of age, wildtype and *oim*/+ femurs were significantly stronger than *oim/oim* femurs, regardless of strain. Outbred wildtype femurs were also stronger than *oim*/+ femurs. By two months of age, wildtype and *oim*/+ femurs were stronger than *oim/oim* femurs regardless of strain. Wildtype femurs were also stronger than *oim/oim* femurs in both strains. At four

months of age, wildtype and *oim*/+ femurs were stronger than *oim/oim* regardless of strain.

Tensile strength (Su) is the corresponding microscopic property to T_{max}. Su describes the strength of the bone material, subtracting out possible geometrical differences. Like T_{max}, Su also increased in all genotypes with age, although *oim/oim* remained weaker than wildtype and *oim*/+ at all ages (Figure III-3b). Su increased with age in wildtype femurs between one and two as well as one and four months of age, regardless of strain. *Oim*/+ femurs also increased between one and four months of age in both strains. *Oim/oim* femurs increased in tensile strength between one and four as well as between two and four months of age in the C57 strain. Within the outbred strain, *oim/oim* femurs increased in tensile strength between one and two and one and four months of age. Strain differences were only seen in *oim/oim* femurs at two months of age, with outbred *oim/oim* being stronger than C57 *oim/oim*.

Genotype differences also mimicked those seen in T_{max}. At one month of age, both wildtype and *oim*/+ femurs were stronger than their *oim/oim* counterparts in both strains. At two months of age wildtype femurs had stronger material than their *oim/oim* littermates in both strains, while *oim*/+ femurs in the C57 strain were also stronger than *oim/oim* femurs. By four months of age, the difference in strength between wildtype and *oim/oim* was more pronounced. Wildtype femurs of both strains were stronger than their *oim/oim* littermates, while *oim*/+ femurs of the outbred strain were also stronger than their *oim/oim* counterparts.

The last measure of bone biomechanical strength was energy until failure (U), a measure of the ability of the bone to absorb energy prior to fracturing. At all ages,

oim/oim femurs were not able to absorb as much energy before fracturing as wildtype or *oim*/+ (Figure III-3c). Additionally, at two months of age wildtype femurs from both strains were able to absorb more energy than *oim*/+ femurs. This was also true for the C57 strain at four months of age. Strain differences were seen with outbred femurs, which were always able to absorb more energy than their C57 counterparts. Wildtype animals showed strain-associated differences at one and two months of age. *Oim*/+ femurs from the outbred strain were able to absorb more energy than C57 *oim*/+ femurs at all ages, while *oim/oim* femurs showed strain differences only at four months of age. The only age-associated changes in energy until failure were in C57 wildtype between one and four months of age and in outbred *oim*/+ femurs between two and four months of age. Though U did increase with age in outbred wildtype femurs, substantial intersample variation prevented the p value from reaching significance (p value= 0.1).

The next two parameters describe the stiffness of the femurs. Torsional stiffness (Ks) is a macroscopic property and shear modulus of elasticity (G) is a microscopic property. Strain differences in Ks were only seen at four months of age in *oim*/+ femurs (Figure III-4a). Ks increased in *oim*/+ femurs at all ages within the C57 strain, but only between one and two as well as one and four months in the outbred strain. *Oim/oim* femurs had increased Ks at all ages regardless of strain. The Ks of wildtype femurs increased at all ages within the C57 strain, while wildtype femurs in the outbred strain increased between one and two as well as one and four months of age. Differences in Ks due to genotype were seen between outbred wildtype and *oim/oim* femurs at one and four months. At two months of age, outbred wildtype and *oim*/+ femurs were stiffer than *oim/oim*. This was also true of C57 wildtype and *oim*/+ femurs at two and four months.

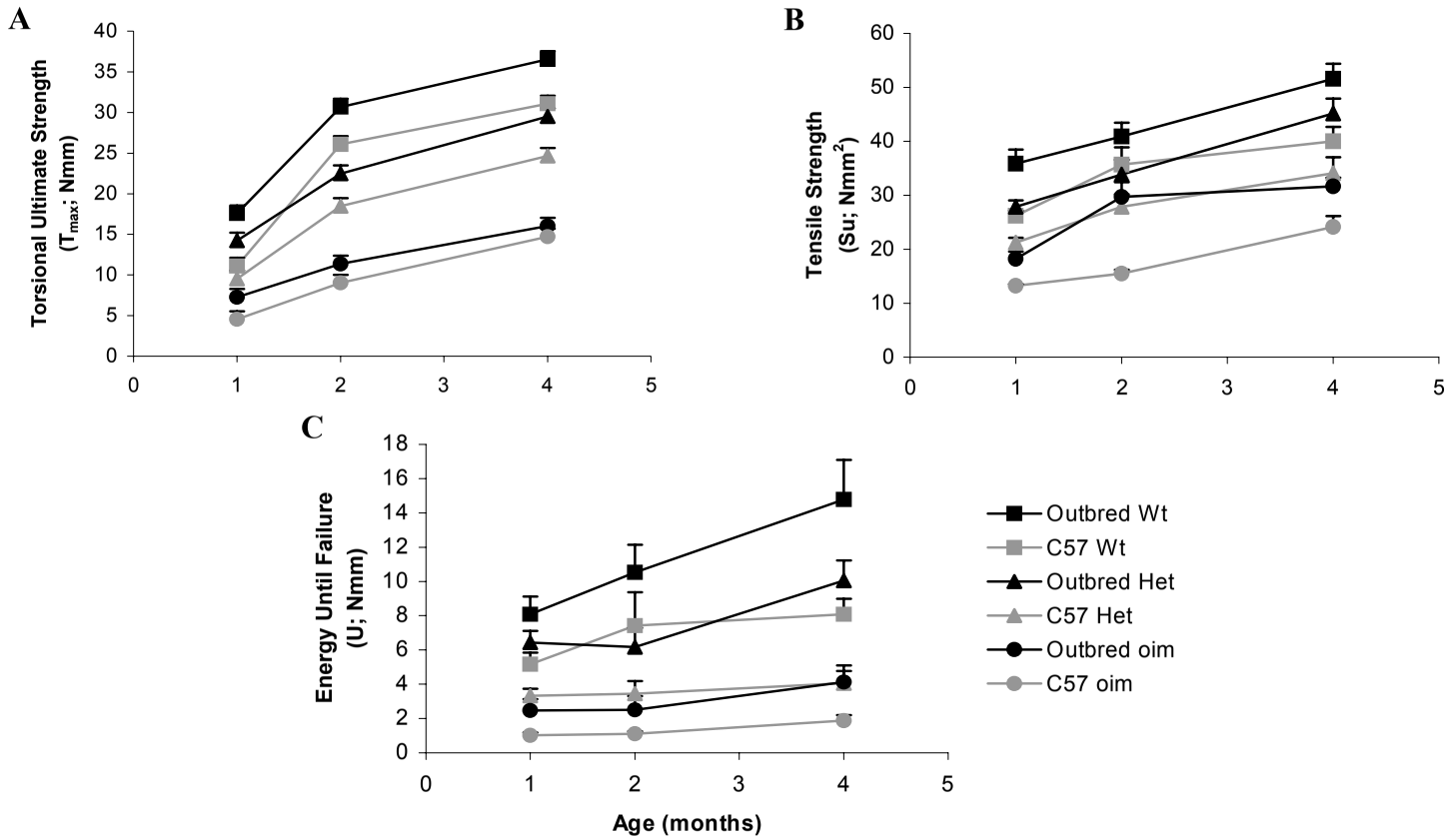

Figure III-3. Biomechanical integrity as determined by T_{max}, Su and U improves with age in both strains and in all genotypes, although *oim* remains weaker than wildtype at all ages. T_{max} had strain differences in Wt (1 month). Wt and *oim*/+ femurs of both strains were stronger than *oim/oim* femurs (all ages). Su had strain differences in *oim/oim* femurs (2 months). Wt femurs of both strains had a higher Su than *oim/oim* femurs (2 and 4 months); C57 Wt femurs also had a higher Su than *oim/oim* femurs at 1 month. U showed strain differences in Wt animals (1 and 2 months) and in *oim/oim* animals (4 months). Wt and *oim*/+ femurs of both strains were able to absorb more energy than *oim/oim* femurs (all ages). p values <0.05. [per age class Wt, n=8-12; Het, n=6-12; *oim*, n=6-12]

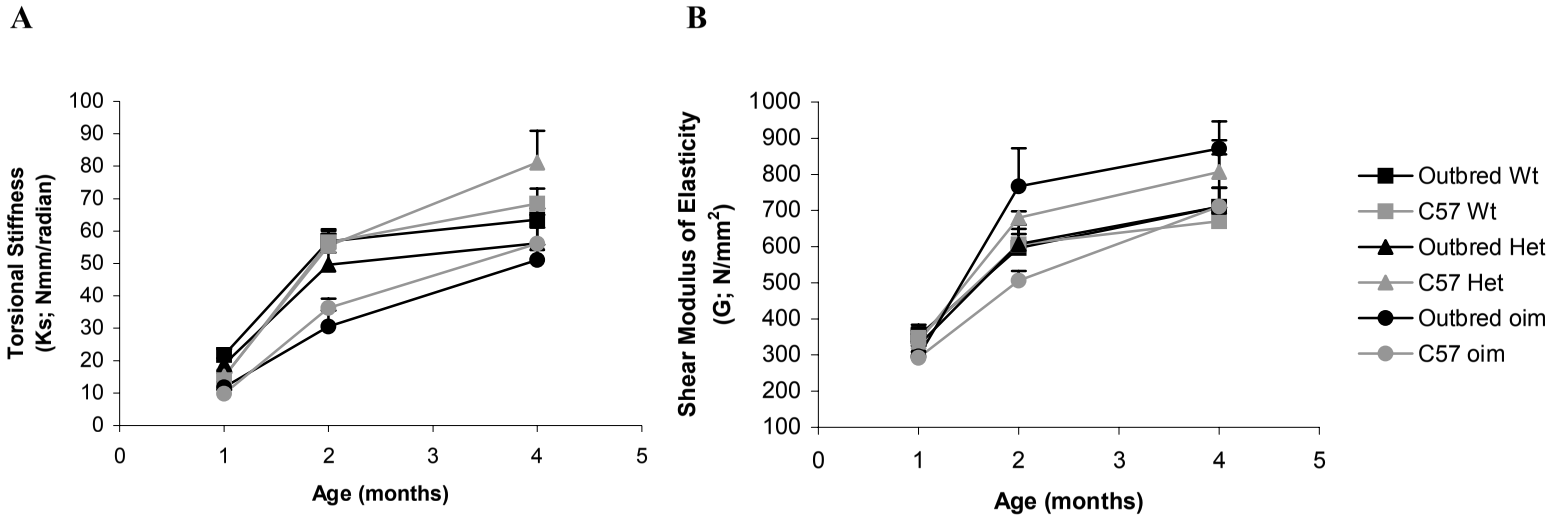

Figure III-4. Stiffness increased in both strains and in all genotypes with age. Strain differences were not seen, regardless of genotype or at any age. Wt femurs of both strains had increased Ks compared to *oim/oim* femurs (2 and 4 months). Outbred Wt femurs were also stiffer than *oim/oim* femurs (1month). G showed strain differences in *oim/oim* femurs (2 months). G did not demonstrate genotype differences at any age or in either strain. p values <0.05. [per age class Wt, n=8-12; Het, n=6-12; *oim*, n=6-12]

Shear modulus of elasticity (G) describes the stiffness of the bone material only. The same strain differences seen in Ks were also seen for shear modulus in *oim*/+ femurs (Figure III-4b). Additionally, *oim/oim* femurs also showed strain differences in G at two months of age. Age-associated changes in shear modulus were seen in wildtype, *oim*/+ and *oim/oim* femurs of both strains between one and two as well as between one and four months of age. *Oim*/*oim* femurs of the C57 strain also showed differences between two and four months of age. Genotype differences were not seen at one month of age in either strain. At two months of age, the only genotype-associated difference was between *oim*/+ and *oim/oim* femurs of the C57 strain. By four months of age, the only difference between the genotypes was in the outbred strain, between *oim*/+ and *oim/oim* femurs.

Mineral composition of tibias as determined by neutron activation analysis

Several minerals demonstrated associations with bone biomechanical integrity at four months of age, including fluoride, magnesium, sodium and zinc. These minerals were then further analyzed at one and two months of age. Phosphorus, calcium and potassium as well as the Ca/P ratio were also analyzed at one and two months of age. These minerals did exhibit strain, genotype and age changes, although the significance of these changes is unclear, and due to their lack of association between the levels of these minerals and bone biomechanical integrity, they will not be discussed further. Fluoride and magnesium both negatively associate with bone biomechanical integrity. Fluoride associates with bone strength regardless of strain in an age-specific manner (Figure III-5). At a given age, the higher the fluoride level, the weaker the bone. However, older animals had increased levels of fluoride as well as increased strength. Magnesium also

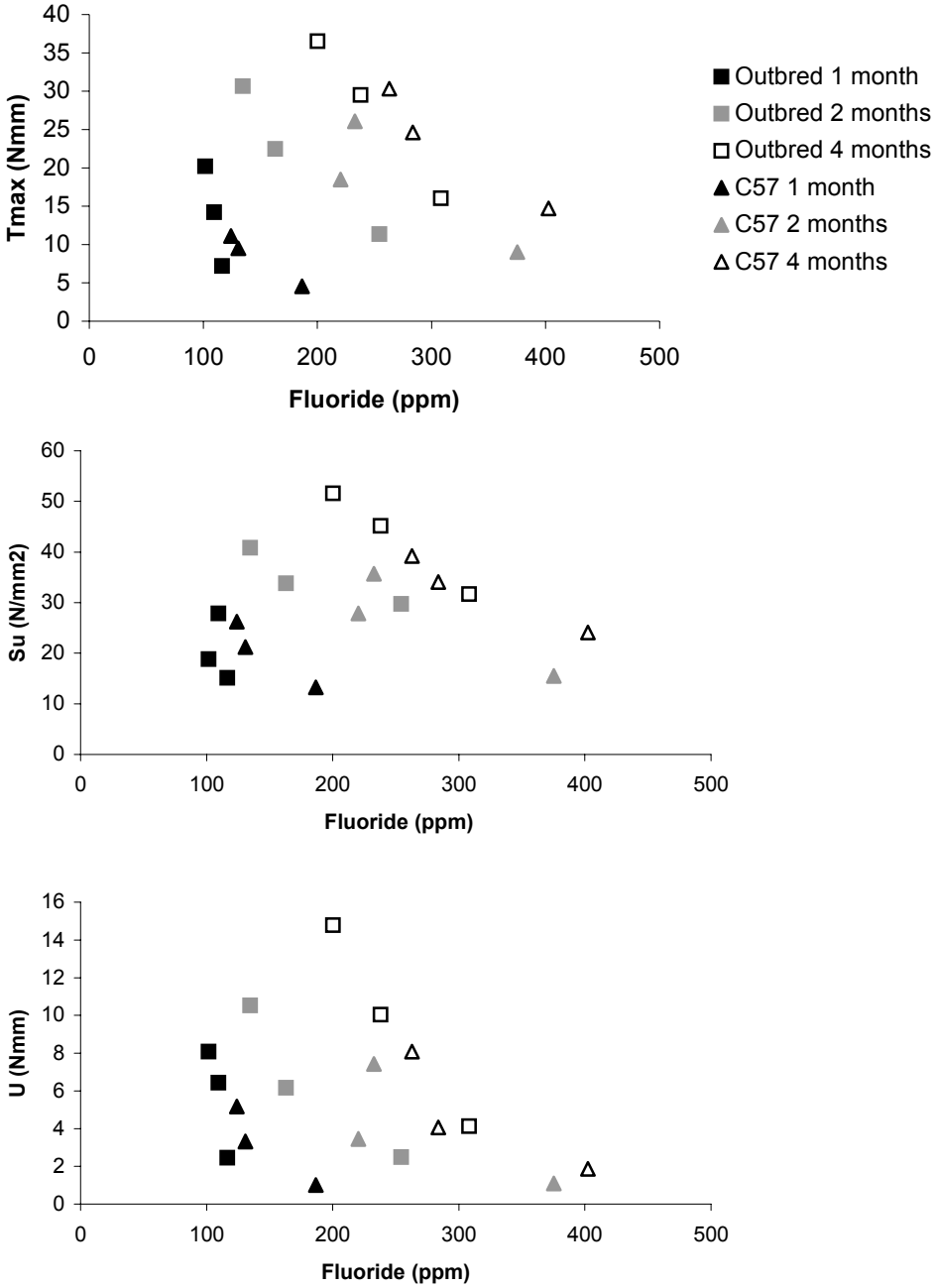

Figure III-5. Association of fluoride levels and femoral biomechanical integrity. Fluoride negatively correlates with T_{max}, Su and U. The higher the level of fluoride, the weaker the bone, regardless of strain. [n=7-8 of each strain/genotype/age]

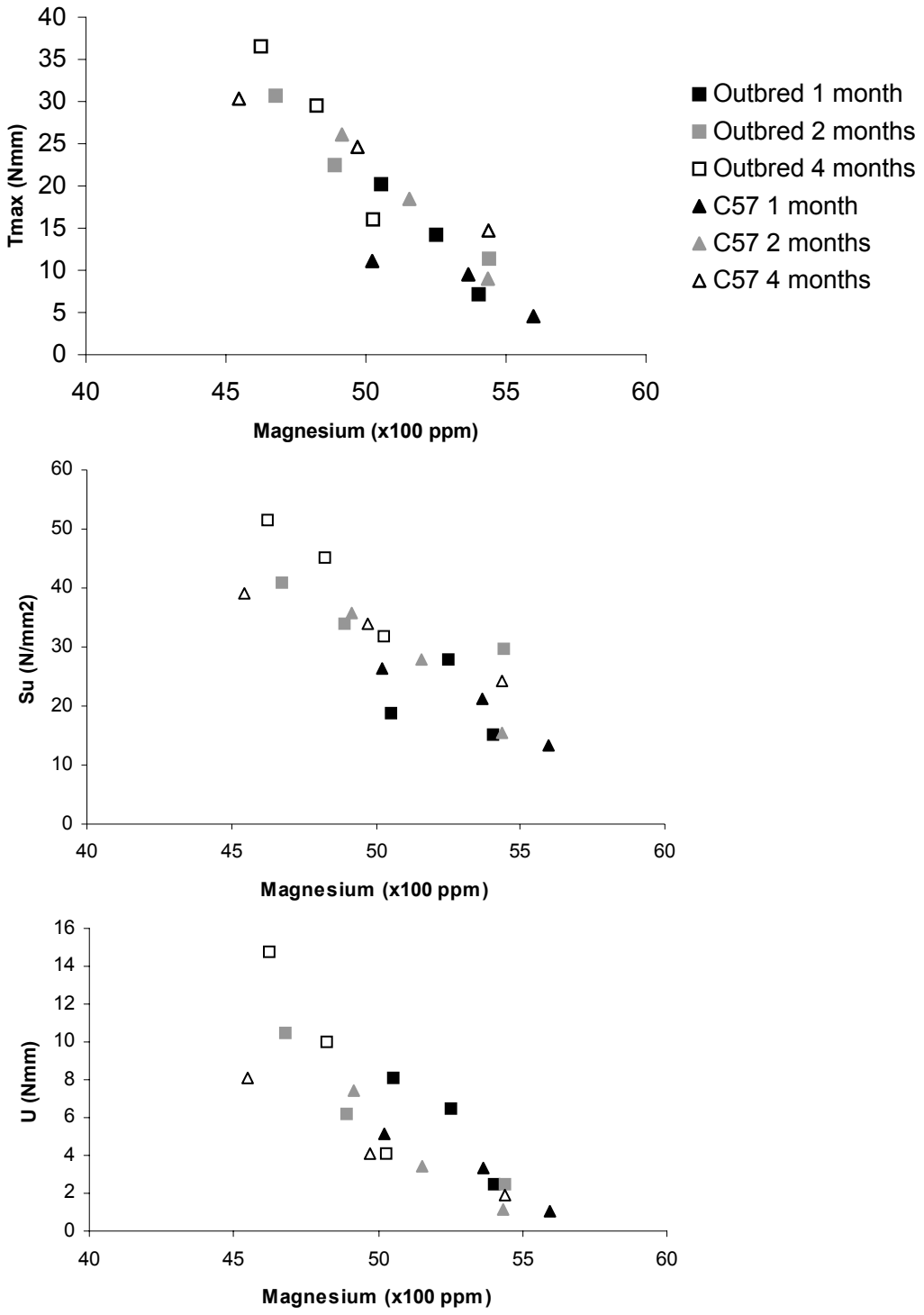

Figure III-6. Association of magnesium and bone biomechanical integrity. Magnesium negatively correlates with T_{max}, Su and U. The higher the level of magensium, the weaker the bone, regardless of strain. [n=7-8 of each strain/genotype/age]

