Functional phenotyping of bone
a hierarchical assessment of bone failure characteristics

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Functional phenotyping of bone: a hierarchical assessment of bone failure characteristics

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# Table of contents

Acknowledgements........................................................................................................................................... v
Summary................................................................................................................................................................ v
Résumé .............................................................................................................................................................. xi
Conventions...................................................................................................................................................... xiv

## 1 Introduction..................................................................................................................................................... 1

1.1 Bone............................................................................................................................................................. 1
1.2 Osteoporosis ................................................................................................................................................. 4
1.3 The mouse as a model.................................................................................................................................. 6
1.4 Phenotypical characterization...................................................................................................................... 10
1.5 Objectives of the study ............................................................................................................................... 18
1.6 Bibliography ............................................................................................................................................... 22

## 2 Whole bone mechanics .............................................................................................................................. 29

2.1 Femoral neck stiffness and strength critically depend on bone alignment – a parametric study in a mouse inbred strain .................................................................................................................... 31
2.2 Differential effects of bone structural and material properties on bone competence in C57BL/6 and C3H/He inbred strains of mice ................................................................. 47
2.3 Bone morphometry highly predicts cortical bone stiffness and strength but not toughness in an inbred mouse model of high and low bone mass.............................................. 67
2.4 Tissue modulus derived from beam theory is biased by bone size and geometry: implications for the use of three-point bending tests to determine bone tissue modulus ................................................................................................................... 85

## 3 Hierarchical investigation of bone strength .............................................................................................. 103

3.1 Functional microimaging: a hierarchical investigation of bone failure behavior... 105
3.2 Intermediary comments ............................................................................................................................ 131

## 4 Bone ultrastructure....................................................................................................................................... 133

4.1 Time-Lapsed Assessment of Microcrack Initiation and Propagation in Murine Cortical Bone ..................................................................................................................................................... 135
4.2 Canal network characteristics are strong predictor of microdamage accumulation in bone.............................................................. 157
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Summary

Although osteoporotic fracture is a common and expensive healthcare problem, the factors responsible for patient’s susceptibility to fracture remain incompletely understood. Bone mineral density is strongly associated with bone strength, but its use to assess fracture risk in all patients has been limited. The potential addition of geometric and material properties, at multiple hierarchical levels, may improve fracture risk predictions significantly.

During the past decades, the mouse has evolved to the most important model organism in the field of modern biomedical research. This is due to two principal reasons: on the one hand, the successful application of various transgenic technologies, and on the other hand, the strong homology between mice and men. With the efficient techniques given to geneticists, they are nowadays able to identify locations on chromosomes, which play a major role in the process of modeling and remodeling of bone. Nevertheless, the geneticists need quantitative endpoints to accomplish that work. Micro-computed tomography (μCT) has proven to be well suited for assessing and quantifying a variety of three-dimensional microstructural bone phenotypes, also referred to as bone morphometry.

The gold standard to determine bone strength is a functional, mechanical test. Such a test gives several measures of bone competence, like stiffness, ultimate force, ultimate displacement and work to failure. Due to their small size these tests are challenging in murine bone; compared to data from larger animals the precision is often lower. Accurate mechanical and material data on bone strength are still lacking for the mouse.

For this reason, this thesis focused on improved prediction of bone mechanical properties. The main aims of this study were to experimentally assess bone mechanical properties, such as absolute strength, bone stiffness and post-yield behavior, and to relate these mechanical properties to bone overall bone geometry as well as to bone micro- and ultrastructure. To fulfill these objectives, an integrative approach for hierarchical investigation of bone was developed, working at different scales of resolution, ranging from the whole bone to its ultrastructure.

At the organ level, the influence of boundary conditions on the testing of whole murine femora was evaluated and the optimization of sample preparation and alignment increased significantly the reproducibility of the mechanical tests. Axial compression and three-point bending tests preformed on two murine inbred strains, C57BL/6 (B6) and C3H/He (C3H), revealed that C3H mechanical bone phenotype is less variable than in B6. Comparing the mechanical parameters with bone morphometry, it was shown that whole bone
morphometrical indices, such as bone volume, cortical thickness and cross-sectional area predicted almost 80% of bone stiffness, strength and yield force. For the femoral neck, cortical thickness explained 83% of bone strength when femoral head was axially loaded. Our investigation showed that bone post-yield behavior could not be explained by morphometry.

Accurate mechanical parameters, compared to microstructural finite element (µFE), showed that the classical Euler-Bernoulli beam theory underestimated significantly the bone Young’s modulus, when estimated from three-point bending tests. Stiffness was measured mechanically and Young’s modulus was calculated based on the µFE analyses. The low aspect ratio of murine femora excludes the use of classical beam theory, without applying correcting factors. It was also shown that reported murine inbred strain-specific differences in tissue modulus as derived from three-point bending tests are largely an effect of geometric differences, not accounted for by beam theory.

Direct mechanical testing provides detailed information on overall bone mechanical and material properties, but fails in revealing local properties such as local deformations and local contribution of bone micro- and ultrastructure on bone failure. Therefore, we incorporated several imaging methods in our mechanical setups in order to get a better insight into bone deformation and failure characteristics. Whole bone testing was recorded with high-resolution and high-speed cameras. The movies showed fracture initiation and bone whitening at high stress regions. This also helped to remove erroneous samples and with that to increase, though, the power and the relevance of the experiments. At a microstructural scale, image-guided failure assessment (IGFA) uncovered, in a three-dimensional manner, fracture initiation and propagation as well as local deformation of cancellous bone. It also showed that femoral neck of B6 and C3H fractured differently when the femoral head was loaded axially.

The poor prediction of bone post-yield behavior obtained with classical bone morphometry, together with the whitening processes observed before bone fracture, motivated the investigation of bone microcrack initiation and propagation at an ultrastructural scale. Synchrotron radiation based micro-computed tomography (SRµCT) permits visualization of bone porosity features with a resolution of 1 µm and below. We successfully imaged osteocyte lacunae, canals, uncracked ligament bridges and mineralized collagen fibers directly in 3D. Furthermore, the contribution of these ultrastructural entities on the microcrack initiation and propagation was showed. Canal voids were larger in C3H than in B6. In C3H, they were therefore able to initiate and determine the orientation of microcracks. Osteocytic lacunae did not initiate microcracks, but their distribution provided guidance for the propagation of microdamage. Microdamage accumulated more and faster in C3H than in B6.
This difference at a submicron scale can explain the higher brittleness and the lower toughness of C3H observed at the macroscopic level.

In conclusion, a new hierarchical approach for functional investigation of bone was developed. It allowed relating bone structural properties at different scales, ranging from whole bone to bone ultrastructure, to the overall bone competence. In the future, this might help the development of better tools and measures for clinically predicting bone strength.
Résumé

Bien que les fractures ostéoporotiques soient un problème de santé fréquent et coûteux, les facteurs responsables du risque de fracture chez les patients restent peu compris. La densité minérale de l’os est fortement associée à la résistance osseuse, cependant, sa capacité à prédire le risque de fracture chez tous les patients est limitée. La prise en compte de propriétés géométriques et matérielles, à de multiples niveaux hiérarchiques, peut potentiellement améliorer de manière significative les prédictions de risques de fracture.

Au cours des dernières décennies, la souris est devenue un des principaux modèles d’investigation de l’ère moderne de la recherche biomédicale. Ceci est dû à deux principales raisons: d’une part, la réussite de différentes techniques transgéniques et, d’autre part, la forte homologie entre la souris et l’homme. Grâce à des technologies efficaces, les généticiens sont aujourd’hui dans la mesure d’identifier des sections de chromosome qui ont un rôle majeur dans le processus de modelage et de remodelage des os. Néanmoins, les généticiens ont besoin de repères quantitatifs pour accomplir leur travail. La micro-tomographie (μCT) a déjà prouvé à maintes reprises son efficacité dans l’évaluation et la quantification en trois dimensions de phénotypes de la microstructure osseuse. Ce procédé est aussi appelé morphométrie.

Afin de déterminer la résistance des os, la pratique standard est le test mécanique. Un tel test fournit plusieurs mesures de la compétence osseuse, telles que la rigidité, la résistance ultime, le déplacement ultime ainsi que le travail jusqu’à la rupture. A cause de la petite taille des os de souris, les tests mécaniques sur de tels os sont bien plus ardu que sur des os d’animaux plus grands et leur précision est souvent inférieure. De ce fait, des données précises sur les propriété matérielles et mécaniques des os de souris font encore défaut.

Pour cette raison, le but de cette thèse était d’améliorer la prédiction des propriétés mécaniques de l’os. Les objectifs principaux de ce travail étaient d’évaluer de manière expérimentale, les propriétés mécaniques osseuses, telles que la résistance ultime, la rigidité, et le comportement plastique, ainsi que de comparer ces propriétés mécaniques à la géométrie macroscopique de l’os, à sa microstructure et à son ultrastructure. Afin d’atteindre ces objectifs, une approche hiérarchique d’investigation intégrale de l’os a été développée en travaillant à différentes échelles de résolution, allant de l’os entier à son ultrastructure.

Au niveau de l’organe, nous avons analysé l’influence des conditions extérieures lors d’un test de femurs de souris. Nous avons aussi optimisé la préparation et l’alignement des échantillons, ce qui a amélioré la reproduicibilité des tests mécaniques de façon significative.
Les tests de compression axiale ainsi que les tests de flexion à trois points sur deux types de souris de souches consanguines, C57BL/6 (B6) et C3H/He (C3H), ont permis d’identifier que les propriétés mécaniques de C3H sont moins variables que celles de B6. En comparant les paramètres mécaniques avec la morphométrie osseuse, nous avons également démontré que les indices morphométriques de l’os entier, telle que le volume osseux, l’épaisseur cortical et l’aire de la section ne prédisent pas moins de 80% de la rigidité, de la résistance et de la force maximale élastique. Dans le col du fémur, l’épaisseur corticale a prédit 83% de la résistance osseuse lorsque la tête du fémur était contrainte axialement en mode de compression. Nos investigations ont montré qu’il n’était pas possible d’expliquer le comportement plastique de l’os en ne considérant que sa morphométrie.

En comparant des tests mécaniques de la plus haute précision avec des éléments finis microstructuraux (μFE), nous avons montré que la théorie classique des faisceaux d’Euler-Bernoulli sous-estimait de manière significative le module de Young de l’os, lorsque celui-ci était dérivé d’un test de flexion à trois points. Nous avons mesuré mécaniquement la rigidité et calculé le module de Young à l’aide des μFE. Le faible rapport longueur/épaisseur des fémurs de souris exclut l’utilisation de la théorie classique des faisceaux sans l’aide de facteurs de correction. Nous avons aussi montré que les grandes différence dans le module de Young de diverses souches consanguines, dérivé de test de flexion à trois points, étaient largement dues aux différences géométriques; ces dernières n’étant pas prises en compte par la théorie des faisceaux.

fémur des souches consanguines B6 et C3H s’initiaient et se propageaient différemment lors de tests de compression axiale chez chaque souche.

La faible prédiction du comportement plastique obtenue avec les méthodes classiques, additionnée au phénomène de blanchiment avant la fracture, ont motivé nos investigations sur l’initiation et la propagation des microfissures à l’échelle de l’ultrastructure osseuse. Atteignant une résolution de 1 μm et en dessous, la micro-tomographie basée sur les radiations du synchrotron (SRμCT) a déjà permis la visualisation de certaines caractéristiques de la porosité osseuse. Avec cette méthode, nous avons réussi à imager les lacunae d’ostéocytes, les canaux, les ponts ligamenteux ainsi que les fibres de collagène minéralisées de l’os en trois dimensions. De plus, la contribution de ces entités ultrastructurelles sur l’initiation et la propagation des microfissures a pu être dévoilée. Les canaux des C3H étaient plus grands que ceux des B6 et, de ce fait, étaient capables d’initier et de déterminer l’orientation des microfissures. Les lacunae d’ostéocyte, eux, n’initiaient pas de microfissures, mais leur distribution guidait leur propagation. Les microfissures s’accumulaient en plus grande quantité et plus vite chez les C3H que chez les B6. Cette différence au niveau de l’ultrastructure a permis d’expliquer la plus grande friabilité et la plus faible tenacité des os de C3H, observées au niveau macroscopique.

En conclusion, une nouvelle approche hiérarchique d’investigation de l’os a été développée dans ce travail. Elle a permis de relier les propriétés structurelles à différentes échelles, de l’os entier à son ultrastructure, à la compétence globale de l’os. Cette nouvelle approche pourrait éventuellement contribuer au développement de meilleurs outils et moyens de mesure quant à la prédiction clinique de la résistance osseuse.
Conventions

In this thesis, we used the following convention for the coordinate system:
- the x-axis is the medio-lateral axis
- the y-axis is the anterio-posterior axis
- the z-axis is the proxo-distal axis
Chapter 1

Introduction

1.1 Bone

The skeleton is primarily composed of two major constituents: cartilage and bone. Bone itself is a “hard” tissue compared to soft tissues, like tendons or ligaments. The hardness and stiffness of bone is caused by calcium phosphate, which is deposited in the bone matrix in the form of a crystalline hydroxyapatite (HA). Other matrix constituents are collagen and various proteins.

Cartilage covers, in its hyaline form, the articulating surfaces of bone that comprise joints. It is a relatively soft tissue with a low surface friction, but will not be discussed further, although it has many things in common with bone regarding its constituents and cell lineage.

Bone is a unique material, its structure is intricately organized and exhibits complex biological and mechanical characteristics, such as anisotropy, inhomogeneity and viscoelasticity. Furthermore, it is capable of reacting in an adaptive manner to a variety of environmental stimuli.

At the macrostructural level, two basic arrangements of bone tissue are present: cortical and trabecular. The shaft of long bones (diaphysis) and the thin shell covering the vertebrae are composed of cortical bone. Trabecular bone can be found in the interior of the distal and proximal ends of long bones (epiphyses) and in the interior of vertebral bodies (Fig. 1.1).
The stiffness and strength of cortical bone are mainly controlled by the mineralization of the matrix, the porosity and, in remodeling vertebrates, the lamellar arrangement of the osteons. The relationship between apparent stiffness, porosity and mineral content was recognized early on and investigated by Currey [2] as well as by Schaffler and Burr [3]. The strength of cortical bone is controlled by the same factors as the stiffness, but additionally by the loading mode. Cortical bone is stronger in compression than in tension for both longitudinal and transverse directions. Transverse specimens are less strong than longitudinal ones (Table 1.1). The material properties of cortical bone also vary with age [4].

Table 1.1: Representative mechanical properties of human cortical bone [5]

<table>
<thead>
<tr>
<th>Direction</th>
<th>Modulus [GPa]</th>
<th>Tensile ultimate stress [MPa]</th>
<th>Compressive ultimate stress [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longitudinal</td>
<td>17.4</td>
<td>133</td>
<td>195</td>
</tr>
<tr>
<td>Transversal</td>
<td>9.6</td>
<td>51</td>
<td>133</td>
</tr>
<tr>
<td>Shear</td>
<td>3.51</td>
<td>69</td>
<td>69</td>
</tr>
</tbody>
</table>

Trabecular bone exhibits a broader spectrum of mechanical properties than cortical bone, which is due primarily to the spatial and temporal variations in bone mass and morphometry. The mechanical properties of trabecular bone are controlled predominantly by the bone volume density (BV/TV) and the micro-architecture (morphometry) at the tissue...
1.1 Bone level. The architecture is the result of the spatial arrangement and shape of interconnecting trabeculae. These can be idealized as cylindrical (rod-like) or plate-like structures (Fig. 1.2).

Figure 1.2: Trabecular bone structure (human lumbar vertebra) with heterogeneous architecture; plate-like and rod-like structure can be observed.

Strength and stiffness can vary by up two orders of magnitude within a single bone, solely due to changes in density [6]. The apparent elasticity for instance can vary from approximately 10 Mpa to 2 Gpa, depending on the anatomic site and age. Hence, trabecular bone is more compliant than cortical bone. The Young’s modulus (E), as well as strength (σ), depends considerably on the apparent bone density (ρ). Typically, power-law function like $E = C*\rho^N$ are used to describe these relationship, in which the exponent $N$ has a value of approximately 2 [6]. Hence, a reduction of the apparent bone mass, as it occurs in aging or osteoporotic bone, causes a severe loss of bone stiffness and strength. The different architectures of trabecular bone that can be found result in an anisotropy of the corresponding elastic properties. Because trabecular bone is both anisotropic and heterogeneous, it is difficult to define “standardized” elastic properties [7].
1.2 Osteoporosis

The disease

Osteoporosis is a disease characterized by an excessive decrease in bone mass which leads to an increased susceptibility to fracture and skeletal deformation. The World Health Organization [8] defined osteoporosis as bone density 2.5 standard deviations below the mean for young white adult women [9]. Whilst a decrease in bone density can reduce the overall strength of bone, it is not the only factor which contributes to increased susceptibility to fracture, the quality of bone is also important. Bone quality is determined not only by bone mass (as measured by bone density) but also, amongst other factors, by the micro-architecture of bone, the crystal size and shape, the brittleness, the connectivity of the trabecular network, the vitality of bone cells, the ability to repair microcracks and the structure of bone proteins.

A common misconception about this disease is that it is considered to only afflict females, but the prevalence in men also increases exponentially with age. The rise in hip fracture rate occurs about 10 years earlier in women than in men. By the age of 90, about 17% of males have had a hip fracture, compared to 32% of females [10]. Additional to the obvious costs on health, osteoporosis is a global problem and carries with it significant socio-economic costs. This is illustrated by the IOF audit report “Call to Action” published in 2001, which claims that osteoporosis costs national treasuries in the EU over 4.8 billion euro annually in hospital healthcare alone. The following statistics, published by the National Osteoporosis Foundation, elaborate further on the impact of osteoporosis:

1) One in two women and one in four men over age 50 will have an osteoporosis related fracture in her/his remaining lifetime.

2) A woman's risk of hip fracture is equal to her combined risk of breast, uterine and ovarian cancer.

3) Osteoporosis is responsible for more than 1.5 million fractures annually.

4) An average of 24 percent of hip fracture patients aged 50 and over dies in the year following their fracture.

5) The estimated national direct expenditures (hospitals and nursing homes) for osteoporotic and associated fractures in the US was $17 billion in 2001 ($47 million each day) and the cost is rising.
1.2 Osteoporosis

The future: targeting genes which define osteoporosis

Genes contain the sequence for proteins, which are the building block of our bodies and help to regulate the operation of every organ. Knowledge about the genes involved can lead to advances in understanding and treatment of a disease, for example gene and protein expression can be regulated such that the desired phenotype is enhanced, moderated or even eliminated. In the case of osteoporosis the identification of a gene or set of genes which regulate bone strength would represent a huge step in allowing science to control bone strength and potentially lead to a cure for osteoporosis.

The introduction of new technology to measure bone mass helped provide the first clues that osteoporosis is a heritable disease. BMD studies of mother daughter pairs, twins and large cohorts estimated the heritability of this trait to be between 50% and 70%. This finding led most investigators to conclude that the phenotypic variation in BMD was caused by the action of a limited number of genes with discrete effects. Although data from these studies failed to yield major genes that defined osteoporosis, such studies, combined with genome wide scanning of multigenerational families, inferred that the genetic influence on bone acquisition was both complex and polygenic. In addition to this complex multifactorial nature of genetic influences, two other factors further complicate the search for osteoporosis genes. 1) Whilst there maybe polygenic determinants which have a direct influence on BMD these determinants themselves are influenced by genes that do not have a direct effect on BMD, i.e. epistasis. 2) There are numerous environmental factors that may modulate expression of one or more genes, i.e. nutrition, hormonal interactions, the mechanical environment and life style factors. These same technologies also led to the realization that BMD is a complex trait and is not the only influential factor. Analysis of bone micro-architecture has identified “bone quality” as another influential component and is a term which incorporates additional factors such as bone turnover rate, trabecular spacing and trabecular connectivity as well as microarchitecture, mineralization, microcracks and damage, distribution of the three main cell types in bone (osteoclasts, osteoblasts, and osteocytes), and collagen properties.

With so many aspects contributing to the strength of bone eluding the genes responsible presents a sizeable task and cannot be done by studying human biology alone. The study of genetics in humans is limited to some degree by the tremendous heterogeneity among population, as well as multiple genetic, heritable and environmental determinants of the target phenotype. If the challenge is to be realized a more controllable genetic model is required. In the last decade, the mouse as a model for skeletal phenotype has become increasingly
important. The biological similarities between man and mouse make this small animal the ideal experimental surrogate and in the context of comparative genomics this small animal greatly increases the likelihood that candidate genes and effective therapies will be found.

1.3 The mouse as a model

Many features of human biology at the cell and molecular levels are shared across the spectrum of life on earth; our more advanced organism-based characteristics are shared in a more limited fashion with other species. At one extreme are a small number of human characteristics (brain functions and behavior) that are shared by no other species or only by primates. But at a step below there is a whole set of characteristics, which are shared in common only with mammals. In this context, the importance of mice in genetic studies was first recognized in the biomedical fields of immunology and cancer research, for which a mammalian model was essential. Although it has been obvious that many other aspects of human biology and development should be amenable to mouse models, until recently, the tools just did not exist to allow for a genetic dissection of these systems.

The movement of mouse genetics to the forefront of modern biomedical research was catalyzed by the recombinant DNA revolution, which began over 30 years ago. With the ability to isolate cloned copies of genes and to compare DNA sequences from different organisms came the realization that mice and humans as well as all other placental mammals are even more similar genetically than they were thought to be previously. An astounding finding has been that all human genes have counterparts in the mouse genome which can almost always be recognized by cross-species hybridization. Thus, the cloning of a human gene leads directly to the cloning of a mouse homolog which can be used for genetic, molecular, and biochemical studies that can then be extrapolated back to an understanding of the function of the human gene. Although the haploid chromosome number associated with different mammalian species varies tremendously, the haploid content of mammalian DNA remains constant at approximately three billion base pairs. It is not only the size of the genome that has remained constant among mammals; the underlying genomic organization has also remained the same as well. Large genomic segments (on average, 10-20 million base pairs) have been conserved virtually intact between mice, humans, and other mammals as well. In fact, the available data suggest that a rough replica of the human genome could be built by simply breaking the mouse genome into 130-170 pieces and pasting them back together again in a new order [11, 12]. Although all mammals are remarkably similar in their
1.3 The mouse as a model

Overall body plan, there are some differences in the details of both development and metabolism, and occasionally these differences can prevent the extrapolation of mouse data to humans and vice versa [13]. Nevertheless, the mouse has proven itself over and over again as being the experimental animal model par excellence for studies of nearly all aspects of human genetics.

Besides the strong homology in the genome, the mouse is, among mammals, ideally suited for genetic analysis also for several other reasons. First it is one of the smallest mammals known, second it has a short generation time, in the order of 10 weeks from being born to giving birth. Third, females breed prolifically in the laboratory with an average of 5-10 pups per litter. Fourth, an often forgotten advantage is the fact that fathers do not harm their young and that laboratory-bred strains are relatively docile and easy to handle. Finally, investigators are even able to control the time of pregnancies.

Manipulation of the mouse genome and micro-analysis

The close correspondence discovered between the genomes of mice and humans would not have been sufficient to drive researchers into mouse genetics without the simultaneous development, during the last decade, of increasingly more sophisticated tools to study and manipulate the embryonic genome. Today, genetic material from any source (natural, synthetic or a combination of the two) can be injected directly into the nuclei of fertilized eggs; two or more cleavage-stage embryos can be teased apart into component cells and put back together again in new "chimeric" combinations; nuclei can be switched back and forth among different embryonic cytoplasmas; embryonic cells can be placed into tissue culture, where targeted manipulation of individual genes can be accomplished before these cells are returned to the embryo proper. Genetically altered live animals can be obtained subsequent to all of these procedures, and these animals can transmit their altered genetic material to their offspring.

Progress has also been made at the level of molecular analysis within the developing embryo. With the polymerase chain reaction (PCR) protocol, DNA and RNA sequences from single cells can be characterized and enhanced versions of the somewhat older techniques of in situ hybridization and immuno-staining allow investigators to follow the patterns of individual gene expression through the four dimensions of space and time.

Finally, with the automation and simplification of molecular assays that has occurred over the last several years, it has become possible to determine chromosomal map positions to a very high degree of resolution. Genetic studies of this type are relying increasingly on
extremely polymorphic microsatellite loci to produce anchored linkage maps, and large insert cloning vectors, to move from the observation of a phenotype to a map of the loci that cause the phenotype, to clones of the loci themselves. All of these techniques provide the scientific community with the ability to search for answers to the many questions posed. This will invariably lead to more questions, but the potential is there to elucidate the mechanisms of many diseases and realize effective treatments.

The mouse and osteoporosis

Rodent models for testing hypotheses to skeletal disorders are not new. In fact this is how many of the established treatments came to market. The ovariectomised rat is a well established tool and was used to test how estrogen deprivation affects the bone remodeling unit. At the forefront of technology today is the mouse model. Numerous mouse models exist, each of which attempt either identify or evaluate candidate genes associated with osteoporosis.

Inbred strains of mice and quantitative trait loci

Inbred strains of mice combined with the power of breeding strategies are used to identify the gene or the combination of genes responsible for a particular phenotype. This method is being used to uncover genes which regulate BMD.

Inbred strains are developed by repeated matings between siblings for at least 20 consecutive generations, which results in nearly 100% homozygosity at all alleles across the mouse genome, i.e. all mice have identical alleles thus an identical genetic makeup. (i.e. they could all be considered as identical twins). Many different inbred strains exist, each strain with differing sets of phenotypic characteristics. One such difference is the wide variation in BMD among inbred strains. By crossing these strains in accordance with various breeding strategies (so producing hybrid strains) regions of the genome responsible for phenotypic variation (Quantitative Trait Loci, QTL) can be determined via statistical analysis methods. These statistical methods (QTL analysis) correlate the phenotypic variation with genotypic variation across the hybrid population. QTL analysis, while lifting the ‘statistical fog’ surrounding conventional quantitative genetics [14], provides a powerful magnifying lens for deciphering the chromosome regions regulating complex traits. In this context, the introduction of QTL analysis in quantitative genetics can be compared with the introduction of the optical microscope in cell biology.
1.3 The mouse as a model

By crossing two inbred strains of mice results in hybrid F1 mice (termed the first filial generation) that are completely heterozygous and genetically identical with each other at all genetic loci. Intercrossing F1 progeny results in F2 mice in which there is independent segregation and random assortment of unlinked genes (Fig. 1.3).