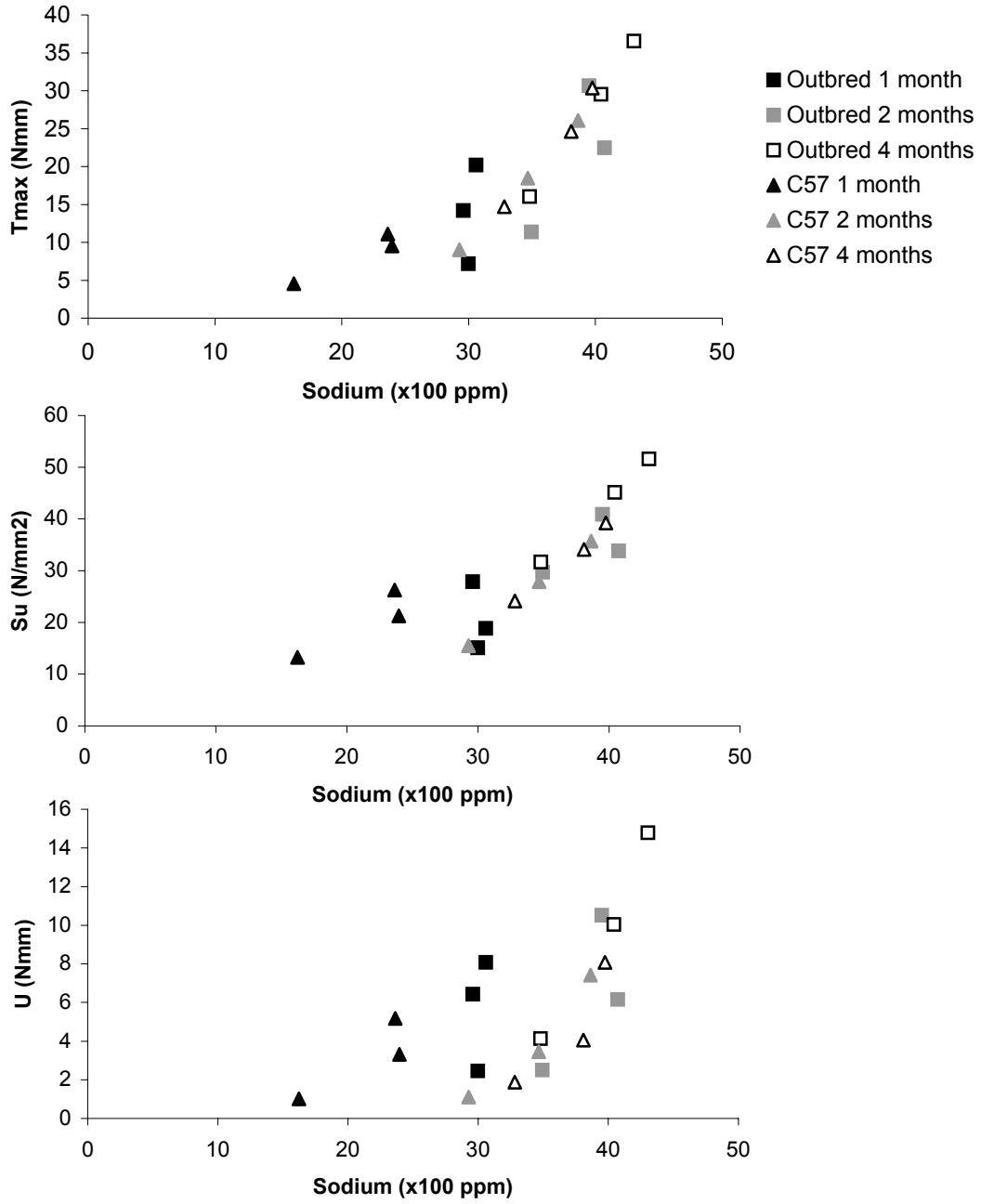

Figure III-7. Association of sodium and bone biomechanical integrity. Sodium positively correlates with T_{max}, Su and U. The higher the level of sodium, the stronger the bone, with the exception of one month bones from the outbred strain. [n=7-8 of each strain/genotype/age]

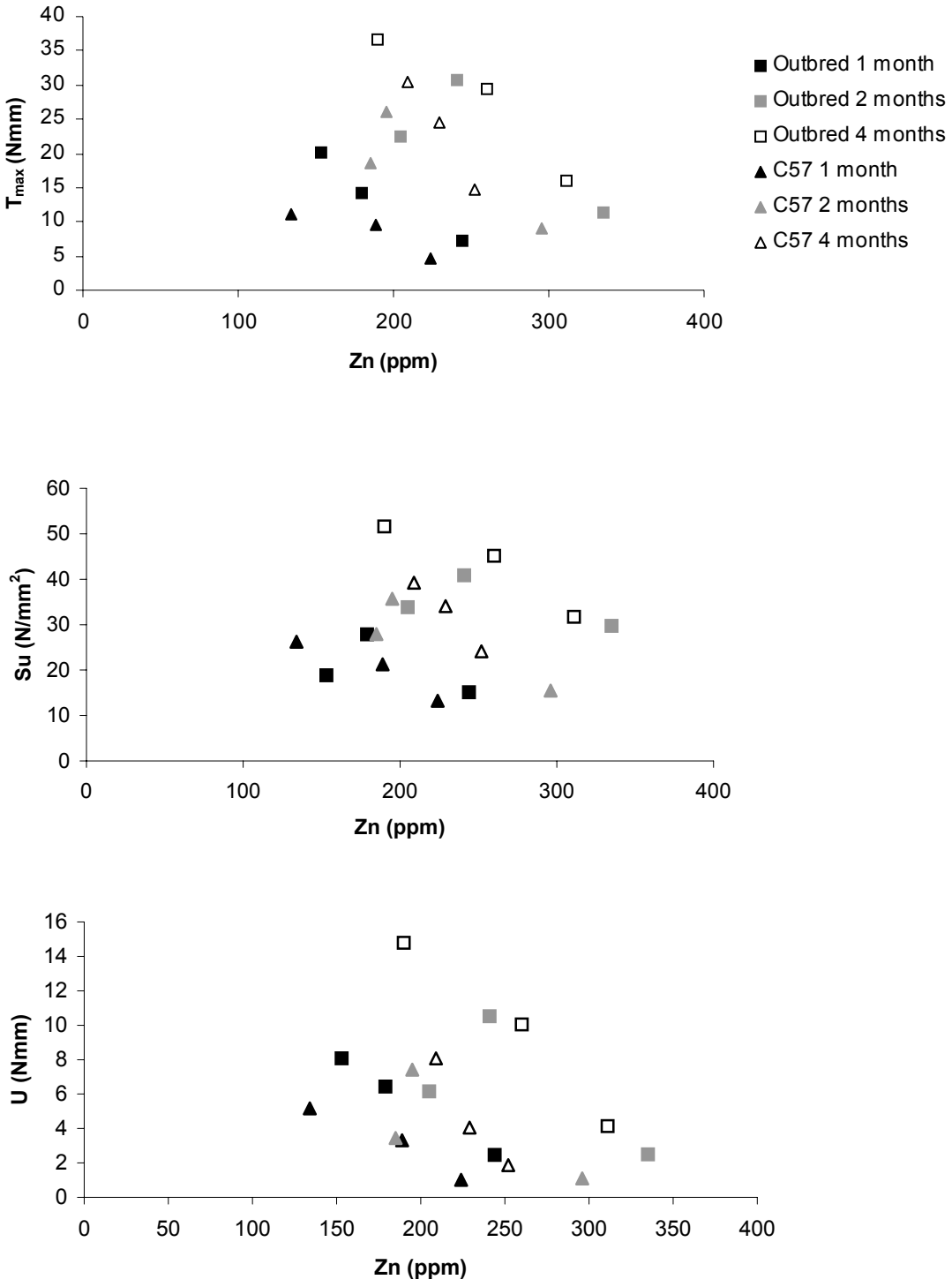

Figure III-8. Association of zinc and bone biomechanical integrity. Zinc negatively correlations with T_{max}, Su and U. The higher the level of zinc, the weaker the bone, regardless of strain. [n=7-8 of each strain/genotype/age]

negatively associates with bone strength regardless of strain (Figure III-6). The lower the magnesium level, the stronger the bone. For example, samples from four month-old animals had the lowest magnesium levels and highest T_{max}, Su and U values.

The next mineral to be analyzed was sodium. Unlike the previous minerals, sodium positively associates with bone strength. For this mineral, the greater the quantity of sodium, the stronger the bone. The one exception was in outbred animals at one month of age, which did not show an association between sodium and bone strength.

The final mineral to be analyzed was zinc. Zinc was negatively associated with bone strength with low zinc content associated with stronger bones (Figure III-8). However, this association was not seen at two months of age, regardless of strain. Additionally, at one month of age outbred animals did not show this association.

Oim/oim femurs contain less hydroxyproline than wildtype femurs at all ages

Hydroxyproline is an amino acid unique to collagen and, therefore, can be used as a relative measure of the amount of collagen in a sample. *Oim/oim* femurs contained less hydroxyproline than their wildtype counterparts at all ages, regardless of strain (Figure III-9). Additionally, *oim/oim* femurs contained less hydroxyproline than *oim*/+ femurs of both strains at four months of age. *Oim*/+ femurs from the C57 strain at two months of age also contained less hydroxyproline than their wildtype counterparts. Strain differences in hydroxyproline content were only seen between four month-old wildtype femurs with C57 femurs having more hydroxyproline than outbred wildtype femurs. Age-associated changes in hydroxyproline content were seen with two and four month femurs having more hydroxyproline than one month-old femurs of all genotypes within

the outbred strain. Within the C57 strain, age-associated increases in hydroxyproline content were seen in wildtype femurs between one and two and one and four months of age. *Oim*/+ femurs demonstrated differences in hydroxyproline content between one and two months of age while *oim/oim* femurs had more hydroxyproline at four months compared to one month of age.

Pyridinoline Crosslink Quantitation: A Marker of Bone Turnover

Pyridinoline crosslinks demonstrate age, strain and genotype associated differences. Within the C57 strain, all animals had significantly more pyridinoline at two months of age than at one or four months of age, regardless of genotype (Figure III-10). This was also true in outbred wildtype animals. The pyridinoline profile was different in *oim*/+ and *oim/oim* animals from the outbred strain. *Oim*/+ animals only had significant differences between one and four months of age and *oim/oim* animals did not have age-associated differences in pyridinoline content. Strain differences were only seen at four months of age and only in *oim/*+ and *oim/oim* animals. Genotype differences in pyridinoline content were only seen between wildtype and *oim*/+ animals of the outbred strain at two months of age.

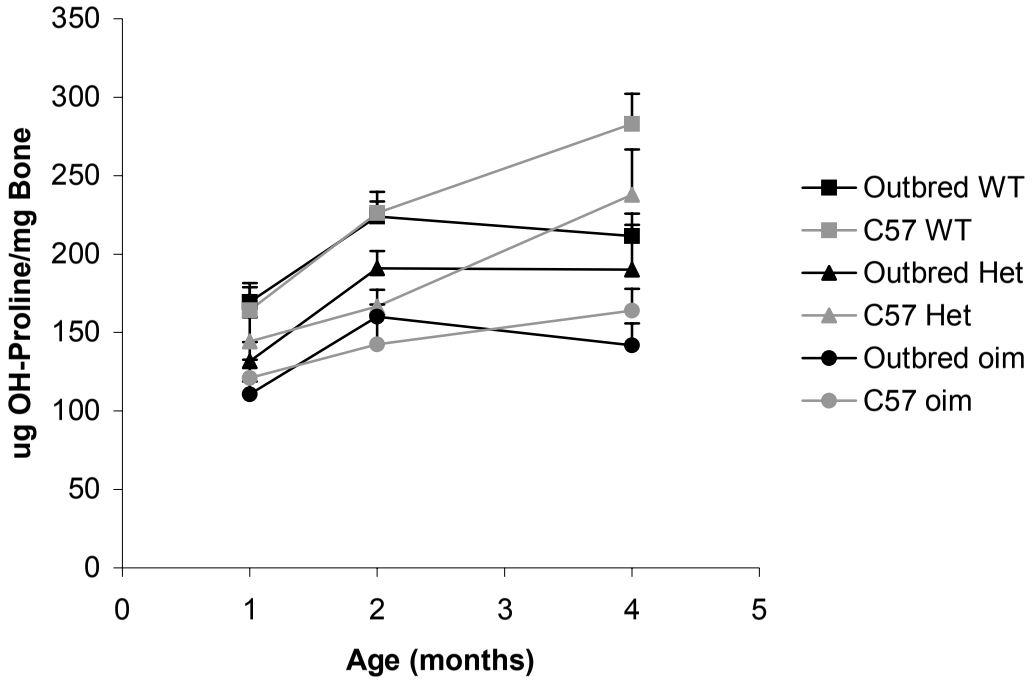

Figure III-9. C57 animals demonstrate age-related increases in hydroxyproline content at all ages while outbred animals only show an increase at two months of age with a decrease at four months of age. Strain differences were seen in Wt animals (4 months). Wt femurs of both strains had increased hydroxyproline content compared to *oim/oim* femurs at all ages. p values <0.05. [per age class Wt, n=7-13; Het, n=8-10; *oim*, n=7-13]

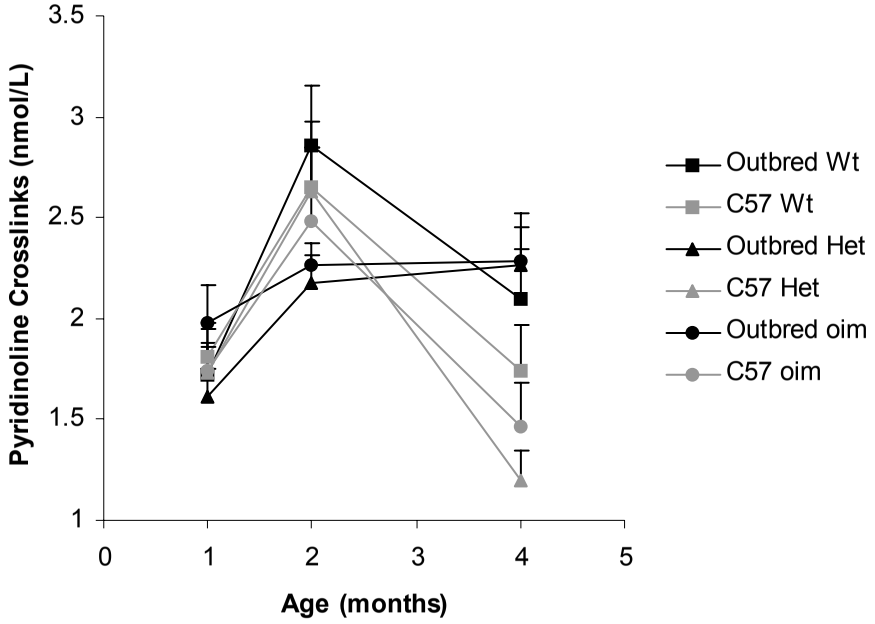

Figure III-10. Pyridinoline content of serum at one, two and four months of age. Strain differences were seen in *oim*/+ and *oim/oim* animals (4 months). Genotype differences were not seen at any age. p values <0.05. [per age class Wt, n=7-9; Het, n=7-9; *oim*, n=5-8]

DISCUSSION

The goal of the study presented here was to determine the effect of the *oim* mutation and genetic background on the biomechanical integrity of bone throughout development. Previous studies on normal and *oim* bone have focused on those changes occurring later in life and have found substantial differences between young and old bone. However, the effect of the *oim* mutation on bone throughout development has not been done.

Several previous studies have focused on changes in bone strength at various ages in several mouse models of OI. McBride et al demonstrated that the peak torque of *oim/oim* bones was significantly reduced as compared to both wildtype and *oim*/+ animals at six months of age. However, at 12 months of age, *oim/oim* and *oim*/+ animals had the same peak torque, but both were still greatly reduced compared to wildtype (McBride, Shapiro et al. 1998). It is thought that bone from OI patients plays "catch-up" with increasing age in an attempt to correct for the reduction in the amount and quality of the bone (Ramser, Villanueva et al. 1966).

The Brtl and Mov-13 mice are models of OI that have also shown age-associated changes in bone quality. Both of these models carry mutations in the α1(I) chain and are studied as heterozygotes due to the deleterious nature of their defects. The Brtl model is a glycine to cysteine substitution in the α1(I) chain leading to OI type VI (Forlino, Porter et al. 1999). The Mov-13 model is a transgenic model carrying a murine retrovirus in the first intron of the COL1A1 gene resulting in a null allele and an OI type I phenotype (Bonadio, Saunders et al. 1990). Femurs from Brtl mice had reduced maximum load compared to wildtype at three months of age (Kozloff, Carden et al. 2004). However, by

12 months of age, Brtl femurs were able to resist the same loads as wildtype animals (Kozloff, Carden et al. 2004). Stiffness was also improved at six months of age, with Brtl animals having similar levels to wildtype (Kozloff, Carden et al. 2004). This is consistent with observations that post-pubertal fracture number tends to decreased in OI patients (Paterson, McAllion et al. 1984). The Mov-13 model also demonstrated age-associated improvements in bone strength as well as geometry. 15 week-old animals showed matrix deposition on the periosteal surface as well as increased cortical bone area and bending moment of inertia as compared to both femurs from wildtype and 8 week-old Mov-13 mice (Bonadio, Jepsen et al. 1993). These studies demonstrate that despite diverse OI-causing mutations, these animals show age-associated improvements in bone quality occur, which may improve their quality of life. The differences between the aging profile of the *oim* and Brtl animals may be due to several differences between the two models. First, *oim/oim* animals are homozygous for the mutation while Brtl animals are heterozygous. Secondly, the nature of the mutation causing the disease is different between the two models. The Brtl model carries a glycine to cysteine substitution in the α1(I) chain, while the *oim* model is a functional α2(I) null due to a single nucleotide deletion leading to the production of homotrimeric type I collagen. Lastly, the type of OI the mutations causes is important, as type III patients typically do not experience the degree of post-pubertal improvement in fracture rate seen in type I/VI patients.

In this study, animals of all genotypes and both strains increased in body mass with age, although *oim/oim* animals of both strains at four months of age had similar body mass to wildtype animals of the same strain at one month of age. Additionally, *oim/oim* animals were able to improve their biomechanical integrity with age. *Oim/oim*

animals of both strains at four months of age had similar profiles as *oim*/+ animals of the same strain at one month of age for T_{max}, Su and U. This indicates that, over time, *oim/oim* animals are somewhat able to compensate for their weaker bone at both the material and whole bone levels, although they are not able to reach wildtype levels. However, slight changes in biomechanical integrity may translate to large improvements in the quality of life for OI patients.

When the mineral phase of the bone was examined, several minerals were found to associate with biomechanical integrity. Fluoride, magnesium and zinc were all negatively associated with femoral bone strength, while sodium was positively associated. Fluoride, sodium and zinc all increased with age, which might be explained by the higher than average mineralization density found in OI patients described by Rauch, et al (Rauch 2006). It is thought that the thinner type I collagen fibrils found in OI patients leaves more intermolecular space to be filled with mineral. As the animal ages, this mineral filled space may become more pronounced than at younger ages. This is seen by age-associated increased in calcium and phosphorus in animals of all genotypes and in both strains.

Fluoride was unique from the other minerals in that it associated with bone strength in an age-dependent manner, with distinct fluoride profiles at each time point. Magnesium also demonstrated a negative association with bone strength, although the range of magnesium levels leading to differences in bone strength was very small. This suggests that subtle changes in magnesium levels may have a large impact on the strength of the bone. Magnesium levels decrease with age, thus allowing the crystal size to increase along with the biomechanical integrity of the bone (Boskey, Rimnac et al. 1992).

The final mineral to associate with bone strength was zinc. Zinc is also negatively associated with bone biomechanical integrity in an age-dependent manner. The increased levels of zinc seen in the older animals may be a reflection of the reduced bone turnover seen post-puberty. The variable levels of zinc seen at two months of age, regardless of strain, may also be a reflection of the specific stage of puberty the bone is in at the time of testing. In conclusion, trace mineral composition, especially fluoride, magnesium and sodium, appear to play an important role in determining biomechanical femoral strength, regardless of genotype or strain and should be investigated further.

The main difference in collagen content, as measured by hydroxyproline content, was seen between strains. Outbred animals of all genotypes reached their peak hydroxyproline content at two months of age, while C57 animals of all genotypes continued to accumulate collagen until four months of age. In these animals, continued accumulation of collagen will also allow for continued accumulation of mineral. Additionally, pyridinoline crosslinks were highest at two months of age, regardless of strain or genotype. C57 animals, as well as outbred wildtype animals, experienced a sharp reduction in the amount of pyridinoline between two and four months of age. This is probably a reflection of the changes in bone remodeling associated with adolescence. Two and four months of age in mice correspond to puberty and post-puberty, respectively, in humans. Puberty is a time of rapid growth while bone remodeling slows dramatically during postpuberty (Specker 2006). The combination of the accumulation of both mineral and organic phases of the bone and a decrease in bone turnover should increase the biomechanical integrity of the bone.

In conclusion, several age-related differences exist in *oim/oim* animals that may allow the animals to compensate for their weaker bone material. However, *oim/oim* animals still remain weaker than wildtype animals regardless of strain or age. C57 animals are also weaker than outbred animals of like genotypes at all ages. This suggests that genetic background plays a role in determining the severity of the *oim* mutation. In addition, although *oim/oim* animals are able to compensate to some degree for their weaker bone material, they are not able to attain wildtype bone biomechanical integrity with age. Taken together, these studies indicate the importance of genetic background in determining phenotypic severity, but that the presence of the *oim* mutation and the age of the animal are the ultimate determinants of phenotypic severity.

CHAPTER IV

THE G610C MOUSE MODEL OF OSTEOGENESIS IMPERFECTA: A VIABLE NEW MOUSE MODEL OF OI

This chapter is in preparation for submission to the Proceedings of the National Academy of Science

A COL1A2 G610C MUTATION IN HUMANS AND MICE

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ABSTRACT

Osteogenesis imperfecta (OI) is a genetically and clinically heterogeneous disorder characterized by impaired biomechanical properties of type I collagen-containing tissues, such as bone. Most OI-causing mutations occur in one of the two type I collagen genes, COL1A1 or COL1A2. More than 850 distinct mutations have been reported, with the majority being single glycine substitutions within the triple helical domain and limited to single individuals and families.

Recently, a large Amish kindred was identified in which 64 individuals carry an autosomal dominant cysteine for glycine substitution at position 610 (G610C) in the COL1A2 gene. Affected individuals are classified as having mild OI though they demonstrate significant variation clinically in their self-reported fracture number and bone mineral density measurements.

To elucidate the mechanism causing this variation, a knock-in mouse carrying the G610C mutation was created. As a component of the characterization of this model, the skeletal phenotype was evaluated in femurs from wildtype, G610C/+ and G610C/G610C animals for geometric parameters using μCT imaging and biomechanical properties using torsional loading to failure. G610C/G610C femurs were significantly weaker than wildtype for the biomechanical parameters torsional load, tensile strength and energy until failure. In all cases, G610C/+ exhibited intermediate features, although not significant. μCT analysis did not reveal genotypic differences in femoral geometry.

Taken together, these studies indicate the OI-causing G610C COL1A2 mutation impairs biomechanical properties of bone, weakening both structural and material properties without altering geometry.

INTRODUCTION

Osteogenesis imperfecta (OI) is a genetically and clinically heterogeneous disease characterized by impaired biomechanical properties of type I collagen-containing tissues, such as bone, tendon, skin, and teeth. More than 850 unique OI-causing mutations have been reported, with most being in one of the two type I collagen genes, proα1(I) collagen gene (COL1A1) or proα2(I) collagen gene (COL1A2) (Dalgleish 2006). Most of these mutations are considered private, being limited to one or a few individuals or families.

OI can be subdivided into four categories based on clinical manifestation (Sillence, Senn et al. 1979). Type I OI is the mildest form, characterized by normal stature with an increased number of pre-pubertal fractures (Byers 1993; Marini 1998). Type II OI is perinatal lethal due to pulmonary insufficiency (Byers 1993). Type III OI is the most severe form compatible with life. Type III OI patients are often wheelchair bound and have progressive bowing of the long bones, short stature, blue-grey sclera and reduced biomechanical integrity manifested by numerous fractures throughout life (Byers 1993). Type IV is the most variable exhibiting a range of severities of the bone phenotype in between types I and III. These patients have mild to moderate bone deformity with normal stature (Byers 1993).

The lack of a large human population carrying the same mutation has been a limiting factor in studying the impact of specific mutations on determining clinical severity of the disease. Recently, a large Amish kindred (Figure IV-1) was identified through the Amish Family Osteoporosis study in which 64 individuals in 37 nuclear families carry an autosomal dominant cysteine for glycine substitution at position 610

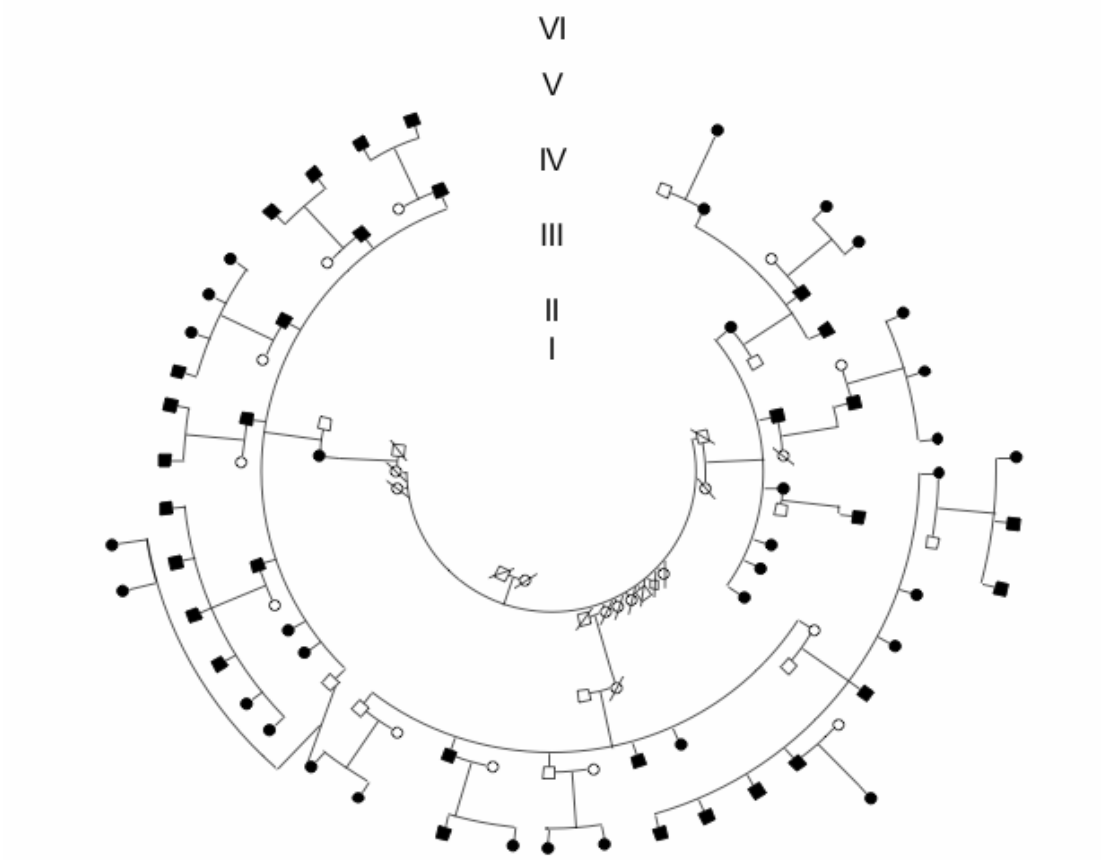


Figure IV-1. COL1A2 Gly-610-Cys Mutation Pedigree. Note the common ancestor pair from which the 63 identified carriers of the G610C mutation are descended. Provided by Dr. Daniel McBride, University of Maryland-Baltimore.

□ Unaffected
■ G610C
□ Male
○ Female
⊠⊘ Unavailable for study

(G610C) in the COL1A2 gene. This mutation leads to OI type I/IV, characterized by an increased number of fractures and reduced bone mineral density. Even though all the individuals carry the same mutation, they exhibit a broad range of phenotypic severity as determined by self-reported fracture number (0-7) and bone mineral density at the hip and spine (+2 standard deviations to –5.5 standard deviations).

To elucidate the mechanism causing this variation, a knock-in mouse carrying the G610C COL1A2 mutation was created by Dr. Daniel McBride (University of Maryland-Baltimore). A 13 kb genomic clone containing the appropriate segment of the COL1A2 gene was isolated from a lambda phage library. Site-directed mutagenesis was used to alter the GGT codon to TGT. The construct was then electroporated into the 129Sv/Ev embryonic stem cell line. G418 antibiotic selection was used to identify clones carrying the construct and these surviving clones were then subjected to Southern blot analysis to identify G610C carrying cells. These G610C positive cells were injected into C57BL/6J blastocysts. The resulting chimeras were then bred to C57BL/6J animals to produce founder animals and repeatedly backcrossed to wildtype C57 animals to ensure the animals used in experiments were on an inbred C57 background. After seven backcross generations, the animals are considered to be <99% identical except for the transferred region. These animals were then used as breeding pairs to propagate the line. Genomic DNA sequence analysis confirmed the planned codon change at the DNA level (Figure IV-2a) while RT-PCR sequence analysis (Figure IV-2b) demonstrated expression of the mutant allele. The glycine to cysteine substitution in the α2(I) chain provides a unique opportunity to identify and quantitate the mutant allele at the protein level, as cysteine is not normally found in the triple helical portion of type I collagen. To determine if the

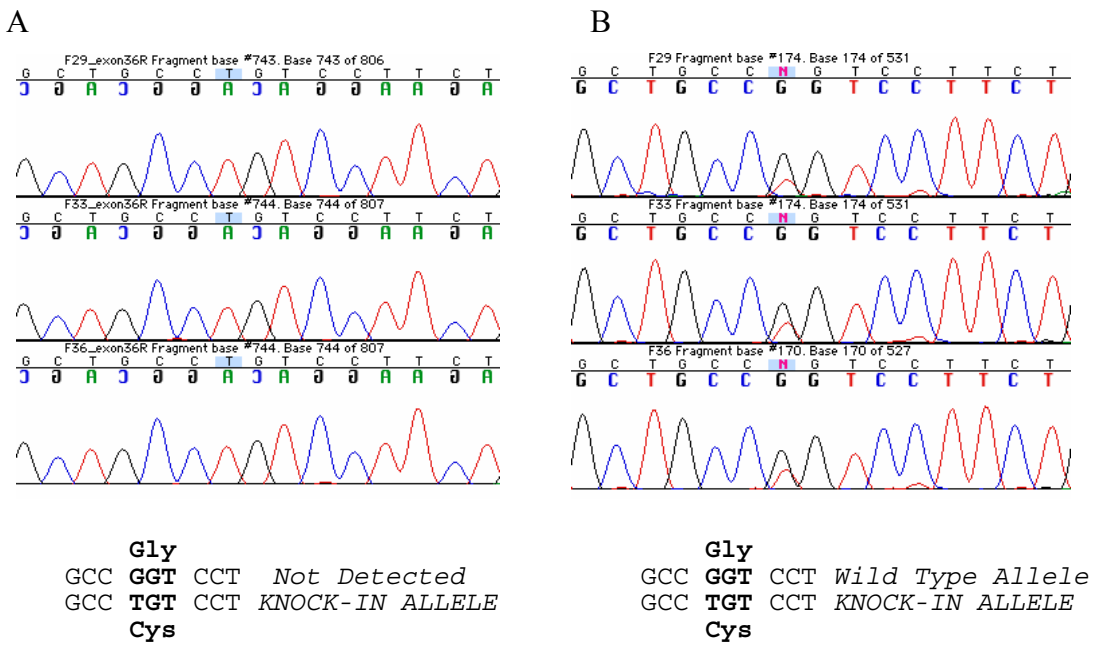


Figure IV-2. The G610C mutant allele is seen at both the DNA and RNA levels.

A) Genomic DNA was isolated from founder mouse tail tips and subjected to PCR. The PCR was designed to only amplify mutant allele. B) RT-PCR using total RNA isolated from founder mice demonstrates the expression of both the mutant and normal allele at the RNA level. The PCR was designed to amplify both mutant and normal alleles. Provided by Dr. Daniel McBride, University of Maryland-Baltimore.

mutant allele was present in tissue, Dr. Daniel McBride isolated type I collagen from tail tendon of wildtype, G610C/+ and G610C/G610C animals, and using the Cy5 fluorescent dye showed the presence of both α1(I) and α2(I) chains in type I collagen from all samples analyzed. Cysteine labeling demonstrated tissue expression of the abnormal allele in the α2(I) chains of both G610C/+ and G610C/G610C animals (Figure IV-3a). Control protein was isolated from wildtype littermates and from the Brtl model of OI. Since type I collagen does not normally contain cysteine, the absence of cysteine labeling in tendon from wildtype animals is to be expected. The Brtl model of OI carries a glycine to cysteine mutation in the α1(I) chain of type I collagen and, therefore, serves as a positive experimental control, demonstrating cysteine labeling in the α1(I) chain.

Collagen from G610C/+ and G610C/G610C tail tendon also showed altered α1(I):α2(I) ratios (Figure IV-3b). Control collagen was isolated from the Brtl model and shows the expected ratio of approximately 2:1. Wildtype collagen also shows the expected ratio. Collagen from G610C/+ mice has a slightly higher ratio at 2.6-3.0. However, collagen from G160C/G610C mice have a much higher α1(I):α2(I) ratio at 7.9 and 8.5. This can be seen visually on the gel, as collagen from G610C/G610C animals have a much fainter α2(I) band compared to wildtype and G610C/+ animals.

In order to better understand these altered α1(I):α2(I) ratios, collagen from wildtype, G610C/+ and G610C/G610C animals was subjected to differential scanning calorimetry to determine the identities of the collagen species (Figure IV-4). As expected, wildtype collagen showed only normal heterotrimeric type I collagen, made of two α1(I) chains and one α2(I) chain [$\alpha1(I)_2\alpha2(I)$]. Collagen from G610C/+ animals

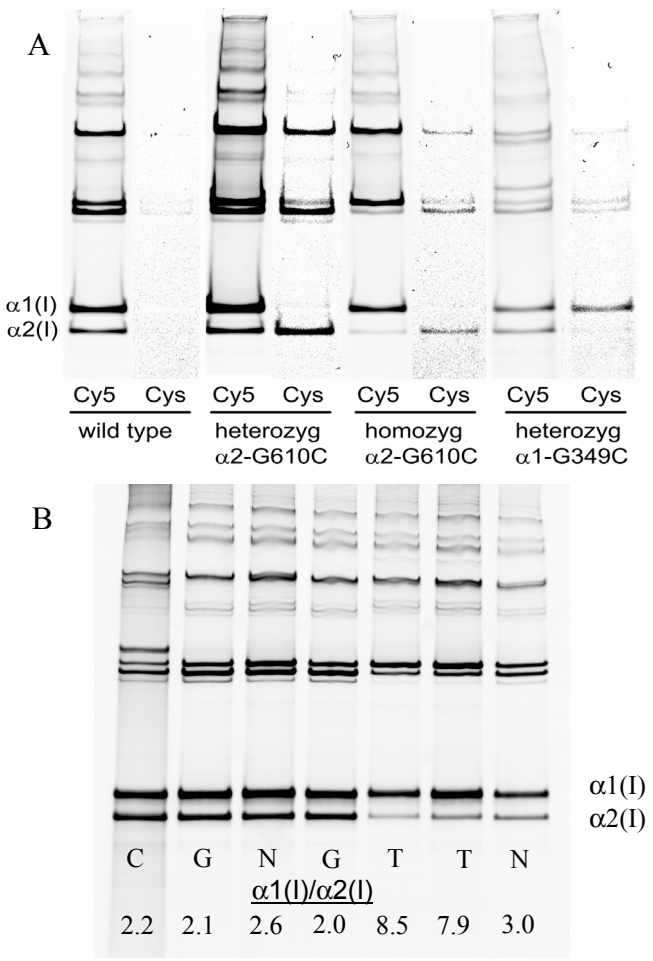


Figure IV-3. Protein analysis of tail tendon isolated from wildtype, G610C/+ and G610C/G610C mice. A) Fluorescent labeling of collagen with Cy5 or Cys by BODIPY-L-Cys demonstrates the G610C protein is incorporated into collagen extracted from heterozygous and homozygous tail tendon. Type I collagen isolated from the Brtl IV (G349C) mouse, which carries a glycine to cysteine mutation in the α1(I) chain is used as an experimental control. **B:** Tris/acetate 3-8% gradient gel shows altered a1(I)/a2(I) ratios in type I collagen extracted from heterozygous and homozygous tail tendon. C: Brtl Control, G: WT, N: G610C/-, T: G610C/G610C. Provided by Dr. Daniel McBride, University of Maryland-Baltimore.