![Diagram](image)

Figure 1.3: Schematic representation of crossing two inbred strains to produce an F2 generation exhibiting independent segregation and random assortment of unlinked genes. The histogram shows the variation of a specific phenotype across the F2 population (bone mineral content in this case).

The determination of QTL’s associated with a phenotype is made possible through the use of molecular markers. Molecular markers are essentially identifiable physical locations on a chromosome (e.g. gene) whose inheritance can be monitored. Markers can be expressed regions of DNA (genes) or some segment of DNA with no known coding function but whose pattern of inheritance can be determined. These markers define a DNA map, i.e. a multitude of reference point which can be used to help identify QTL’s. Via this molecular profiling and statistical analysis the association between a phenotype and a marker genotype can be tested. For most species, an adequate coverage of the genome can be achieved with approx. 100–150 marker loci evenly spaced along the chromosomes. Once a marker density of approx. one marker/20 cM is reached, it becomes more profitable to increase the number of progenies rather than the number of markers to increase the accuracy of QTL detection [15]. For the mouse there are 8000 genotypic markers that are polymorphic across all strains.

Micro-computed tomography (μCT) is a new emerging alternative technique to non-destructively image and quantify trabecular bone in three dimensions that has been pioneered in the Institute for Biomedical Engineering (ETH Zürich). The μCT revolution has provided
investigators with the opportunity to study many phenotypic characteristics associated with osteoporosis and bone quality, i.e. the ability to quantify trabecular bone volume, trabecular spacing and trabecular number provide more phenotypes for QTL analysis. This will most certainly contribute to a greater understanding of bone strength and the associated physical and genetic determinants.

Inbred strains when crossed can provide an invaluable tool for locating and enumerating QTL’s, moreover the gene x gene interaction in the F2 population resulting in phenotypic values that are greater or less than the progenitor strain phenotype can offer critical insight into the complex genetic influence on a specific phenotype. However the initial QTL’s may reside in chromosomal regions up to 40 centimorgans (cM = recombination distances between specific markers), areas of the chromosome with thousands of potential candidate genes. Fine mapping and congenic construction define narrower regions of the chromosome and allow for positional cloning and gene sequencing to take place.

The previous paragraphs describing the use of mice and breeding strategies are intended, only, to give the reader a basic insight into the power of the mouse as a tool for exploring human diseases. Despite major efforts only very few genes defining osteoporosis have been cloned. Still, inbred, recombinant inbred and congenic strains, as model systems, offer a wealth of information related to the acquisition and maintenance of peak bone mass.

1.4 Phenotypical characterization

Functional characterization

Experimental analyses of skeletal structures are generally performed to measure strength and stiffness associated with the applied conditions [16]. In the case of a phenotypical characterization they are used to quantify the influence of the genotype and/or environmental conditions on the functional properties of the skeleton.

Biomechanical testing can be separated in two major fields: analyses of tissue or whole bone specimens. The former is used to evaluate the mechanical behavior at the tissue level directly, whereas the latter is performed to characterize the whole bone structure or to extract tissue estimates from overall behavior.

The majority of biomechanical testing is conducted using tissue specimens yielding estimates of mechanical tissue properties. The methods applied are the same as those used to study the mechanical properties of typical engineering materials, such as metals, ceramics or
1.4 Phenotypical characterization

wood, for instance. They are therefore based on basic mechanics knowledge and its terminology. Typically tension-, compression- or bending-tests are conducted using standardized specimen geometry such as cylinders, cubes or beams. These specimens have a size of several millimeters [16-19]. Compression cylinders commonly have a length and diameter of about 12 and 8 mm, respectively, whereas tension specimens are of bigger size. A homogeneous material distribution is the principal underlying assumption allowing the determination of “averaged” material properties. The measurement of whole bone characteristics is under influence of various factors such as size and shape of the bone, tissue-strength, -architecture, -composition, load application, load distribution, and others (Fig. 1.4). These influences can only be eliminated and tissue properties can only be estimated reliably if extensive knowledge about these influencing factors exists and can be incorporated into the analysis.

Figure 1.4: General influencing factors that control the experimentally determined properties of whole bone specimens. Femur under compression (left) and femur under 4-point bending (right).

Femoral bending test

Due to the small size of the murine skeletal elements, commonly only whole bone tests, such as femoral bending or tibial bending are performed [18, 20]. These tests are similar to the standardized 3-point bending test used to analyze beams of homogeneous tissue with plane parallel surfaces. The 3-point bending test is an uncomplicated experimental analysis
requiring minimal expertise and hardware. The distance between the lower supports used to test mouse femora or tibiae is between 4 and 10 mm [21-23] depending on the mouse strain and the animal age. The vertical load is applied at the anterior surface of central diaphysis creating a linearly varying bending moment and constant shear force along the loaded femoral axis. The maximum bending moment occurs right at the load application point causing peak compression stresses at the anterior and tensile stresses at the posterior surface. Therefore, only the femoral region close to the load application point (diaphysis) is tested significantly (Fig. 1.5).

Figure 1.5: Femoral 3-point bending test showing a murine femur placed between two lower supports, 6 mm apart, and one upper support used to apply the vertical load.

The tissue properties derived from the bending tests are based on the beam-bending theory, which assume homogeneous material properties, a constant cross sectional area, a constant moment of inertia and a large aspect ratio (length/width ratio). These assumptions are not met for the femur or tibia of any mammal, and calculated tissue properties should therefore be treated with care. A recent study from Schriefer et al. [24] points out that bone with the highest aspect ratio and largest cortical thickness to diameter ratio, should be used to improve the accuracy of estimating the Young’s modulus.
1.4 Phenotypical characterization

Torsion and femoral neck test

Femoral torsion tests and compressive femoral neck tests are performed only occasionally [20, 25, 26]. In a torsion test, the distal and proximal regions of the femur are embedded in pods while the torsion load is applied via axial rotation (Fig. 1.6). Stiffness, ultimate load as well as failure load can be measured. The data obtained can be used in combination with cross sectional properties (e.g. cross sectional moment of inertia) to derive estimates of tissue characteristics. Similarly to the bending test, estimations are based on severely simplified mechanical models, which assume constant cross sections and homogeneous tissue properties. These assumptions can introduce an error up to 40% into the shear modulus estimation as reported by Levenston et al. [27].

![Figure 1.6: Femur tested under torsion through axial rotation of its proximal end. Proximal and distal part embedded in pods.](image)

In a femoral neck test, the distal portion of the femur shaft is embedded in a pod while a vertical compressive load is applied on the femoral head creating bending and shear forces in the femoral neck (Fig. 1.7). The measured failure load indicates the load carrying capacity of the cortical and trabecular arrangement in the femoral neck, but cannot be used to derive any biomechanical tissue properties.
Figure 1.7: Murine femur under vertical compression. An axial load is applied on the femoral head while the distal part of the bone is embedded.

Microstructural finite element analysis

Finite element analysis (FEA) has become an essential part of biomechanical investigations focused on better understanding the structure-function relationships associated with tissue adaptation and failure. With the recent introduction of microstructural FE models generated from computer reconstructions of trabecular bone, it is now possible to calculate loads at the microstructural or tissue level. These models have shown that predicted tissue stresses and strains in bone specimens can differ considerably from those estimated from apparent level stresses and strains for the material as a continuum [28, 29]. However, since the in situ loading conditions for these specimens are not exactly known, these models are not adequate for the determination of realistic loading conditions in bone tissue [29].

Microstructural FE models were used to calculate the complete stiffness characteristics of bone samples and related that to the three-dimensional architectural organization of the bone specimens and to ultrasound measurements [30, 31]. In a similar approach the development of architecture and mechanical stiffness characteristics in juvenile trabecular bone was determined [32]. Stresses and strains were also calculated to estimate the loading conditions that bone cells experience and used it to calculate bone adaptation [33, 34]. This model can explain the emergence and maintenance of trabecular architecture as an optimal mechanical structure, as well as its adaptation to alternative external loads.
1.4 Phenotypical characterization

In these studies cubes of bone were used, cut out of a larger bone. However, since the *in situ* loading conditions for these specimens are not exactly known, these models are not adequate for the determination of realistic loading conditions in bone tissue. For this reason, there is a need for full-scale models, including the cortical shell and realistic boundary conditions to model the musculoskeletal interface, resulting in an improved prediction of individual bone strength as an indicator for fracture risk or osteoporosis. Recently, tissue loading conditions were calculated in trabeculae of a microstructural model of a canine proximal femur [35] and a human proximal radius[36]

Ulrich *et al.* [37] took this approach one step further and used the same technology to analyze an *in vivo* microstructural model of the distal radius measured with high-resolution 3D-QCT. This was the first time that tissue-level stresses and strains were estimated in a living human subject.

Morphometrical characterization

Morphometry, with respect to biological and medical sciences, is the quantitative analysis of the form of organisms and their parts. In the fields of bone biology and biomechanics, it is used to describe the shape of bones at a macroscopic scale, and the architecture of bone tissue and bone cells at a microscopic scale.

In the past, the establishments of any relationship that could predict apparent biomechanical properties of bone, such as stiffness and strength using the bone mineral density (BMD), was one of the central objectives in the field of bone biomechanics. But the addition of architectural parameters improved the predictive power of the regression functions using BMD alone [38-40]. In early studies, two morphometrical parameters received the primary attention for the description of trabecular bone: relative bone volume and anisotropy. Relative bone volume (BV/TV) is the ratio of tissue volume total volume. It is equivalent to the corresponding bone area/total area ratio (B.Ar/T.Ar), which can be directly measured from 2D histological sections [41]. The anisotropy of trabecular bone architecture is an important characteristic, since it represents the principal directions of the trabecular orientation and exhibits a strong impact on the anisotropic stiffness and strength of bone, which was recognized early on by Raux *et al.* [42], Townsend *et al.* [43] and Goldstein [44]. The quantitative description of the anisotropy was first proposed by Whitehouse [45] when he introduced the concept of mean intercept length (MIL), which can also be expressed alternatively by the mean bone length [46] developed by Smit [47].
Chapter 1. Introduction

The introduction of µCT into the field of skeletal biology and biomechanics provided the research community with powerful analysis tool [48-53]. µCT is a non-destructive imaging technique which allows to achieve images at very high resolution (~5 µm). From two-dimensional projection, a fully three-dimensional image can be reconstructed. The advantage of three-dimensional images is that they enable assessment of truly three-dimensional parameters which may not be correctly computed from two-dimensional sections. Such indices can be used to estimate the influence of bone architecture on the mechanical competence of bone.

Although microstructural FEA provides accurate measures of bone mechanical properties, it does not yield information on which structural properties contribute most to the mechanical bone strength and if an ideal treatment could be designed, which architectural features should be targeted first; bone density, trabecular thickness or separation, connectivity or the structure model index. Several studies have illustrated that the inclusion of architectural properties of bone quality will increase the predictability of mechanical strength in trabecular bone [54-56]. In two recent studies on whole bones [18, 57], it was possible to improve also whole bone strength predictions by including micro-structural morphometric indices. From these studies we have also learnt that mechano-structure relationships, meaning which parameter or sets of parameters best determine mechanical bone competence, might well depend on the type of mechanical test used, the pathological state of the bone, and the type of treatment. But it is also clear that the inclusion of microstructural properties can greatly improve (as much as 21%) the prediction of mechanical bone strength. Nevertheless, these studies only demonstrated the importance of architectural bone properties in a statistical sense. They do not explain the real physical contribution of the microarchitecture to the mechanical failure behavior of bone. The so far presented techniques developed for three-dimensional trabecular bone morphometry apply to porous structures as a whole but not to its individual elements. A recent project [58] in contrary aimed at the computation of local morphometric parameters of individual elements and to link them to global failure. Especially, the ability to decompose the bone microarchitecture to extract individual structure features, such as trabecular rods and plates, show high potential to perform local bone morphometry. Local in that sense means that we are now able to quantify individual trabeculae with respect to their volume, thickness, orientation and type of structure. Earlier experimental studies, using an image-guided failure assessment technique, showed that failure of an individual trabecula can lead to global bone failure and that bone failure can be predicted best using a “weakest link of the chain” approach. Local bone morphometry
1.4 Phenotypical characterization

allowed identifying such “weak” trabeculae and therefore improved predictive power in the
determination of bone strength and failure behavior. It was also found that a 10% change in
local thickness (in the rods only) was responsible for a three fold increase in mechanical
strength of osteoporotic bone where changes in bone density were only linearly related to
bone strength in the low density (osteoporotic) regime [58].

Image-guided failure assessment (IGFA)

The strength of a bone can be determined from a direct mechanical test. It provides
information on global bone failure. However, questions as where failure is initiated, and how
failure is influenced by trabecular architecture, cannot be answered. For this reason, we have
developed an image-guided technique that utilize micro-compression in combination with
micro-computed tomography and allows direct three-dimensional visualization and
quantification of fracture progression on the microscopic level [59].

The IGFA device includes two steel load platens, a moment-relief bearing and an
integrated load cell. Since μCT imaging utilizes low-energy X-rays, the measurement window
in the micro-compression device (12 mm in diameter) is made of a radiolucent, highly stiff
plastic to allow X-ray penetration of the device. The internal load chamber fits wet or dry
bone specimens with maximal diameters of 9 mm and maximal lengths of 22 mm. The micro-
compressor has an outer diameter of 19 mm and a total length of 65 mm in order to fit into the
μCT gantry. Axial compressive displacements are progressively applied to the contained
specimen, while simultaneously measuring nominal stress. The specimen is μCT imaged after
each strain step; so that deformation of the three-dimensional microstructure of the bone can
be observed directly as the specimen is compressed beyond yield. The stiffness of the bone is
determined from the slope of the linear portion of the resultant stress-strain curve.
Comparisons with data from microstructural FE models based on image data from these test,
permit the determination of tissue modulus. Furthermore, these tests will allow us to directly
measure yield strain in murine bone.
1.5 Objectives of the study

Introduction

The gold standard to determine bone strength is a *functional*, mechanical test. Such a test gives several measures of bone strength such as ultimate force, ultimate displacement and work to failure. Due to their small size these tests are even more challenging in murine bone; compared to data from larger animals the precision is often lower [60]. An important limitation for our purposes is that these tests are laborious, hence, not applicable to test the large numbers of bones needed in genetic studies (commonly 1,000 bones per study). Therefore, a non-destructive, high-throughput technique to determine bone strength is currently being implemented in the Institute for Biomechanics. Nevertheless, accurate mechanical and material data on bone strength are still lacking. That is why we proposed to focus on bone mechanical properties investigation in this doctoral thesis. We uncovered bone properties such as absolute strength of whole bones or cortical samples as well as bone stiffness and postyield behavior. Fracture location and propagation were also analyzed and with this new input, we should be able to create more accurate and realistic μFE-models. Another point of great interest was bone damage at the microscopic and nanoscopic levels. We proposed to investigate the effects of the known, but barely understood process of bone microcracking. We looked into the porosity of bone and examined how this ultrastructure influences bone microdamage.

Specific aim 1: Determining bone mechanical properties with direct mechanical testing

Accurate positioning for mechanical testing of murine bone

Inbred strains of mice make useful models for studies of genetic effects on bone structure. Sensitive and precise methodologies to determine both genotype and phenotype are critical when evaluating genetic effects. The femoral neck and the vertebral body are relevant and sensitive sites for studying the degree of osteopenia [61]. The gold standard for determining bone strength is direct mechanical testing. Since murine bones are very small, preparation and positioning of the samples are of utmost importance for ensuring reproducibility of the tests. In **Chapter 2**, alignment systems were designed and evaluated for accurate positioning of murine femora and murine vertebrae in a mechanical testing device. Parametrical studies were also performed in order to investigate the influence on
1.5 Objectives of the study

reproducibility of misaligned samples. Further, all the mechanical tests were recorded with high-resolution and high-speed cameras. The feedback of these cameras gave a good insight into the reproducibility of the tests, especially alignment and positioning accuracy, and into the outer deformation and failure behavior of the tested bones.

Bone mechanical properties predicted by bone cross-sectional geometry

Engineering principles predict that bone cross-sectional geometry is an important determinant of breaking strength. In Chapter 2, cross-sectional geometry at the mid-diaphysis and the neck region of murine femora in C57BL/6 and C3H/He inbred strains was assessed by mean of desktop micro-computed tomography. The same bones were then tested under three-point bending or femoral head compression in order to measure their mechanical properties. Comparing the mechanical results with the cross-sectional morphometry, the influence of bone geometry on bone mechanical parameters was assessed. Specifically, we better determined the role of bone structure and geometry in the process of bone failure behavior.

Mechanical parameters as input for computational mechanics

In Chapter 2, the performed three-point bending and axial loading tests helped to determine accurate elastic and postyield properties of the femoral mid-diaphysis and femoral neck that were then incorporated into the $\mu$FE-models. Indeed, biomechanical testing is a straight-forward procedure but has its limitations, as it is a destructive test, indicating that a sample can only be tested once. Furthermore, these tests are prone to errors, especially in view of the small size of murine bones, and they are laborious, hence, not applicable to test the large numbers of bones needed in genetic studies. Therefore, a functional phenomics approach, which is a non-destructive and high-throughput technique to determine bone strength, is currently being developed in the group, but outside this thesis. Nevertheless, the highly detailed and precise mechanical testing developed in the thesis will help to validate the computational mechanics approach. This will be done by testing different inbred mice, i.e. C57BL/6J and C3H/HeJ progenitor’s strains. Mechanical tests will show mechanical properties differences between these groups. A long-term goal of this type of research will be to show that the $\mu$FE-models also compute such differences in a similar fashion.
Specific aim 2: Developing an integrative approach for hierarchical bone investigation

As already mentioned, biomechanical testing is the gold standard to determine bone competence. In Chapter 3, we incorporated several imaging methods in our mechanical setups in order to get a better insight into bone deformation and failure mechanisms. Our aim was to develop an integrative approach for hierarchical bone investigation, working at different scales of resolution ranging from the whole bone to its ultrastructure. This approach explained how microstructural and even ultrastructural damage in bone lead to changes in bone competence and in the worst scenario to bone failure.

Imaging of bone biomechanical testing at the macroscopic level

The use of high-resolution and high-speed cameras increased drastically the amount of information assessed from a mechanical bone test. This was especially important when dealing with very small bones such as the murine femur. Here the feedback of the camera in the process of aligning and positioning the samples was indispensable for reproducibility. Global failure behavior and fracture location was visualized with high temporal resolution.

Image-guided failure assessment at the microscopic level

Complementary to intrinsic material properties, bone microstructure both in trabecular and cortical bone influences bone strength and failure mechanisms significantly. For this reason, we developed an image-guided failure assessment technique, also referred to as functional microimaging, allowing direct time-lapsed 3D visualization and computation of local displacements and strains for better quantification of fracture initiation and progression at the microscopic level.

The existing internal load chamber was modified so that whole murine bones could be tested. Stepwise compressive IGFA tests, analog to direct mechanical tests, were then performed on murine femora. The power of IGFA in comparison with direct mechanical testing is that IGFA uncovers the local deformations and strains inside the bone structure.

Looking at local deformations and this especially at loading or support points between sample and classical material testing machine, IGFA permits to correct the measured total deformation of the sample. Indeed, classical tests do not take in account that local deformations form at contact points between load and samples. These local deformations are then automatically added and the measured total deformation is wrong.
1.5 Objectives of the study

Image-guided failure assessment in the submicron domain

In bone research micro-computed tomography has become a powerful and a widely used tool, to assess bone density, mineralization and architecture using conventional X-ray tubes [50, 53], and even smaller features [62], i.e. the osteocyte and osteoclast lacunae and microcrack distribution using synchrotron radiation µCT (SRµCT). SRµCT and µCT are well suited because they are both noninvasive techniques and real 3D data sets of the anisotropic bone structures are retrieved.

Due to its high resolution, the investigation of different mouse inbred strains by means of SRµCT allowed a better insight into micro-damage behavior than with a classical desktop µCT. The discernment of microcracks improves the understanding of failure behavior.

In Chapter 4, we set up an experiment that explored the initiation, propagation and damage accumulation of microcracks in murine cortical bone under dynamic loading in two varieties of mouse inbred strains, C3H and B6. The main goal of this study was to analyze microcracks in murine cortical bone at the sub-micrometer level. In these experiments, we investigated the contribution of bone canular network and osteocytic cell lacunae to the formation and propagation of microcracks in murine cortical bone under compressive loading. We could then answer such question as: Do osteocytic lacunae act as stress-risers? Does osteocyte volume vary at different locations and in different strains? Does this affect microcracks propagation? What is the effect of the canular network on microcracks initiation and propagation? The outcome of this research is crucial for the understanding of bone strength.

Due to its high resolution, the investigation of different mouse inbred strains by means of SRµCT uncovered bone ultrastructure and microcrack behavior. Resolution of 1 μm and better has made it possible to analyze trabecular architecture and local tissue properties [62]. Moreover, new features such as bone vasculature and even single osteocytes within the bone tissue are now detectable in a fully nondestructive and three-dimensional fashion [63]. A previous study [64] has shown that both strains, B6-lit/lit and C3.B6-lit/lit, have very similar overall bone porosity, but canals and osteocytic lacunae are differently distributed. Mechanical test have also shown differences in bone mechanical properties of these two strains. A new challenge was to relate these microscopic features with the initiation, propagation and damage accumulation of microcracks and show how microcracks contributed to the overall bone competence. Understanding how bone reacts under loading at a nanoscopic level gave new clues for whole bone strength and failure behavior.
1.6 Bibliography


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1.6 Bibliography


1.6 Bibliography


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Chapter 2

Whole bone mechanics
2.1 Femoral neck stiffness and strength critically depend on bone alignment – a parametric study in a mouse inbred strain

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Abstract

Biomechanical tests of human femora have shown that small variations of the loading direction result in significant changes in measured bone mechanical properties. However, the heterogeneity in geometrical and bone tissue properties make human bones not well suited to assess reproducibly the effects of loading direction on stiffness and strength. In order to precisely quantify the influence of loading direction on femoral neck stiffness and strength, we tested femora from C57BL/6He (B6) inbred mice. Very low variability in bone properties between each animal can be expected. In order to accurately position the femora for biomechanical testing, we developed an image-based alignment protocol. We then investigated the loading direction influence on femoral neck stiffness and strength. First, an aluminum femoral phantom was tested under compression with different loading directions. The benefits of testing such a phantom are the homogeneous material properties and excellent experimental reproducibility. Finally, we performed compressive tests under different loading directions on B6 mice until failure of the femoral neck. With the newly developed alignment protocol the reproducibility error of the axial alignment was 1.45°. The new protocol also significantly reduced variability of measured stiffness to less than 13%. Both tests, with the aluminum phantom and the murine bones, showed a strong dependence of stiffness on loading direction: a 5° change in loading direction resulted in almost 30% change in stiffness. Murine
bones testing also revealed a variation in strength due to loading direction: $5^\circ$ change in loading direction resulted in 8.5% change in strength. In conclusion, this study showed the importance of precise sample positioning for testing murine femoral neck and the extreme sensitivity of this site regarding loading direction. These results strongly indicate that femur alignment also has to be closely controlled when performing mechanical tests of the femoral neck in human bone.

**Keywords:**
Femoral neck, mouse strain, biomechanical testing, aluminum phantom, loading direction

**Introduction**

Many elderly people suffer from osteoporosis, a skeletal disorder characterized by compromised bone strength leading to an increased risk of fracture. Osteoporosis primarily induces bone fragility resulting from decreased bone mass, altered microarchitecture, and impaired bone quality [1]. Any bone can be affected, but of special concern are fractures of the hip, the spine and the wrist. Vertebral fractures have serious consequences, including loss of height, severe back pain, and deformity. A hip fracture almost always requires hospitalization and major surgery. It can impair a person's ability to walk unassistedly and may cause prolonged or permanent disability and even death. Fractures of the femoral neck mainly occur in two situations. The first and most common one is under impact loading during a fall. The second configuration is under non-traumatic loading. In both configurations, fracture risk is much higher for people suffering from osteoporosis.

Since the femoral neck is a relevant and sensitive site for studying the degree of osteopenia, many researchers have investigated this location. The gold standard to determine bone strength associated with susceptibility to fractures is biomechanical testing [2-8]. It has been shown that, in addition to bone structural parameters such as bone density, bone architecture and bone geometry, also the loading configuration plays a non-negligible role in determining bone competence. The direction of the applied load on the femur may be an important factor in the etiology of hip fractures. In an ex-vitro study on human femora, Pinilla et al. [9] showed that a moderate variation in the fall-related loading angle significantly reduced the failure load of the proximal femur. They found that failure load decreased by 24% as the loading angle changed from $0^\circ$ to $30^\circ$. Using in vivo QCT-based modeling of human femora, similar findings [10] showed that the bending strength of the femoral neck varied significantly for different loading directions.
2.1 Femoral neck stiffness and strength critically depend on bone alignment

Although tests on human bones are the most pertinent when studying human bone properties and diseases such as osteoporosis, the heterogeneity of the populations makes them not well suited to precisely assess the effects of loading direction on bone strength experimentally. The results are affected by differences in geometry and material properties between the samples. Indeed, the mechanical and material properties variability in human samples is often high, since they originate from people having different sizes, different ages, different lifestyles and different genetic backgrounds. This heterogeneity can be largely avoided using animal models, especially when using inbred strains [11-17]. Animal models complement and extend human studies by allowing close control of environmental factors, by expanding the characterization of phenotypes underlying bone strength and by facilitating breeding strategies to identify genetic linkage [18]. Raising inbred strains for mechanical studies allows having populations with exactly same age, identical genotypes and constant environmental factors including diet and daily activity. This results in very low variability in bone properties. Consequently the difference in mechanical properties as measured in experimental testing can be assumed to mostly depend on variations in the boundary conditions. Rat and mouse inbred strains with relevant biological phenotypes related to disease models may provide important genetic clues that will improve the efficiency of identifying genes underlying bone strength.

In this work, we hypothesized that femoral stiffness and strength at the hip site are dependent on the loading angle. The specific aim of this study was to quantify the influence of sample positioning on stiffness and strength in the femoral neck. Specifically, we investigated inbred female C57BL/6He mice. This inbred strain is commonly used in bone phenotype studies [1, 19-27].