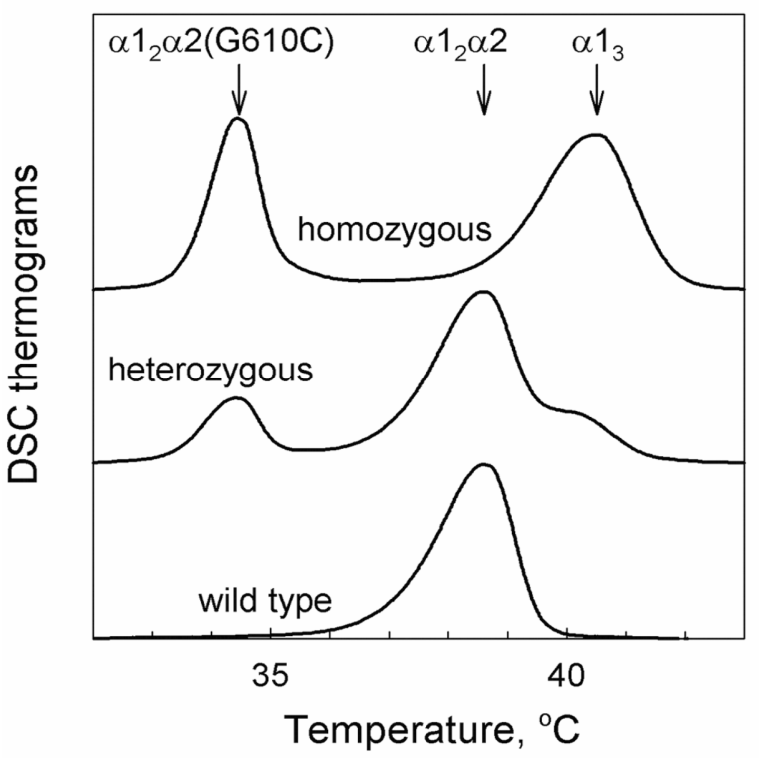


Figure IV-4. Protein analysis demonstrates three species of type I collagen. Differential scanning calorimetry demonstrates that wildtype animals produce only normal $\alpha1(I)_2\alpha2(I)$ heterotrimeric type I collagen whereas mice carrying the G610C mutation produce abnormal $\alpha2(I)$ chains as well as $\alpha1(I)_3$ homotrimeric type I collagen. Note the absence of normal heterotrimer in G610C/G610C. Provided by Dr. Daniel McBride, University of Maryland-Baltimore.

showed both the expected collagen species: normal $\alpha1(I)_2\alpha2(I)$ collagen and mutant G610C collagen [$\alpha1(I)_2\alpha2(G610C)$]. An unexpected finding was the deposition of a small amount of homotrimeric type I collagen [$\alpha1(I)_3$] in the G610C/+ animals. When collagen from G610C/G610C animals was analyzed, two collagen species were seen, in approximately the same concentration.

As the hallmark of OI is reduced biomechanical integrity of bones, the next goal and our role in the characterization of newly created G160C mouse was to evaluate changes in bone geometry and bone biomechanical integrity. We predicted that G610C/+ and G610C/G610C mice would have reduced biomechanical integrity as most individuals carrying the G610C allele were found to have low bone mineral density and an increased number of fractures. However, all of the people identified as carrying the G610C mutation carried only one copy of the mutant allele and so it was unknown if carrying two copies of the mutant allele would have a more severe phenotype than G610C/+ animals. This model also provided a unique opportunity to study whether the G610C mutation has a gene dose effect.

METHODS

Animals

Heterozygote G610C animals were cared for in an AAALAC accredited facility at the University of Maryland-Baltimore. Animals had ad libitum access to standard rodent food and water and cared for in compliance with an approved University of Maryland Animal Care and Use Protocol. Genotypes (Wt, G610C/+, G610C/G610C) were determined by direct sequencing of DNA samples obtained from tail snips.

Experimental Design

Animals (Wt n=5, G610C/+ n=8, G610C/G610C n=7) were sacrificed at four months (peak bone mass) of age via CO_2 asphyxiation. Both femurs were removed, cleaned of soft tissue attachments, wrapped in strips of sterile gauze soaked in sterile 1X PBS and stored at -80^{o} C.

μCT Analysis

Geometric parameters were defined from right femurs by μCT scan analysis (MicroCAT II, ImTek, Inc., Knoxville, TN) prior to *ex vivo* torsional loading to failure. Please refer to page 58 for a detailed description of these analyses.

Torsional Loading to Failure

Bone biomechanical parameters were analyzed using a combination of μCT analyses and torsional loading to failure. Please refer to page 60 for a detailed description of the experimental set-up and analyses. One exception: femurs for torsional loading to failure were potted using strips of parafilm instead of paper washers.

Statistical Analyses

All statistical analyses were done using the SAS program (SAS Institute Inc., Cary, NC). The analysis of variance was analyzed as a split plot in space. The main plot contained the effect gene and the sub-plot contained the effects side (left versus right) and gene x side. There was no significant difference due to side or gene x side. If the F test was significant for gene, Fisher's least significant difference (LSD) was used to

determine mean differences. A polynomial orthogonal contract analysis was performed to test for linear and/or quadratic gene dose effect.

To compare G610C to *oim*, the data was analyzed as a completely randomized design in which treatments were arranged as a 2 x 3 factorial (2 strains, 3 genotypes). Mean differences were determined using Fisher's least significant differences (LSD).

RESULTS

G610C femurs do not exhibit alterations in geometry

To determine if the G610C glycine to cysteine substitution in the α2(I) collagen chain caused alterations in femoral geometry, femurs from four month-old animals underwent μCT analysis. Measurements included total femur length, medio-lateral diameter, anterior-posterior diameter, marrow cavity diameter, and cortical bone width. None of these parameters were significantly different between wildtype controls, G610C/+ heterozygotes or G610C/G610C homozygotes (Table IV-1).

Femurs from G610C animals have reduced biomechanical integrity

To determine biomechanical integrity, the same femurs that underwent μCT analyses were then subjected to torsional loading to failure. The combination of μCT analysis and torsional loading allows for the separation of macroscopic whole bone properties from microscopic material properties. Macroscopic properties describe the bone as a whole, including any geometrical adaptations the animal may have made. Microscopic properties have been normalized to subtract out the geometric contribution and thus describes only the material properties of the bone.

Table IV-1. Summary of geometrical properties of femurs from 4-month old wildtype (+/+), heterozygote (G610C/+) and homozygote (G610C/G610C) mice as determined by μCT analysis.

	Femur Length (mm)	ML[a] Diameter (mm)	AP[b] Diameter (mm)	ML[a] Endosteal Diameter (mm)	Marrow Cavity Diameter[c] (mm)	Cortical Bone Width[c] (mm)
Wt N=5	15.5 ± 0.727	1.69 ± 0.171	1.34 ± 0.109	0.676 ± 0.215	0.550 ± 0.154	0.482 ± 0.052
G610C/+ N=8	15.2 ± 0.446	1.69 ± 0.111	1.34 ± 0.085	0.741 ± 0.086	0.635 ± 0.061	0.440 ± 0.034
G610C/G610C N=7	15.4 ± 0.588	1.70 ± 0.065	1.39 ± 0.066	0.715 ± 0.051	0.609 ± 0.043	0.469 ± 0.031
P value	0.8013	0.9735	0.4422	0.6510	0.2608	0.1506

All measurements except femur length were taken at the mid-shaft slice. [a]ML: mediolateral; [b]AP: anterior-posterior; ML: medio-lateral; [c]Marrow cavity diameter and cortical bone width values are averages of measurements taken in the ML and AP directions

Torsional ultimate strength (T_{max}) is a macroscopic property that measures the ultimate torsional force needed to break the bone. The corresponding microscopic property is tensile strength (Su), which measures the ability of the material to resist forces without breaking. Torsional stiffness (Ks) is the macroscopic property that describes the stiffness, or ability to resist torsion without breaking, of the whole bone while shear modulus of elasticity (G), a microscopic property, describes the elasticity of the bone material. The final macroscopic property is energy until failure (U), which describes the amount of energy the bone absorbs until it fractures.

T_{max} was significantly reduced (45%) in G610C/G610C animals compared to wildtype (Figure IV-5a). Tensile strength (Su), the corresponding microscopic property, showed similar results. G610C/G610C tensile strength was reduced by 51% as compared to wildtype (Figure IV-5b). For both of these parameters, heterozygote femurs had intermediate values to wildtype and G610C, thought not statistically significant. When gene dosage effect was analyzed, both T_{max} and Su exhibited linear effects.

The next biomechanical parameter, energy until failure (U), had a similar profile to T_{max} and Su. Energy until failure was significantly reduced (63%) in G610C/G610C femurs relative to wildtype femurs (Figure IV-5c). G610C/+ heterozygotes had intermediate values for U relative to wildtype and G610C/G610C homozygote femurs, though not statistically significant. This parameter also showed a linear gene dosage effect.

Stiffness had a very different profile than T_{max} or Su. Neither torsional stiffness (Figure IV-6a), a macroscopic property, or shear modulus of elasticity (Figure IV-6b), the microscopic property, were different between the genotypes. Heterozygote and G610C

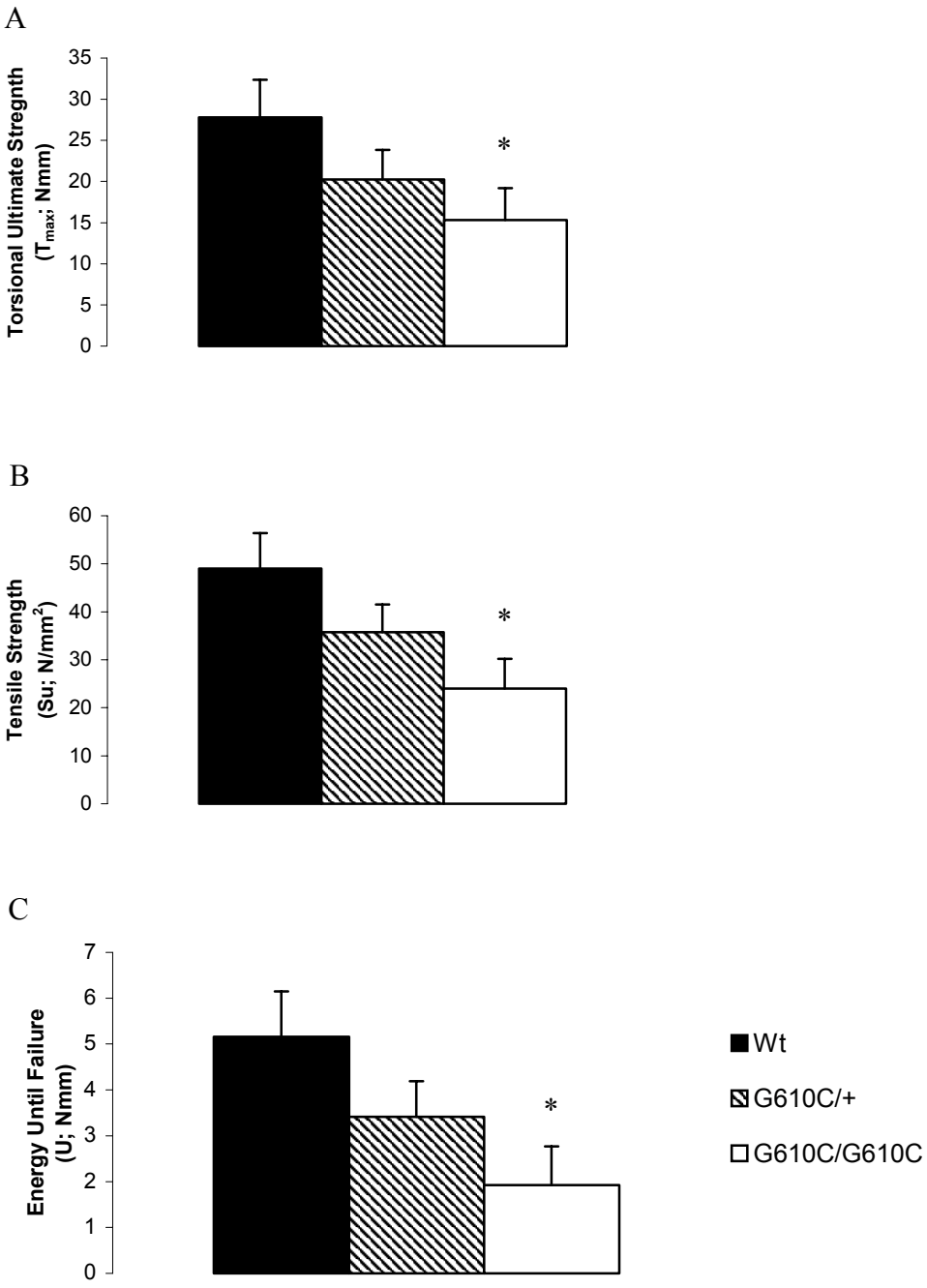


Figure IV-5. Biomechanical integrity of Wt, G610C/+ and G610C/G610C femurs as determined by torsional loading to failure. * p value <0.05 compared to wildtype. [Wt, n=5; G610C/+, n=8; G610C/G610C, n=7]

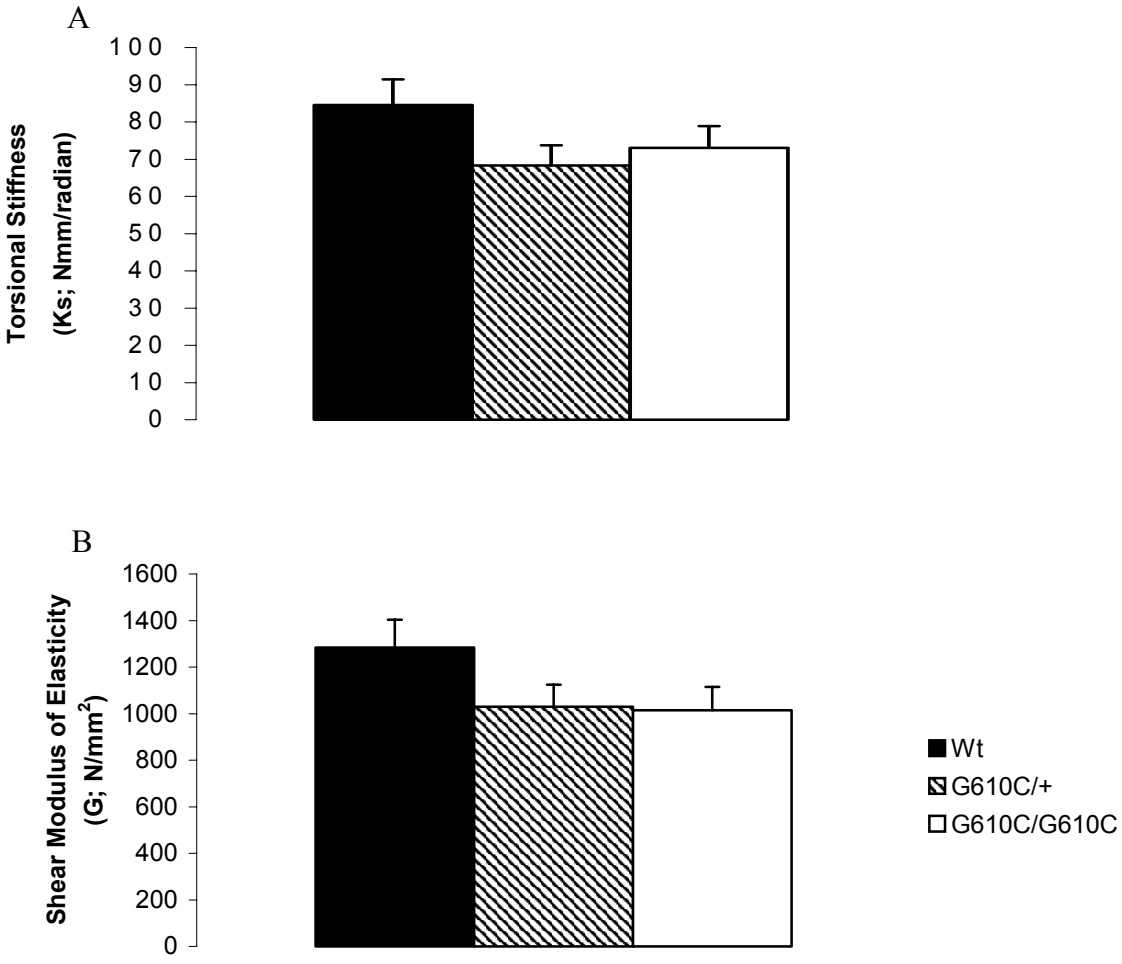


Figure IV-6. Femoral stiffness of Wt, G610C/+ and G610C/G610C femurs as determined by torsional loading to failure. [Wt, n=5; G610C/+, n=8; G610C/G610C, n=7]

homozygote femurs did exhibit some reduction in Ks and G as compared to wildtype, though it was not significant.

Comparison of G610C femurs to C57-*oim* femurs

The G610C mouse is a model of OI types I/IV while the *oim* mouse is a model for OI type III. Since both mouse strains are maintained on the same C57BL/6J background, we were able to directly compare alterations in the geometrical and biomechanical parameters due to the different mutations. We hypothesized that femurs from animals carrying the type III OI-causing *oim* mutation would have lower biomechanical integrity than femurs from mice carrying the type I/IV OI-causing G610C mutation.

When geometry was evaluated, there were no geometric differences in femurs from the different genotypes for the G610C strain. The *oim* mutation, however, did have shorter femurs, smaller marrow cavity diameters and a smaller polar moment of area as compared to wildtype (Figures II-7, 8, 10). As geometry did not impact the G610C mutation, further analyses were focused on the biomechanical parameters.