Material and Methods

Experimental tests were performed to quantify the effect of loading direction on biomechanical parameters of the femoral neck. First, we tested an aluminum phantom of the murine femur, in which the femoral head was loaded under different directions. The aluminum phantom permitted an excellent control of the testing variability, because this test was not affected by variations in material properties nor geometry. Second, we developed an alignment device as well as a new positioning protocol for murine femora. After developing this protocol we performed identical tests as with the aluminum phantom to investigate the influence of changing loading directions on real bone femoral neck stiffness and strength.
Mechanical testing of the aluminum phantom

An aluminum phantom of a mouse femur was machined using laser technologies (Fig. 2.1). The phantom was embedded in polymethyl methacrylate (PMMA) and mounted on a ball-socket joint support. This support allowed compressive tests of the femoral head with different angles of inclination in frontal and sagittal planes (Fig. 2.1). The tests were performed on a materials testing machine (1456, Zwick GmbH & Co.). We preloaded the sample with 1 N, the loading rate was 0.5 mm/s and the maximal load was limited to 25 N in order not to exceed the elastic limit of the phantom and to prevent permanent damage while repeating the loading test for each angular configuration. For each orientation of the phantom, tests were repeated three times. During the compressive loading of the aluminum phantoms, we measured force and displacement from which stiffness was computed. Due to the symmetry in the geometry of the aluminum phantom, in the sagittal plane, only positive inclination angles were tested from 0° to 45° with 5° increments. In the frontal plane, the inclination was varied from -45° to 45°, with 5° increments.

![Figure 2.1: Left: Aluminum phantom of a murine femur. Right: phantom embedded in PMMA and mounted on the ball-socket joint support. Here, the phantom is tested under a positive loading angle of 20°](image)

Sample alignment

For sample alignment, we dissected left and right femora from nineteen fresh frozen 16-week old C57BL/6He (B6) female mice. Use of mice in this research project was reviewed and approved by the local IACUC. Femora were cut above the condyles, resulting in a length
2.1 Femoral neck stiffness and strength critically depend on bone alignment

of 11±0.5 mm. Using a stereomicroscope, the femora were positioned in a custom made Plexiglas alignment device (Fig. 2.2). Online registration of the bones in the alignment device was made with a CCD camera attached to the stereomicroscope and connected to a personal computer. We quantified bone alignment from the camera image on the computer screen. Quantitative assessment of bone alignment was performed in three measurements. First, we measured the angle $\alpha$ in the transverse plane of the femoral neck in the alignment device (Fig. 2.3). The reference points to define this angle were the third trochanter and the center of the femoral head. Then we determined the angles $\beta$ and $\gamma$ of the femoral shaft in the frontal and sagittal planes, respectively. To measure these two angles, we used as reference points the top of the main trochanter and the center of the shaft diameter at the cut position (Fig. 2.3). Finally, the absolute angle $\delta$ between the vertical axis and the femoral shaft was trigonometrically computed from $\beta$ and $\gamma$. After the bones were positioned, they were embedded with cyano-acrylate glue (Superglue, UHU Schweiz AG, Schönenwerd, Switzerland) into aluminum bone holders which could then be rigidly fixed in the testing device.

**Figure 2.2**: Left: Exploded view of the alignment device with ten murine femora ready to be aligned and ten aluminum cylinders where the femora will be glued into. Right: Detail of the alignment device with aligned bones clamped between the two upper Plexiglas plates.
Figure 2.3: The angular alignment of each femur was measured in three planes. Left: Angular alignment $\alpha$ in the transverse plane; Center: angular alignment $\beta$ in the frontal plane; Right: Angular alignment $\gamma$ in the sagittal plane. Anatomical landmarks were used as reference points to define femur orientation: $A$: third trochanter; $B$: center of the femoral head sphere; $C$: center of the femoral shaft at the cut position; $D$: top of the trochanter.

Biomechanical testing of murine femoral neck

For biomechanical testing, we dissected left and right femora from twenty-nine fresh frozen 16-week B6 female mice. The animals were stored at -20°C and thawed at room temperature just before dissection of the femora. Left and right femora were meticulously prepared in exactly the same way as described in section Sample alignment for mechanical testing. The femora were then mounted on a ball-socket joint support, which allowed compressive tests of the femoral head with different angles of inclination in the frontal plane (Fig. 2.4). The 58 samples were divided into five groups. Each group was tested with a different inclination angle in the frontal plane ranging from $-10^\circ$ to $10^\circ$, with $5^\circ$ incrementing. The samples were randomly distributed to each group. Originally, each group had similar
2.1 Femoral neck stiffness and strength critically depend on bone alignment

numbers of left and right femora, but due to loss of samples during the experiments, the population in each group varied finally from 8 to 13 samples. Some samples were broken during the positioning preparation and others were excluded from the testing results due to bad embedding. The mechanical tests consisted of loading the femoral heads until fracture of the femoral neck occurred, using the same materials testing machine as for the aluminum phantom tests. The samples were pre-loaded with 1 N and load-displacement curves were recorded at a crosshead speed of 0.5 mm/s until failure. Stiffness was calculated as the slope of the linear part of the load–displacement curve. Strength was also measured as the maximum of the load–displacement curve.

![Figure 2.4: One B6 right femur positioned in the materials testing machine under a negative loading angle of -5°.](image)

**Statistical analysis**

In both aluminum phantom and murine bone testing, regression lines were computed to show the linear relationship between stiffness and angular position. In murine bone testing, such a regression line was also computed to illustrate the relationship between bone strength and angular position. The error in positioning alignment using the new developed device was calculated with the root mean square (RMS) formula. Stiffness and strengths were compared between the five murine bone groups, with inclination angles ranging from -10° to 10°, by Single way ANOVA with a Least Significant Difference (LSD) post hoc analysis. All
statistical analysis was performed with the statistical program: SPSS 13.0 (The Apache Software Foundation)

Results

The compressive loading tests of the aluminum phantom showed an excellent reproducibility of the three measurements for each angle (CV = 0.4%). The relationship between stiffness and angular position in the sagittal plane was linear (Fig 5, $R^2 = 1$, $p < 0.01$); 5° off-axis loading resulted in a stiffness loss of 8.5%. In the frontal plane, the stiffness had a maximum at 20° inclination (Fig. 2.5). Around the neutral position of 0°, between -5° and 10°, the stiffness related linearly to inclination angle ($R^2 = 0.99$, $p < 0.01$) and a change in inclination of 5° indicated a change in stiffness of 50%.

Figure 2.5: Stiffness of the aluminum phantom in the sagittal plane (top) and in the frontal plane (bottom). The reproducibility of the measurements was so good that the error bars fall within the data point. (top): the data were linearly distributed ($R^2 = 1.00$, $p < 0.01$). Due to symmetry of the aluminum phantom, measurements were performed only for positive angles. (bottom): The data were linearly distributed around the neutral position [-5°; 10°] ($R^2 = 0.99$, $p < 0.01$). Stiffness was maximal at 20° inclination.
2.1 Femoral neck stiffness and strength critically depend on bone alignment

The Plexiglas alignment device was developed to simultaneously align 10 left or 10 right murine femora. Using this new device, the angle in the transverse plane was $\alpha = 90.05^\circ \pm 3.23^\circ$ (mean ± sd) and the axial angle relatively to vertical was $\delta = 89.57^\circ \pm 1.5^\circ$ (mean ± sd). The reproducibility errors (RMS) of $\alpha$ and $\delta$ were 3.17° and 1.45°, respectively.

For the murine femora we found a significant influence of the inclination angle on the measured bone stiffness and strength in the frontal plane. Stiffness was significantly different between each group ($p < 0.05$) (Table 2.1). The relationship between stiffness and angular position showed a strong linearity ($R^2 = 0.98$, $p < 0.01$), just as for the aluminum phantom. Thus, the bones and the aluminum phantom demonstrated similar linear trends. The femoral neck stiffness rose linearly with the absolute femoral shaft angle $\delta$ (Fig. 2.6). A 5° inclination in axial positioning around the neutral position of the B6 femora caused a 28.5% change in stiffness.

Further, bone strength also showed a linear relationship with angular position ($R^2 = 0.86$, $p < 0.01$). A 5° inclination in axial position around the neutral position of the B6 femora caused a 8.5% change in strength. So, strength was also affected by change in angular position, but less than stiffness. Further, strength was less variable than stiffness and ranged from 12.8 to 17.5N with a maximum CV of 10.5% at the neutral position, 0° (Fig. 2.6, Table 2.1).

<table>
<thead>
<tr>
<th>Groups (loading directions)</th>
<th>-10°</th>
<th>-5°</th>
<th>0°</th>
<th>5°</th>
<th>10°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiffness: Mean ± SD (N/mm)</td>
<td>29.91±3.73</td>
<td>37.01±4.28</td>
<td>51.65±6.34</td>
<td>60.03±7.78</td>
<td>65.86±7.4</td>
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<tr>
<td>Stiffness: CV (%)</td>
<td>12.47</td>
<td>11.57</td>
<td>12.27</td>
<td>12.96</td>
<td>11.24</td>
</tr>
<tr>
<td>Strength: Mean ± SD (N)</td>
<td>13.18±1.32</td>
<td>12.8±0.83</td>
<td>15.95±1.68</td>
<td>15.9±1.47</td>
<td>17.53±1.81</td>
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<tr>
<td>Strength: CV (%)</td>
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<td>6.45</td>
<td>10.54</td>
<td>9.27</td>
<td>10.33</td>
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<td>8</td>
<td>8</td>
<td>9</td>
<td>13</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.1: Femoral stiffness and strength of the femoral necks measured in five groups loaded under five different angles ($\beta$) in the frontal plane. Stiffness and strength of the murine bones depended strongly on loading direction. All groups were significantly different for stiffness ($p < 0.05$), as assessed by single way ANOVA test with LSD post hoc analysis.
Figure 2.6: (top) Stiffness of aluminum phantom plotted against the loading angle $\beta$ in the frontal plane. The data are linearly distributed around the neutral position [-5°; 10°] ($R^2 = 0.99, p < 0.01$). (middle) B6 femoral neck stiffness plotted for the five tested loading angles $\beta$ in the frontal plane. The data were linearly distributed around the neutral position ($R^2 = 0.98, p < 0.01$). (bottom) B6 femoral neck strength plotted for the five tested loading angles $\beta$ in the frontal plane. The data were linearly distributed around the neutral position ($R^2 = 0.86, p < 0.01$).
Discussion

The aim of this study was to investigate the influence of sample alignment on experimentally determined bone stiffness and strength. We showed that accurate alignment is of utmost importance for accurate and precise determination of bone stiffness and strength; a 5° deviation from perfect vertical alignment led to a 28.5% error in estimated stiffness. The effects on strength were less dramatic, but with a change of 8.5% per 5° remained still considerable. In order to limit alignment errors, we developed and designed a new sample alignment protocol which was able to position murine femora with an absolute error (RMS) of only 1.45°.

For our parametric analyses we used a mouse inbred strain, which has the advantage that the individuals are genetically identical, hence, the femora are very similar. But even femora from inbred mice show some biological variability. To exclude any variability, we also used an aluminum phantom of a mouse femur. Its material properties are well known and remain constant through a whole test series as long as the deformations stay within the elastic domain. Therefore, the variations in the tests results were only depending on the changes in loading direction and were not disturbed by any other parameters.

The head of the aluminum phantom was loaded under different directions. As expected, reproducibility of the phantom tests was close to perfection (CV = 0.4%). The changes in stiffness due to variations of the loading direction were remarkably pronounced: 5° inclination relative to the vertical position in the frontal plane induced a stiffness change of 50%. The same inclination in the sagittal plane resulted in 8.5% difference in stiffness compared to vertical loading. For the same inclinations, the metallic phantom showed more marked variations in stiffness than the murine bones. Its custom machined geometry as well as its different material properties made it stiffer than murine bones. For such geometry, a stiffer material reacts stronger to variations in loading direction. Nevertheless, the phantom showed very similar behavior to different loading configuration as real bones. Furthermore, the perfect reproducibility of the tests on one unique sample made this model a very useful tool for our parametric research. Indeed, the changes in stiffness were directly related to changes in loading directions without any additional disturbance due to differences of geometry or material properties from one test to the other.

Stiffness was maximum when the bones were tested under 20 degrees (Fig. 2.5) The existence of a maximum coincides well with theories which maintain that femoral geometry and structures are optimized for very precise loading configurations [28-31], and suggest that
overall geometry of the femur is adapted to best resist physiological loading. At this maximum stiffness angle, the maximal bending moment along the femoral shaft had a minimum. This supports the hypothesis that anatomy and function of the hip mechanical system is optimized in a way that limits the highest bending stresses in the femoral shaft [30, 32, 33]. Moreover, Bergmann et al. [30] showed that the loading directions in the human femur for all daily activities varied in a very small range. In this study, we demonstrated a somewhat different view; the femur seems to have an optimized geometry for one specific loading direction. For this loading direction, the femur behaves stiffer and bending stresses in the whole bone are lower.

The alignment device resulted in an angular reproducibility error (RMS) of 3.17° in the transverse plane for the murine femora. This error is not so important for typical vertical compressive tests of the femoral neck, since, in this configuration, the femur is vertically oriented and the mechanical setup is axis symmetric around the vertical axis. Of much more importance is the error (RMS) in the axial alignment angle $\delta$ of 1.45°. Taking the linear regression equation as calculated from stiffness against $\beta$ (Fig. 2.6), a 1.45° axial error resulting from the alignment procedure was then responsible for a 5% error in stiffness. This means that when testing femoral neck stiffness, 5% of variability is explained by the misalignment and the rest comes from actual differences in bone geometry and material properties. The use of our alignment device showed clear improvements in reproducibility as compared to other studies. The stiffness coefficient of variation (CV) of the bone mechanical tests laid between 11 and 13% for the different inclinations groups (Table 2.1). These values mean that 6 to 8% of the variability come from differences in bone geometry and materials properties between the samples, since 5% are due to sample misalignment in the mechanical setup. This CV of 11 to 13% is much lower, hence, better, than previously reported for this site in mice from the same inbred strain [22]. This previous study reported a CV of 27.5%; more than two times higher than in our study. Not only the stiffness, but also the strength had a smaller CV in our study (Table 2.1). For different loading angles, the CV of the strength was between 6.5% and 10.5%, which was better than the 13.5% in the aforementioned study [22]. These numbers demonstrate that a good alignment significantly lowers variability in stiffness and strength measurements.

Our experimental tests revealed that small variations in alignment induced large changes in the measured stiffness. Around the neutral position of 0°, we measured a change in stiffness of almost 30% for 5° axial inclination in the frontal plane (Fig. 2.6). Small variations in alignment also provoked significant changes in bone strength; a 5° inclination in the frontal
2.1 Femoral neck stiffness and strength critically depend on bone alignment

plane was responsible for 8.5% change of bone strength. This narrow relationship between loading directions and stiffness or strength is particularly relevant, since femoral bone competence in inbred mice is a phenotype that is widely assessed. In such studies, large populations of mice are usually experimented within the same strain or between different strains. Comparing results between predefined groups and showing significant differences between treated or untreated animals, between different strains or between different models is often the main goal of these studies. Reducing variability in the results within each group makes the results more powerful, increases the significance of the study and therefore its impact. In the case of femoral bone testing, it is exactly what we achieved in reducing the variability caused by bad sample positioning.

Additionally, this study showed a predictable influence of the loading angle on sample stiffness. In the sagittal plane, the phantom showed a linear dependence between stiffness and loading angle ($R^2 = 1.00, p < 0.01$) (Fig. 2.5). In the frontal plane, around the neutral position from $-5^\circ$ to $10^\circ$, stiffness also linearly correlated to inclination angle ($R^2 = 0.99, p < 0.01$) (Fig. 2.5). Similar linear correlations were found for B6 femoral neck stiffness and loading angle around the neutral position ($R^2 = 0.98, p < 0.01$) (Fig. 2.6). Strength also linearly correlated to inclination ($R^2 = 0.86, p < 0.01$). Hence, we are now able to predict the influence of loading direction on femoral stiffness and strength. We showed this predictable influence for murine bone testing. With the commonly accepted hypothesis that the murine model is an excellent model for bone testing and phenotyping [1, 19-27], such a predictable influence of loading inclination on bone stiffness is likely to exist in the case of human femora, too.

The advantages of using an animal model are that the costs are lower and that many more samples can be tested than in human studies. Nevertheless, the results of the current study are of importance also for tests performed on human femora. There is no reason to assume that what we found for murine femora should not be valid for human femora as well. Results of our study imply then that such an angular dependence of the stiffness must also be present in human bones. Future studies, determining bone mechanical properties of healthy and osteoporotic human femora, should therefore include very accurate and reproducible sample preparation and alignment protocols, similar to the ones presented in this publication. In combination with results from Pinilla et al. or Carpenter et al. [9, 10] this study highlights the importance of loading direction in femoral neck mechanical testing. Changes in loading direction lead to drastic and predictable changes of bone structural capacity. Exact angles need therefore to be reported in order to be able to compare different studies.
In summary, the femoral neck is a very sensitive site regarding load direction in femoral head compressive tests. Small alignment errors generate significant changes in the femoral stiffness and strength. Furthermore, loading direction on the femoral head showed a very predictable influence on femoral stiffness. This influence plays a key role in femoral biomechanical testing, since different loading directions can potentially have higher effects on mechanical results than intrinsic differences in bone mechanical and material properties. This study was able to quantify these effects in a murine model and also highlighted the importance of accurate control of loading direction in human femoral neck testing.

Acknowledgements

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References

2.1 Femoral neck stiffness and strength critically depend on bone alignment


2.2 Effects of bone structural and material properties on bone competence

2.2 Differential effects of bone structural and material properties on bone competence in C57BL/6 and C3H/He inbred strains of mice

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Abstract

The femoral neck is a relevant and sensitive site for studying the degree of osteopenia. From a mechanical perspective, osteopenia is characterized by reduced bone stiffness and strength leading to an increased fracture risk. Engineering principles predict that bone structural parameters, like cross-sectional geometry, are important determinants of bone mechanical parameters. Mechanical parameters are also directly affected by the material properties of the bone tissue. However, the relative importance of structural and material properties is still unknown. Inbred strains of mice make useful models to study these aspects. Therefore, the aim of this study was to compare bone competence and structural parameters of both low bone mass C57BL/6 (B6) and high bone mass C3H/He (C3H) strains in order to better determine the role of bone structure and geometry in bone failure behavior. Murine femora of 12- and 16-week-old B6 and 12- and 16-week-old C3H inbred strains were mechanically tested under axial compression of the femoral neck. In order to assess the structural properties, we performed 3D morphometric analyses in five different compartments of the mouse femur using micro-computed tomography (μCT): full bone, trabecular bone in the metaphysis, cortical bone in the diaphysis and trabecular and cortical bone in the neck. The mechanical tests revealed that B6 femora became stiffer, stronger, and tougher from 12 to 16 weeks, while bone brittleness stayed constant. C3H bone stiffness increased, but strength
remained constant, toughness decreased and bone became more brittle. These age effects indicated that B6 did not reach peak bone properties at 16 weeks of age while C3H did reach skeletal maturity before 16 weeks of age. Our investigations showed that the strength of the femoral neck in the B6 strain was explained to 83% by cortical thickness at this location; in contrast, in C3H none of the mechanical properties of the femoral neck were explained by bone structural parameters. We conclude that the relative contributions of bone structural and material properties on bone strength are different in B6 and in C3H. We hypothesize that these different contributions are related to differences at the ultrastructural level of bone that affect bone failure.

**Keywords:**
Inbred strains, biomechanics, bone strength, bone structure, mouse, bone quality

**Introduction**

Femoral neck fractures are becoming an alarming clinical and social problem worldwide. Due to the advances in modern medicine and an ever-growing life expectancy in the general population, trauma centers are increasingly faced with a substantial age-related case load [1-3]. Furthermore, hip fractures are associated with a high level of patient morbidity and mortality. It is reported that about 25% of subjects die within one year after the fracture event [4, 5] and that only about 60% of subjects completely regain normal physical activity [6]. Therefore, much clinical attention has been centered on the prevention of femoral neck fractures. Prevention therapies, based on hormone or bisphosphonate intake, have been shown to be effective. However these remain expensive and have associated side effects [7]. Subsequently, efforts have been made to identify those subjects at high risk of fracture and, consequently, in need of secondary prophylaxis. The primary cause of hip fracture is osteoporosis, a disease that reduces bone density below that level needed for the mechanical support of normal activities [8]. Attempts at identifying individuals at risk of hip fracture have involved identifying those with critically reduced levels of bone density. The procedure requires that the clinician obtains a dual energy X-ray absorptiometry scan of the patient’s proximal femur. Bone mineral density (BMD) is measured at various regions of interest and compared with the mean femoral BMD of a healthy population (chosen as the reference level). However, the two density distributions, of patients at-risk and age-matched controls, have been found to overlap by large amounts, reducing the accuracy of classification to about 65% [9].
2.2 Effects of bone structural and material properties on bone competence

These problems have led many to attempt to improve patient classification by considering other parameters besides bone density. Factors such as subject’s life-style, coordination, and proximal femur anatomy, have been identified as being influential in the occurrence of femoral neck fracture [10]. In particular, the effect of various proximal femur geometric measurements has been investigated [11-13]. Nevertheless, even though the femoral anatomy has been demonstrated to be important in the prediction of fractures, the relative role of each geometric measurement remains controversial [14, 15].

The use of the mouse as a model for human musculoskeletal diseases has increased in popularity as the mouse genome has been well characterized. The advantages of the mouse model are that various strains have been observed to exhibit disease state characteristics similar to those found in humans and that the mouse is easily accessible to manipulation of the genetic makeup by either gene knockout, gene overexpression (transgenes), or genetic breeding strategies [16]. With the exception of identical twins, the genetic background in humans varies significantly from one individual to another, making studies of genetic involvement in a given bone phenotype in humans difficult. Well-characterized animal lines, such as murine inbred strain, with phenotypes related to certain aspects of human osteoporosis are therefore used as an approach to study more homogeneous populations.

In previous biomechanical studies of murine bones, diaphyseal bending strength [17-23] and torsional strength [24, 25] have been measured. However, the femoral neck is clinically a more interesting measuring site than the diaphysis. Studies on rabbit, rat and mouse bones have shown that the evaluation of femoral neck strength is a good indicator of bone fragility [26-31]. In these studies, the femora have been loaded at the femoral head in a direction parallel to the femoral shaft axis, simulating single-legged stance in humans.

Despite the wide variety of clinical methods available today for assessing bone properties, nondestructive imaging methods such as peripheral quantitative computed tomography (pQCT) and DXA have limited use for evaluation of microstructural parameters due to the small size of murine bones. Histomorphometry, despite its very high resolution, is a destructive and time-consuming method. Alternatively, micro-computed tomography (μCT) is fully nondestructive and well-suited for assessing truly three-dimensional (3D) microstructural bone properties [32-37]. Previous studies have shown μCT to be an accurate technique with close correlations between microtomographic and histomorphometric measurements of static structural bone metrics in various applications [38-42].

Adult C3H/HeJ (C3H) and C57BL/6J (B6) mice are similar in body size and weight and their bones are of similar external size, but show significantly different morphological
and compositional bone traits [43-45], such as adult peak bone density and cross-sectional area [46, 47]. More importantly, these two mice have often been identified as a model system for high (C3H) and low (B6) bone mass phenotypes [48] and with that as a model to study genetic factors in osteoporosis. In the present study, we evaluated the microstructure and mechanical properties in the femoral neck of the two inbred strains B6 and C3H. We hypothesized that microstructural properties would predict mechanical behavior of the femoral neck differently in the two mouse strains. Therefore, our aim was to compare femoral neck competence and morphometric parameters of both strains in order to better determine the role of bone structure and geometry in the process of bone failure at this precise location. For this purpose, murine femoral heads were loaded in axial direction and a compartmental morphometric analysis of the femoral diaphysis, metaphysis and neck was performed. As far as we know, there have been no earlier attempts to evaluate the morphometric properties of the femoral neck compartment by means of μCT.

Material and Methods

Animal model

For this study, we used two inbred strains, B6 and C3H. All mice were female and raised at Harlan (Horst, The Netherlands). They were sacrificed by CO₂ inhalation at 12 or 16 weeks. The animals were then stored at -20°C and thawed at room temperature just before dissection of the femora. Eight femora from 12-week-old B6, 8 femora from 16-week-old B6, 20 femora from 12-week old C3H and 12 femora from 16-week old C3H were dissected. Use of mice in this research project was reviewed and approved by the local authorities for all levels of investigation.

Morphometric analysis

After removing soft tissue, each bone was measured using desktop micro-computed tomography (μCT, μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) equipped with a 5 μm focal spot X-ray tube as a source. A 2D charge-coupled device, coupled to a thin scintillator as a detector, permitted acquisition of 20 tomographic images in parallel. The long axis of the femur was orientated orthogonal to the axis of the X-ray beam [49]. The X-ray tube was operated at 50kVp and 160μA. The integration time was set to 100 milliseconds. Scans were performed at a nominal resolution of 20μm in all three spatial dimensions (medium resolution mode). Two-dimensional CT images were reconstructed in 1024 x 1024 pixel matrices from 1,000 projections using a standard convolution-backprojection procedure with a Shepp and Logan filter. Images were stored in 3D arrays with an isotropic voxel size of
2.2 Effects of bone structural and material properties on bone competence

20 μm. Then, they were rotated in a standard orientation and a constrained 3D Gaussian filter was used to suppress partly the noise in the volumes ($\sigma = 1.2$ and support = 1).

Morphometric analyses were performed in five different compartments. Compartment I included the full femur, compartment II contained the trabecular bone in the distal metaphysis, and compartment III comprised a 1-mm-thick slab in the midshaft. These three compartments were determined, generated and analyzed in the same way as described earlier [49]. For the purposes of this study, two new compartments were defined. Compartment IV comprised the cortical bone of the femoral neck and compartment V included the trabecular bone of the femoral neck. These new compartments were generated fully automatically using IPL scripts (Scanco Medical, Bassersdorf, Switzerland) based on distance transformation (DT) [50] and on classical erosion and dilation algorithms. Since the femoral neck is thinner than the femoral head and the femoral diaphysis, the neck region was defined as the region between the head and the diaphysis with thickness below a fixed threshold value. The threshold value was determined relatively to the average cortical bone thickness of the full femur compartment. This new type of adaptive mask permitted to isolate the neck region in an straightforward and reproducible way, independently of the geometrical differences between the individual samples (Fig. 2.7).

For segmentation, the threshold values were set to 22.4% of the maximum gray scale value, as previously described [51], for the full femur, diaphysis cortical and cortical neck compartments and to 16.0% for the the diaphyseal trabecular and trabecular neck compartments.