When the two mutations were analyzed for T_{max}, *oim* and G610C homozygote femurs both showed 52% and 45% reduction in torsional ultimate strength, respectively, as compared to their wildtype littermates (Figure IV-7a). *Oim/oim* and G610C/G610C femurs did not have significant differences in T_{max}. The same was true for the microscopic parameter tensile strength (Figure IV-7b). Both *oim* and G610C homozygotes had significantly reduced Su, 38% and 51%, respectively, compared to their wildtype littermates. Again, there were no significant difference between Su of

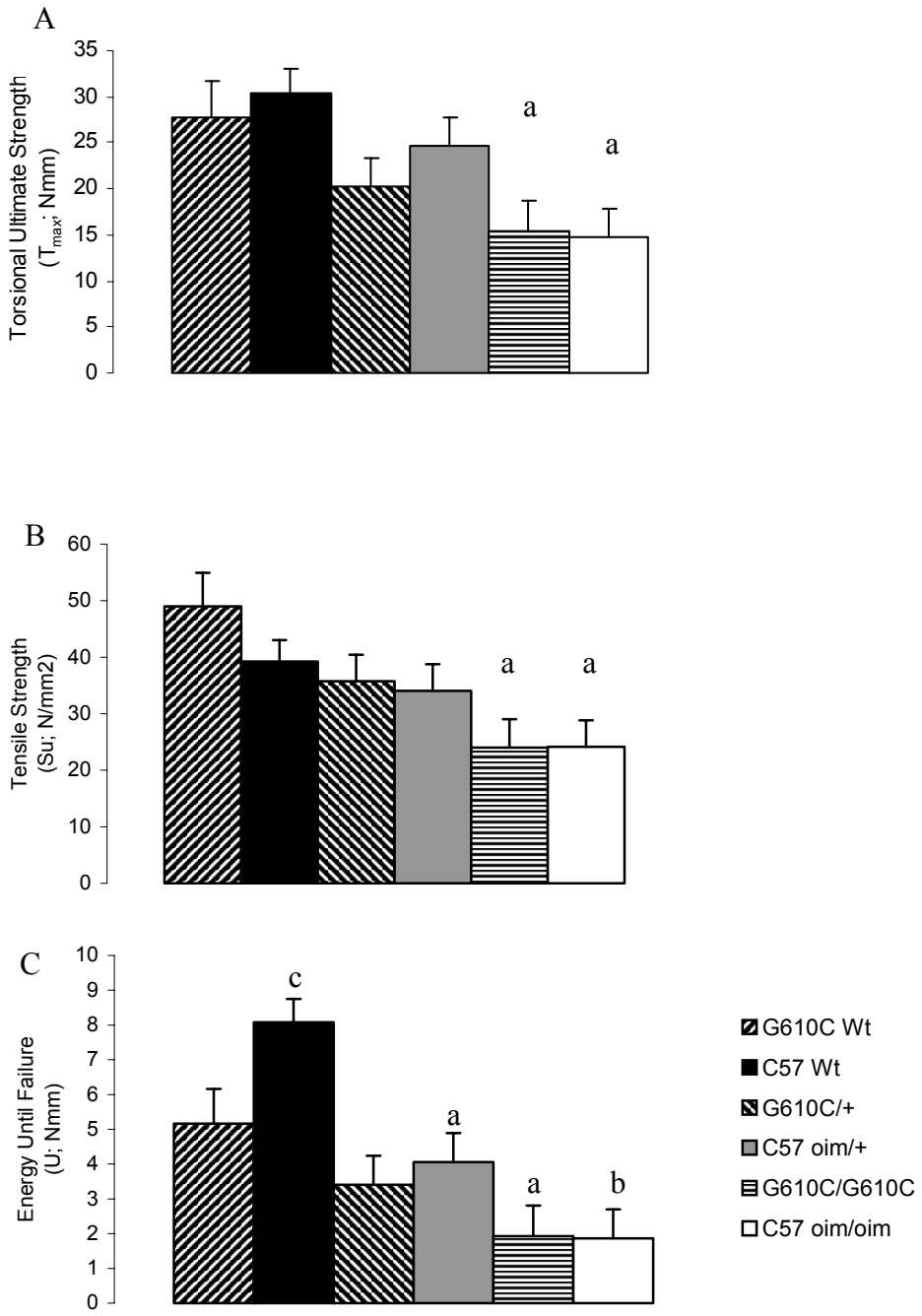


Figure IV-7. Comparison of biomechanical integrity of femurs from C57-*oim* and C57-G610C mice. [a] p value <0.05 compared to Wt littermates; [b] p value <0.0001 compared to Wt littermates; [c] p value <0.05 compared to G610C Wt. [G610C Wt, n=5; C57 Wt, n=12; G610C/+, n=8; C57 *oim*/+, n=8; G610C/G610C, n=7; C57 *oim/oim*, n=8]

homozygote *oim* and G610C femurs. The next parameter, energy until failure, exhibited the greatest reduction for both mutations. Femurs from wildtype littermates absorbed 77% more energy before fracturing than *oim* homozygotes (Figure IV-7c). Wildtype femurs absorbed 63% more energy prior to fracturing than their G610C/G610C littermates. There was also a significant difference between wildtype littermates from the two mouse lines.

When stiffness parameters, torsional stiffness and shear modulus of elasticity, were examined, no differences were seen between Wt and *oim* or Wt and G610C/G610C. Differences in stiffness between *oim* and G610C/G610C were also not seen. However, there was a significant difference between wildtype littermates from the two mouse lines.

DISCUSSION

Current mouse models of OI include the *oim* and Brtl IV models. The most extensively studied model is the osteogenesis imperfecta murine (*oim*), a naturally occurring mouse model of OI originally identified at Jackson Laboratory due to its small size and bone deformities. This model is the most severely affected of any of the viable mouse models due to a spontaneous nucleotide deletion in the proα2(I) collagen (COL1A2) gene, producing an abnormal protein product that is unable to be incorporated into the mature collagen molecule (Chipman, Sweet et al. 1993; McBride and Shapiro 1994). Therefore, these animals are functionally null for the proα2(I) collagen chain and produce homotrimeric type I collagen, consisting of three α1 chains [$\alpha1(I)_3$]. As a result of the *oim* mutation, homozygote mice have bowing of the long bones and reduced biomechanical integrity, similar to that of a human OI type III patient (Pihlajaniemi,

Dickson et al. 1984; Chipman, Sweet et al. 1993), who also produced only homotrimeric type I collagen. Clinical features of this patient included numerous spontaneous fractures as well as short stature, severe deformities and bowing of the limbs (Nicholls, Osse et al. 1984). The biggest disadvantage of this model is that although the phenotype mimics that seen in humans, the type of mutation and mode of inheritance are extremely rare. Most OI-causing mutations are glycine substitutions, not deletions causing homotrimeric type I collagen production. The *oim* mutation also has an autosomal recessive mode of inheritance, which is unusual in the human OI population where most mutations are dominant.

The Brtl IV mouse model is a relatively new transgenic mouse model that carries a glycine to cysteine substitution at position 349 of the COL1A1 gene, leading to the production of mutant α1 chains (Forlino, Porter et al. 1999). This mouse was created to mimic the type IV OI phenotype of a human patient (Sarafova, Choi et al. 1998). This patient showed short, bowed limbs prenatally and had her first femur fracture at two weeks of age. By age five, the child had suffered seven femur fractures and had undergone intermedullary rodding (Sarafova, Choi et al. 1998). The Brtl IV model is the result of a glycine substitution, the most common molecular cause of OI in the human population. However, the exact mutation carried by these animals has only been identified in one patient. This model also has some drawbacks as some of the heterozygote animals died in the perinatal period due to respiratory insufficiency for unknown reasons (Forlino, Porter et al. 1999). The maintenance of this mutation on a mixed genetic background also makes genotype-phenotype correlations difficult.

The G610C mouse has distinct advantages over the *oim* and Brtl IV mouse models of OI. It is the first animal model of OI to have the same genotype and phenotype as a large human OI population. Both the *oim* and Brtl models are based on human mutations, but their mutations are only present in one individual (Chipman, Sweet et al. 1993; Sarafova, Choi et al. 1998). The G610C is also the first model in which both mouse and extensive human data and numerous human fibroblast cell lines are available, allowing direct comparison between the two species. The ability to do direct mouse-human comparison will be particularly advantageous for testing drug therapies. Both the G610C and Brtl models result from glycine substitutions, the most common type of OI-causing mutation (Dalgleish 2006), and although the G610C mouse carries the mutation in the α2(I) chain and the Brtl mouse carries the mutation in the α1(I) chain, the study of both of these models may help determine the role of each of the chains in bone biomechanical integrity. The G610C model has several advantages over the Brtl and *oim* models. First, unlike the Brtl model, the G610C model is maintained on an inbred background making the study of modifier genes possible. Secondly, the G610C model results from the most common type of OI-causing mutation, a glycine substitution, while the unique nature of the *oim* mutation does not directly apply to the majority of the human OI population.

Unlike the *oim* mutation, the G610C mutation did not alter femoral geometry. G610C/G610C femurs were virtually unchanged in length as compared to their wildtype littermates, whereas *oim*/*oim* femurs were statistically shorter than their wildtype littermates. This difference may be a reflection of the type of OI each model represents. The G610C mutation is a model for OI type IV, in which patients do not necessarily

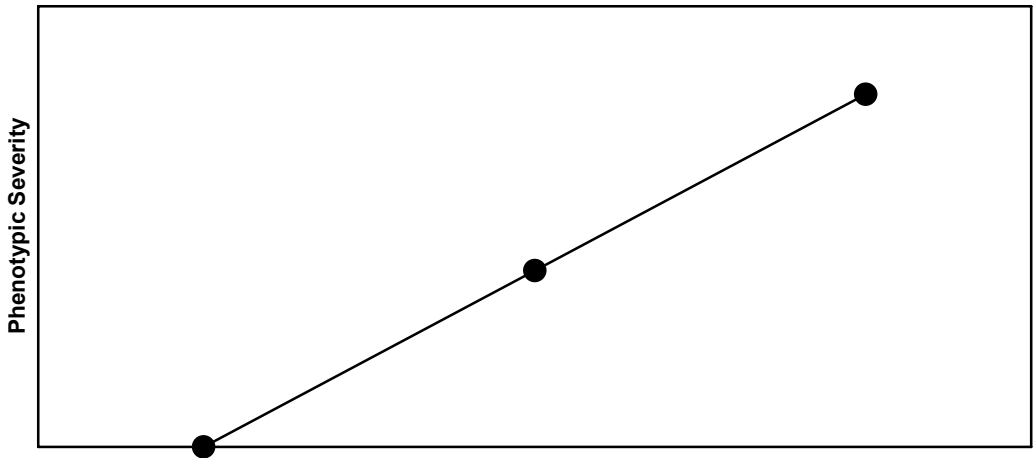
exhibit changes in stature. Conversely, the *oim* mutation represents OI type III in which patients are much shorter than other aged-matched children. Also, G610C/+ and *oim*/+ heterozygotes were significantly different from each other in all geometrical parameters except femur length and cortical bone width. These differences may be due to 1) the mixture of normal and mutant collagen types and 2) the identity of the collagen types within this mixture. G610C/+ animals carry two types of α2(I) chains, normal and $\alpha2(I)_{G610C}$. *Oim*/+ animals carry only one type of α2(I) chain, but half of their collagen is normal heterotrimeric collagen while the other half is the abnormally expressed homotrimeric type I collagen.

When biomechanical parameters were compared between the C57-*oim/oim* and C57-G610C/G610C, no differences were seen. This was surprising as the *oim* mutation mimics the more severe phenotype of human OI type III while the G610C mutation is known to cause OI type IV in the human population. However, it should be noted that in the human population, only G610C/+ heterozygote patients have been identified to date and diagnosed with OI types I/IV. If two G610C/+ patients were to produce a G610C/G610C offspring, it would be expected that the child would have a more severe phenotype than the parents, perhaps more similar to human OI type III, which is seen in the study presented here. However, the lack of differences between homozygote *oim* and G610C animals may be due in part to the presence of the homotrimer in the G610C animals. The unexpected presence of the $\alpha1(I)_3$ collagen may be a consequence of reduced α2(I) collagen or related to the presence of the neomycin cassette, which has not yet been removed from the mice. When human fibroblasts from G610C carriers as well as collagen from G610C/G610C mice no longer carrying the cassette were examined, no

homotrimeric type I collagen was seen (Daniel McBride, personal communication). Once the neomycin cassette has been removed, these protein and biomechanical experiments should be repeated. The homotrimer may be causing a more severe phenotype in conjunction with the presence of the G610C mutation than the mutation alone. When human fibroblasts from G610C carriers as well as collagen from G610C/G610C mice tails no longer carrying the cassette were examined, no homotrimeric type I collagen was seen (Daniel McBride, personal communication).

Gene dosage effect of the G610C mutation was analyzed to determine if observational data that one mutant allele gives a less severe clinical outcome than two mutant alleles. For biomechanical parameters where G610C/G610C animals were significantly different from wildtype animals, a polynomial orthogonal contract analysis was performed to test for linear and/or quadratic gene dose effect. A linear gene dose effect suggests that a linear relationship exists between the number of mutant alleles and the severity of the disease. A quadratic gene dosage effect suggests that the presence of one mutant allele is either better or worse than zero or two mutant alleles. For the biomechanical parameters T_{max}, Su and U, a linear gene dosage effect was seen (Figure IV-18), suggesting that G610C/+ animals have a less severe phenotype than G610C/G610C animals.

The G610C mouse model of OI is the first model to mimic a large human OI population in both genotype and phenotype. The present study showed that homozygosity for the COL1A2 G610C loci is not lethal and produces a gene dose effect. Heterozygosity for the G610C allele has a biomechanical phenotype intermediate to wildtype and homozygotes. These animals also have a greater phenotypic variability



	Wt	G610C/+	G160C/G610C
G610C Alleles	0	1	2
Wt Alleles	2	1	0

Figure IV-8. The G610C allele has a gene dose effect on T_{max}, Su and U. One copy of the mutant allele is sufficient to show disease characteristics. However, the addition of a second mutant allele induces a more severe disease phenotype.

than homozygotes, mimicking the human condition. Homozygosity for the G610C mutation does not alter bone geometry, torsional stiffness or shear modulus of elasticity (G). Two copies of the G610C mutation significantly reduce ultimate torsional strength, tensile strength and energy until failure. Future studies will need to be done once the neomycin cassette is removed to determine if the presence of the homotrimeric type I collagen contributes to the phenotype of the G610C mice.

The G610C mutation will also be bred on to another inbred mouse strain, DBA/2J, known to have high, but phenotypically normal bone mineral density (Beamer, Donahue et al. 1996). The femurs from these animals will then be analyzed as described in this study. The mineral composition of femurs from both strains will also be analyzed using neutron activation analysis to determine if the G610C mutation, genetic background or a combination of the two alters trace mineral content of the bones. By comparing the same mutation on different genetic backgrounds, modifier genes within the genetic background may be able to be identified that contribute to the clinical variability seen in the human G610C population. The *oim* mutation is also being bred on to the DBA/2J strain and maintenance of the *oim* and G610C mutations on the same inbred backgrounds (both C57 and DBA) will also allow for future examination of the effect of the specific OI-causing mutation on the clinical outcome of the disease.

Taken together, these studies indicate the G610C mutation leads to alterations in type I collagen, weakening both macroscopic structural properties and microscopic material properties without appearing to impact the geometric properties of the femur. These properties make the G610C mouse a viable model in which to study gene dose effects as well as mechanisms impacting the phenotypic severity of OI.

CHAPTER V

PERSPECTIVES AND FUTURE DIRECTIONS

The aims of the research presented in this dissertation were 1) to analyze the role of genetic background, and the modifier genes contained therein, in determining phenotypic severity of the *oim* mutation at peak bone mass, 2) analyze the effects of aging on the clinical outcome of the *oim* mutation, and 3) compare two different OI-causing mutations, a glycine substitution and a functional α2(I) null. Toward this end, the results presented here demonstrate that 1) bone geometry and biomechanical integrity are influenced by genetic background, 2) genetic background also influences mineral-specific changes in bone mineral composition and alters collagen content, 3) the *oim* mutation overrides the differences induced by genetic background and is the ultimate predictor of clinical outcome, 4) bone biomechanical integrity improves post-puberty and 5) homozygosity for the G610C and *oim* mutations yield similar phenotypic severities.

During the course of these studies, additional questions arose. The following four objectives are aimed at further understanding the role of genetic background in determining phenotypic severity of the *oim* mutation, as well as studies aimed at developing therapies to alleviate the symptoms of OI and improve the quality of life for patients.

Objective 1. Does the phenotypic severity of the *oim* mutation lessen when the mutation is present on an inbred DBA/2J mouse strain known to have high, but phentoypically normal, bone mineral density?

Rationale. The studies presented in this dissertation, in addition to studies on multiple strains of inbred mice, have shown differences in bone strength and quality in the absence of any disease causing mutation. A study using 11 strains of inbred mice found that DBA/2J (DBA) animals had 21% more bone mineral density than C57BL/6J (C57) animals (Beamer, Donahue et al. 1996). Geometric differences in bone were also seen between the two strains, with DBA animals having a smaller marrow cavity diameter with a corresponding increase in cortical bone width (Beamer, Donahue et al. 1996) as well as an overall smaller cross-sectional area (Akhter, Iwaniec et al. 2000). Consistent with these changes in femoral geometry, DBA animals demonstrated increased biomechanical integrity compared to C57 animals (Akhter, Iwaniec et al. 2000). These studies show that DBA animals have changes in geometry and mineral density that confers an increased biomechanical integrity.

The main goals within the field of OI, as well as in other bone diseases such as osteoporosis, are to increase bone biomechanical integrity in an effort to reduce the number of fractures sustained throughout the lifetime and, thereby, increase the quality of life for these patients. Studies of inbred strains of mice with naturally occurring differences in bone strength carrying the same *oim* mutation may provide insight into the mechanisms governing genotype-phenotype correlations as well as the role of genetic background and modifier genes in determining phenotypic severity. Changes in gene expression between the different strains may lead to the increased biomechanical strength, and these changes in gene expression may be potential targets for drug therapies.

Approach. To produce the DBA-*oim* line, the *oim* mutation will be bred from the outbred B6C3Fe background to the inbred DBA background. The congenic DNA background of heterozygote DBA-*oim* animals to be used as breeding pairs will be confirmed as described on page 57. Wildtype, heterozygote and homozygote DBA-*oim* animals will be genotyped as previously described (Phillips, Bradley et al. 2000).

Animals will be sacrificed at one, two and four months of age and tissues harvested as described on page 57. A similar experimental plan as described in this thesis will be used to test both femurs and tibias from these animals. Briefly, femurs from each of the three genotypes will undergo μCT analysis to determine femoral geometry as described on page 58 prior to *ex vivo* torsional loading to failure as described on page 60. Tibias will be used to determine bone mineral composition as outlined on page 66. Serum will be used to assess if changes in markers of bone turnover exist. If differences in bone quality are found, microarray or quantitative trait loci (QTL) studies can then be used to determine if differences in gene expression exist between the strains as well as between wildtype and *oim* animals of each strain. These differences in gene expression will then be analyzed to determine if these genes play a role in determining parameters known to impact bone strength.

Anticipated Results and Potential Problems. As the μCT, torsional loading to failure, mineral composition and serum markers of bone turnover analyses have previously been done in our laboratory, we do not anticipate any technical difficulties. We hypothesize that the DBA line will have differences in geometry based on previous studies (Akhter, Iwaniec et al. 2000) and improved biomechanical integrity (Beamer, Donahue et al.

1996) compared to the C57 line. We further expect the addition of the *oim* mutation to the DBA line will result in a less severe phenotype than that seen in the C57-*oim* animals. We also expect differences in bone mineral composition, both between wildtype and *oim* animals within a strain, but also between DBA-*oim* and C57-*oim* animals. The DBA line is also predicted to have an increased amount of collagen than the C57 line as well as decreased markers of bone turnover. These studies may help elucidate the mechanisms underlying the differences in bone strength seen in inbred strains of mice as well as those differences leading to the phenotypic variation seen within the OI population. These studies will also be foundational for future microarray analyses aimed at identifying specific modifier genes impacting the clinical severity of the *oim* mutation. These genes may present possible therapeutic targets aimed at enhancing positive bone qualities while minimizing negative bone qualities.

Objective 2. To examine the relationship between the osteoblast and surrounding matrix and the mechanism governing mineralization.

Rationale. Osteoblasts are the cells responsible for synthesizing and laying down the extracellular matrix, including type I collagen (Favus 1993) and, therefore, mutations affecting type I collagen may also impact these cells. And because type I collagen and hydroxyapatite crystals interact to form a unique composite material, alterations in type I collagen impact the mineral phase of the bone. *Oim* animals have less mineral as seen by reduced bone mineral content and density measurements (Fratzl, Paris et al. 1996). The mineral they have is made of thinner and less well aligned crystals than wildtype animals (Fratzl, Paris et al. 1996). Changes are also seen in the mineral composition of femurs

between *oim* and wildtype animals. These differences may reflect the impact type I collagen mutations have on mineral accretion in bones and teeth.

Approach. We will excise calvaria from wildtype and *oim* mice at one month of age and harvest the osteoblasts. These osteoblasts will then be grown on tissue culture plates coated with either heterotrimeric type I collagen harvested from wildtype tail tendon or homotrimeric type I collagen harvested from *oim* tail tendon and allowed to mineralize. Mineral resulting from these cultures will be collected and subjected to instrumental neutron activation analysis as previously described. The same type of study can be done using osteoblasts from G610C animals to study mineralization in a milder variant of OI. These studies will be particularly useful as they can be compared to data from the human patients carrying this mutation.