Morphometric traits were determined using a direct 3D approach [52] in each of the five different analysis compartments. For the whole bone, only apparent volume density (AVD) was assessed, which is the number of bone voxels, defined by the thresholding procedure, divided by the number of all voxels within the outer contour describing the bone envelope. Parameters determined in the metaphyseal trabecular and neck trabecular bone included bone volume density (BV/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N), and connectivity density (Conn.D). Seven geometrical parameters, including total volume (TV), cortical bone volume (Ct.V), bone surface area (BS), bone volume density (Ct.V/TV), bone surface density (BS/TV), bone surface to volume ratio (BS/BV), cortical average thickness (Ct.Th) were assessed in the 1-mm-thick cortical volume in the diaphysis and in the femoral neck cortical bone. Two further parameters were computed in the diaphyseal cortical compartment: anterior-posterior diameter (APD) and average cross-sectional area (T.Ar).
Figure 2.7: a) Micro-CT images of each femur were used to automatically isolate the femoral neck for morphological analysis. On the right: compartment IV, femoral neck cortical bone and compartment V, femoral neck trabecular bone. b) Difference in cross-sectional geometries of the proximal femur between B6 and C3H are clearly visible. B6 shows more trabecular structures, while cortical bone in C3H is thicker.

Biomechanical testing

For testing, the femora were cut above the condyles, resulting in a length of 11±0.5 mm. After a rigorous alignment of the samples, which ensured an axial reproducibility error of only 1.5° [53], the femora were embedded with cyano-acrylate glue (Superglue, UHU Schweiz AG, Schönenwerd, Switzerland) into aluminum bone holders which could then be rigidly fixed in the testing device. Compressive loading was applied at the femoral head in a custom made loading device, which was integrated in a materials testing machine (1456, Zwick GmbH & Co.) (Fig. 2.8). Load-displacement curves were recorded at a crosshead speed of 0.5 mm/s [45]. From these curves, bone strength (maximum force), stiffness (slope
2.2 Effects of bone structural and material properties on bone competence

of the linear part of the curve), brittleness (deformation to failure; the smaller the deformation is, the more brittle the sample is) and toughness (work to failure) were assessed.

Figure 2.8: A left femur of a C3H mouse positioned in the materials testing machine and loaded axially.

Statistical analysis

Both strain and age groups were compared at the mechanical and morphometric levels using single way ANOVA with a Least Significant Difference (LSD) post hoc analysis where the significance level was set to \( p < 0.05 \). Relationships between mechanical and morphometric parameters were computed by single and multiple linear regression analyses. All statistical analyses were performed with MS Excel 2003, the GNU statistical package R (Version 2.4.0, http://www.r-project.org) and the statistical program: SPSS (Version 13.0, The Apache Software Foundation, Chicago, USA).

Results

In B6, all the mechanical values, except failure deformation, increased significantly with age \( (p < 0.01) \) (Fig. 2.9 and Table 2.2). Femora became stronger, stiffer and tougher,
but bone brittleness did not change. The C3H strain behaved differently. Stiffness and brittleness significantly increased (p < 0.01). Strength remained constant and toughness decreased (p < 0.01) (Fig. 2.9 and Table 2.2).

![Graphs of Stiffness, Strength, Deformation, and Toughness](image)

**Figure 2.9:** Stiffness, strength, deformation and toughness in the four groups of animals.

Linear regression analyses between the mechanical parameters and the morphometric indices of the five compartments were performed. In B6, strength was best explained Ct.V/TV (67%, p < 0.001) and Ct.Th (83%, p < 0.001) as measured in the neck compartment (Fig. 2.10). The other mechanical parameters from B6 were not predicted by any morphometrical parameter of any compartment. Similar analyses for the C3H strain demonstrated that morphometry did not significantly predict any mechanical parameter. Linear regression showed regressions of $R^2 < 0.5$ in each case. Particularly the morphometric indices that were good indicators for the femoral neck strength in B6, bone volume density and cortical thickness of the femoral neck cortical compartment, influenced bone strength in C3H only very weakly ($R^2 < 0.15$) (Fig. 2.11).
2.2 Effects of bone structural and material properties on bone competence

Table 2.2: Neck morphometrical and mechanical results. Data presented as mean ± SD. s strain significantly different, a age significantly different (oneway ANOVA, post-hoc LSD, p < 0.01). Morphometry: neck cortical bone volume (Ct.V), neck cortical bone volume density (Ct.V/TV), neck cortical thickness (Ct.Th) and neck average cross-sectional area (T.Ar). Mechanics: strength (Fu), stiffness (S), deformation to failure (d) and work to failure (U).

<table>
<thead>
<tr>
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<th></th>
<th>C3H</th>
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<td></td>
<td>12 week</td>
<td>16 week</td>
<td>12 week</td>
<td>16 week</td>
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<td>0.84 ± 0.10s</td>
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<td>Ct.Th (mm)</td>
<td>0.18 ± 0.01sa</td>
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<td>T.Ar (mm²)</td>
<td>0.43 ± 0.06sa</td>
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<tr>
<td>Fu (N)</td>
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<td>16 ± 1.7a</td>
<td>16.9 ± 2.0a</td>
<td>16.9 ± 3.0</td>
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<td>S (N/mm)</td>
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<td>51.2 ± 6.9sa</td>
<td>57.3 ± 17.5sa</td>
<td>89.7 ± 9.2sa</td>
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<td>d (mm)</td>
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<td>0.29 ± 0.03s</td>
<td>0.30 ± 0.07a</td>
<td>0.20 ± 0.05s</td>
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<tr>
<td>U (Nmm)</td>
<td>1.7 ± 0.5s</td>
<td>2.7 ± 0.6s</td>
<td>3.0 ± 0.8sa</td>
<td>2.0 ± 0.7a</td>
</tr>
</tbody>
</table>

Figure 2.10: Femoral neck morphometrical parameters (left: Ct.V/TV, R² = 0.67; right: Ct.Th, R² = 0.83) showed a good correlation with femoral strength in B6 inbred strains of mice.
Correspondences in morphometric indices between the different compartments were also investigated in each strain. Very high correlations between indices of compartment I, III and IV were found in B6. Bone volume density of cortical bone in the femoral neck correlated very well with the apparent bone density of the full bone and with the bone volume density of the diaphysis cortical bone (Fig. 2.12a and 2.12b). Similarly, cortical thickness of the femoral neck correlated significantly with cortical thickness of the diaphysis (Fig. 2.12c). Since full bone AVD, diaphyseal Ct.V/TV and Ct.Th correlated well with neck cortical properties in B6, they were also good predictors for bone strength in B6 (0.78 < $R^2$ < 0.82). In C3H, the geometry of the femoral neck compartment did not correlate significantly with the morphometric indices of any other compartment.

In order to even better predict B6 strength from structural parameters, multiple linear regression analyses including morphometric indices of the compartments I, III and IV were also computed. Adding full AVD and diaphysis Ct.Th to neck Ct.Th could not better explain B6 strength than neck Ct.Th alone. In C3H, multiple linear regression analyses including different compartments only slightly improved the predictive power of morphometry on mechanics, but correlations did not reach significance.
2.2 Effects of bone structural and material properties on bone competence

Figure 2.12: Correlation of cortical bone volume density in the femoral neck with full bone apparent volume density (a) and diaphyseal cortical bone volume density (b), respectively. Correlation of femoral neck cortical thickness with diaphyseal cortical thickness (c).

Discussion

In this study, mechanical tests of B6 and C3H inbred strains of mice were performed by axial compression at the femoral head. Our tests revealed that B6 femora became stiffer, stronger, and tougher from 12 to 16 weeks, whereas bone brittleness stayed constant. C3H behaved differently in this time lap: stiffness increased, but strength remained constant, toughness decreased and bone became more brittle. We used \( \mu \)CT to assess structural bone parameters, including those at the femoral neck, which to our knowledge, has not been done before. Our results showed that mechanical strength of the femoral neck in the B6 strain was explained to 83% by the cortical thickness at this location; in contrast, none of the mechanical properties were explained by bone morphometry in the C3H strain.

The different evolutions of mechanical properties in both strains with aging confirmed previous studies showing that B6 do not reach material and mechanical peak bone properties before 20 weeks [54, 55]. Indeed, bone mechanical properties, such as strength, stiffness and toughness increased between 12 and 16 weeks. On the other hand, our results for C3H suggested that skeletal maturity was already reached before 16 weeks, since strength stayed constant, bone became more brittle and toughness decreased. The increase in brittleness and the reduction of toughness are typical characteristics of bone aging after maturity [56-58]. Bone is a natural composite comprising mineral (mainly hydroxyapatite), organic (mostly type I collagen) and water phases [59]. Jepsen reported that changes of the bone matrix were associated with aging [57]. The aging bone becomes more porous, locally more highly mineralized and accumulates more microdamage. These changes are central to the evolution of bone brittleness. Similarly, Wang et al. showed that the mechanical integrity of the
collagen network deteriorates with increasing age and correlates significantly with the decreased work to failure, that is to say with the decreased toughness of aged bone [58].

In the B6 strain, only one bone mechanical parameter, strength, was significantly influenced by bone geometry and microstructure. In the femoral head compressive loading configuration, bone strength only depends on material and geometrical properties intrinsic to the femoral neck, since the failure happens in the neck. The other parameters, stiffness, brittleness and toughness are dependant of the whole bone behavior under axial loading. Indeed, not only the neck, but also the femoral diaphysis deformed during the test. Consequently, these mechanical parameters rely on a more complicated process than strength, including compressive, bending and shear constraints in the whole bone. This explains, to some extent, why the femoral neck properties only predicted the neck strength. In order to consider the whole bone, other compartments in the mid-diaphysis and the distal metaphysis as well as the full bone (compartment I to III) were investigated, but they did not increase the predictive power of this study. Our results suggested consequently that mechanical parameters, except strength, were not predictable, in this loading configuration, considering only geometry.

This study showed that the cortical compartments were better predictors than the trabecular ones. Bone strength in the femoral neck of the B6 was mainly determined by cortical bone density and cortical thickness. The neck trabecular compartment showed only few bone and a low number of trabeculae. Murine proximal femur contains proportionally thicker cortical bone and less dense trabecular network than in humans [60]. In the neck trabecular compartment, we saw that the trabecular network was concentrated on both ends of the neck, close to the shaft and to the head (Fig. 2.7). However, the neck fractures occurred mostly in the middle of the neck, where no trabecular bone was present and where, therefore, the neck was the weakest. At this location, only cortical bone could determine strength.

Cortical neck morphometric parameters explained bone strength in B6, but not in C3H. C3H morphometric properties varied a lot less with age than those of B6 which was one of the reasons why correlations between morphometry and strength were lower in C3H than in B6 (Table 2.2). This implies that strength in C3H bone, much more than in B6, is determined not only by structural but also by other bone properties, often referred to as bone quality [61-63]. The term ‘bone quality’ incorporates the effects of ultrastructural properties such as mineralization, microporosity, microdamage, the distribution and the activity of the three main cell types in bone (osteoclasts, osteoblasts, and osteocytes), and collagen quality. Biomechanics of the bone tissue will not be fully understood, as long as the contribution and
interaction of these parameters to bone strength will not be completely identified, analysed and quantified. There is already a number of studies that have investigated different contributions to bone strength. Highly mineralized bone is stiffer, more brittle and less tough than lowly mineralized bone [57, 64]. The increase of overall porosity in cortical bone is also responsible for a decrease of bone mechanical properties [65, 66], whereas the quality of the collagen matrix was shown to predominantly determine the toughness of bone [58, 67-70]. Further, it was reported that bone cells had different levels of activities in various inbred strains resulting in different bone tissue properties, especially in the amount of minerals and in the remodelling rates [71, 72]. Finally, microdamage accumulation is central to the strength, toughness, brittleness and fatigue resistance of bone. For a composite material like bone, failure is the end result of a damage accumulation process [57, 73, 74]. The high brittleness of C3H compared to B6 can be explained, to some extent, by higher bone mineral density [75], but also suggests that C3H bone is more porous and accumulates more microdamage. Investigation of cortical porosity and microdamage initiation and propagation in mouse inbred strains may help uncovering the processes at ultrastructural scales which lead to bone failure, and consequently may further our understanding of bone failure behaviour.

This study demonstrated differences in mechanical properties of the femoral neck under axial loading between the two inbred strains B6 and C3H. Bone strength in the B6 was explained, to great extent, by the cortical thickness at the femoral neck, while in C3H no morphometric parameter could predict it. We conclude that the relative contributions of bone structural and material properties on bone strength are different in B6 and C3H. We hypothesize that these different contributions are related to differences at the ultrastructural level of bone that affect bone failure.

Acknowledgements

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2.2 Effects of bone structural and material properties on bone competence


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2.3 Bone morphometry highly predicts cortical bone stiffness and strength but not toughness in an inbred mouse model of high and low bone mass

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Abstract

Osteoporosis is a disease of decreased extrinsic bone strength, which leads to an increased risk of fracture. Bone strength is not only determined by the density of the bone, but also by other factors. Engineering principles predict that for bone, cortical cross-sectional geometry is an important determinant of breaking strength. Inbred strains of mice make useful models to study bone properties. In this study, cross-sectional geometry in the diaphysis of murine femora in a low bone mass (C57BL/6J) and a high bone mass (C3H/HeJ) inbred strain was assessed by means of desktop micro-computed tomography. The same bones were then tested under three-point bending in order to measure their mechanical properties. Mechanical tests were imaged with a high-resolution and high-speed camera in order to visually examine the test progression. We investigated the influence of cortical bone geometry on bone mechanical properties. Specifically, our aim was to compare bone competence and cortical morphometric parameters of both B6 and C3H strains in order to better determine the role of bone structure and geometry in the process of bone failure behavior.

Our results showed that the C3H strain is a more reproducible model regarding bone morphometric and mechanical phenotypes than the B6 strain. Bone strength, stiffness, yield force, yield displacement and toughness as well as morphometric traits were all significantly
different between each strain, while post-yield displacement was not. It was found that bone volume, cortical thickness and cross-sectional area predicted almost 80% of bone stiffness, strength and yield force. Nevertheless, cortical bone post-yield behavior such as bone toughness could not be explained by morphometry. These results in combination with the observed post-yield whitening confirmed the important contribution of ultrastructure and microdamage in the process of overall bone failure behavior, especially in the post-yield phase.

**Keywords:**
Inbred strains, biomechanics, bone strength, bone structure, bone quality

**Introduction**

The number of osteoporosis-related hip fractures is expected to increase from 1.7 million in 1990 to 6.3 million in 2050 [1]. Given the mortality, disability, and cost associated with fragility fractures, identifying the factors that contribute to fracture risk has become increasingly important for improved diagnosis, treatment, and prevention [2]. Peak bone mass, which is defined by genetic and environmental factors [3], has been postulated to be an important risk factor. However, measures of bone mass and bone density have been inconsistent predictors of fracture risk [4-8]. Although altered bone quality has been recognized as an additional determinant of fracture risk [9-11], the genetic and environmental contributions to variations in bone quality, the correlation between bone quality and fracture risk, and the relationship between bone quality and bone mass are poorly understood. Bone quality is a generic term that refers to a wide spectrum of tissue mechanical properties such as elastic modulus, strength, toughness, creep, and brittleness. The specific tissue mechanical properties that contribute to bone strength are not well understood [12].

With the exception of identical twins, the genetic background in humans varies significantly from one individual to another, making studies of genetic involvement in a given bone phenotype in humans difficult. The use of animal models with relevant biological phenotypes related to disease models may provide important genetic clues that will improve the efficiency of identifying genes underlying bone strength. Well-characterized animal lines with phenotypes related to certain aspects of human osteoporosis can be used as an approach to study more homogeneous populations in which isolation of candidate chromosomal regions and genetic loci should be faster and more efficient [13]. Animal models complement and extend human studies by allowing close control of environmental factors by expanding the
characterization of phenotypes underlying bone strength and by facilitating breeding strategies to identify genetic linkage. Of particular value are experimental approaches using inbred animals. Although individuals within an inbred strain are genetically identical, genetic differences exist between different inbred strains. Where there are differences in bone strength between two inbred strains, one can identify the genetic differences that are linked to the variation in bone strength phenotypes [14]. Adult C3H/HeJ (C3H) and C57BL/6J (B6) mice are similar in body size and weight and their bones are of similar external size, but show significantly different morphological and compositional bone traits [15-17], such as adult peak bone density and cross-sectional area [18, 19]. Most importantly, these two mice have often been identified as a model system for high (C3H) and low (B6) bone mass phenotypes [20] and with that as a model to study genetic factors in osteoporosis.

In the current study, we evaluated the microstructure and mechanical properties of cortical bone in the femur of inbred strains B6 and C3H. We hypothesized that bone properties were predicted by cortical bone morphometry. We asked the question, whether these predictions was influenced by genetic background and whether they were different for pre- and post-yield mechanical properties. Our aim, therefore, was to compare bone competence and cortical morphometric parameters of both strains in order to better determine the role of bone structure and geometry in the process of bone failure behavior.

Material and Methods

Animal model

For this study, we used two inbred strains, where C57BL/6He (B6) represented the low bone mass strain and C3H/He (C3H) displayed the high bone mass phenotype. Twenty femora from 16-week-old B6 and 20 femora from 16-week old C3H were were imaged by means of micro-computed tomography (μCT) and tested in three-point bending. Each series of 20 bones was composed of 10 femora from male mice and 10 femora from female mice. The mice were raised and sacrificed at the Jackson Laboratory (Main, USA). Bones were sized and prepared at the Jackson Laboratory and kept in alcohol for the oversee travel. Use of mice in this research project was reviewed and approved by the local authorities.

Morphometric analysis

Each bone was measured using desktop micro-computed tomography (μCT, μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) equipped with a 5 μm focal spot X-ray tube as a source. A 2D charge-coupled device, coupled to a thin scintillator as a detector, permitted acquisition of 20 tomographic images in parallel. The long axis of the femur was orientated
orthogonal to the axis of the X-ray beam. The X-ray tube was operated at 50kVp and 160μA. The integration time was set to 100 milliseconds. Scans were performed at a nominal resolution of 20μm in all three spatial dimensions (medium resolution mode). Two-dimensional CT images were reconstructed in 1024 x 1024 pixel matrices from 1,000 projections using a standard convolution-backprojection procedure with a Shepp and Logan filter. Images were stored in 3D arrays with an isotropic voxel size of 20 μm. Then, they were rotated in a standard orientation and a constrained 3D Gaussian filter was used to suppress partly the noise in the volumes (σ = 1.2 and support = 1). The images were then segmented to distinguish bone voxels from non-bone voxels using a global threshold (22.4% of maximum possible gray scale value) as previously described [21]. A 1-mm analysis region, situated at 55% of the length from the proximal side was defined as the analysis region [22] (Fig. 2.13). At this location, only cortical bone can be found. Morphometric traits were determined using a direct 3D approach [23]. Fourteen morphometric parameters, including total volume (TV), cortical bone volume (BV), bone surface area (BS), bone volume density (BV/TV), bone surface density (BS/TV), bone surface to volume ratio (BS/TV), cortical average thickness (C.Th), anterior-posterior diameter (APD) and average cross-sectional area (T.Ar) were assessed in the 1-mm-thick cortical volume in the diaphysis. Bending moments of inertia were also computed: bending moment of inertia with respect to the anterior-posterior axis (I_{AP}), bending moment of inertia with respect to the medial-lateral axis (I_{ML}), biaxial area moment of inertia (I_{xy}), polar moment of inertia (J) and principal moments of inertia (I_{max} and I_{min}).
2.3 Effects of bone structural and material properties on bone competence

Figure 2.13: μCT images of femur. Geometric traits were determined on a 1-mm region situated at 55% of the length from the proximal side. Difference in cross-sectional geometries between C3H and B6 are clearly visible.

Biomechanical testing

Before testing the 40 bones were removed from alcohol and rehydrated during 24 hours in phosphate buffered saline solution. Load was applied midway between two supports that were 6 mm apart, exactly in the region where the morphometric analysis was performed. The femora were positioned so that the loading pin was applying a force at a location on the shaft situated at 55% of the length from the proximal side. The femora were lying freely on the supports and the 1 N preload oriented them so that the load was applied in the anterior-posterior direction (Fig. 2.14a). Load-displacement curves were recorded at a crosshead speed of 0.5 mm/s. Bone stiffness, strength, yield force, yield displacement, post-yield displacement as well as toughness were derived from these curves as described previously [17, 24]. Tests were imaged with a high-speed and high-resolution camera (AOS Technologies, Daetwil, CH). The image size was set to 1280 x 1024 pixels and the frame rate to 62.5 frame/s. Between 200 and 300 high-resolution images were recorded per single test.
Figure 2.14: a) Murine femur preloaded with 1 N. The supports are 6 mm apart and the loading pin applies the force at a location on the shaft situated at 55 % of femoral length as measured from the proximal side. b) In three-point bending tests, fracture initiated under tension. c) The black arrow shows bone whitening before the fracture initiates.

Statistical analysis

Both strains were compared at the mechanical and morphometric levels using Student unpaired t-tests. Relationships between mechanical and morphometric parameters were computed using single and multiple linear regression analyses. Significance level for all analyses was set to p < 0.05. All statistical analyses were performed with Excel 2003 (Microsoft, Redmond, USA) and the GNU statistical package R (Version 2.4.0, http://www.r-project.org)
2.3 Effects of bone structural and material properties on bone competence

Results

The coefficients of variation (CV) of all morphometric traits were smaller for C3H than for B6 (Table 2.3). Furthermore, our measurements revealed that both strains were significantly different for all the parameters except post-yield displacement. Comparison between the mechanical and morphometric parameters showed that the morphometric parameters typically varied less than the mechanical ones within each inbred strain. CV’s were smaller (<12%) for morphometric parameters, while moments of inertia and mechanical parameters ranged from 10 to 47%, except for the post-yield parameters which were between 40 and 93%.

Table 2.3: Morphometric parameters: Mean ± SD (CV). * p < 0.05 (student unpaired t-test), ** p < 0.01 (student unpaired t-test), *** p < 0.001 (student unpaired t-test)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B6</th>
<th>C3H</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV (mm³)</td>
<td>1.61 ± 0.15 (9.52%)</td>
<td>1.29 ± 0.11 (8.36%)</td>
</tr>
<tr>
<td>BV (mm³)</td>
<td>0.70 ± 0.08 (11.47%)</td>
<td>1.00 ± 0.09 (9.24%)</td>
</tr>
<tr>
<td>BS (mm²)</td>
<td>3.72 ± 0.41 (11.07%)</td>
<td>2.04 ± 0.15 (7.28%)</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>43.50 ± 2.86 (6.57%)</td>
<td>77.32 ± 2.61 (3.37%)</td>
</tr>
<tr>
<td>BS/TV (mm⁻¹)</td>
<td>2.31 ± 0.12 (5.25%)</td>
<td>1.58 ± 0.08 (5.22%)</td>
</tr>
<tr>
<td>BS/BV (mm⁻¹)</td>
<td>5.35 ± 0.58 (10.86%)</td>
<td>2.05 ± 0.17 (8.36%)</td>
</tr>
<tr>
<td>APD (mm)</td>
<td>1.10 ± 0.04 (3.45%)</td>
<td>1.02 ± 0.03 (2.90%)</td>
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<tr>
<td>C.Th (mm)</td>
<td>0.18 ± 0.01 (7.83%)</td>
<td>0.35 ± 0.03 (7.38%)</td>
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<tr>
<td>T.Ar (mm²)</td>
<td>0.78 ± 0.09 (11.00%)</td>
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<tr>
<td>IML (mm⁴)</td>
<td>0.13 ± 0.03 (20.06%)</td>
<td>0.10 ± 0.01 (10.61%)</td>
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<td>IAP (mm⁴)</td>
<td>0.22 ± 0.05 (23.49%)</td>
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<td>Ixy (mm⁴)</td>
<td>0.04 ± 0.02 (47.40%)</td>
<td>0.02 ± 0.01 (37.93%)</td>
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<tr>
<td>J (mm⁴)</td>
<td>0.35 ± 0.07 (21.57%)</td>
<td>0.29 ± 0.05 (18.00%)</td>
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<tr>
<td>Imax (mm⁴)</td>
<td>0.18 ± 0.06 (24.97%)</td>
<td>0.20 ± 0.04 (21.62%)</td>
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<tr>
<td>Imin (mm⁴)</td>
<td>0.11 ± 0.02 (15.61%)</td>
<td>0.10 ± 0.01 (12.20%)</td>
</tr>
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</table>

The information gained from the high-resolution and high-speed camera revealed the way fractures occurred. The images showed well how bone fractures initiated at the posterior, tensile, side of the bone (Fig. 2.14b). Other relevant information delivered by this visualization technique was the exact positioning of the femora in three-point bending after preload. It provided an easy and elegant way to ascertain that bones, simply put on supports,
were positioned correctly and that they rotated in a very reproducible way after preload. Furthermore, imaging helped to discriminate outliers and permitted to determine if variability in the results was caused by problems in the experimental set-up. Even if a reasonable force versus displacement curve was measured, visualization of the failure process could sometimes identify measurement errors. Indeed, two tests on B6 and one test on C3H were excluded because the samples slipped or did not position appropriately after preloading. The high resolution images recorded also showed whitening effect found in bone as a consequence of overloading (Fig. 2.14c).

The average curves of the three-point bending tests were computed (Fig. 2.15). Female and male bones were pulled, since there was no significant difference between genders. All coefficients of variation (CV) were smaller for C3H than for B6 (Table 2.4), as it was the case for the morphometric parameters. Our experiments also revealed that both models were significantly different for stiffness, strength, yield force, yield displacement and toughness. Only for post-yield displacement, the two strains did not show significant differences. In both strains, the results were much more reproducible for strength, stiffness, yield force and yield displacement than for the post-yield parameters, such as post-yield displacement and toughness.

![Figure 2.15: Average load-displacement curves from three-point bending tests for B6 and C3H.](image)

*Figure 2.15: Average load-displacement curves from three-point bending tests for B6 and C3H.*
2.3 Effects of bone structural and material properties on bone competence

Table 2.4: Biomechanical parameters of the three-point bending tests. * $p < 0.05$, ** $p < 0.001$ (student unpaired t-test). Data are presented as mean ± SD (CV). Stiffness (N/mm): slope of the linear part of the curve before the yield point. Strength (N): ultimate force. Yield force (N): loading force at the yield point. Yield displacement (μm): deformation at the yield point. Post-yield displacement (μm): deformation between the yield point and the failure displacement. Toughness (Nmm): Work to failure or area below the load-displacement curve until total failure.