These studies will allow us to test several aspects of mineralization. First, by comparing the mineral made in tissue culture with the mineral made *in vivo* we will be able to determine if this system is valid for the study of type I collagen mutations and their effect on mineralization. This will also allow us to do microarray studies to determine if the osteoblasts function differently in wildtype and *oim* animals. Second, we will be able to study the interplay between the osteoblast and type I collagen. If the wildtype osteoblasts plated on the homotrimeric collagen produce wildtype mineral, we can deduce that the osteoblast is the major determinant in the mineralization of bone. Conversely, if the wildtype osteoblasts plated on homotrimeric collagen produce mineral like that seen in the *oim* mouse, it will tell us that the ECM and type I collagen have a major role in regulating bone mineralization. Thirdly, we can also see if *oim* really makes less bone. It is known that *oim* animals have reduced bone mineral density, but it

is not known if this is due to reduced bone synthesis or because of some other mechanism. By quantitating the amount of mineralization in tissue culture, we will be able to determine whether osteoblasts from *oim* animals are able to produce the same quantity of bone as osteoblasts from wildtype animals. Next, we will be able to study the translation of the α2(I) procollagen chain. It is known that *oim* animals produce homotrimeric type I collagen lacking the α2(I) chain and that these animals do produce α2(I) mRNA. However, it has been postulated, but not shown, that these animals may be able to either translate the mutant α2(I) mRNA into protein which is rapidly degraded or that the mRNA is not able to be translated at all. Lastly, we will be able to study the rates of collagen synthesis in wildtype and *oim* osteoblasts. We know that *oim* animals produce less type I collagen than wildtype animals. It is currently unknown whether this is due to 1) reduced production of type I collagen by the osteoblast, 2) unstable and rapidly degraded collagen or 3) less efficient incorporation of homotrimeric collagen into the matrix.

Anticipated Results and Potential Problems. We do not anticipate technical difficulties with the osteoblast harvesting or culture maintenance as Dr. Sarah Dallas at the University of Missouri-Kansas City has done these types of procedures before and is willing to teach us. We hypothesize that mineral produced in tissue culture will accurately mimic mineral produced *in vivo*. However, it is possible that the artificial environment of tissue culture will adversely effect mineralization. We also believe that homotrimeric type I collagen will influence mineralization as studies done on *oim* mice have shown abnormal mineralization and mineral composition. Further, we believe that

osteoblasts from *oim* mice will produce less bone than wildtype animals due to differences in the efficiency of mineralization. Upon analysis of α2(I) mRNA, we expect to find that it is translated into protein, but that the inability of the chain to be incorporated into the collagen leads to its degradation. We also expect to find reduced rates of collagen synthesis and secretion in *oim* animals due to the abnormal temporal and spatial expression of homotrimeric type I collagen.

Objective 3. To determine if the bone phenotype seen in *oim* animals can be improved by increased physical activity, such as swimming or walking on a treadmill, which can be translated into lifestyle changes for OI patients.

Rationale. Exercise is proposed to induce changes in bone by the mechanostat theory. This theory describes a mechanism by which the bone responds to changes in loading with alterations to bone architecture (Skerry 2006). These changes in loading can be positive, as in the case of chronic exercise, or negative, as seen with patients on long-term bed rest. If the force is moderate and sustained over a long period of time, it can induce these changes in either bone mass or geometry that will better allow the bone to resist these forces, ultimately resulting in a stronger bone (Skerry 2006). In the case of OI, the bone material is hypothesize to be too stiff and, therefore, cannot properly respond to mechanical stimuli (Rauch 2006), although muscles from OI patients or *oim* mice have not been studied. In these patients, a long-term exercise regimen may provide protection against fractures by chronically applying strain to the bone and, potentially, induce the positive effects seen in animal studies.

There are several lines of evidence that suggest exercise may improve bone strength and quality, although studies on the effect of exercise on OI patients or the *oim* mouse have not been done. Studies using ovarectomized rats found that an exercise program consisting of running the rats on a treadmill produced increased bone mineral density in both femoral and vertebrae as well as reduced bone resorption and increased bone formation compared to ovarectomized, non-exercised animals (Gala, Diaz-Curiel et al. 2001). Another study using non-ovarectomized rats found that loaded ulnas induced changes in bone geometry and structural properties and that these changes were sufficient to increase bone biomechanical parameters without changes to bone material properties (Warden, Hurst et al. 2005). Additional studies using the myostatin knock-out mice, which have abnormally large muscles due to disregulated muscle growth, have also been shown to have increased bone mineral density as well as increased bone biomechanical parameters (Hamrick 2003; Hamrick, Samaddar et al. 2006). These studies will be discussed further in objective 4.

The opposite effect has been seen in astronauts returning from space flight. Bone loss in these individuals resembles bone loss in the normal aging skeleton or in non-weight bearing situations (Doty 2004). The lack of gravity in space reduces the stress on the bone, decreasing collagen secretion, ultimately feeding back to the osteoblast to reduce collagen synthesis and bone formation (Doty 2004). Taken together, these studies have shown that regular exercise increases bone mineral density while space flight induces osteoporosis due to a net loss of bone.

Approach. Outbred wildtype, *oim*/+ and *oim/oim* to be swum or treadmilled will be aged to 2 ¾ months of age and weighed prior to each exercise session. Control animals of each genotype not undergoing exercise will be weighed once weekly for the duration of the study and allowed *ad libitum* access to food and normal cage activity. Mice undergoing swimming will be swum until four months of age with a maximum swim time of 30 minutes/day. Mice undergoing treadmilling will undergo an acclimation period prior to walking at 10 m/minute on a 7^{o} incline for 30 minutes per day, 5 days a week until they are four months of age. We will start this study using only wildtype, *oim*/+ and *oim/oim* animals from the outbred strain. If these animals are able to withstand this activity, C57 animals of each genotype will then be tested.

Following exercise, muscles from these animals, as well as controls, will be used for either 1) contractile capacity, 2) ATPase activity or 3) histology and collagen quantitation. Animals to be used in the contractile assay will be deeply anesthetized and the gastrocnemius, soleus, plantaris and tibialis anterialis muscles evaluated as previously described (Juppner, Brown et al. 1999). For the ATPase assay, gastrocnemius, quadriceps and soleus muscles will be prepared according to Guth, et al (Guth and Samaha 1970) to detect different fiber types within the muscle slice. The mouse will then be sacrificed via CO_2 asphyxiation, weighed, blood harvested by exsanguination, serum isolated and stored at -80^{o} C. For histology and collagen quantitation, the quadriceps and gastrocnemius muscles will be harvested and the muscle masses taken. Muscle sections for histology will be stained with picrosirius red to determine collagen content as well as hemotoxylin and eosin to assess cell morphology and structure. The hydroxyproline assay will be performed as described by Stegemann, et al (1967).

Femurs and tibias from exercised and control animals will also be analyzed for changes in bone geometry and biomechanical strength as well as histological changes. Bones to be used for μCt analysis and torsional loading to failure will be harvested, stored and tested as described previously. If changes in geometry or biomechanical integrity are seen, bone sections will be analyzed for histomorphometrical changes as described above. The hydroxyproline assay will also be done as previously described.

Anticipated Results and Potential Problems. As our lab has recently completed a pilot study swimming mice, we do not anticipate any technical difficulties with this aspect. We hypothesize that *oim* animals will be able to withstand the rigors of swimming, but that the buoyancy of swimming may not be sufficient to induce measurable changes in muscle mass or histology, bone geometry or bone biomechanical properties. The treadmill study will be done under the direction of Dr. Dongsheng Duan, so we do not anticipate any technical difficulties with this portion. One major concern is that the *oim* animals, especially C57-*oim* animals, will be unable to tolerate the weight-bearing forces induced by treadmilling. Should this be the case, these animals will be exercised for a shorter period of time or at a lower speed. We hypothesize that walking will increase muscle mass and pull on the bone, altering bone geometry and improving biomechanical integrity. Exercise should induce a switch from fast twitch to slow twitch fibers.

Objective 4. To determine if inhibition of myostatin will improve the bone phenotype seen in *oim* mice.

Rationale. There is currently no cure for patients with OI and the only treatments available are aimed at reducing fracture and increasing the quality of life. However, studies have not been done on the muscle pathology or the effect on the bone of increasing muscle mass in either OI patients or the *oim* mouse. A single paper was found in the literature in which a two year-old boy presented with extreme muscle weakness preventing him from walking. Further investigation found an osteopenic skeleton with signs of healing fractures and gel electrophoresis identified a type I collagen migration abnormality leading to an OI type IV diagnosis (Boot, de Coo et al. 2006). Another study examined muscle strength and exercise capacity in OI type I patients. This study found that patients exhibited muscle weakness and reduced exercise tolerance, but did not determine if this weakness was due to the sedentary lifestyle that often accompanies OI or to true disease pathology (Takken, Terlingen et al. 2004).

Studies on the myostatin knock-out mouse have found an association between increased muscle mass and improved bone biomechanical integrity. Myostatin is a member of the transforming growth factor (TGF)-β superfamily and is a negative regulator of muscle growth. Myostatin knock-out mice have shown increased muscle mass which results in an increased load on the bone leading to increased bone biomechanical parameters (Hamrick 2003; Hamrick, Samaddar et al. 2006). By breeding myostatin knock-out mice with C57-*oim* mice, we will investigate the possibility that knocking out the myostatin gene will increase muscle mass and improve the quality of bone in the mstn/mstn/*oim*/+ or mstn/mstn/*oim*/*oim* mice.

Approach. Mice heterozygote for both the myostatin and *oim* mutations will be bred to produce mstn/+ (heterozygous for myostatin mutation only), *oim*/+ (heterozygous for *oim* mutation only), mstn/+/oim/+ (heterozygous for both mutations) and mstn/mstn/*oim/oim* (homozygous for both mutations) offspring. Muscle will be collected from animals from each experimental group as well as wildtype littermates and subjected to analyses of muscle quality and function, muscle mass and contractile generating capacity. Bone will also be collected from the same animals and subjected to bone analyses as described above, including μCT analysis, torsional loading to failure, hydroxyproline analysis to quantitate type I collagen and histomorphometrical analyses. Serum will also be collected to evaluate markers of bone turnover.

Anticipated Results and Potential Problems. We anticipate that mstn/mstn single homozygotes will have larger muscles than any of the other genotypes. Furthermore, we hypothesize that mstn/mstn/*oim*/+ and mstn/mstn/*oim/oim* will have larger muscles and improved bone biomechanics compared to *oim/oim* animals. Although unlikely, the increased size of the muscles caused by knocking out the myostatin gene may be too strenuous for the fragile bones of the *oim/oim* animals. Should this be the case, either mstn/+/*oim*/*oim* or mstn/+/*oim*/+ animals will be used. If these studies prove fruitful, the drug MYO-029, a human monoclonal antibody against myostatin (Wyeth Pharmaceuticals), will be a potential treatment option for human OI patients. MYO-029 is currently in a Phase I/II clinical trial for use in treating certain types of muscular dystrophies. Administration of MYO-029 to *oim* mice will also allow us to circumvent any breeding problems or adjust the dose of myostatin to control the effect of the drug on muscle size.

APPENDIX I

ANALYSIS OF TRACE MINERAL COMPOSITION OF THE BRITTLE IV (Brtl) MOUSE

INTRODUCTION

While the overwhelming majority of research into osteogenesis imperfecta has focused on the *oim* mouse model, several other models of OI are starting to emerge. The Brtl IV (Brtl) mouse is a transgenic mouse carrying a GLY349-CYS mutation in one COL1A1 allele (Kozloff, Carden et al. 2004). This mutation leads to the production of mutant α1(I) chains, resulting in a phenotype similar to that of human OI type IV, characterized by bone fragility, an increased number of pre-pubertal fractures, short stature, mild to moderate bone deformity with possible dentinogenesis imperfecta and premature hearing loss (Dalgleish 1997; Kozloff, Carden et al. 2004). This mouse was created to mimic the type IV OI phenotype of a human patient (Sarafova, Choi et al. 1998). Short, bowed limbs were found prenatally with the first femur fracture at two weeks of age. By age five, the child had suffered seven femur fractures and had undergone intermedullary rodding (Sarafova, Choi et al. 1998). She was ambulatory without scoliosis with a height of 50% for that of a two year old and dentinogenesis imperfecta (Sarafova, Choi et al. 1998).

The Brtl animals are capable of producing three types of type I collagen: normal heterotrimeric [$\alpha1(I)_2\alpha2(I)$] collagen, collagen carrying one normal and one mutant α1(I) chain [$\alpha1_{G349C}(I)\alpha1(I)\alpha2(I)$] and collagen carrying two mutant α1(I) chains [$\alpha1_{G349C}(I)_2\alpha2(I)$]. Normal and $\alpha1_{G349C}(I)_2\alpha2(I)$ type I collagen each represent 25% of the total type I collagen found in tail tendon from Brtl mice with the remaining 50% being $\alpha1_{G349C}(I)\alpha1(I)\alpha2(I)$ (Kuznetsova, Forlino et al. 2004). Since type I collagen molecules spontaneously align to form fibrils, these variations in collagen speciation may lead to disruption of the staggered array of the molecules within the fibrils, thus

impacting the biomechanical properties of the tissues containing these fibrils (Kuznetsova, Forlino et al. 2004).

Previous studies indicate changes in bone quality at or around the time of puberty. At the pre-pubertal ages of one and two months, Brtl mice had a lower bone mineral density (BMD) and reduced bending moment and maximum load as compared to age-matched controls (Kozloff, Carden et al. 2004). Cross-sectional area and cortical thickness were also reduced (Kozloff, Carden et al. 2004). However, at the post-pubertal age of 12 months all parameters except BMD were comparable to wildtype (Kozloff, Carden et al. 2004). Raman spectroscopy suggested a reduced mineral-to-matrix ratio in six month, but not two month, Brtl animals (Kozloff, Carden et al. 2004). These data suggest pubertal adaptations, paralleling those seen in human OI type IV patients, in matrix properties without apparent alterations in whole bone properties (Website; Dalgleish 1997; Kozloff, Carden et al. 2004).

Instrumental neutron activation analysis (NAA) provides an exceptionally sensitive method by which to measure trace minerals in the bones of Brtl and age-matched controls. NAA is a non-destructive analysis in which a sample is irradiated to produce a radioactive nuclide, which will decay by emitting γ rays. The number of γ rays emitted indicates the quantity of a particular element, while the energy of the γ rays indicates the identity of the element (Website).

NAA has several unique properties making it a superior method by which to study trace mineral composition. The non-destructive nature of NAA allows for the samples to be used for other downstream applications and the sensitivity of the system allows for

analysis of small samples in which other methods have failed. Other advantages and disadvantages of this system are discussed on page 93.

This study was aimed at further addressing the alterations in mineral seen with Raman spectroscopy. Several minerals were quantitated using NAA, including fluorine, phosphorus and calcium. These data show an altered mineral content in Brtl mice as compared to age-matched controls as well as age-related changes in mineral composition.

METHODS

Animals and Sample Preparation

Mice were generated and sacrificed as previously described (Forlino, Porter et al. 1999; Kozloff, Carden et al. 2004). 10 Wt and 10 Brtl mouse tibias at two and six months of age were evaluated. Tibias were harvested and soft tissue removed prior to being frozen on dry ice and stored at –80^{o} C. Before analyses, the epiphyses were removed and the shaft demarrowed. The samples were then delipified for 10 minutes via acetone sonication (Model G-80-80-1, Laboratory Supplies Company, Hicksville, NY), washed with two water sonications and lyophilized in a Freezemobile 12SL (Virtis, Gardiner, NY) for two and a half hours. The samples were then weighed (Model AT261, Mettler Toledo, Columbus, OH) and loaded in high-density polyethylene vials. A foam spacer was placed on top of the specimen to ensure the similar sample geometry for all tests.

Table AI-1. Instrumental NAA Parameters

Ion	Nuclear Reaction	Photopeak (keV)	Irradiation Time	Decay Time	Count Time	Count Position
F	$F^{19}(n,\gamma)F^{20}$	1633	7 sec	15 sec	30 sec	Face
P	$P^{31}(n,\alpha)Al^{28}$	1779	15 sec	1 min	3 min	Face
Na	$Na^{23}(n,\gamma)Na^{24}$	1368	7 sec	1 min	10 min	1 spin[b]
Mg	$Mg^{26}(n,\gamma)Mg^{27}$	844, 1014	7 sec	1 min	10 min	1 spin
Ca	$Ca^{48}(n,\gamma)Ca^{49}$	3085	7 sec	1 min	10 min	1 spin
K	$K^{41}(n, \gamma)K^{42}$	1524	95 sec	EOI[a]	30 min	1[c]
Zn	$Zn^{68}(n, \gamma)Zn^{69m}$	438	95 sec	EOI[a]	30 min	1[c]

[a] EOI: End of Irradiation Time is the specific time the sample exited the reactor. All samples and SRMs were decay corrected back to this time.
[b] Count position was in a rotating position 2.5 cm from the detector face.
[c] Count position was stationary 2.5 cm from the detector face.

Instrumental NAA

Standards and samples were activated by a thermal neutron flux of $5x10^{13}$ n/cm^2/s for specific times (see Table AI-1) to activate F, Na, Mg, Cl, Ca K and Zn and a 5 x 10^{12} n/cm^2/s flux to activate P. Milk powder, non-fat milk powder and bone ash were used as standards for all elements (NIST SRMs 8435,1549 and 1400, respectively, Bethesda, MD). The samples and standards were counted using a high-resolution gamma-ray spectrometer with a HPGe detector face (EG&G Ortec, Oak Ridge, TN). The spectrometer was connected to a spectroscopy amplifier (Tenelee 244, Oak Ridge, TN) and a loss-free counting module (Model 599, Canberra-Nuclear Data, Meriden, CT). Data acquisition and processing was performed using an ADC (Model 581, Canberra-Nuclear Data) and computer (Digital MicroVax, Maynard, MA).

Statistical Analyses

All statistical analyses were done using SAS (SAS Institute Inc., Cary, NC). All comparisons were analyzed using a general linear model. When heterogeneous variations made it necessary, a log transformation was used to stabilize the variation. If this log transformation failed to stabilize the variation, a ranking procedure was used (Conover and Iman 1982). Mean differences were determined using Fisher's Least Significant Difference (LSD). Means and standard deviations presented are the actual numbers, although p values reflect the transformed or ranked data. All results are presented as mean ± standard deviation. Differences were considered to be statistically significant at p-value ≤ 0.05.

RESULTS

Brtl mice demonstrate an altered bone mineral composition compared to wildtype at both 2 and 6 months of age

Brtl mouse tibias were compared to wildtype mouse tibias for each element at both two and six months of age. The differences observed were mineral and age specific. At two months of age, Brtl mouse tibias had higher levels of phosphorus, magnesium and zinc than wildtype tibias, but lower levels of sodium and potassium. Brtl tibias also had a reduced Ca/P ratio, a measure of phenotypic severity, compared to wildtype at two months of age (Fig. AI-1). At six months of age, Brtl mouse tibias had higher levels of both potassium and zinc than wildtype tibias. No differences in phosphorus, magnesium, sodium or Ca/P ratio were seen between Brtl and wildtype at six months of age. No differences were observed for fluoride or calcium levels between Brtl and wildtype at either two or six months of age (Fig. AI-1).

Brtl and wildtype animals show different age-related alterations in bone mineral composition

In addition to genotype differences, mineral composition also shows age related differences. Similarities between wildtype and Brtl animals were seen for several minerals (Figure AI-2). The amount of fluoride and sodium per mg of bone both increased with age in both wildtype and Brtl animals. Magnesium was decreased in both genotypes with age. Zinc remained unchanged with age in both genotypes.