<table>
<thead>
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<th>C3H</th>
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<tr>
<td>Stiffness (N/mm) **</td>
<td>148.5 ± 28.14 (18.95%)</td>
<td>285.6 ± 40.17 (14.07%)</td>
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<tr>
<td>Strength (N) **</td>
<td>15.63 ± 3.36 (21.49%)</td>
<td>33.33 ± 3.44 (10.32%)</td>
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<tr>
<td>Yield force (N) **</td>
<td>12.17 ± 2.41 (19.79%)</td>
<td>20.94 ± 1.64 (7.81%)</td>
</tr>
<tr>
<td>Yield displacement (μm) *</td>
<td>74.63 ± 13.14 (17.6%)</td>
<td>68.51 ± 4.83 (7.10%)</td>
</tr>
<tr>
<td>Post-yield displacement (μm)</td>
<td>102.39 ± 95.15 (92.93%)</td>
<td>110.07 ± 45.84 (41.65%)</td>
</tr>
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<td>Toughness (Nmm) *</td>
<td>2.98 ± 2.75 (92.83%)</td>
<td>4.26 ± 1.71 (40.25%)</td>
</tr>
</tbody>
</table>

Computing intra-strain correlations of mechanical parameters and morphometric indices, it was found that morphometry was only a poor predictor within each strain; correlations to mechanical parameters were lower than 50%. We therefore pooled both strains. Although mechanical and morphometric parameters were often highly strain dependent and therefore clustered around the mean value of each strain, an inter-strain multiple regression analysis showed that the least clustered morphometric traits, such as BV, C.Th and T.Ar, could predict 87% of stiffness, 85% of yield force and 88% of strength. In order to reduce the gap separating the clusters in the multiple linear regression plots and correct the error in correlation due to the clustering effect, we applied a simplified version of the cluster linear regression method developed by Henning [25] to our data set. A factor for each multiple linear regression was computed. In short, the factor (f) was calculated dividing the x-coordinate of the point in the C3H cluster with the smallest x-coordinate by the x-coordinate of the point in the B6 cluster with the highest x-coordinate. Since the slope of the plots was equal to 1, the factor for y-coordinates was the same as for the x-coordinates. Then, both x and y values of each C3H points were divided by f. Thus a more homogenous population was generated and the contribution of the clustering effect to the high correlation coefficients previously obtained was removed. As expected, the correlation coefficients decreased, but were still significantly high: $R^2 = 0.79$, $R^2 = 0.76$ and $R^2 = 0.77$ for stiffness,
yield force and strength, respectively (Fig. 2.16a-c). Finally, morphometry was only a poor predictor for yield displacement and post-yield parameters, such as post-yield displacement and toughness ($R^2 < 0.50$).

![Graphs showing mechanical properties](image)

**Figure 2.16:** Multiple linear regression analyses revealed that models based on BV, C.Th and T.Ar were good predictors of stiffness, yield force and strength. BV, C.Th and T.Ar predicted 79% of stiffness (a), 76% of yield force (b) and 77% of strength (c).

**Discussion**

In this study, femoral cortical bone morphometry of two characteristic murine strains was assessed. Then, femora were tested under three-point bending to investigate their mechanical competence. In order to better understand the bone failure behavior, analyses of bone competence were conducted combining mechanical and morphometric results.

High resolution imaging of common mechanical tests drastically increased the informative output of the mechanical tests. In an earlier study [26], we showed that, by using a proper image-based aligning procedure and exclusion of unsuccessful tests, the variability was reduced to half when compared to conventional approaches. This is of great value in experimental studies, because reduced variability means increased power in the results. This also indicates that fewer samples and therefore fewer animals are needed to obtain significant results.

Using this image-guided approach, we noticed that, bone surfaces whitened with increasing strain in the post-yield phase (Fig. 2.14c), similar to work on trabecular bone recently reported by Thurner et al. [27]. We hypothesized that the effect seen was due to microcrack formation in these areas, comparable to stress whitening seen in synthetic polymers. Thurner et al. showed that the whitened areas were also of high deformation. They suggested that the detected whitening and also microdamage appeared at points of highest
2.3 Effects of bone structural and material properties on bone competence

strain, which would be consistent with failure initiation in compact bone, at the point of highest local strain [28]. So, visually investigating typical failure locations and fracture propagation, using high-speed video, could give first insight into the bone failure behavior.

In recent years, there has been a considerable emphasis placed on the detailed mechanism whereby the skeleton provides mechanical support for the organism. Extrinsic bone strength is the pivotal parameter describing this aspect of skeletal function. In terms of the determinants of mechanical support, bone density is well recognized, whereas cross-sectional geometry, which is equally as important as bone density, has only achieved recognition of its importance in the last few years. Now, there are multiple studies that have emphasized the importance of bone cross-sectional geometry as a determinant in bone mechanical properties [14, 15, 29-36]. In this study, we investigated bone density and 14 cross-sectional morphometric parameters in cortical of the murine femoral mid-diaphysis. They were all significantly different for the two observed strains. As showed previously, B6 and C3H are well characterized with regards to differences in BV/TV and T.Ar [17-19, 37-39]. These strains appear to be very good models for high and low bone mass, respectively. Our study completed these findings with further significantly different parameters including intrinsic geometrical traits as well as computed moments of inertia (Table 2.3).

Looking at the results of mechanical testing, these experiments revealed that both models were significantly different for stiffness, strength, yield force, yield displacement and toughness. Nevertheless, the 8% difference in post-yield displacement was not significantly different between the two strains, also due to the large standard deviations found for this parameter. Similar to previous studies [12, 15, 17, 36, 40], this indicated that these two inbred mice do not only show different morphometrical phenotypes, but also different mechanical phenotypes. Further, it was found that all the mechanical parameters for C3H were more reproducible than for B6 (Table 2.4), also indicating more homogeneous bone mechanical properties for C3H.

A closer investigation of the morphometric traits revealed that C3H parameters were less variable than B6’s, as it was the case for mechanical properties, confirming that C3H is a strain with more precise traits than the B6, both mechanically and morphometrically. This finding can be of great help when selecting mice inbred strains for phenotyping purposes. In such studies, large populations of mice are usually experimented within the same strain or between different strains. Comparing results between predefined groups and showing significant differences between treated or untreated animals, between different strains or between different models is often the main goal of these studies. Reduced variability in the
results within each group makes the results more powerful, increases the significance of the study and therefore its impact. It also reduces the number of animals needed for significant outcomes.

In this study, we showed that variations in mechanical properties are on average higher than variations in morphometric traits. A reason for this is the higher inaccuracies induced by the mechanical setup. Indeed, the small size of murine bones makes them difficult to test with perfectly reproducible boundary conditions. Therefore, a part of the variation in mechanical results is inherent to the testing setup. This part is much reduced in morphometric analyses, where a very high reproducibility of less than 1% has been shown for cortical bone parameters [22]. A further explanation could come from bone material properties. Bone tissue is a composite material comprised of an organic matrix reinforced with an inorganic mineral phase. The inorganic phase is composed of mineral micro-crystals and the organic matrix of protein (principally collagen) fibers [41]. Because bone is a composite material, its mechanical properties are dependent not only on its cross-sectional geometry, but also on its diverse material components and on its ultrastructure. Here, we mean mostly bone porosity including cortical bone vasculature, osteocytic lacunae as well as the canalicular network and bone microdamage, when referring to cortical bone ultrastructure [42]. It has been shown that bone ultrastructure also has a contribution to failure behavior [42-45]. In a recent study [46], we showed that bone capacity can only be partly explained by bone geometry. Nevertheless, in the present study, we tried to quantify the geometric influence on bone mechanical properties. We performed two types of investigations; an intra-strain (within each strain) and an inter-strain (merging both strains results) analysis. It was found that, intra-strain, morphometry is a poor predictor of bone mechanical properties. The best predictions we could compute using multiple linear regression were lower than 50% in each strain. In the case of strength, stiffness, yield force and yield displacement, this was to be expected, since variations in mechanical and morphometric parameters were relatively small. In other words, these bone phenotypes were well characterized in each strain. For the post-yield parameters, which showed large variations, the lack of correlation indicated that these parameters were mostly independent from bone geometry.

Computing inter-strain multiple linear regression, BV, C.Th and T.Ar were good predictors for stiffness, yield force and strength. No prediction was found for post-yield displacement and toughness. As mentioned previously, multiple linear regression on clustered data can be biased by the cluster effect. The larger the two populations differ for the analyzed parameters, the higher will be the correlation due to the cluster effect. In order to prevent this
2.3 Effects of bone structural and material properties on bone competence

problem, we selected those parameters that clustered the least. When values were more or less linearly distributed, we were able to compute high correlations between pre-yield mechanical parameters (stiffness, yield force and strength) and BV, C.Th and T.Ar ($R^2 = 0.85 – 0.88$). However, still a small gap between both strains could be seen in the linear regression curves. In order to prove that the cluster effect did not act a major influence on the computed correlation coefficients and to correct for this influence, we reduced the gap between both strains using statistical normalization (Fig. 2.16a-c) and verified that the so obtained correlation factors were still high ($R^2 = 0.76 – 0.79$).

In both strains, the mechanical results were more reproducible for strength, stiffness, yield force and yield displacement than for post-yield displacement and toughness. Further, multiple linear regression analysis showed no correlation between the morphometric traits and yield displacement or post-yield mechanical parameters (post-yield displacement and toughness). This also indicated that post-yield behavior is only poorly predicted by microstructural bone morphometry. Comparing the mechanical data with the recorded movies of the tests, whitening as well as fracture initiation happened in the post-yield phase. Whitening indicated that bone failure behavior is governed in its plastic phase by ultrastructural changes including microdamage initiation and propagation. Thus, our study confirmed that microcracking plays an important role in the post-yield failure behavior. It is of great interest to further investigate microdamage in order to better understand the whole failure behavior and to try explaining the large variance in post-yield behavior within each strain. Recent studies [28, 47-51], have explored microdamage initiation and propagation, the relation between ultrastructure and microdamage and the contribution of microdamage to overall fracture initiation and propagation. There was no conclusive finding from these studies and therefore the overall bone failure behavior is still poorly understood and needs further investigation at microscopic and nanoscopic scales in order to uncover the determining properties in post-yield failure behavior.

In conclusion, our work showed that the C3H strain is a more reproducible model regarding bone morphometrical and mechanical phenotypes than the B6 strain. Strength, stiffness, yield force, toughness and morphometric traits were significantly different between each strain, while post-yield displacement was not. Intra-strain mechanical predictions from morphometry were not possible, since bone phenotypes were well characterized in each strain. Inter-strain analyses revealed that the morphometric parameters BV, C.Th and T.Ar predicted almost 80% of the pre-yield parameters such as stiffness, strength and yield force, whereas bone post-yield behavior could not be explained by morphometry. This result, in
combination with the observed post-yield whitening, confirms the importance of bone ultrastructure and its relation to microdamage in order to better understand the overall cortical bone failure behavior.

Acknowledgements

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2.3 Effects of bone structural and material properties on bone competence


2.3 Effects of bone structural and material properties on bone competence


2.4 Tissue modulus derived from beam theory is biased by bone size and geometry: implications for the use of three-point bending tests to determine bone tissue modulus

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Abstract

We used micro-finite element analyses to evaluate the outcomes derived from three-point bending tests. Our findings explain previously reported genetic effects on bone tissue modulus as an effect of differences in bone geometry. The use of three-point bending tests in mouse genetics needs re-evaluation.

Introduction: Current practice to determine bone tissue modulus of cortical bone (E) is to estimate it from three-point bending tests, using Euler-Bernoulli beam theory. However, murine femora are not perfect beams; hence, results can be inaccurate. Our aim was to assess the accuracy of beam theory, which we tested for two commonly used inbred strains of mice, C57BL/6 (B6) and C3H/He (C3H).

Materials and Methods: The three-dimensional structure of male and female B6 and C3H femora (N=20/group) was assessed by micro-computed tomography. For each femur five micro-finite element (micro-FE) models were created that simulated three-point bending tests with varying distances between the supports. Tissue modulus was calculated from beam theory using micro-FE results, and related to the modulus as used in the micro-FE analyses; any deviation is caused by inadequacies in beam theory. An additional set of fresh-frozen
femora (10 B6 and 12 C3H) was biomechanically tested and subjected to the same micro-FE analyses. This latter combined experimental-computational analyses enabled an unbiased assessment of specimen-specific tissue modulus.

**Results:** We found that by using beam theory, tissue modulus was underestimated for all femora. Geometry and size differences caused these large differences in tissue moduli. Owing to the relatively thin cortex, underestimation was markedly higher for B6 than for C3H. Underestimation was dependent on support width in a strain-specific manner. From our combined experimental-computational approach we derived tissue moduli of $12.0 \pm 1.3$ GPa and $13.4 \pm 2.1$ GPa for B6 and C3H, respectively.

**Conclusions:** Micro-FE precisely quantified the underestimation of tissue moduli in murine femora when estimated from beam theory. We showed that reported murine inbred strain-specific differences in tissue modulus as derived from three-point bending tests are largely an effect of geometric differences, not accounted for by beam theory. We suggest a re-evaluation of the tissue properties obtained from three-point bending tests, especially in mouse genetics.

**Keywords:**
Tissue modulus, biomechanical testing, finite element analysis, mouse, femur

**Introduction**

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures [1]. Because the effects of osteoporosis only become apparent when a bone fractures, bone quality is best characterized in terms of bone mechanical properties, such as stiffness and strength. Many factors influence global (also called apparent) bone mechanical properties, such as bone volume, geometry, and intrinsic material properties. Following engineering principles, characteristics that are normally distinguished include structural properties, such as size, shape and trabecular architecture, and tissue level (also called intrinsic) properties, such as tissue modulus. In line with these engineering principles, several studies have investigated bone tissue properties as one of the components influencing bone stiffness and strength. In mice, the most widely used animals in genetics studies, strong effects of genetic background on bone tissue properties have been reported [2-6]; in an analysis of 29 different inbred strains
Tissue modulus derived from beam theory is biased by bone size and geometry

of mice, Wergedal et al. recently reported largely differing tissue moduli, ranging from 3.4 GPa to 10.9 GPa, respectively [6].

In all these animal studies, bone material properties were derived from three-point bending test using Euler-Bernoulli beam theory, which is based on the theory of elasticity. Specifically, tissue modulus ($E$) is derived as [7]:

$$E = \frac{F L^3}{48 d I}$$

where $F$ is the force applied at a location centered between the two supports, $L$ is the width between the two supports, $d$ is the deflection, and $I$ is the second moment of area. Due to the small size of murine bones, this approach is considered to be the gold standard. However, several assumptions underlie the use of beam theory, and these include (a) the beam should be made from a homogeneous, linear elastic, material, (b) the beam should have a uniform cross section, and (c) it should be long in proportion to its depth [8]. These assumptions are well known and have been identified as potential errors in three-point bending tests as it is clear that they are not met for murine femora [2, 9, 10]. Nevertheless, most experimental studies have neglected these conditions, probably because it is as yet unclear how large an error one makes in applying Euler-Bernoulli beam theory to estimate tissue modulus. In a recent study, Schriefer et al [5] tried to estimate the effects of limited aspect ratio (AR), defined as support width divided by specimen diameter. Assuming bones to be hollow cylinders, and using finite element analyses, they derived these errors for two commonly used inbred strains of mice C57Bl/6 (B6) and C3H/He (C3H). They found that underestimation can be large depending on aspect ratio and that it is modulated by cortical thickness; they derived a correction factor of 53% for female B6, and a factor of 15% for female C3H, respectively, when the supports were 10 mm apart; for smaller support width, those correction factors should be even higher. However, bones are not straight, hollow cylinders which was assumed in the derivation of the correction factors. Furthermore, the use of strain-specific correction factors disregards the fact that geometric bone differences do not only occur among inbred strains, but also within inbred strains. In addition, using correction factors based on a priori assumptions on bone geometry (e.g. a hollow cylinder), can cause undesired and non-quantifiable effects. Hence, the adequacy of beam theory to derive tissue modulus from three-point bending tests remains unclear. As a consequence, its current use, e.g. in identifying genes influencing bone properties, is questionable. Therefore, the aim of this study was to determine the precise effects of bone geometry and support width on bone
stiffness and tissue modulus as derived from three-point bending test. Specifically, we assessed those effects for two inbred strains of mice, B6 and C3H.

Methods

Bone stiffness as measured in three-point bending tests depends on bone structure (i.e. shape and internal architecture) and bone tissue level material properties. To assess their specific contributions, we took a combined computational and experimental approach. Bone geometric effects were quantified using computational models with a high level of detail. In combination with experimental biomechanical tests these data enabled to derive tissue modulus in an unbiased way. This combined computational-experimental approach was validated using simple cylindrical beams. The methods are described in the following three sections.

Validation

In order to validate our approach we performed computational analyses similar to Schriefer et al. [5], which we complemented with experimental tests. The experimental tests were performed on cylindrical torlon 4203 (Solvay S.A., Brussels, Belgium) beams with diameters of 1.5 mm, 2.0 mm and 2.5 mm, respectively. These beams were tested in three-point bending with the supports 14.0 mm apart, and were repeated for support widths of 11.9, 10.2, 7.7, 6.0 and 4.0 mm, respectively. Crosshead speed was 0.5 mm/s. Total force was limited to 15 N to stay in the elastic range. Force and deflection were measured; stiffness was calculated as the slope of the force-deflection curve. Tissue modulus was estimated from beam theory (Eq. 1), and related to the tissue modulus as given by the manufacturer.

The computational tests consisted of creating finite element models of cylindrical beams under three-point bending. Support width was arbitrarily set to 10 mm; the thickness of the beam was varied to create models with different aspect ratios. A displacement that equaled 10% of the beam diameter was applied at a point midway between the supports. The models were solved using Marc (MSC Software Corporation, Los Angeles, CA) running on a Superdome System (Hewlett-Packard, Palo Alto, CA) and were used to calculate the force needed to obtain the prescribed displacement. The analyses were repeated for hollow cylinders in which the thickness of the ‘cortex’ was given by the cortical thickness ratio, defined as twice the cortical thickness divided by the beam diameter. The cross-sectional area moment of the beams were calculated and tissue modulus was estimated from beam theory (Eq. 1), and related to the tissue modulus as used in the finite element models (4.14 GPa).
2.4 Tissue modulus derived from beam theory is biased by bone size and geometry

*Murine femoral biomechanics — structural aspects*

The structural aspects (i.e., geometric aspects) of bone stiffness were evaluated for femora from 16-week old inbred strains of C3H and B6, respectively. These two strains are of similar size and weight, and also have femora of similar length, but they differ strongly in their femoral cross-section; where C3H have a thick cortex, cortices in B6 are thin. Femora from both sexes were analyzed. Each group had 10 mice from which left and right femora were analyzed, summing up to eighty femora in total. These bones were sized and prepared at the Jackson Laboratory (Main, USA) and stored in alcohol for the oversee travel. Use of mice for this research project was reviewed and approved by the local IACUC.

The three-dimensional structure of all femora was assessed by micro-computed tomography (µCT40, Scanco Medical, Switzerland) using a 20 µm nominal resolution. After reducing voxel size to 30 µm, the reconstructed images were filtered using a constrained three-dimensional Gaussian filter to partially suppress noise in the volumes ($\sigma = 1.2$ and support = 1). We developed an automated alignment routine that turned the reconstructed femur into a horizontal position, with the condyles pointing downward. In short, the shaft of the femur was automatically extracted. A transformation was then determined such that the principal axes of the inertia tensor coincided with the axes of the reference frame. The µCT data were binarized using a global threshold (22.4% of maximum possible gray scale value) as previously described [11]. Morphometric analysis was performed at the mid-diaphysis at 56% of the femur length when measured from the proximal side, to obtain cortical thickness (Ct.Th), thickness in antero-posterior direction (AP thickness), cortical area (Ct.A), and cross sectional moment of area (I). Visual inspection had identified this position as being the middle of the femoral shaft.

Micro-finite element (µFE) models were generated for each femur using a standard voxel conversion technique, hence, each voxel from the µCT reconstruction was converted to a hexahedral element in the µFE model. Voxel size was 30 µm, hence, the cortex was spanned by 6 and 11 elements for B6 and C3H, respectively. Mesh convergence tests were performed that showed the accuracy of the results (data not shown) when using this voxel size, a result in line with findings in trabecular bone for which it has been shown that accurate results are obtained when the voxel size is less than one-fourth of average trabecular thickness [12]. Boundary conditions were applied that represented a three-point bending test. A prescribed displacement was applied at the anterior side of the femur, at a position of 53% of the length of the femur, as measured from the femoral head. The supports were positioned equal distance from the load application site; nodal displacements at the supports were constrained in vertical
direction. Care was taken that the midpoint of the two supports and the midpoint of the load application site were positioned in one vertical plane. For each femur, μFE analyses were performed simulating support widths of 3, 4, 5, 6, and 7 mm, respectively. To reduce computational time, all bone that extended 1 mm beyond the supports was artificially removed before analysis. Component labeling was performed to assure that no unconnected trabecular fragments (image artifacts) were included in the μFE models. All elements in the μFE models were given an identical, and arbitrary, tissue modulus (E_{FE}) of 1 GPa, and a Poisson ratio of 0.3. The μFE models were used to calculate the force needed to obtain a prescribed displacement, representing 10% of AP thickness. Assuming linear-elastic behavior, the models were solved using an element-by-element method [13, 14] running on a supercomputer consisting of 8 IBM Regatta p690 SMPs for a total of 256 Power4 CPUs. Bone stiffness (S) was calculated as the μFE calculated force divided by the prescribed displacement.

The accuracy of beam theory was tested by relating the tissue modulus as estimated from beam theory (E_{beam}) to the tissue modulus as used in the μFE models (E_{FE} = 1 GPa). To do so, we calculated the tissue modulus as estimated from beam theory in the same way as one would do after an experimental test; hence, tissue modulus was determined using Eq. 1. The data needed as input to this equation were taken from the μFE analyses; hence, F was the force as calculated from the μFE model, L was the support width, and d the prescribed deflection; I_{yy} was the cross sectional moment of area which was directly calculated from one μCT slice at the center of the femoral shaft. In the case where beam theory correctly determines tissue modulus, E_{beam} should equal E_{FE}, hence, ideally, E_{beam} / E_{FE} = 1. Any deviation from 1 can be considered the error due to the use of beam theory.

*Murine femoral biomechanics – material aspects*

Ten femora from 15-week-old female B6 and 12 femora from 15-week old female C3H were tested in three-point bending at room temperature in a custom made loading device, which was integrated in a materials testing machine (1456, Zwick GmbH & Co.). The animals were stored at -20°C and thawed at room temperature just before dissection of the femora. Load was applied midway between two supports that were 6 mm apart. The femora were positioned such that the loading pin was applying a force at a location on the mid-shaft. The femora were lying freely on the supports and a 1 N preload was applied. Load-displacement curves were recorded at a crosshead speed of 0.5 mm/s [15]. Stiffness was calculated as the slope of the linear part of the force-deflection curve.
2.4 Tissue modulus derived from beam theory is biased by bone size and geometry

Before the experimental tests were performed the three-dimensional structure of all femora was assessed by micro-computed tomography at a resolution of 20 µm. Morphometric parameters were calculated from the μCT data, and structural stiffness was determined from μFE analyses simulating three-point bending with a support width of 6 mm, all as described before. In addition to calculating tissue modulus from beam theory ($E_{\text{beam}}$; Eq. 1), the tissue modulus was also calculated based on the μFE analyses by linear scaling, as

$$E_{\text{bone}} = \frac{S_{\text{mech.test}}}{S_{FE}} \cdot E_{FE}$$

where $S_{\text{mech.test}}$ and $S_{FE}$ are the stiffnesses as determined from the biomechanical test and from the μFE analysis, respectively; $E_{FE}$ is the tissue modulus used in the μFE analyses (1 GPa). The linear scaling as presented in Eq. 2 effectively links experimental to computational data and is similar to the methodology to derive tissue modulus from axial compression tests [16, 17]; it is valid because all analyses were linear elastic.

Statistic analysis

Data are presented as mean ± SD. Mechanical and morphometric data for both strains and sexes were compared using Student t-tests. Significance level for all analyses was set to $p < 0.05$. All statistical analyses were performed with Excel 2003 (Microsoft, Redmond, USA).

Results

Validation

The computational analyses revealed that aspect ratio (AR) had a critical effect on the outcomes of three-point bending tests. For perfect cylindrical beams the underestimation for an AR of 5 was 29% (Fig. 2.17a). With smaller AR the underestimation was even larger. For all three tested diameters the experimentally derived tissue moduli closely matched the computationally derived ones (Fig. 2.17a); only for very small aspect ratios a slight deviation between experiment and finite element analyses was found.

The analyses of beams with different cortical thicknesses showed that a decrease of cortical thickness ratio is associated with an increase in the underestimation of tissue modulus. For an AR of 5 and a thickness ratio of 0.6, the underestimation of tissue modulus was 30.1%, compared to 29.0% for a full cross-section beam (Fig. 2.17b). A thickness ratio of 0.3 increased the underestimation to 42.1%.

Murine femoral biomechanics – structural aspects

Morphometric analysis of the diaphyseal region of the femora showed that B6 had thinner cortices than C3H and that their femoral widths were slightly larger (Fig. 2.18; Table...
2.5). Individual differences within strains were small, as identified by the small standard deviations; a small but significant sex difference was found for cortical thickness, cortical thickness ratio, and cross-sectional moment of area in C3H (p < 0.001), but not in B6 (p > 0.05).

Figure 2.17: A. Finite element (FE) analyses showed that tissue modulus as estimated from beam theory was underestimated for full cross-sectional beams and depended on aspect ratio. Experimental finding on 1.5, 2.0, and 2.5 mm beams confirmed our computational findings; B. the underestimation depended on aspect ratio and cortical thickness ratio (Ct.Th ratio), defined as 2•cortical thickness/femoral width; identified are the curves for Ct.Th ratio of 0.2, 0.3, 0.4, 0.5, 0.7, and 1.0, respectively.
2.4 Tissue modulus derived from beam theory is biased by bone size and geometry

The alignment procedure was robust and performed correctly in all cases; all femora were rotated to a similar position as visually checked by three-dimensional reconstruction and visualization.

All mechanical parameters were strongly affected by support width. Structural stiffness decreased with increasing support width (Fig. 2.19a); this decrease was stronger in C3H than in B6. We found that the tissue modulus as estimated from beam theory was much lower than the tissue modulus as applied in the μFE models (Fig. 2.19b). For a 5 mm support, tissue modulus was only 40% of the correct value (i.e. as input in the μFE models) for B6 females; for B6 males, the underestimation of $E_{\text{beam}}$ was even more severe, and was only 34% of $E_{\text{true}}$. Underestimation was less severe for C3H femora, tissue moduli were 68% and 63% of the correct values for C3H females and males, respectively. Just as for the cylindrical beams, the underestimation depended strongly on support width and was more severe as support width decreased.

![Figure 2.18: Top view, frontal view, and several cross sections of a typical male B6 femur (A), and male C3H femur (B), respectively. Also indicated are the volumes tested in three-point bending when using either a 5 mm or 10 mm support width.](image-url)
Chapter 2. Whole bone mechanics

Figure 2.19: A. Stiffness in three-point bending as calculated for male and female B6 and C3H femora using micro-finite element analyses with an arbitrary tissue modulus of 1 GPa; B. For all femora the tissue modulus as determined from beam theory \( (E_{beam}) \) was strongly underestimated; estimated moduli were well correlated to aspect ratio \( (R^2 > 0.99 \text{ for all four groups}) \). Note that error bars representing standard error of the mean are not visible because they fall within the data points.

Table 2.5: Descriptive statistics on the femora evaluated for structural stiffness.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>N</th>
<th>AP thickness (mm)</th>
<th>Ct.Th (mm)</th>
<th>Ct.Th ratios (-)</th>
<th>I ((\text{mm}^4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>Female</td>
<td>20</td>
<td>1.22 ± 0.05</td>
<td>0.17 ± 0.01</td>
<td>0.28 ± 0.02</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>20</td>
<td>1.22 ± 0.05</td>
<td>0.17 ± 0.02</td>
<td>0.28 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>C3H</td>
<td>Female</td>
<td>20</td>
<td>1.11 ± 0.04</td>
<td>0.33 ± 0.02</td>
<td>0.60 ± 0.03</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>20</td>
<td>1.11 ± 0.03</td>
<td>0.36 ± 0.02(^a)</td>
<td>0.66 ± 0.05(^a)</td>
<td>0.11 ± 0.01(^a)</td>
</tr>
</tbody>
</table>

**AP thickness**: femoral width measured in antero-posterior direction;

**Ct.Th**: cortical thickness;

**Ct.Th ratio**: cortical thickness ratio, calculated as \( 2 \times \text{Ct.Th/AP thickness} \);

**I**: cross-sectional moment of area.

Values are mean ± SD

\(^a\) significantly different between males and females \( (p < 0.05) \).

*Murine femoral biomechanics – material aspects*

The 1 N preload oriented all femora into a similar orientation as visually checked on movies made with a high-speed camera during the mechanical tests. Experimentally measured
stiffness was higher in C3H than in B6 (Table 2.6). When estimated from beam theory, tissue moduli were 5.6 GPa and 10.5 GPa for B6 and C3H, respectively (Fig. 2.20). When calculated using μFE calibrated experimental data those values were 12.0 and 13.4 GPa, respectively; hence, the moduli as derived from the experimental-computation approach were about 10% higher for C3H as compared to B6; this difference did not reach statistical significance (p = 0.10).

![Figure 2.20: Tissue modulus for B6 and C3H female femora as determined from experimental testing in combination with beam theory and μFE analysis.](image)

Table 2.6: Mechanical properties for B6 and C3H femora tested in three-point bending, using a support width of 6 mm.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>N</th>
<th>Stiffness (N/mm)</th>
<th>$E_{\text{beam}}$ (GPa)</th>
<th>$E_{\text{beam}}/E_{\text{FE}}$ (-)</th>
<th>$E_{\exp}$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>Female</td>
<td>10</td>
<td>133.5 ± 26.2</td>
<td>5.6 ± 0.5</td>
<td>0.47 ± 0.02</td>
<td>12.0 ± 1.3</td>
</tr>
<tr>
<td>C3H</td>
<td>Female</td>
<td>12</td>
<td>234.7 ± 39.1</td>
<td>10.5 ± 1.7</td>
<td>0.78 ± 0.02</td>
<td>13.4 ± 2.1</td>
</tr>
</tbody>
</table>

*Stiffness: experimentally measured stiffness

$E_{\text{beam}}$: tissue modulus as derived from beam theory and experimental measurement

$E_{\text{beam}}/E_{\text{FE}}$: ratio between derived tissue modulus and true tissue modulus

$E_{\exp}$: bone tissue modulus as derived from μFE analysis and experimental measurement

Asignificantly different between B6 and C3H ($p < 0.05$).
Discussion

Micro-finite element (μFE) analysis has become a widely accepted method for the mechanical evaluation of small bones and bone samples. In this study, we used μFE analysis to accurately quantify the influence of bone geometry on the outcomes of three-point bending tests. The advantage of using μFE was that we could precisely analyze the effects of bone shape on the outcomes of three-point bending tests, without having to make assumptions on bone geometry. We showed that tissue moduli as derived from three-point bending tests are strongly underestimated. We also showed that the extent of underestimation depends on bone size and shape, and that this is different for different inbred strains; tissue moduli for B6 were underestimated markedly more than for C3H. The reason for this underestimation is clear: murine bones are not perfect beams (Fig. 2.18); their geometries deviate from the assumptions underlying beam theory leading to erroneous results when applying beam theory.

The notion that certain requirements must be fulfilled for the use of beam theory is not new. The potentially confounding effects of aspect ratio and cortical thickness ratio on the outcomes of three-point bending test have been described previously [2, 9, 10]. Nevertheless, in three-point bending tests of murine bones these effects have been largely neglected. No standardized experimental set-up has been described in literature; as a result, people have used different experimental set-ups with support width varying from 2.0 mm to 10 mm for murine femora [2, 3, 5, 15, 18]. The short support width leads to relatively constant cross-sectional moment of area, however, at the cost of a very small aspect ratio. The larger support width maximizes aspect ratio, however, it does so at the cost of having a varying cross-sectional moment of area along the beam axis (Fig. 2.18). Both approaches may induce errors. Indeed, this study explained the largely varying estimates for bone tissue modulus that have been reported for the same bone (e.g. the femur) of the same murine inbred strain (e.g. B6) as a consequence of the limitations of current three-point bending tests.

Our experimental data on 10 B6 and 12 C3H femora showed that, when estimated from beam theory, tissue modulus for C3H was nearly twice as high as that for B6. This is in line with several other studies [6, 15, 19, 20], and has traditionally been explained by variations in tissue composition. However, our computational analyses showed that these beam-theory derived estimates of tissue modulus are wrong, because they are affected by bone geometry. With a combined experimental-computational approach we could show that the difference between the moduli was only 10% and that this difference was not statistically significant. Although a larger sample size may prove this to be a true difference, the key finding here is
2.4 Tissue modulus derived from beam theory is biased by bone size and geometry

that the moduli differ to a smaller extent -if at all- than previously reported. Hence, previously reported differences were largely an effect of geometric differences, not accounted for by beam theory. Using the correction factors as determined from our micro-finite element analyses we tried to correct the values as reported in the literature. Instead of using specimen-specific correction factors, as we did for our measured data, we could only use group averages. After correction, the tissue moduli for B6 and C3H were much more similar (Fig. 2.21) and differed only by about 10%, just as in our study. Absolute values vary, though. We expect that these study-specific effects are caused by differences in experimental set-up, such as the speed of the indentor during testing.

![Figure 2.21: Experimental data on tissue moduli as derived from experimental three-point bending tests. Previously reported data showed that tissue moduli were markedly higher for C3H. After μFE correction, these differences largely disappeared.](image)

Our findings have important implications for other inbred strains, too. Several research groups have tested different inbred strains of mice and have noticed inbred strain specific values for tissue moduli. As a consequence, a paradigm has developed stating that bone tissue properties are largely influenced by genes. In this study we showed that the variation in tissue moduli is likely to be caused by variations in bone geometry, and that the true values differ not much, if at all. So whether the paradigm of inbred strain specific tissue moduli will hold, remains to be seen.
Beam theory takes some of the geometric differences between bones into account through the cross sectional moment of area ($I_{yy}$). Therefore, accurate measurements of $I_{yy}$ have become standard in three-point bending analyses of murine bones. However, in this study we showed that in itself an accurate assessment of $I_{yy}$ is of no help: murine femora simply do not fulfill the requirements for accurate use of beam theory, hence, even a perfect assessment of $I_{yy}$ will still result in a strong underestimation of E.

For simple cylindrical beams we were able to validate our computational approach. For all three tested diameters the experimentally derived tissue moduli closely matched the computationally derived ones. This implies, as expected, that the underestimation of tissue modulus as estimated from beam theory is not affected by diameter per se, but only by its ratio to support width.

What do our findings mean to other parameters measured in a three-point bending tests? As long as these parameters are measured directly, such as stiffness (force over displacement) and strength (maximum load until failure) these parameters are correct, because they are not affected by the limitations of beam theory. Nevertheless, it is important to realize that these parameters are affected by support width, as well. It is quite obvious that a larger support width will lead to a lower stiffness. However, it is less intuitive that results obtained at one support width are not necessarily indicative of results obtained at another support width. As an example: at a support width of 3 mm, female C3H femora were 2.4 times stiffer than female B6 femora, whereas at a support width of 7 mm, the C3H femora were nearly as stiff as B6 femora (Fig. 2.19a). These non-intuitive effects make it difficult to propose a preferred support width that should be used as a standard when testing murine femora. As a guiding principle it should be noted that the outcomes are less affected by bone geometry when support width is large. However, this is only true as long as the shaft is in contact with the supports; further enlarging of the support width will introduce effects due to local geometric variations of the femur near the condyles and near the neck region. Indeed, in computational simulations we found larger variability in stiffness and tissue moduli when support width was 8 mm or beyond (data not shown), these were most likely caused by difficulties in determining the proper contact areas between bone and the supports. Hence, we propose a support width of 7 mm. Nonetheless, the limitations of three-point bending should always be kept in mind.

In this study we have clearly shown that the bone tissue moduli as presented in literature are strongly underestimated for murine femora. Still, tissue moduli will likely continue to be of great interest as one of the components determining bone stiffness and
2.4 Tissue modulus derived from beam theory is biased by bone size and geometry

strength. What could be future approaches to derive better estimates of tissue modulus? The use of correction factors based on cylindrical beams, and taking aspect ratio and wall thickness ratio into account, provide a means to reduce the errors made by applying beam theory [5]. Although this will reduce some of the errors, it cannot correct for deviations from perfect circular cross section, nor can it correct for changes in cross section along the femoral axis. The suggestion made by Schriefer et al. [5] that maximizing support width will reduce errors is questionable, because no methodology is available to correct for the strong geometric changes as seen in the femur when using a large support width (Fig. 2.18). A good approach is the inverse FE method that we applied in this study. It takes the specimen-specific variations in bone geometry into account, and allows for an unbiased assessment of tissue modulus. Three additional possible approaches offer potential by measuring tissue modulus more directly. The first approach is to use micro-manufacturing tools to machine small beams from the cortex of murine femora. Those small beams can be tested either in three- or four-point bending tests [21] or in uniaxial tension and compression [22]. To the best of our knowledge such tests have not been performed for bones from C3H and B6 mice, so no comparative data is available. A second approach is to use nano-indentation, which is used to measure elastic properties of bone tissue at a microscopic length scale by making very small indents on polished bone surfaces [23-25]. Reported values for tissue moduli as obtained with nano-indentation are substantially higher (~30 GPa), which is related to testing of dry specimens and to inherently excluding the effects of bone vasculature. Nevertheless, in a relative sense the data should compare to results as obtained from whole bone tests [2]. Indeed, nanoindentation tests have indicated a 10-15% higher tissue modulus for C3H than for B6 [2, 26], similar to the results as obtained in this study using the back-calculated µFE method. A third, and very recent approach is a combination of scanning acoustic microscopy and synchrotron-radiation µCT [27]. As with nano-indentation, this requires polished bone surfaces; furthermore, the specimens need dehydration in alcohol and embedding in polymethylmetacrylate (PMMA). These preparation steps as well as the required image registration between the two measurement techniques could potentially explain the larger difference (~30%) between B6 and C3H reported tissue moduli using this approach.

We conclude that µFE precisely quantified the underestimation of tissue moduli in murine femora estimated from beam theory. For B6 and C3H inbred strains of mice we showed that the tissue moduli as derived from three-point bending tests are largely an effect of geometric differences, not accounted for by beam theory. Our findings indicate that genetic effects on bone tissue modulus are much less strong than previously suggested. We
hypothesize that the large variations found between other inbred strains of mice are also largely affected by these geometric effects, not by tissue compositional variations. To quantify that in more detail, we suggest a re-evaluation of the tissue properties obtained from three-point bending tests, especially in mouse genetics.

Acknowledgements

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References

2.4 Tissue modulus derived from beam theory is biased by bone size and geometry


Chapter 3

Hierarchical investigation of bone strength
Chapter 3. Hierarchical investigation of bone strength
3.1 Functional microimaging: a hierarchical investigation of bone failure behavior

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Abstract

Biomechanical testing is the gold standard to determine bone competence, and has been used extensively. Direct mechanical testing provides detailed information on overall bone mechanical and material properties, but fails in revealing local properties such as local deformations and strains and does not permit quantification of fracture progression. Therefore, we incorporated several imaging methods in our mechanical setups in order to get a better insight into bone deformation and failure characteristics on various levels of structural organization. Our aim was to develop an integrative approach for hierarchical investigation of bone, working at different scales of resolution ranging from the whole bone to its ultrastructure. Inbred strains of mice make useful models to study bone properties. We investigated bone properties in C3H/He (C3H) and C57BL/6 (B6), two strains known for their differences in bone phenotype. At the macroscopic level, we used high-resolution and high-speed cameras which drastically increased the amount of information obtained from a biomechanical test. The new image data proved especially important when dealing with very
small bones such as murine femora. It allowed to visualize global failure behavior and fracture initiation with high temporal resolution. At the microscopic level, bone microstructure, i.e. trabecular architecture and cortical porosity, are known to influence bone strength and failure mechanisms significantly. For this reason, we developed an image-guided failure assessment technique, also referred to as functional microimaging, allowing direct time-lapsed three-dimensional visualization and computation of local displacements and strains for better quantification of fracture initiation and progression. While the resolution of typical desktop micro-computed tomography is around a few micrometers, highly brilliant X-rays from synchrotron radiation permit to explore the sub-micrometer world. This allowed, for the first time, to uncover fully nondestructively the 3D ultrastructure of bone including vascular and cellular structures and to investigate their role in the development of bone microcracks with an unprecedented resolution. The differences in bone mechanical properties observed macroscopically in C3H and B6 were explained, to some extent, by their differences in microstructural architecture and porosity assessed with high-resolution techniques.

We conclude that functional microimaging, i.e. the combination of biomechanical testing with non-destructive and high-resolution 3D imaging and visualization are extremely valuable in studying bone failure mechanisms. Functional investigation of microcrack initiation and propagation will lead to a better understanding of the relative contribution of bone mass and bone quality to bone competence, especially to post-yield failure behavior.

**Keywords:**
Bone strength, mouse strain, biomechanical testing, Micro-computed tomography, hierarchical imaging

**Introduction**

Bone belongs to some of the best investigated biological materials due to its primary function of providing skeletal stability. Bone is susceptible to different local stimuli including mechanical forces and has great capabilities in adapting its mechanical properties to the changes in its environment. Nevertheless, aging or hormonal changes can make bone lose its ability to remodel appropriately, with loss of strength and increased fracture risk as a result. Due to the emergence of accurate and precise bone densitometry over the last two decades, bone mass has become a primary endpoint in osteoporosis diagnosis and monitoring. Strong correlations between bone mass and mechanical properties of trabecular bone have been demonstrated for large populations using power-law regressions [1-7]. However, changes in
3.1 Functional microimaging

mass can only partially explain the variation of trabecular bone strength of individuals leaving sometimes up to 90% of the strength variation unexplained [8]. Thus, accurate diagnosis in a clinical environment solely based on bone densitometry is difficult. With up to 90% unexplained variation, other factors such as bone microarchitecture, bone cell distribution, bone remodeling, the distribution of microcracks or microdamage and the quality of the underlying organic bone matrix must play important roles too. These other actors are often referred to as bone quality [9]. However, the relative contribution of bone mass and bone quality is still poorly understood.

The gold standard to determine bone competence is direct mechanical testing of bone. Direct mechanical testing provides detailed information on overall bone mechanical and material properties, but fails in revealing local functional properties such as local deformations and strains or quantification of fracture progression. In order to get a better insight into local bone deformation and failure characteristics, we combined mechanical testing with imaging to uncover these local properties. The variety of biomedical imaging techniques makes it possible to work with a wide range of resolution scales, from the macroscopic down to the nanoscopic scale.

Bone tissue has been studied extensively with micro-computed tomography (μCT) and synchrotron radiation based μCT (SRμCT) [10-12]. Tomography using X-rays is a well established technique that is also applied to large systems such as bodyparts or whole organs of human beings [13] and to small systems below micrometer scale such as individual biological cells [14]. Because X-ray based tomography uses the same physical principles at different levels of spatial resolution, CT, μCT and SRμCT allow investigations of tissues and organs in a hierarchical fashion – from human, to organ, to tissue and down to the individual cell covering more than 6 orders of magnitude in size. Moreover, μCT and SRμCT are non-destructive techniques allowing multiple time-lapsed imaging of samples.

At the microscopic level, bone samples can be imaged in 3D using μCT between subsequent micro-compression steps. Bone fracture is a time-dependent, non-linear event including high local deformations and local trabecular fractures. Although some work on bone failure characteristics has been done, basic knowledge of local trabecular failure is still lacking. In estimating the risk of spontaneous fractures, an extended understanding of the failure behavior of trabecular bone is essential. For this reason, our group has developed an image-guided failure assessment (IGFA) technique, also referred to as functional microimaging, allowing investigation of micro-architectural deformations as well as direct time-lapsed 3D visualization and quantification of fracture progression at the microscopic
level [15]. This technique has recently been validated as compared to classical continuous mechanical testing [16, 17].

SRμCT resolutions of 1 μm and better have made it possible to analyze tissue properties in the submicron domain [18, 19]. It is now possible to uncover, in a three dimensional fashion, the ultrastructure and to investigate microcrack initiation and progression in bone samples under load. Indeed, bone mass and bone architecture are certainly the dominant parameters determining bone strength. However, to determine the strength of the actual bone tissue, cellular effects such as bone mineral density variation or cell lacunae size and distribution must be investigated. Recent results show that bone vasculature is a major contributor to local tissue porosity, and therefore can be directly linked to the mechanical properties of bone tissue [20].

In the current study, all the investigations were performed on murine bones. Indeed, well-characterized mouse lines with phenotypes related to certain aspect of human osteoporosis can be used as an approach to study more homogenous populations in which isolation of candidate chromosomal regions and genetic loci should be faster and more efficient. Animal models complement and extend human studies by allowing close control of environmental factors, by expanding the characterization of phenotypes underlying bone strength and by facilitating breeding strategies to identify genetic linkage [21, 22].

Adult C3H/He (C3H) and C57BL/6 (B6) mice are similar in body size and weight and their bones are of similar external size, but show significantly different morphological and compositional bone traits [21, 23], such as adult peak bone density and cross-sectional area [24, 25].

The specific aim of this study was to develop and implement an integrative approach to hierarchically investigate bone and its mechanical properties, working at different scales of resolution ranging from the whole bone to its ultrastructure. We hypothesized that there would be differences in bone mechanics between the two strains, B6 and C3H, on all levels of resolution.

Material and Methods

**Animal model**

For this study, we used two inbred strains, C57BL/6He (B6) and C3H/He (C3H). All mice were female and raised at Harlan (Horst, The Netherlands). They were sacrificed by CO2 inhalation at 16 weeks. The animals were then stored at -20°C and thawed at room
3.1 Functional microimaging

temperature just before dissection of the femora. Use of mice in this research project was reviewed and approved by the local authorities for all levels of investigation.

*Hierarchical bone model*

Within this study, we followed a hierarchical description of the bone, as previously described [20]. For this purpose, we distinguished three levels on different length scales. On the organ level, the whole femur was considered and cortical bone was considered to be compact. At the tissue level, we considered bone microstructure such as trabecular bone. Cortical bone was still considered to be compact, but micro-fractures were distinguished. Finally, at the cellular level, the cannular network, the osteocyte lacunar system and microdamage were distinguished within cortical bone.

*Imaging of bone biomechanical testing at the organ level*

At the organ level, we used imaging tools to obtain additional information from two types of mechanical tests on murine femora: compression of the femoral head and 3-point bending tests. In both loading configurations, tests were imaged with a high-speed and high-resolution camera (AOS Technologies, Daetwil, CH). The image size was set to 1280 x 1024 pixels and the frame rate to 62.5 frame/s.

Compression tests on 50 femoral heads were performed on B6 mice. Left and right femora were meticulously prepared and positioned for mechanical testing with an axial maximum alignment error of 1.5°. The femora were then rigidly fixed in a materials testing machine (1456, Zwick GmbH & Co.). The mechanical tests consisted of loading the femoral heads until fracture of the femoral neck occurred. The samples were pre-loaded with 1 N and load-displacement curves were recorded at a crosshead speed of 0.5 mm/s [26]. Between 200 and 300 high-resolution images were recorded per single test.

Ten B6 and 12 C3H femora were tested in three-point bending at room temperature in a custom-made loading device, which was integrated in the materials testing machine (1456, Zwick GmbH & Co.). Load was applied midway between two supports that were 6 mm apart. The femora were positioned such that the loading pin was applying a force at a location on the shaft situated at 55 % of the length of the femur as measured from the proximal side. The femora were laying freely on the supports and the 1 N preload oriented them so that the load was applied in the anterior-posterior direction. Load-displacement curves were recorded at a crosshead speed of 0.5 mm/s. Bone stiffness, strength, yield force, yield displacement, post-yield displacement as well as toughness were derived from these curves as described
previously [26, 27]. Between 200 and 300 high-resolution images were recorded per single test.

Image-guided failure assessment at the tissue level

We performed two types of time-lapsed experiments imaged with a desktop µCT (µCT 40, Scanco Medical AG, Bassersdorf, Switzerland): femoral head micro-compression tests and three-point bending tests on two B6 femora using IGFA. The image resolution for these tests was 20 µm. The complete compression experiment included 18 steps of 54 µm before catastrophic failure of the femoral neck. In three-point bending, we loaded the bone in 14 steps up to total failure.

In order to reach a higher resolution than with the desktop µCT, a loading device was designed to perform IGFA using synchrotron radiation based µCT (SRµCT) [12]. We repeated the aforementioned femoral head compressive test with this setup on two B6 and two C3H mice. These tomography experiments were performed at the Material Science (MS) beamline of the Swiss Light Source (SLS) at the Paul Scherrer Institut (PSI). For the IGFA experiments, a photon energy of 20 keV was used. The pixel size in each of the recorded projections was 3.5 µm. These data were subsequently binned by a factor of two prior to the reconstruction in order to increase the signal-to-noise-ratio. At each compression step during IGFA, every sample was scanned 3 times at different heights, since the X-ray beam height was less than 2 mm. The tomography setup had a field of view [28] of 7 x 7 mm². Thus, the tube made of Torlon 4203 (Solvay S.A., Brussels, Belgium), a poly-amid-imide, around the sample with an outer diameter of 10 mm was not entirely in the FOV of the detector, hence, local tomography was performed.

Image-guided failure assessment at the cellular level

Femora were dissected from two genetically distinct inbred strains of mice (7 C3H and 7 B6 femora) and embedded at both ends into polymethyl methacrylate cement (PMMA) (Fig. 3.7b). We created a round notch in the mid-diaphysis using a high-precision blade saw (Isomet 5000, Bühler LTD, Lake Bluff, Michigan, USA) (Fig. 3.7b). The notch width was 750 µm. This notch was expected to create a weakened region in the cortical bone, where the microcracks would initiate and propagate while the bone was loaded. Before imaging, the samples were stored in saline solution. At the MS Beamline of the SLS, samples were then placed into a custom-made loading device. This loading device comprised an X-ray translucent loading chamber; and the sample was loaded under compression with a precision
3.1 Functional microimaging

screw. To prevent any torsional constraint due to screwing, a ball-bearing was included into the loading device (Fig. 3.7a and 3.7b). The samples were then loaded in a stepwise manner with 1% strain incremental steps. The loading process was deformation controlled. Between each step a high-resolution 3D image (700 nm nominal resolution) was acquired with the SrμCT. Because of the small field of view [28] of 1.4 mm x 1.4 mm local tomography was performed. For each 3D image, in total 1001 projections were acquired over a range of 180 degrees at a photon energy of 17.5 keV. The data was reconstructed using filtered backprojection. In this study, the extraction of the cortical void spaces included a combination of different image processing procedures (IPL, Scanco Medical AG, Bassersdorf, Switzerland), including morphological operators. They were optimized to extract the canal network, the osteocyte lacunar system and the microcracks within cortical bone as three separate phases. For each phase, a set of morphometrical parameters were measured. Canular volume density (Ca.V/Ct.V), canular spacing (Ca.Sp), canular unit volume (Ca.V/Ca.N) and canular average length (Ca.Le) were measured from the canular network. Finally, microdamage was quantitatively determined by the following parameters: crack total volume (Cr.V), crack average thickness (Cr.Th) and crack number per unit cortical bone (Cr.N/Ct.TV).

Statistical analyses

Morphometric indices and mechanical parameters strains were compared between both murine strains using two-tailed Student’s t-test. All statistical analyses were performed with MS Excel 2003, the GNU statistical package R (Version 2.4.0, http://www.r-project.org) and the statistical program: SPSS (Version 13.0, The Apache Software Foundation, Chicago, USA).

Results

Imaging of bone biomechanical testing at the organ level

High-resolution and high-speed imaging during compression testing on femoral heads allowed recording of deformations and fractures. We distinguished three characteristic types of fractures. The most frequent one was the oblique fracture going from the proximal side of the femoral head to the distal portion of the neck-shaft intersection (N = 28) (Fig. 3.1a). The second most common fracture type we observed was the so-called trochanteric fracture (N = 13), where the fracture includes a part of the greater trochanter (Fig. 3.1b). There were also a few vertical fractures which were parallel to the femoral shaft (N = 8) (Fig. 3.1c). Also in the
three-point bending configuration, the additional information gained from the high-resolution and high-speed camera revealed ways how fractures occurred. The images showed that bone fractures initiated at the posterior, tensile, side of the bone (Fig. 3.1d).