Figure A1-1. Genotype Differences at 2 and 6 Months of Age

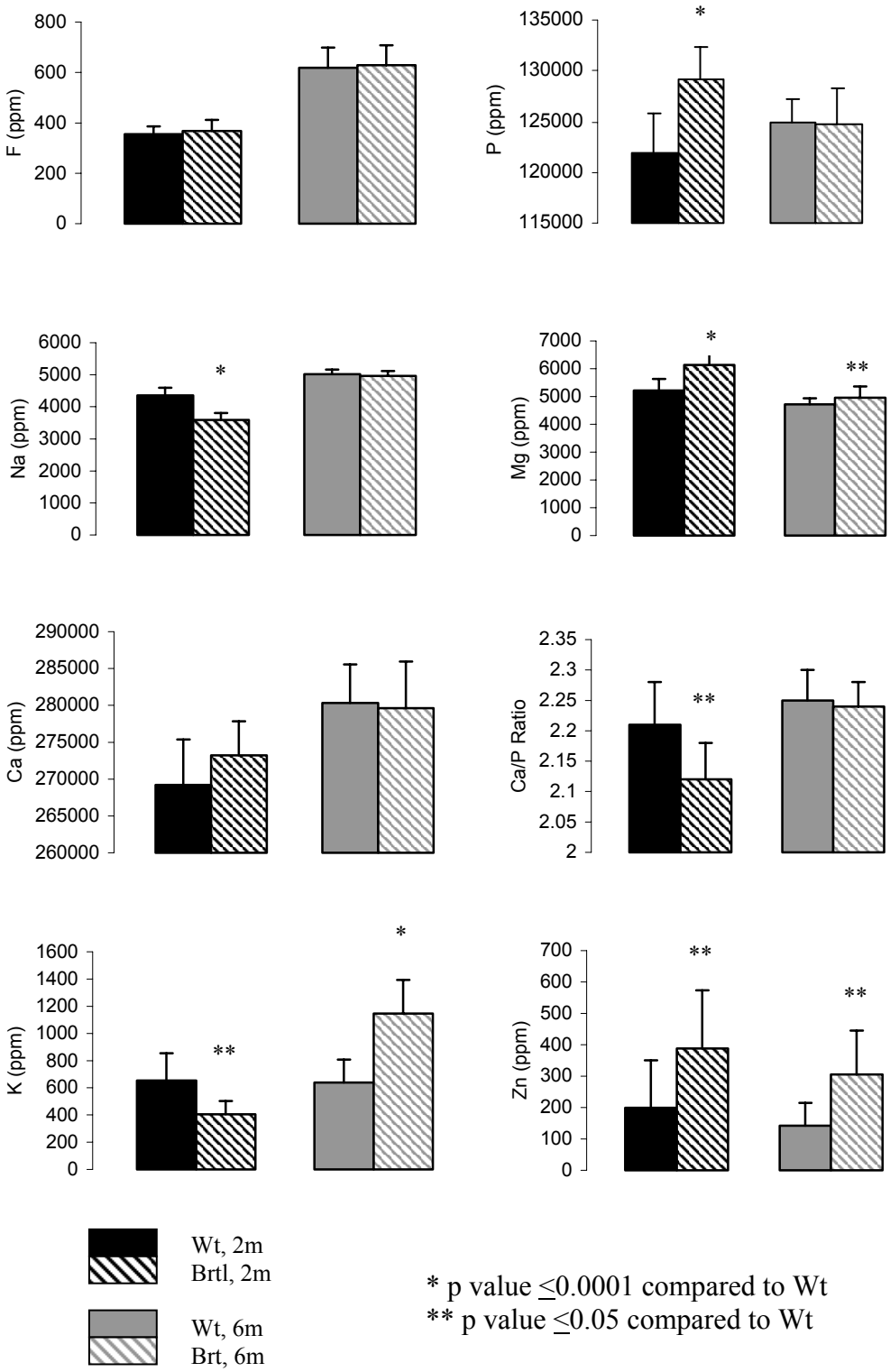
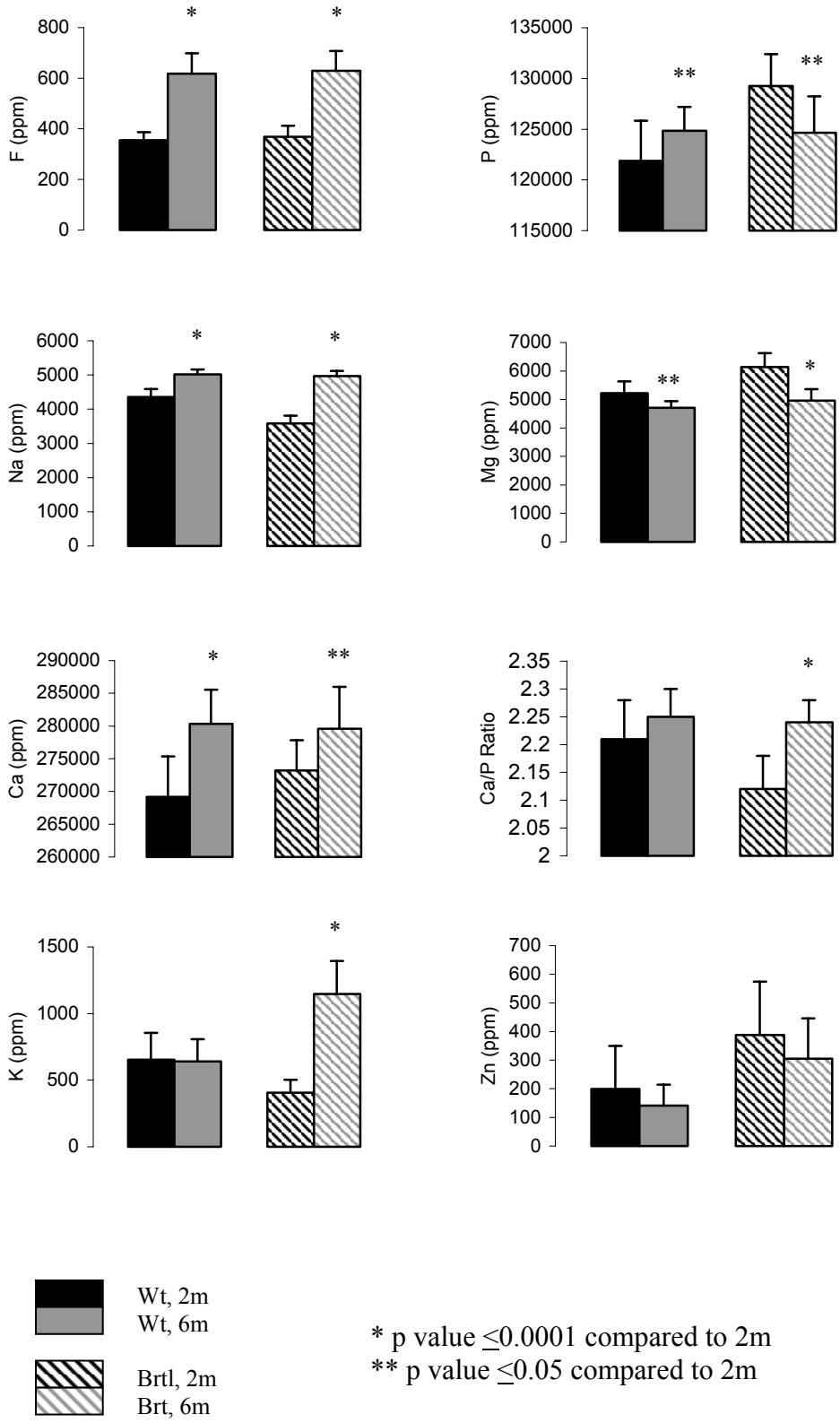


Figure A1-2. Age Related Differences Between 2 and 6 Months of Age

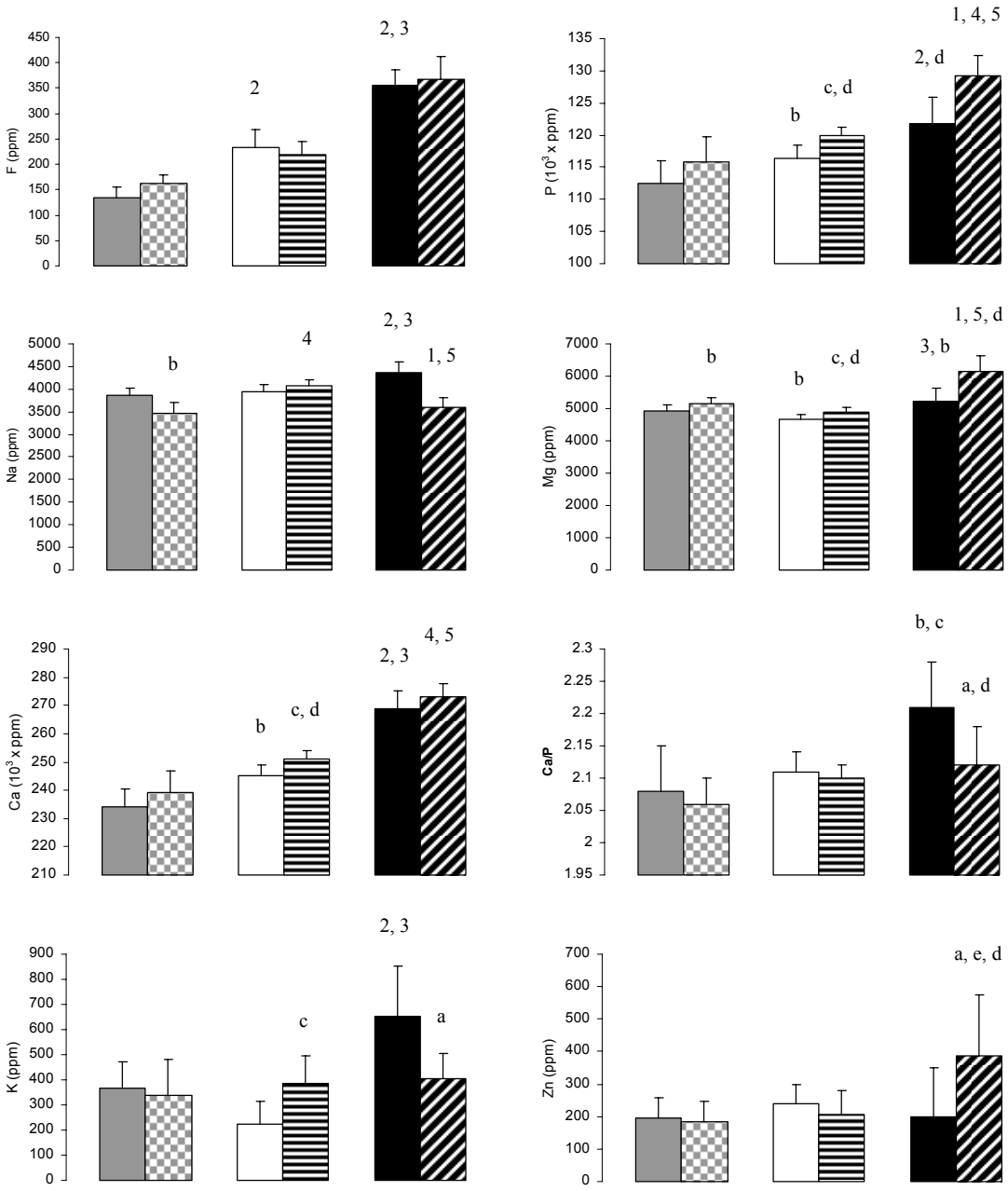


Interestingly, some differences in genotypic response to age were seen for phosphorus, potassium and Ca/P ratios. Phosphorus increased in wildtype, but decreased in Brtl animals between two and six months of age. Potassium and Ca/P ratios were unchanged at two months of age for wildtype, but both were increased in Brtl animals at six months of age. Only calcium showed an increase in wildtype animals between two and six months of age while remaining unchanged in Brtl animals (Fig. AI-2).

Brtl mice have a different bone mineral composition than other models of OI

When compared to outbred (B6C3Fe) and C57 *oim* heterozygote mice, the Brtl mice also had a different mineral composition (Figure AI-3). Brtl had consistently higher levels of a given mineral than heterozygotes from either of the other two strains. This was true of the wildtype animals of each of these strains as well; wildtype littermates of the Brtl animals had higher levels of mineral than either of the C57BL or B6C3Fe backgrounds. *Oim* animals were not compared as they are homozygous for the *oim* mutation and the Brtl animals are heterozygous. This study suggests that the identity of the specific mutation has a role in determining bone mineral composition. However, genetic background is also a strong contributor, as differences in wildtype animals of each strain showed very different mineral profiles. Taken together, these data indicate a strong role for both genetic background and specific mutation in determining mineral composition of tibias.

Figure AI-3. Genetic Background and Mutation Impacts Mineral Composition at 2 Months of Age



C57BL Het
C57BL WT
Outbred Het
Outbred WT
Brtl
Wt (Brtl)

Compared to Brtl Wt: p value [a] ≤ 0.05, [1] ≤0.0001
Compared to C57 Wt: p value [b] ≤0.05, [2] ≤0.0001
Compared to Outbred Wt: p value [c] ≤0.05, [3] ≤0.0001
Compared to C57 Het: p value [d] ≤0.05, [4] ≤0.0001
Compared to Outbred Het: p value [e] ≤0.05, [5] ≤0.0001

DISCUSSION

The aim of this study was to determine if the Brtl mutation caused an alteration in bone mineral composition and, if so, do these alterations change with age. Additionally, the Brtl mutation was compared to outbred and C57 heterozygotes, which carry one copy of the *oim* mutation, to assess the role of genetic background and specific mutation on mineral composition. To this end, tibias from two and six month Brtl and wildtype animals underwent neutron activation analysis. This technique is ideal for these types of studies because it enables quantitation of individual trace elements, instead of total mineral weights of other techniques like ashing. It is also non-destructive, allowing the same samples to be analyzed multiple times for multiple minerals. It is also very sensitive, easily quantifying as little as 0.1-1000 ng of an element, although this is element specific (www.missouri.edu./~murrwww).

When wildtype and Brtl animals were compared at two and six months of age, Brtl animals demonstrated a different mineral composition than wildtype animals. Differences between wildtype and Brtl animals at two months of age were seen in phosphorus, sodium, magnesium, potassium, zinc and the Ca/P ratio. At six months of age, the only differences that remained were in magnesium, potassium and zinc, indicating an age related shift in bone composition. The elevated level of phosphorus seen in Brtl tibias at two months of age may be an effort by the animal to increase bone mineralization in response to the altered type I collagen as hyperphosphatemia has been documented to induce mineralization of soft tissues (Hruska and Lederer 1999). Sodium was reduced in Brtl animals at two months of age, although the role of sodium in the bone is unknown. The Brtl mutation also caused an increase in the amount of magnesium

at both two and six months of age. Increases in magnesium have been shown to decrease crystal size and perfection, thus decreasing the biomechanical integrity of the bone (Boskey, Rimnac et al. 1992).

The Ca/P ratio is often used to describe the severity of OI within the human population. The lower the ratio, the more severe the clinical manifestations. Brtl tibias at two months of age exhibit a reduction in the Ca/P ratio, while Brtl animals at six months of age have a Ca/P ratio similar to wildtype. This is consistent with previous studies showing post-pubertal adaptations in the Brtl mice that improve their bone strength (Kozloff, Carden et al. 2004).

The final two minerals to be analyzed were potassium and zinc. Potassium was reducing in Brtl mice at two months of age, but increased at six months of age. As the bones of Brtl animals improve with age, this finding is not surprising. Potassium is thought to contribute to an alkaline environment that may be protective to the bone (Tucker 2003). The theory that an acidic environment may lead to bone loss has been supported by several short term studies in both men and women (Frassetto, Todd et al. 2000; Buclin, Cosma et al. 2001; Sellmeyer, Stone et al. 2001). Diets high in phosphorus, chloride and several amino acids, but low in fruits and vegetables and vitamin C are thought to lead to reduced bone mineral density and increased fracture risk (Tucker 2003) while alkalizing nutrients inhibit bone resorption (Buclin, Cosma et al. 2001). High levels of zinc have been shown to decrease bone turnover and the elevated levels of zinc seen in Brtl animals at both two and six months may be an attempt by the animals to maintain the bone they have, even though it may be weaker than wildtype.

When age-related alterations in mineral composition were evaluated, several changes were observed. However, the mineral profile at six months of age is more like wildtype than at two months of age. This implies an improvement in mineral composition with age, consistent with biomechanical improvement seen at six months of age. Wildtype animals showed increases in fluoride, phosphorus, sodium and calcium with a decrease in magnesium. The increases in fluoride and sodium may simply reflect accumulation of these minerals with increased exposure in the diet. The increases in phosphorus and calcium along with the decrease in magnesium may reflect the improved bone quality as seen by improved biomechanical integrity (Kozloff, Carden et al. 2004). The observed increased in potassium in Brtl tibias at six months of age may also play a protective role for the bone (Tucker 2003). The higher Ca/P ratio seen in Brtl tibias from animals at six months of age correlated will with the improvement seen in biomechanical analyses as a reduced Ca/P is seen in OI patients (Cassella and Ali 1992).

To determine if genetic background and/or specific OI-causing mutation impacts mineral composition, Brtl tibial mineral composition was compared to *oim* heterozygotes maintained on two genetic backgrounds at two months of age. Brtl mice demonstrated a consistently higher variability in the amount of any give mineral as seen by an increased standard deviation. Minerals that showed mutation, but not strain, increases were phosphorus, magnesium and calcium regardless of genetic background. As expected, both mutations decreased the Ca/P ratio, indicating a more severe phenotype than their wildtype counterparts. Strain, but not mutation, specific differences were seen in zinc. The *oim* caused a decrease in zinc, regardless of genetic background, while Brtl mice caused a significant increase. Fluoride, sodium and potassium showed both mutation and

strain specific alterations in mineral composition. The amount of these elements was specific to the genetic background as well as the identity of the mutation.

Taken together, these data suggest that the Brtl mutation alters bone mineral composition at both two and six months of age. However, the mineral profile at six months of age is more like wildtype than at two months of age, implying an improvement in mineral composition with age. Comparison of the Brtl mutation to the *oim* mutation suggests the specific OI-causing mutation helps determine bone mineral composition in a mineral specific manner. However, genetic background seems to be the predominant factor in determining mineral levels, as Brtl animals, as well as their wildtype littermates, had consistently higher levels of any given mineral than outbred or C57 animals.

APPENDIX II

ANALYSIS OF TRACE MINERAL COMPOSITION OF THE PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1) OVER-EXPRESSION MOUSE

This chapter is in preparation for submission to the Journal of Bone and Mineral Research.

TRANSGENIC OVER-EXPRESSION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 RESULTS IN AGE-DEPENDENT AND GENDER-SPECIFIC CHANGES IN FEMORAL STRENGTH AND MINERALIZATION

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INTRODUCTION

The plasminogen activator inhibitor (PAI-1) is a molecule known to be involved in the cardiovascular system. The plasminogen activator (PA) normally converts zymogen plasminogen into plasmin, which is involved in several downstream pathways, including extracellular matrix remodeling (Daci, Verstuyf et al. 2000). PAI-1 interacts with tissue-type (tPA) and urokinase (uPA) plasminogen activators to inhibit the plasminogen activator (Daci, Verstuyf et al. 2000). In addition to its role in the cardiovascular system, PAI-1 is also produced by bone cells (Daci, Verstuyf et al. 2000). Previous studies have found that PAI-1 knock-out mice were protected from estrogen deficiency-induced trabecular bone loss (Daci, Verstuyf et al. 2000). When one week old tPA and uPA double knock-out mice were examined, these animals were found to have increased bone formation, possibly due to the accumulation of noncollagenous proteins of the bone matrix (Daci, Everts et al. 2003). Although PAI-1 knock-outs are protected from bone loss associated with estrogen loss, this protection is only seen in trabecular bone. Cortical bone density was similar between wildtype mice and PAI-1 knock-out mice four weeks post-ovarectomy (Daci, Verstuyf et al. 2000). Taken together studies of the PAI-1 knock-out mice suggest that decreased PAI-1 in humans could confer protection from menopause-induced bone loss in trabecular bone.