Another relevant information delivered by this visualization technique was the exact positioning of the femora in three-point bending after preload. It provided an easy and elegant way to ascertain that bones, simply put on supports, were positioned correctly and that they rotated in a very reproducible way after preload. Furthermore, imaging helped to discriminate outliers and permitted to determine if variability in the results was caused by problems in the experimental set-up. Even if a reasonable force versus displacement curve was measured, visualization of the failure process could sometimes identify measurement errors. Indeed, tests where samples slipped in their holder due to bad embedding or with fractures which happened at an unusual location, i.e. femoral head fracture (Fig. 3.2a), could easily be classified as erroneous tests and excluded from further analysis. In the femoral head loading configuration, stiffness, deformation and work to failure coefficients of variation (CV) were reduced nearly by half when removing the outliers. Bone strength CV was not significantly improved excluding the bad tests. Detailed visualization of these tests also permitted to visualize local deformations that were not uncovered by simple analysis of the test outputs such as femoral head deformation under compression (Fig. 3.2b) or local cortical deformations at the supports in three-point bending (Fig. 3.2c). In three-point bending tests, the average local deformations in B6 and C3H were 70 µm and 20 µm, respectively. The high-resolution images also showed whitening effect found in bone as a consequence of overloading (Fig. 3.1 and 3.3).

Finally, we computed the results of the 3-point bending tests. Bone strength, stiffness, yield force and yield displacement as well as post-yield parameters, such as post-yield displacement and toughness, were statistically different for B6 and C3H (Table 3.1).
3.1 Functional microimaging

Table 3.1: Biomechanical results of the three-point bending tests. Data are presented as mean ± SD (CV). Stiffness (N/mm) = slope of the linear part of the curve before the yield point. Strength (N) = ultimate force. Yield force (N) = loading force at the yield point. Yield displacement (μm) = deformation at the yield point. Post-yield displacement (μm) = deformation between the yield point and the failure displacement. Toughness (Nmm) = Work to failure or area below the load-displacement curve until total failure.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>C3H</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Stiffness (N/mm)</td>
<td>125.6 ± 27.2 (21.6 %)</td>
<td>289.6 ± 18.9 (6.5%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Strength (N)</td>
<td>15.1 ± 1.6 (10.5%)</td>
<td>30.8 ± 1.5 (4.9%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Yield force (N)</td>
<td>10.3 ± 1.6 (15.3%)</td>
<td>19.6 ± 1.4 (7.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Yield displacement (μm)</td>
<td>75.7 ± 7.5 (10%)</td>
<td>85.3 ± 6.6 (7.7%)</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Post-yield displacement (μm)</td>
<td>233.3 ± 70.9 (30.4%)</td>
<td>185 ± 36.6 (19.8%)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Toughness (Nmm)</td>
<td>5.26 ± 2.26 (43%)</td>
<td>7.23 ± 1.68 (23.3%)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
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Figure 3.1: Three characteristic types of femoral neck fractures. (a) oblique, (b) trochanteric and (c) vertical type. (d) In three-point bending, fracture initiates under tension. Black arrows show whitening.
Chapter 3. Hierarchical investigation of bone strength

Figure 3.2: (a) Bad fracture occurring in the femoral head instead of femoral neck. (b) Local deformation of the femoral head at the loading pin contact surface. The femoral head sphere is clearly flattened (black arrow). (c) Local deformation of cortical bone in the femur midshaft in a three-point bending test (black arrow).

Figure 3.3: The black arrow shows bone whitening before the fracture initiates.

Image-guided failure assessment at the tissue level

Performing IGFA allowed a good insight into the structural failure mechanism of whole inbred murine femora under compression. We could follow the deformation of the trabeculae and the formation of cracks in the cortical bone as well as in the trabecular bone (Fig. 3.4a). The mechanical properties of the bone were derived from the load-displacement
3.1 Functional microimaging

diagram. 20 N strength and 75 N/mm stiffness were calculated for a 16-week old B6 femur (Fig. 3.4b). These results are similar to results obtained with continuous testing methods [26].

Figure 3.4: (a) Assessment of femur deformation pattern under axial loading. Initial, intermediate and final steps of IGFA testing on a B6 mouse femur. (b) Typical force–displacement diagram for compression testing at the femoral head.

In order to visualize trabecular structure in more detail, as well as to better identify crack initiation and crack growth, IGFA was also performed using SRμCT. Femoral head compression tests were imaged at 3.5 μm resolution. Despite a longer imaging time and a more complicated setup than common IGFA, images assessed by SRμCT showed more details with better quality (Fig. 3.5). Fracture initiation and propagation were well depicted in such experiments. Tests on B6 and C3H showed different fracture behavior. In the C3H samples, no trabecular bone could be found in the femoral neck, but the neck was formed with a thicker cortex than in B6. Our analysis revealed that the fracture initiated were the ratio of bending moment relative to the cross sectional moment of inertia was the highest. Bone failed at the location where the tensile stresses were the highest in cortical bone.

In the same fashion as with the high-resolution and high-speed camera, IGFA showed relevant details of the tests that could not be deduced from the quantitative output alone. In addition to uncover the structural failure mechanism, IGFA also imaged the local deformations at the interface between bone samples and loading setups such as femoral head deformation under compression (Fig. 3.6a) or local cortical deformations at the supports in three-point bending (Fig. 3.6b).
Figure 3.5: Compression and fracture at the proximal femur. a) C3H left femur, b) C3H femoral neck, c) B6 left femur, d) B6 femoral neck.
3.1 Functional microimaging

Figure 3.6: (a) Local deformation of the femoral head at the loading pin contact. Formation of a notch due to the loading pin (grey circles). (b) Local deformation of cortical bone in the femur midshaft in a three-point bending test (white arrow).

Image-guided failure assessment at the cellular level

Initiation and propagation of microcracks were imaged at a sub-microscopic resolution in a non-destructive manner with SRµCT. This experiment showed microcrack initiation with increasing strain. Cortical bone of a B6 sample showed no microcrack at 1% strain. At 2%, microcracks appeared at several locations (Fig. 3.7c). At 1% strain in a C3H sample, we could observe a crack at very early stage. The crack was hardly visible at the used resolution (Fig. 3.7c). Loading the sample further to 2%, the microcrack propagated, reaching the bone surface. Further analyses revealed that microcrack propagation usually initiated at vessels or bone surfaces and were not stopped by the lacunae; microcracks went through these osteocytic cavities (Fig. 3.8a). Precisely, 40% of microcracks in C3H initiated at vascular surface while the rest initiated at the inner cortical bone surface. In B6, all the microcracks initiated at the inner cortical bone surface. SRµCT images at 700 nm resolution permitted superb 2D and 3D visualizations of uncracked ligament bridging as well as mineralized collagen fibrils, which are thought to be of extreme importance in bone toughening mechanism (Fig. 3.8b, 3.8c and 3.9). Finally, we were able for the first time to extract the canal network, the osteocyte lacunae and the microdamage, as three separate phases within cortical bone (Fig. 3.10). Morphometric analyses of each phase revealed that the cannular volume and the cannular length in C3H were significantly higher than in B6, while the cannular spacing remained constants Consequently, more microdamage accumulated in C3H (Tables 3.2 and 3.3).
Figure 3.7: (a) The loading device with a translucent chamber and a precision screw at the top. (b) Murine femur embedded into PMMA and the internal setup with a ball joint. (c) SRμCT reconstructed slices of B6 cortical bone close to the notch; nominal resolution: 700 nm, binned; 1% apparent strain; microcracks are not visible, (d) 2% apparent strain; visible microcracks initiate. (e) SRμCT reconstructed slices of C3H cortical bone close to the notch; nominal resolution: 700 nm; 1% apparent strain, a tiny microcrack is visible (black arrow); (f) 2% apparent strain, the microcrack has propagated.

Figure 3.8: (a) Crack initiation in the vessel and the microcrack propagates through two lacunae. (b) Uncracked ligament bridging along the crack path (arrows). (c) 3D imaging of C3H cortical bone at the same location as (a) and (b). Blow-up: 3D representation of an uncracked ligament bridge.
3.1 Functional microimaging

Figure 3.9: SRμCT reconstructed slice of B6 cortical bone at 1% strain with a microcrack and a microfracture. Nominal resolution: 700 nm. Uncracked ligament bridges intersecting the microcrack (black arrows) and mineralized collagen fibrils spanning the microfracture (white arrow).

Figure 3.10: (left) 3D representation of osteocyte lacunae (yellow), canal network (red) and microcracks (green) in C3H cortical bone. (right) Same image as (left) without the lacunae. The microcracks initiated at the vasculature surface.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>C3H</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Ca.V/Ct.TV (%)</td>
<td>0.67 ± 0.50</td>
<td>1.4 ± 0.45</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Ca.Sp (%)</td>
<td>0.11 ± 0.03</td>
<td>0.13 ± 0.02</td>
<td>0.08</td>
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<tr>
<td>Ca.V/Ca.N (10^3 μm^3)</td>
<td>7.5 ± 3.1</td>
<td>54.7 ± 32.6</td>
<td>&lt; 0.001</td>
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<tr>
<td>Ca.Le (mm)</td>
<td>0.20 ± 0.16</td>
<td>0.80 ± 0.70</td>
<td>&lt; 0.05</td>
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</table>

Table 3.3: Microcrack morphometric indices. More cracks and longer cracks in C3H than in B6 at 1% compressive strain. Data are presented as mean ± SD. Cr.Th (μm) = average crack thickness. Cr.Le (mm) = average crack length. Cr.N/Ct.TV (mm^-3) = crack number per unit cortical bone.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>C3H</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Cr.Th (μm)</td>
<td>2.5 ± 0.9</td>
<td>2.6 ± 0.8</td>
<td>0.43</td>
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<tr>
<td>Cr.Le (mm)</td>
<td>0.11 ± 0.05</td>
<td>0.29 ± 0.19</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Cr.N/Ct.TV (mm^-3)</td>
<td>3.4 ± 1.3</td>
<td>7.6 ± 3.0</td>
<td>&lt; 0.005</td>
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</tbody>
</table>

Discussion

In this paper, we described an integrative approach for hierarchical bone investigation, working at different scales of resolution ranging from the whole bone to its ultrastructure. We combined several imaging methods with standard mechanical testing tools in order to get a better insight into bone deformation and failure mechanisms. Mechanical properties in two mouse inbred strains, B6 and C3H, were investigated and the differences in bone properties were assessed at all scales.

Macroscopic imaging of common mechanical tests, such as compression tests on proximal femur or three-point bending tests, drastically increased the informative output of these tests. We improved the experimental reproducibility by easily recognizing outliers and removing bad samples (Fig. 3.2a). As a consequence, variability of the tests was reduced up to 50% for mechanical parameters. This is of great value in experimental studies, because
3.1 Functional microimaging

reduced variability means increased power in the results. This also means that fewer samples and therefore fewer animals are needed to obtain significant results.

Looking at local deformations at loading or support points, visualization with high-speed, high-resolution cameras permitted to correct the measured total deformation of the sample. Indeed, classical tests do not take into account that local deformations form at contact points between load and samples (Fig. 3.2b and c). In a normal setup, these local deformations are automatically, but unintentionally, added to the measured total deformation. In the 3-point bending tests, local deformations at the supports were 3 times higher for B6 than C3H. This was explained by thicker cortical bone in C3H and higher hardness of C3H cortical bone [29].

Moreover, bone surface was seen to whiten with increasing strain (Fig. 3.1 and 3.3), similar to work on trabecular bone recently reported by Thurner et al. [11]. We hypothesize that the effect seen is due to microcrack formation in these areas, similar to stress whitening seen in synthetic polymers. Previous study showed that the whitened areas are also of high deformation. Thurner et al. hypothesized that microdamage and associated whitening appear at points of highest strain, which would be consistent with failure initiation in compact bone, which was found to be at the point of highest local strain [30]. Hence macroscopic investigation of typical failure locations and fracture propagation could give a first insight into bone failure behavior.

All the mechanical parameters for C3H were more reproducible than for B6 (Table 3.1), as evidenced by their lower CV. This means that this murine model has more homogeneous bone mechanical properties than B6. Further, these experiments also revealed that both models were significantly different for stiffness, strength, yield force, yield displacement, post-yield displacement and toughness. C3H was less tough and more brittle (shorter post-yield deformation) than B6. Comparing the mechanical data with the recorded movies of the tests, whitening as well as fracture initiation clearly happened in the post-yield phase. The high brittleness of C3H compared to B6 suggested that C3H bone is more porous and therefore initiates and accumulates more microdamage [22, 31]. So, we hypothesize that microcracking plays an important role in the post-yield failure behavior; hence, that it is of great interest to investigate it further in order to better understand the whole failure behavior. For these reasons, we extended our investigations and compared B6 and C3H at the tissue and cellular levels.

Moving to microscopic scales and investigating bone at a tissue level allowed further observations on why and how bone fails. As discussed before, bone mass alone does not
entirely explain variations in bone strength and competence. It has been well established that prediction of bone strength can be greatly improved by including microarchitectural parameters in the analysis [32-35]. Therefore, IGFA is a very powerful tool in visualizing and quantifying local behavior of bone microstructure under load. This technique gave insight into whole bone failure behavior with its deforming internal microstructure during femoral head compression or three-point bending tests (Fig. 3.4 – 3.6). Adding to this tool a novel image analysis approach to compute local displacements and strains in the compressed structures [36], close tracking of very local structural changes, such as single trabecular deformation is now possible.

During femoral head compression, IGFA showed that fracture occurred were the ratio of bending moment relative to the cross sectional moment of inertia was the highest. In other words, bone failed at the location where the stresses were the highest in cortical bone (Fig. 3.4 and 3.5). In B6, whose neck contained more trabecular bone than C3H neck, the fracture initiated and propagated in the region of the neck where trabecular bone density was lower (Fig. 3.5).

Exploration of bone in the submicron domain is a necessity when investigating bone competence, because the so-called bone ultrastructure, comprising vasculature, osteocytic lacunae and microdamage, is thought to play an important role in bone material and mechanical properties as well as in bone remodeling processes. As mentioned before, bone is an inhomogeneous material with a complex hierarchical microstructure [37-40] that can be considered at several dimensional scales. With SRμCT, we are now able to visualize the bone ultrastructure. In order to fully understand bone competence, bone as a material has to be characterized first. And there, the ultrastructure certainly plays a key role. Bone porosity and the distribution of its different entities (vessels, lacunae, canaliculae and microdamage) are factors that directly influence material properties [41]. Furthermore, bone ultrastructure is meant to be partly responsible for bone remodeling and has active contributions to mechanisms such as mechanotransduction; osteocytes are believed to be distributed inside bone in a way to best sense the local environment and load applied on bone [41-44].

A prerequisite for the full understanding of bone mechanics and the modeling of bone’s mechanical behavior is that the mechanisms that govern the deformation and failure of the tissue in the post-yield region are clarified. Of course, bone microdamage plays a major role in the post-yield behavior, but we also imaged other processes that are thought to be important, in particular post-yield bone toughening mechanisms. We observed two sorts of such processes; uncracked ligament bridging and mineralized collagen fibers, which both
3.1 Functional microimaging

spanned the cracks (Fig. 3.8 and 3.9). Uncracked ligament bridging was defined by Nalla et al. [45] as a toughening mechanism involving two-dimensional uncracked regions along the crack path that can bridge the crack on opening. The microcracking can in principle lead to toughening since it can cause dilation and increase the compliance of the region surrounding the crack. In other words, the uncracked ligament bridges increase resistance to fracture by sustaining part of the applied load that would otherwise contribute to crack advance. The image resolution achieved in this study permitted superb visualizations in 2D and 3D of uncracked ligament bridges. For the first time, uncracked ligament bridges were imaged in three dimensions at a nominal resolution of 700 nm (Fig. 3.8). The 3D visualization revealed that the uncracked ligament bridges are not two dimensional uncracked regions along the crack path, as related previously [40, 45], but have proper three dimensional shapes. Their thickness varied between 1-3 μm. SRμCt also uncovered bundles of mineralized collagen fibrils which spanned the cracks (Fig. 3.9). Mineralized collagen fibrils are the basic building blocks of bone tissue at the supramolecular level [46]. Uncracked ligament bridging is deemed to be the predominant toughening mechanism in cortical bone, while mineralized collagen fibrils, much smaller, may play a secondary role in little cracks [47].

Using SRμCT, this study showed for the first time microcrack initiation and propagation (Fig. 3.7c). Our model of static loading together with non-destructive imaging permitted to visualize the same region of bone before and after microcrack initiation. Further crack propagation could also be imaged up total bone failure. Due to its non-destructive and 3D nature, this new tool permitted to relate bone ultrastructure to bone failure behavior in an unprecedented manner. We could investigate where the microcracks initiated and how they propagated in murine cortical bone. Our results showed that 40% of microcracks in C3H initiated at vascular surface while the rest initiated at the inner cortical bone surface. In B6, all the microcracks initiated at the inner cortical bone surface. These observations suggested that the vessels acted as stress raisers which favored the initiation of microcracks in the C3H.

In our investigations, it appeared that osteocyte lacunae played another role in microcrack initiation and propagation than vasculature. Microcracks did not seem to initiate at the lacunae surface, but our data showed that cracks propagated through the lacunae. The lacunar voids, more exactly their distribution, guided the microcrack paths. Several previous studies suggested that osteocyte lacunae are stress concentrators [48-50] with average surrounding strain 1.5-4.5 times greater than the strain applied on bone [51-53]. These higher stresses and strains should then drive the microcracks paths across the cortical bone; and this is precisely what we observed in our data.
To the best of our knowledge, this study presented for the first time a volumetric visualization, at 700 nm nominal resolution, of cortical bone with different elements of porosity, vasculature, osteocyte lacunae and microdamage, separated as three separate phases (Fig. 3.10). This opens a new scope for volumetric analysis. Applying morphometric analysis on each phase, we were able to quantify, semi-automatically, cortical porosity and microdamage at very high resolution and in three dimensions. We believe that this new tool is of extreme importance when comparing different samples. Comparison of bone ultrastructure and microdamage accumulation between healthy and unhealthy donors or from different species is now possible. In our case, comparing ultrastructures from two murine inbred strains, with different genetic backgrounds, revealed further important clues on the role of porosity in microdamage initiation and propagation and on the overall bone failure behavior.

This paper presented for the first time an integrative image-guided approach to measure and analyze bone failure initiation and propagation in a fully non-destructive way, and even more importantly, in a hierarchical fashion ranging from macro to nano; from organ to cell. In this study, we were able to observe at the organ level how overall fractures initiate and propagate. At the tissue level, we examined how the bone internal microstructure behaves when bone is under load. Local deformations and microarchitectural changes were observed and analyzed. Submicrometer tomography permitted to look into the ultrastructure of bone. The difference of failure behavior between two inbred strains, observed macroscopically, could be explained, to some extent, investigating bone properties at smaller scales. Especially, the different post-yield behaviors of B6 and C3H were related to differences in cortical porosity and microdamage accumulation.

The developed integrative approach to functionally and hierarchically determine bone competence is very powerful. The time-lapsed high resolution 3D imaging of bone under load will provide better understanding of the bone failure processes by non-invasive assessment of local deformation, fracture initiation and fracture propagation, which cannot be assessed with other techniques in an integrative fashion.

Acknowledgements

This work was supported through ETH Intramural Funding (TH 00124/41-2631.5) and the Swiss National Science Foundation (FP 620-58097.99 and PP-104317/1). We thank Paul Lüthi for machining several parts indispensable for our loading device and Amela Groso for her help and support at the Materials Science (MS) Beamline of the Swiss Light Source (SLS).
3.1 Functional microimaging

References


3.1 Functional microimaging


3.1 Functional microimaging


3.2 Intermediary comments

At this stage of the thesis, we have gone from bone macroscopic mechanical and material properties to bone ultrastructure. The two next papers will focus on bone ultrastructure and its influence on microdamage initiation and propagation.

Before diving into this very small world, I would like to make the reader aware of some aspects of this work which were discussed in the five previous papers and still need some more precisions. The following paragraphs results mainly from discussions with colleagues, supervisors and experts I had after finishing the writing of all the papers.

First, up to now, I used the term brittleness to express the deformation to ultimate failure. The smaller the deformation was, the more brittle the bone was. This definition is true as long as we consider bone as a continuum material. When regarding bone as a porous material, one has to be careful when using this term. Indeed, in Chapter 4, we will see that bone accumulating more microdamage tends to fail under less deformation. In the next papers, where these processes are described, I continued to define this type of bone as more brittle, following the definition valid for continuum, but a material that accumulates microdamage before failing is not brittle, rather ductile. The reader must keep in mind that the use of the term brittleness is exact only at the macroscopic level and is to some extent misused in the next chapter. This is actually an essential point of this thesis. Our work showed that the type of bone which looks more brittle at the macroscopic level is, actually, a type of bone which accumulates more microdamage. This is a very important finding of this thesis and will be explained in more details in the following chapter.

Discussing our papers with experts, we also came to an interesting discussion about whitening in 3-point bending. Whitening occurred close to the loading point, on the anterior side. At this location, the sample was locally loaded under compression. There, the strains were the highest, since local deformations due to the indenter added to bending deformations. Nevertheless, the failure initiated at the opposite side of the femoral shaft, the posterior side, under tensile. This confirms the fact that bone is weaker under tensile than compression. Hence, failure did not initiate where bone was whitening as it is described in the previous paper. Whitening accumulated where highest stresses were located, but the fracture occurred were the total tensile stresses exceeded bone strength. This seems to be in contradiction with our previous affirmation that fracture initiates where microdamage accumulates, but in this special case of 3-point bending, we have a mixed loading configuration and bone is weaker under tensile. So, it was not exact to say that, in 3-point bending, fracture initiated where
Chapter 3. Hierarchical investigation of bone strength

microdamage accumulated, but this did not invalidate the relation between microdamage and fracture, since there was a complex regime of loading. In the next chapter, the loading configuration is less complex than for 3-point bending and therefore the relation between microcrack and bone failure will be more obvious.

Finally, it has been often mentioned in this work that uncracked ligament bridging played an important role in bone toughening mechanisms. In our work, we were able to visualize these uncracked ligament bridging linking both sides of the cracks, but we never investigated their mechanical properties nor their role in the toughening process. We did not attribute any mechanical role to these ligaments, but we only referred to previous studies which tried to demonstrate it. The main achievement of our work in this domain was the ability to localize and visualize these interesting features using SRμCT. We believe that combining this visualization technique with appropriate mechanical experiments will help to investigate the affective role of uncracked ligament bridging in bone competence.
4.1 Assessment of microcrack initiation and propagation

Chapter 4

Bone ultrastructure
4.1 Time-Lapsed Assessment of Microcrack Initiation and Propagation in Murine Cortical Bone

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Abstract

Aging or hormonal changes can make bone lose its ability to remodel appropriately, with loss of strength and increased fracture risk as a result. In addition to the amount of mineral i.e. calcium in bone, other parameters, usually referred to as bone “quality”, are responsible for bone strength. Bone quality includes microarchitecture, microcracks and damage, remodeling, distribution of the three main cell types in bone (osteoclasts, osteoblasts, and osteocytes), and collagen properties. It has been hypothesized that osteocyte lacunae and the canular network affect microcrack initiation and propagation. Due to its high resolution, bone assessment by means of synchrotron radiation-based micro-computed tomography (SRμCT) allows an unprecedented and non-invasive insight into microcrack behavior. Ultrastructural features such as bone canals and even single osteocytes within the bone tissue are now detectable in a fully nondestructive and three-dimensional fashion. A new challenge, and the specific aim of this study, was to relate these microscopic features with initiation and propagation of microcracks.
Chapter 4. Bone ultrastructure

Femora were taken from two genetically distinct inbred strains of mice (C3H/He and C57BL/6) and embedded at both ends into polymethyl methacrylate cement. We created a round notch in the mid-diaphysis and then loaded the samples axially under compression, from 0 to 4% strain, in a stepwise manner with 1% strain steps. Between each step a high-resolution 3D image (700 nm nominal resolution) was acquired using SRµCT. A tool to automatically classify measured porosity as bone canular network, osteocyte lacunae or microcracks, was developed and volumetric analyses of the three phases were conducted.

For a typical C3H/He sample, canular, lacunar and microcrack densities were 1.9%, 2.1% and 0.2%, respectively. Due to its 3D nature SRµCT imaging allowed to visualize the volumetric extent of microcracks. At 2% apparent strain the average microcrack thickness was 2.0μm. We observed initiation and propagation of microcracks, under increasing apparent bone strain. Microcracks initiated at canal and at bone surfaces; the osteocyte lacunae provided guidance to the microcracks. Moreover, SRµCT images permitted a superb visualization of uncracked ligament bridging, which are thought to be of extreme importance in bone toughening mechanisms. We conclude that this study showed the power of three dimensional visualization and quantification of cortical bone porosity and microdamage at high resolution. We observed that a microcrack can appear as a linear crack in one plane, but as a diffuse crack in a perpendicular plane, hence, we postulate the necessity of 3D imaging techniques to unravel microcrack initiation and propagation and their effects on bone mechanics. We believe that these new investigation tools will be very useful to further enhance our understanding of bone failure mechanisms.

Keywords:
Microcracks, cortical bone ultrastructure, Synchrotron light based micro-computed tomography, canals, osteocytic lacunae

Introduction

Many of the elderly people suffer from osteoporosis resulting in a decrease in bone strength, which often leads to fracture. For decades it was thought that bone strength is mainly dependent on the amount of mineral i.e. calcium in bone [1, 2]. However, it has been shown that the mineral content only explains bone strength to a limited extend. Thus, for better understanding of bone as a material, and consequently of osteoporosis as the major bone disease, also other parameters have to be included into the analysis of bone strength. These other parameters are usually referred to as bone “quality” and include bone architecture,
小微裂纹的起始和扩展的评估

4.1 Assessment of microcrack initiation and propagation

turnover, damage accumulation, remodeling, collagen cross-linking and mineralization. This present work focused on microdamage, more precisely on microcracks, which are thought to play an important role in bone fracture behavior but also in other phenomena such as bone remodeling, mechanotransduction or bone toughening mechanisms.