In order to more closely examine the role of PAI-1 on bone quality and strength, a PAI-1 overexpression mouse was created and previously described by Eren, et al (Eren, Painter et al. 2002). At 32 weeks of age, wildtype mice had a significant reduction in both torsional ultimate strength and tensile strength. PAI-1 KO mice, however, were protected from this age-associated reduction in bone strength (Sarah Nordstrom, personal

communication). 32 week-old PAI-1 female KOs also exhibited an increased bone mineral density as compared to their age-matched wildtype littermates. Osteocalcin and TRACPb levels were also measured as markers of bone formation and resorption, respectively. And while PAI-1 female KOs at 32 weeks of age did exhibit a reduction in osteocalcin levels, the lack of PAI seems to protect the animals from the same degree of loss seen in age-matched wildtypes (Sarah Nordstrom, personal communication). TRACP b levels were unchanged at 32 weeks of age in female KOs as compared to their wildtype littermates. These studies demonstrate the protective function of PAI-1 overexpression against bone loss and weakness in female mice at 32 weeks of age.

As bone is made of both an organic and mineral phases, we next wanted to examine the mineral composition of femurs from PAI-1 overexpressing mice. Several minerals were quantitated using instrumental neutron activation analysis (NAA), including fluorine, phosphorus and calcium. These data show an altered mineral content in PAI-1 mice as compared to age-matched controls.

METHODS

Animals and Sample Preparation

Femurs from 10 male and 10 female Wt and PAI-1 mice were evaluated at both 16 and 32 weeks of age. Femurs were prepared as described on page 57.

NAA

Standards and samples were tested as described on page 66 and in table II-1.

Statistical Analyses

All comparisons statistical analyses were done using SAS (SAS Institute Inc., Cary, NC). All comparisons were analyzed using a general linear model. When heterogeneous variations made it necessary, a log transformation was used to stabilize the variation. If this log transformation failed to stabilize the variation, a ranking procedure was used (Conover and Iman 1982). Mean differences were determined using Fisher's Least Significant Difference (LSD). Means and standard deviations presented are the actual numbers, although p values reflect the transformed or ranked data. All results are presented as mean ± standard deviation. Differences were considered to be statistically significant at p-value ≤ 0.05.

RESULTS

PAI-1 mice exhibited altered bone mineral composition as compared to age and gender matched controls

PAI-1 mouse femurs were compared to wildtype femurs from animals of the same age and gender for each element. The differences observed were mineral specific. Fluoride, magnesium, and calcium were altered in both males and females at both 16 and 32 weeks of age. Fluoride was significantly lower in PAI-1 femurs as compared to their wildtype littermates (Figure II-1; notation "a"). Magnesium was significantly lower in femurs from PAI-1 females at 16 weeks of age and from males at both 16 and 32 weeks of age. Conversely, PAI-1 femurs from females at 32 weeks of age had more magnesium and than wildtype controls. Calcium had a similar profile as magnesium, except PAI-1 femurs from 16-week male and female animals had less calcium than wildtype femurs,

but at 32 weeks of age, both male and female PAI-1 femurs had more calcium than wildtype. Phosphorus, chloride and the Ca/P ratio were only changed in PAI-1 femurs from 32 week-old males. PAI-1 males had significantly more phosphorus and chloride as well as an elevated Ca/P ratio compared to their wildtype littermates. The final three elements, sodium, potassium and zinc, were altered in gender and age specific manners. Sodium was elevated in 32-week old, but not 16-week old, PAI-1 femurs from both males and females as compared to wildtype controls. Potassium was elevated in 16-week old, but not 32-week old, femurs from PAI-1 mice as compared to wildtype controls. Finally, zinc was significantly reduced in femurs from PAI-1 males at both 16 and 32 weeks of age.

PAI-1 mice exhibited age-associated changes in bone mineral composition as compared to genotype and gender matched controls

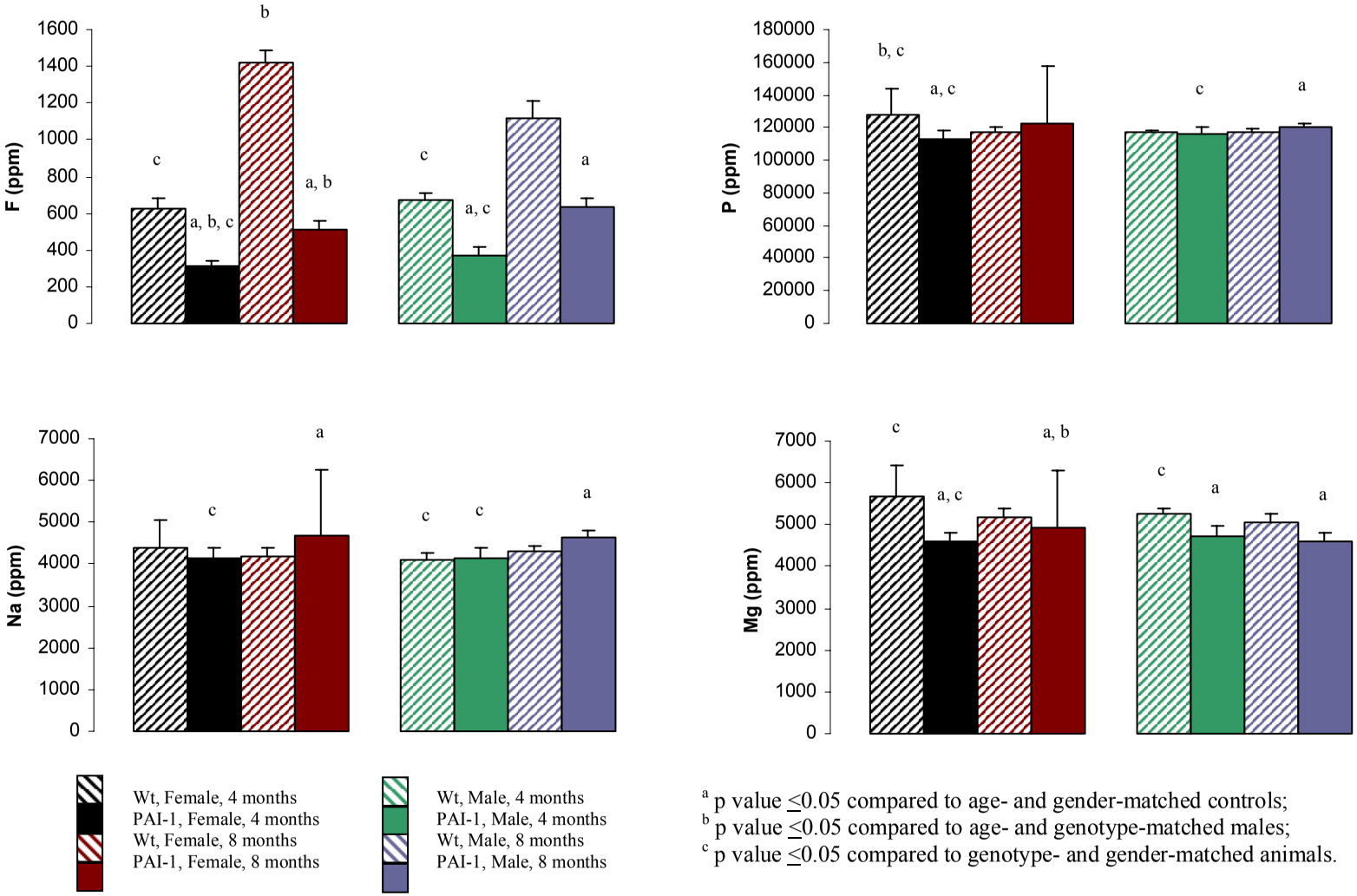
Fluoride was significantly increased in femurs from 32-week old animals as compared to genotype and gender matched 16-week old animals (Figure II-1, notation "c"). Phosphorus and calcium were significantly reduced in femurs from wildtype females at 32-weeks of age compared to 16-weeks of age. Conversely, both phosphorus and calcium were significantly elevated in femurs from PAI-1 femurs from both males and females at 32-weeks of age compared to 16-week old animals. Sodium was elevated in PAI-1 femurs from male animals at both 16- and 32-weeks of age, as well as in femurs from PAI-1 females. Magnesium was significantly reduced in wildtype femurs from both males and females, while magnesium was only reduced in PAI-1 females. Chloride was only altered in PAI-1 males, with 32-week old femurs having significantly more chloride

than 16-week old femurs. Zinc demonstrated age-associated changes with wildtype females having significantly more zinc at 32 weeks of age than at 16 weeks of age. When the Ca/P ratio was compared for age-related differences, femurs from both male and female PAI-1 animals at 32 weeks of age had significantly higher ratios than their 16-week old counterparts. Wildtype animals did not demonstrate any alterations in the Ca/P ratio associated with age. Age-associated changes in potassium were not seen.

Gender differences in mineral composition

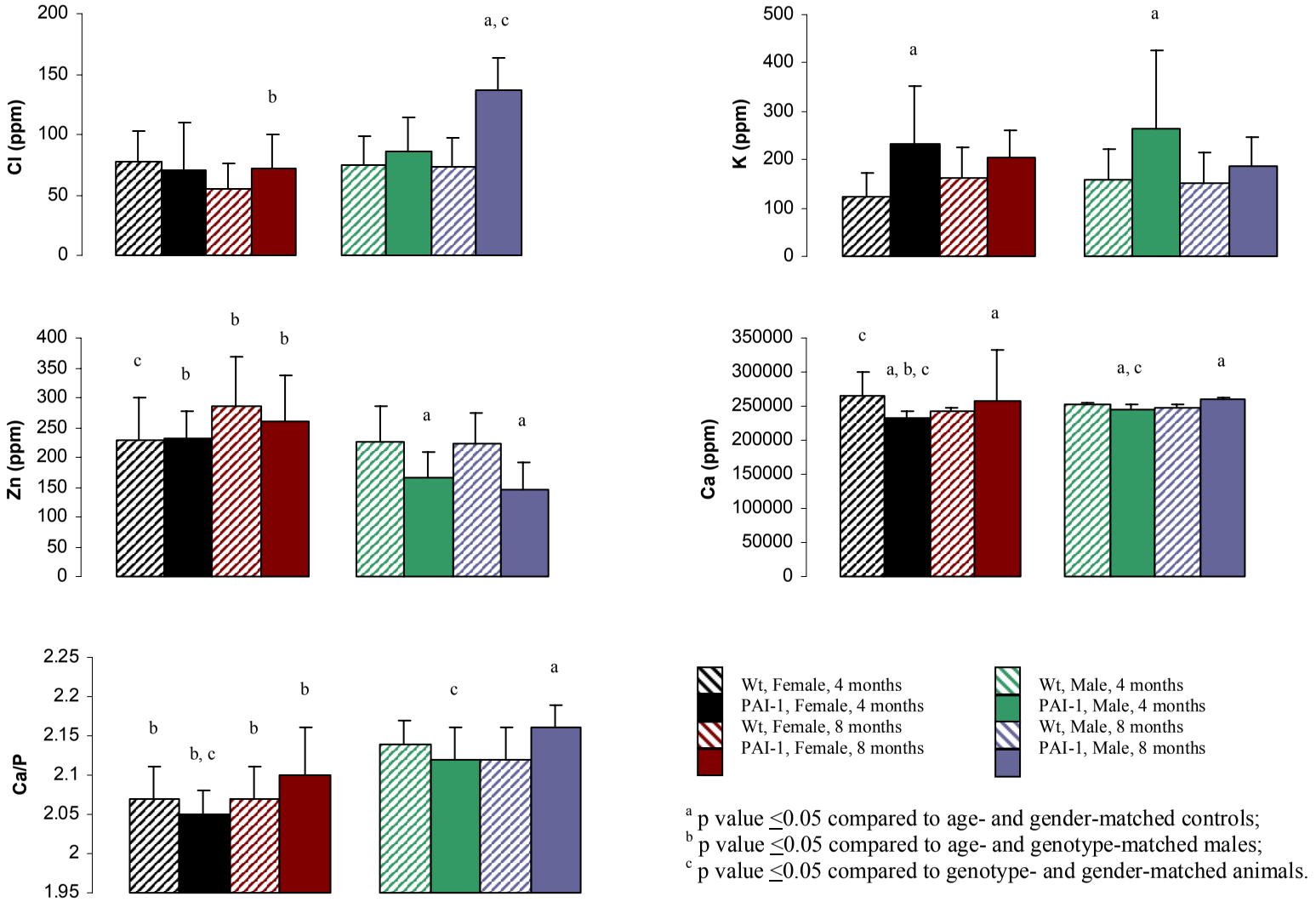
In wildtype animals at 32 weeks of age, wildtype males had significantly more fluoride than their female counterparts. However, the over-expression of PAI-1 caused females at both 16 and 32 weeks of age to have lower fluoride levels than their male littermates (Figure II-1; notation "b"). Differences in phosphorus, magnesium, chloride and calcium were limited to only one genotype or one age point. Phosphorus was only different between wildtype males and females at 16 weeks of age. Magnesium and chloride were only different in PAI-1 32-week old femurs, with females having significantly more magnesium than males. Calcium only showed gender differences in PAI-1 femurs from 16-week old animals. The Ca/P ratio demonstrated gender-specific differences in both wildtype and PAI-1 femurs at both 16 ad 32 weeks of age, with males exhibiting an increase ratio as compared to females. Zinc was significantly elevated in femurs from female wildtype animals at 32 weeks of age, as well as in PAI-1 femurs from 16- and 32-week old animals. Gender-associated differences were not seen in sodium or potassium.

Figure AII-1. Mineral Composition of Femurs From Male and Female Wildtype and PAI-1 Over-expression Mice at 16 and 32 weeks of Age



[a] p value ≤0.05 compared to age- and gender-matched controls;
[b] p value ≤0.05 compared to age- and genotype-matched males;
[c] p value ≤0.05 compared to genotype- and gender-matched animals.

Figure AII-1, Continued



[a] p value ≤0.05 compared to age- and gender-matched controls;
[b] p value ≤0.05 compared to age- and genotype-matched males;
[c] p value ≤0.05 compared to genotype- and gender-matched animals.

DISCUSSION

Studies on the PAI-1 over-expressing mouse have found that over-expression of the PAI-1 gene, especially in the context of estrogen deficiency, protects the animal from age-related bone loss. Consistent with these findings, PAI-1 animals also exhibit several changes in bone mineral content that appear to increase the quality of bone. These minerals include fluoride, phosphorus and magnesium. Several gender and age differences were also seen in bone mineral content. The significance of these findings is unclear, as differences in bone strength due to age and gender have not been documented.

In the current study, fluoride was significantly reduced in PAI-1 femurs as compared to wildtype. Fluoride is known to have biphasic effects, being beneficial at low doses but detrimental at high doses (Cheng, Bader et al. 1995). Riggs, et al showed that fluoride therapy in postmenopausal osteoporotic women caused thickening of the trabeculi, but induced loss of cortical bone, putting the bone at risk for fracture (1994). Consistent with this, association of relatively low levels of fluoride in PAI-1 femurs as compared to wildtype corresponds with stronger bones as seen by larger T_{max} and Su values (Sarah Nordstrom, personal communication). Conversely, *oim/oim* animals, known to have impaired biomechanical integrity, have relatively high levels of fluoride.

Phosphorous and calcium are the main components of hydroxyapatite crystals and play a crucial role in bone mineralization. Both of these minerals exhibited similar profiles; when the level of one mineral decreased, the level of the other mineral also decreased. However, calcium exhibited more genotype related changes than phosphorus. The Ca/P ratio has been used in humans as an indicator of the severity of the disease. The lower the ratio, the worse the clinical severity (Cassella, Garrington et al. 1995).

Consistent with this, female mice have lower Ca/P ratios than their male counterparts, regardless of age or genotype. PAI-1 males at 32 weeks of age also have a higher Ca/P ratio than their wildtype counterparts and these differences are mimicked in the biomechanical parameters.

Magnesium also showed significant differences between genotypes with femurs from PAI-1 overexpressing animals having reduced levels of magnesium at both 16 and 32 weeks of age. As reduced magnesium is known to increase crystal size and perfection (Boskey, Rimnac et al. 1992), the reduction in magnesium seen in this study would be expected to improve bone biomechanical properties, which was demonstrated by torsional loading to failure (data not shown). Consistent with this, *oim/oim* animals have relatively high levels of magnesium.

Potassium was elevated in PAI-1 femurs at 16 weeks of age. Potassium is thought to contribute to an alkaline environment that may be protective to the bone (Tucker 2003), which is supported by the increased biomechanical integrity of PAI-1 bones (data not shown). The theory that an acidic environment may lead to bone loss has been supported by several short term studies in both men and women (Frassetto, Todd et al. 2000; Buclin, Cosma et al. 2001; Sellmeyer, Stone et al. 2001).

Sodium and chloride were both elevated in PAI-1 femurs. Sodium content was elevated in PAI-1 over-expressing femurs of both sexes as 32 weeks of age while chloride was only elevated in PAI-1 femurs from males at 32 weeks of age. Although the role of sodium within the bone is unclear, the higher levels of sodium seen in the PAI-1 femurs seem to confer some additional strength to the bone, as these bones have increased breaking and tensile strength as compared to wildtypes. This is consistent with

data seen in the *oim* model, where increased amounts of sodium were associated with increased biomechanical integrity. The role of chloride in the bone is unknown.

Zinc has been shown to have positive effects on bone growth and can stimulate osteoblasts to proliferate and differentiate as well as inhibit osteoclast formation (Igarashi and Yamaguchi 2001). The reason for the lower levels of zinc seen in PAI-1 males is unclear, as this should be detrimental to the animal.

The observed changes in bone mineral content indicate a positive role for the PAI-1 gene in improving bone quality. This is especially true in the context of estrogen deficiency, when females are prone to accelerated bone loss as compared to age-matched males. By over-expressing the PAI-1 gene, female mice are better able to stave off this bone loss, maintaining bone biomechanical integrity similar to that seen prior to estrogen decline. Taken together, both torsional loading to failure and bone mineral composition studies indicate that the PAI-1 gene provides protection for the bone from reductions in bone quality due to estrogen deficiency.

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VITA

Stephanie Michelle Carleton was born on April 6, 1979 in Wichita, KS. She is the only child of Ken and Carolyn Carns of St. Charles, MO. She attended Francis Howell Senior High School in Weldon Springs, MO before attending Lindenwood University in St. Charles, MO. There she obtained a Bachelor of Science in Biology with a minor in chemistry. Under the guidance of an outstanding undergraduate advisor, Dr. L. Rao Ayyagari, Stephanie was awarded a Howard Hughes Undergraduate Research Fellowship at the University of Iowa-Iowa City for the summer of 2000. Following graduation from Lindenwood, Stephanie worked at Monsanto before beginning graduate school at the University of Missouri-Columbia in the Genetics Area Program where she was award a Life Sciences Fellowship. Just before beginning graduate school, Stephanie married her high school sweetheart, Kevin Carleton, who works for Lowes Co. Stephanie has decided to stay in Columbia to complete her post-doctoral fellowship with her current advisor, Dr. Charlotte Phillips. Kevin and Stephanie hope to return to the St. Louis area following completion of this fellowship.