Small cracks are known to exist in normal bones in humans and animals in vivo: these vary in length from 10 μm to 1 mm and are typically elliptical in shape with dimensions 100-500 μm [3-7]. There is considerable interest in these cracks, for a number of reasons. Firstly, they can grow to cause fractures in bone (known clinically as ‘stress fractures’): a practical problem which occurs in human subjects (e.g. athletes, dancers and army recruits) and in animals (e.g. racehorses, battery chickens) [8-11]. Secondly, it is known that these cracks are constantly being repaired by the body: if this repair process is suppressed (by drug treatments or certain medical conditions) then the incidence of stress fractures increases [12-14]. There is compelling evidence that the network of living cells in our bones is capable of detecting the presence of small cracks and repairing them if necessary. Increased stress over long periods of time (e.g. a relatively active lifestyle) causes our bones to increase in strength and thickness; it is not known how this process works but it is hypothesized that our bodies can detect the presence of damage in the form of cracks and take appropriate action to reduce stress levels [15-19]. These phenomena have provided the motivation for many studies to measure and characterize small cracks and to understand the mechanisms of their propagation in this material. To date, most studies have focused either on detecting cracks formed in vivo in humans and animals [5, 7, 20] or on experiments to study the initiation and propagation of cracks in specimens of excised bone during either brittle fracture [21-23] or fatigue [24-26]. Some brittle-fracture studies [27-29] have shown significant crack extension before failure in tests at constant deflection rate, with associated increases in toughness (i.e. R-curve behavior). Microscopic observations have shown that the micromechanisms of crack propagation and toughening are complex and involve contributions from several different factors, including microdamage zones ahead of the crack tip and bridging actions across the crack faces [30-32].

Bone in large mammals possesses a microstructure with features such as osteons and vascular canals on a size scale of the order of 20–100 μm and an ultrastructure consisting of lamellae which contain collagen fibrils and hydroxyapatite crystals, on a size scale of the order of 1 μm and below. Rodent bone is simpler: neither microcracks nor osteons are normally present in rat and mouse cortical bone [33] and therefore one degree of complexity is removed. The absence of microcracks permits a better control of microcrack initiation and propagation for in vivo and in vitro experiments. Therefore, rodent bone allows to directly
relate microcrack initiation and propagation with bone porosity features such as bone canular network and osteocyte lacunae distribution. Bone canular network is a generic term comprising vasculature as well as resorption voids induced by bone remodeling.

Recently, it was suggested that bone microvasculature, osteocytes and osteocyte canalicular network, as major contributors to local tissue porosity, can also be directly linked to the mechanical failure properties of bone tissue [34-38].

Up to now, microdamage has mainly been analyzed qualitatively and quantitatively with 2D techniques such as histology and scanning electron microscopy [32, 39-46]. Confocal microscopy permits 3D visualization but only close to the sample surface. In contrast, the whole sample thickness can be visualized in 3D with synchrotron radiation-based micro-computed tomography (SRμCT) allowing for extensive 3D analyses. Due to its high resolution, bone assessment by means of SRμCT allows better insight into ultrastructural void spaces and their contribution to the failure behavior of bone, especially the initiation, propagation and accumulation of microcracks. Resolutions of 1 μm and better have made it possible to analyze tissue properties in the nano domain. Moreover, new features such as bone microvasculature and even single osteocytes within the bone tissue are now detectable and can be analyzed in a fully nondestructive and three-dimensional fashion [47]. Therefore, the aims of this work were to describe this new 3D method and to discuss in details the advantages of this technique over the classical methods used so far. Some preliminary results on microcrack initiation and propagation during mechanical overloading obtained with SRμCT in the murine model are presented.

Material and Methods

Animal model

For this study, we used two inbred strains, where C57BL/6 (B6) represented the low bone mass strain and C3H/He (C3H) displayed the high bone mass phenotype. All mice were female and raised at Harlan (Horst, The Netherlands). They were sacrificed by CO₂ inhalation at 16 weeks. The animals were then stored at -20°C and thawed at room temperature just before dissection of the femora. Two femora from 16-week-old B6 and 2 femora from 16-week old C3H were dissected. Use of mice in this research project was reviewed and approved by the local authorities for all levels of investigation.

Experiment

After dissection, femora were embedded at both ends into polymethyl methacrylate cement (PMMA). We created a round notch in the mid-diaphysis using a high-precision blade
4.1 Assessment of microcrack initiation and propagation

saw (Isomet 5000, Bühler LTD, Lake Bluff, Michigan, USA) (Fig. 4.1 and 4.2). The notch width was 750 μm. This notch was expected to create a weakened region in the cortical bone, where the microcracks would initiate and propagate while the bone was loaded. Before imaging, the samples were stored in saline solution.

Two B6 and two C3H samples were scanned on a common desktop micro-computed tomography system (μCT) (μCT40, Scanco Medical, Switzerland) using a 6 μm nominal resolution and three times frame averaging in order to increase the signal to noise ratio. From those scans, micro-finite element (μFE) models were generated for each femur, around the notch region, using a standard voxel conversion technique; hence, each voxel from the μCT reconstruction was converted to a hexahedral element in the μFE model. Component labeling was performed to assure that no bone residuals from the sawing of the notch were included in the FE models. Boundary conditions, which represented an axial compression test, were applied. A prescribed displacement, representing 1% of the length between both embedded ends, was applied at the top of the notch region (Fig. 4.1). All elements were assigned a Young's modulus of 5.0 GPa, and a Poisson ratio of 0.3. Assuming linear-elastic behavior, the models were solved using an element-by-element method [48, 49] running on a super computer consisting of 8 IBM Regatta p690 SMPs for a total of 256 Power4 CPUs. The models were used to calculate the strain field around the notch, resulting from the prescribed 1% strain.

![Figure 4.1](image-url)  
*Figure 4.1: The mid-diaphyseal region with the notch was modeled with μFE. Boundary conditions were applied representing an axial compression test. A prescribed displacement of 1% of the length between both embedded ends was applied at the top of the notch region.*
The samples were then placed into a custom-made loading device, which fitted into the positioning setup of the Materials Science (MS) Beamline of the Swiss Light Source (SLS). This loading device comprised an X-ray translucent loading chamber. The sample was loaded under compression with a precision screw. To prevent any torsional constraint due to screwing, a ball bearing was included into the loading device (Fig. 4.2).

Figure 4.2: (a) Murine femur embedded into PMMA, (b) 3D microtomography image of the notch in the femur, (c) (left) the loading device with a translucent chamber, a precision screw at the top and (right) the internal setup with a ball joint.

The samples were then loaded in a stepwise manner as described elsewhere [50]. The loading process was displacement controlled and ranged from 0 to 4% strain, with 1% strain steps. Between each step a high-resolution 3D image (700 nm nominal resolution) was acquired with the SRμCT. For each 3D image, in total 1001 projections were acquired over a range of 180 degrees at a photon energy of 17.5 keV. The data was reconstructed using filtered backprojection.

For segmentation and further quantitative analysis of bone porosity and microcracks, component labeling and morphologic operations were applied to the data. The technique of negative imaging [51] was applied in this study to assess the porosity within cortical bone. In this context negative imaging denotes the technique to first measure the matrix of a porous structure using CT, and subsequently, to extract the enclosed porosity as a negative imprint of the surrounding matrix. In this study, the extraction of the cortical void spaces included a combination of different image processing procedures (IPL, Scanco Medical AG, Bassersdorf, Switzerland), including morphological operators. They were optimized to extract the canal network, the osteocyte lacunar system and the microcracks within cortical bone as three
4.1 Assessment of microcrack initiation and propagation

separate phases. For each phase, a set of morphometrical parameters were measured. Canular volume density (Ca.V/Ct.V), canular thickness (Ca.Th) and canular volume (Ca.V) were measured from the canular network. The osteocytic lacunae were characterized by lacunar volume density (Lc.V/Ct.V), average lacunar thickness (Lc.Th) and lacunar volume (Lc.V). Finally, microdamage was quantitatively determined by the following parameters: crack volume density (Cr.V/Ct.TV), average crack thickness (Cr.Th) and crack volume (Cr.V).

Results

The results of the μFE models showed that around the notch the strains parallel to loading direction ranged between -1.56% and 0.11% (negative value for compressive strain) (Fig. 4.3). The highest strains were found close to the notch demonstrating that the notch created a region with high strains in this portion of the diaphysis. This augmented the chance of microcracks initiating and propagating in this region. At the notch, the bone diaphysis was mainly loaded under compression. Knowing the stress and strain distribution around the notch allowed having an insight into the mechanics around the notch. With this information, it was then possible to relate the microcrack initiation and propagation with compressive loading along the main axis of the femoral mid-shaft. The planes of the observed cracks in both murine strains were always parallel to the loading axis.

Figure 4.3: Strains parallel to the loading axis calculated with μFE model of the notch region. The maximum strains (calculated as 1.56 times apparent strain) are close to the notch.
Initiation and propagation of microcracks were imaged at a submicron resolution in a time-lapsed and non-destructive manner. This experiment showed microcrack initiation while increasing the sample strain. Cortical bone of a B6 sample showed no microcrack at 1% strain (Fig. 4.4a). At 2%, microcracks appeared at several locations (Fig. 4.4b). At 1% strain in a C3H sample, we could observe a crack at a very early stage (Fig. 4.4c). The crack was hardly visible at the 700 nm resolution. Loading the sample further to 2%, the microcrack propagated, reaching the bone surface (Fig. 4.4d). The analysis revealed that microcracks propagation was usually not stopped by the lacunae; microcracks went through these osteocytic cavities (Fig. 4.4d). Our observations showed that the microcracks initiated either at the canal surface (Fig. 4.5) or at the bone surface (Fig. 4.6). The osteocyte lacunae provided guidance to the microcracks in their progression. The lacunar distribution even had the capability to change the orientation and the direction of the microcracks. (Fig. 4.6).

![Figure 4.4: (top) SRμCT reconstructed slices of B6 cortical bone close to the notch. Nominal resolution: 700 nm. (a): 1% apparent strain, no microcrack, (b): 2% apparent strain, microcrack initiation. (bottom) SRμCT reconstructed slices of C3H cortical bone close to the notch. Nominal resolution: 700 nm. (c) 1% apparent strain, microcrack (black arrow), (d) 2% apparent strain, microcrack propagation.](image)
4.1 Assessment of microcrack initiation and propagation

Figure 4.5: SR\(\mu\)CT reconstructed slices of C3H cortical bone. Nominal resolution: 700 nm. (a) Region close to the notch; the small spots are osteocyte lacunae and the bigger ones are canals, (b) Magnification of identified region in Fig. 4.5a: crack initiation in the canal, (c) Uncracked ligament bridging along the crack path (arrows), (d) The microcrack propagates through two lacunae.

Figure 4.6: (From left to right) Sequence of SR\(\mu\)CT reconstructed cross sectional slices of B6 cortical bone. Nominal resolution: 700 nm. The distance between each slice is around 20 \(\mu\)m. This sequence shows how microcracks go through the osteocyte lacunae and how these lacunae provide guidance to the crack. From slice 2 to slice 3, the lacunae distribution changes the direction of the microcrack wake. On slice 2, the arrow shows the beginning of the new crack orientation. On slices 3 and 4, the dashed line reminds the original crack orientation.

SR\(\mu\)CT images at 700 nm resolution permitted superb visualizations of uncracked ligament bridging. We showed uncracked ligament bridges in 2D reconstructed slices (Fig. 4.5c and 4.7) as well as in 3D segmented images (Fig. 4.8). Furthermore, the high resolution achieved at the SLS permitted to visualize another feature also responsible of bone toughening; undamaged filaments, which were bridging the crack, consisting of mineralized collagen fibrils (Fig. 4.7).
Figure 4.7: SRμCT reconstructed slice of B6 cortical bone at 1% (a) and 2% (b) strain with a microcrack and a microfracture. Nominal resolution: 700 nm. Uncracked ligament bridges intersecting the microcrack (black arrows) and mineralized collagen fibrils spanning the microfracture (white arrows).

Figure 4.8: 3D reconstruction of C3H cortical bone at the same location as in Figure 4.5. Blow-up: a 3D representation of an uncracked ligament bridge.

Another advantage of 3D SRμCT imaging was that it allowed to visualize the microcracks in different planes, e.g. in the microcrack wake plane or perpendicular to it; hence, it was possible to observe microcracks in all orientations. We observed a striking difference in the appearance of the same crack when visualized in different planes (Fig. 4.9).
4.1 Assessment of microcrack initiation and propagation

In one plane, one microcrack looked linear and very confined, while in the perpendicular plane, the same crack appeared totally diffuse.

Finally, applying the technique of negative imaging permitted for the first time to extract the canal network, the osteocyte lacunar system and the microcracks, as three separate phases within cortical bone using a single data set (Fig. 4.10). In C3H, vascular, lacunar and microcrack average densities were 1.39%, 1.27% and 0.66%, and in B6, 0.67%, 1.27% and 1.04%, respectively, at an apparent strain of 2%. Average microcrack thickness was 2.0 μm. As an example of the three phases analysis capabilities, morphometric parameters of one C3H sample (density, average thickness and average volume of canals, canaliculi and microdamage), which characterize each of these porosity features in cortical bone, are summarized in Table 4.1.

Figure 4.9: SRuCT reconstructed slices of B6 cortical bone at 2% strain. (left) Linear microcrack in the x-y plane, (right) The same microcrack as in (left), but represented in the y-z plane. Diffuse pattern (white arrow).
Figure 4.10: (left) 3D representation of osteocyte lacunae (yellow), canal network and microcracks (green) in C3H cortical bone. (right) Same image as (left) without the lacunae. The microcracks appear to initiate from the canal surface.

Table 4.1: 3D morphometrical parameters in C3H (n = 1) at 0%, 1% and 2% overall strain. Ct.V: Cortical bone volume; Ca.V: Canal network volume; Ca.Th: average thickness of canal network; Lc.V: Total lacunar volume; Lc.Th: Average lacunar thickness; Cr.V: Total crack volume; Cr.Th: Average crack thickness.

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Discussion

The aims of this study were to describe a new method to investigate, non-destructively and in three dimensions, microdamage initiation and propagation in cortical bone as well as presenting some preliminary findings. To reach our aims we used murine cortical bone, because murine bone does not include osteons nor any lamellar structure. This makes this type of bone less complex than human or other bigger mammalian bone. By removing one degree
4.1 Assessment of microcrack initiation and propagation

of complexity, we could investigate easier and in more detail the single influence of bone ultrastructural features such as cortical bone canular network or osteocyte canaliculi on microdamage initiation and propagation.

This study showed microcrack initiation and propagation, using SRµCT (Fig. 4.4). Our model of static loading together with non-destructive imaging permitted to visualize the same region of bone before and after microcrack initiation. The SRµCT method was able to separate pre-existing microcracks from induced one by loading the sample. Further crack propagation could also be imaged up to microfracture formation and total failure of bone. This is an extraordinary tool to relate bone ultrastructure to bone failure behavior. We could investigate where the microcracks initiated and how they propagated in murine cortical bone. Our preliminary results showed that microcracks usually initiated at canular surface and in some case at the outer or inner cortical bone surface. These observations indicated that the canals could be considered as voids around which stresses are higher when bone is under load. Canals acted as stress raisers which favored the initiation of microcracks. Our data would, however, also allow an opposing hypothesis: it could also be interpreted that canals, instead of initiating the microcracks, stopped their propagation. The canal, with its big size in comparison to microcracks, could then absorb all the energy concentrated in the microcrack propagation and prevent it to continue expanding. More data of the same nature should bring an exact answer on the role of canals in microdamage initiation and propagation in cortical bone.

Our data suggested that the osteocyte lacunae played another role in microcrack initiation and propagation than the canals. The microcracks did not seem to initiate at the lacunae surface, but our investigation showed that cracks propagated through the lacunae. The lacunar voids, more exactly their distribution, guided the microcrack paths. Some previous studies suggested that osteocyte lacunae are stress concentrators [37, 52, 53] with average surrounding strain 1.5-4.5 times greater than the strain applied on bone [36, 38, 54]. These higher stresses and strains would then drive the microcracks paths across the cortical bone.

The µFE model showed the distribution of strains parallel to loading direction. Axial compression of the sample resulted in a compressive stresses and strains in the region around the notch (Fig. 4.3). All the cracks observed in this study were linear and parallel to the loading axis. This was in accordance with previous studies where it was claimed that in region subject to tensile strains, the bone showed predominantly focal regions of diffuse microdamage, while in compressive strain regions, the tissue developed linear microcracks.
Further, it has also been hypothesized that under compression the linear crack wakes tend to be parallel to the loading direction [55, 56].

Investigating the crack orientation, we have shown the benefit of approaching the bone microdamage problem with a three dimensional, non-destructive imaging approach; particularly when looking at microcrack initiation and analyzing the crack path as well as its interactions with canals or osteocyte canaliculi volumetric distribution. 3D imaging also provided helpful information on the bridging mechanism. A further advantage of 3D visualization over the usual 2D methods is the possibility to visualize bone ultrastructure and microdamage in different planes and through the whole bone sample. With SRμCT, we looked at microcracks in three dimensions, but also slice-wise in different planes (Fig. 4.9). Such an approach is not possible with standard histology. We saw how different the same linear crack looked in one plane and the plane orthogonal to that plane. On one plane, the microcrack was very well defined and confined, while, in the plane parallel to the crack wake, the same microcrack looked very diffuse. As already mentioned, we only observed linear cracks with their wake parallel to the loading axis. But, if we looked at the microcracks slicing parallel to their wake, they mostly had a diffuse appearance (Fig. 4.9). These surprising observations contradicted, to some extent, the commonly accepted idea that there are two distinct types of microdamage, linear microcracks and diffuse microdamage [45, 55-58]. Actually, our murine model only contained rodent cortical bone, while the other studies involved osteonal bone. The fact that we only observed linear microcracks does not exclude the existence of diffuse damage in osteonal bone. But, the way a microcrack shape changed from very confined to totally diffuse depending on the slicing plane implies that one should be very cautious when looking at microcracks in 2D and defining them as linear or diffuse. We believe that there is a good chance to misclassify a linear microcrack in the diffuse type, since the shape of linear microcracks in parallel slices is very similar to the so-called diffuse microdamage. We also postulate that 3D investigation of osteonal bone is absolutely necessary to be able to clearly distinguish linear from diffuse damage and to have a complete answer on differences in morphology and mechanics between linear microcracks and diffuse damage.

A prerequisite for the full understanding of bone mechanics and the modeling of bone’s mechanical behavior is that the mechanisms that govern the deformation and failure of the tissue in the post-yield region are clarified. Of course, bone microdamage plays a major role in the post-yield behavior, but we also imaged others processes that are thought to be important, in particular post-yield bone toughening mechanisms. We observed two sorts of
4.1 Assessment of microcrack initiation and propagation

such processes; uncracked ligament bridging and mineralized collagen fibers, which both spanned the cracks (Fig. 4.5, 4.7 and 4.8). Uncracked ligament bridging was defined by Nalla et al. [59] as an extrinsic toughening mechanism involving two dimensional uncracked regions along the crack path that can bridge the crack on opening. The uncracked ligament bridges increase resistance to fracture by sustaining part of the applied load that would otherwise contribute to crack advance. The image resolution achieved in this study permitted superb visualizations in 2D and 3D of uncracked ligament bridges. For the first time, uncracked ligament bridges were imaged in three dimensions at a nominal resolution of 700 nm (Fig. 4.8). The 3D visualization revealed that the uncracked ligament bridges are not two dimensional uncracked regions along the crack path, as reported previously [28, 59], but have proper three dimensional shapes. Their thickness varied between 1-3 μm. SRμCT also uncovered bundles of mineralized collagen fibrils which spanned the cracks (Fig. 4.7). Mineralized collagen fibrils are the basic building blocks of bone tissue at the supramolecular level [60]. Uncracked ligament bridging is deemed to be the predominant toughening mechanism in cortical for microcrack propagation, while mineralized collagen fibrils, much smaller, may play secondary role in little cracks [61].

This study presents a volumetric visualization, at 700 nm nominal resolution, of cortical bone with different elements of porosity, canals, osteocyte lacunae and microdamage, separated as three separate phases. This opens a new scope for volumetric analysis of cortical porosity and microdamage (Table 4.1). We believe that this new tool will be very important when comparing different samples. Comparison of bone ultrastructure and microdamage accumulation between healthy and unhealthy donors or from different species is now possible. In our case, comparing ultrastructures from two murine inbred strains, with different observable genetic backgrounds, will reveal further important clues on the role of porosity in microdamage initiation and propagation and on the overall bone failure behavior.

Former studies supported various theories on the ways bone remodeling works and how it is initiated [33, 62-64]. Different models of mechanotransduction were developed where osteocyte sense the strains surrounding the lacunae when bone is loaded or where the fluid flow around the osteocyte processes in the canaliculi is activated under loading. In both types of models, microdamage would play an important role. In the case of the osteocytes sensing strains, microdamage could change the loading configuration around the lacunae [65] or even, as we saw in this work, disrupt the lacunae. Microcracks could also rupture the canaliculi containing the osteocyte process and perturb the fluid flow [34, 66, 67]. More work has to be done to really understand the way bone remodeling is activated and how
mechanotransduction works. Furthermore, the role of microdamage in these different processes is still not well understood. We strongly believe that with further improvement, especially in resolution, the 3D imaging capabilities of SRμCT will reveal important clues on the manner that microcracks affect osteocytes lacunae and how they disrupt the canalicular network.

This study had a number of limitations. SRμCT was time consuming and did not allow to perform a large study with many samples, such as high-throughput phenotypic studies. Furthermore, the resolution achieved with this technique was not as high as with scanning electron microscopy [68]. A better resolution would permit to visualize microcracks at earlier stages, as well as the interaction between cracks and the canalicular network. Another limitation of this study was the loading regime. We could only perform static loading. Performing fatigue test would be closer to physiological loading modes. Finally, our model showed rodent bone, which was described above as an advantage for looking at individual interactions between microdamage and bone ultrastructure, but is different from human bone which is characterized by osteonal remodeling. In order to achieve direct comparisons with human bone, another model with osteonal bone should be investigated.

In summary, this study showed the great power of three dimensional visualization and quantification of cortical bone porosity and microdamage at high resolution. Further, it was shown how microcracks initiated and propagated within bone ultrastructure under time-lapsed loading. SRμCT was able to uncover in 2D and in 3D very small features such as uncracked ligament bridging and mineralized collagen fibrils which are thought to play important roles in bone toughening mechanisms. SRμCT allowed to visualize the volumetric extent of microcracks. We observed that a microcrack can appear as a linear crack in one plane, but as a diffuse crack in a perpendicular plane. Hence, we postulate the necessity of 3D imaging techniques to unravel microcrack initiation and propagation and their effects on bone mechanics. Finally, we developed a semi-automatic tool to separate bone porosity and microdamage in three different phases and to morphometrically quantify each of them. This tool will be very useful in further studies to compare different cortical bone samples.

Acknowledgements

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References


4.1 Assessment of microcrack initiation and propagation


4.1 Assessment of microcrack initiation and propagation


4.2 Effects of bone structural and material properties on bone competence

4.2 Canal network characteristics are strong predictor of microdamage accumulation in bone

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Abstract

Loss of bone density or bone mass, long considered as the major cause of bone fracture, actually fails in explaining bone strength. Other architectural parameters succeeded in explaining bone strength and bone stiffness, but bone post-yield and bone failure behaviors are still poorly understood. Studies on murine inbred strains showed differences between strains in bone toughness and brittleness which were not related to differences in bone density or in bone architecture. In order to better understand bone post-yield behavior and consequently bone failure behavior, this current study aimed to investigate cortical bone ultrastructure and to relate cortical porosity to microdamage initiation and propagation in C57BL/6 (B6) and C3H/He (C3H); two murine inbred strains known for their differences in bone phenotype. Notched murine femora of B6 and C3H were loaded axially under compression in a stepwise manner. Between each loading step, 3D images, with nominal resolution of 700 nm, were acquired by means of synchrotron radiation micro-computed tomography. Bone porosity was divided in three phases: osteocyte lacunae, canular network and microdamage. Morphometric indices for each phase were determined and quantified. Then, the role of lacunae and canals in the initiation and propagation of microdamage was assessed. Lacunar density and lacunar spacing was similar in both strains. C3H cortical bone
showed higher canular volume and larger canal units (defined as one main canal with its ramifications of second order) than B6. C3H also accumulated more (in term total volume and number of cracks) and faster (in term of compressive strain) microdamage until failure. In both strains, lacunae did not initiate microcracks, but their distribution provided guidance to the microcrack paths. In C3H, large canals initiated microcracks. The canal units supported the propagation of microcracks, since the microcracks tended to bridge the canal units ramifications in order to absorb the high stress around the canals voids. As a consequence, cracks propagated parallel to the canals in C3H. In B6, where the canal units were smaller, microcracks propagated oblique to the canals. Linear regression analyses showed a strong correlation between the number of cracks per unit of cortical bone volume and the canal unit volume ($r = 0.87$, $p = 0.001$). This direct relation between canular porosity and microdamage accumulation explains to great extent the differences in bone post-yield between B6 and C3H. We conclude that the large canal units in C3H are responsible for more and faster microdamage accumulation than in B6, hence, C3H cortical bone is more brittle than B6 bone. We hypothesize that by an identical mechanism the resorption induced cavities in elderly human bone make this type of bone more prone to microcrack initiation and accumulation; this again would increase bone brittleness, and explain the higher incidence of bone fractures in the elderly.

**Keywords:**
Inbred strains, bone quality, cortical porosity, canal, osteocytic lacuna, microcrack

**Introduction**

Over the years, loss of bone mineral or bone mass has been considered the major cause of age-related bone fractures [1, 2]. However, the large overlap in bone density that exists between healthy individuals and patients who sustain bone fractures suggests that low bone density is not the only reason for the weakening of bone [3]. A former study reported that the risk of bone fracture for older women (average 75 years old) is about 7%, whereas such a risk is only 1% for much younger individuals (average 45 years old), although they have a similar bone density level [4]. In addition, it has been found that, although elderly black Gambian women also experience loss of bone mass, they rarely suffer osteoporotic bone fractures as compared with their white counterparts [5].

Bone microstructure and geometry have significant influences on the mechanical properties of bone as an organ [6, 7]. The changes in geometrical properties of bone influence
4.2 Effects of bone structural and material properties on bone competence

the amount of loading the bone can carry. In addition to the changes in the actual cross-sectional area, the spatial distribution of bone is important in determining mechanical response. Bone cross-sectional area is a significant factor in resistance to tensile or compressive axial loads whereas moment of inertia and polar moment of inertia determine the bending and torsional rigidity of the bone, respectively. It is, however, noteworthy that bone strength is not only dependent on the cross-sectional geometrical properties of the bone but is also affected by ultrastructural parameters including intra-cortical porosity [7-11]. In particular, it has been shown that, similar to cross-sectional parameters [12-19], ultrastructural parameters exhibit age-related changes and affect the propensity of bone to fracture [7, 20]. These observations suggest that age-related changes in geometrical, microstructural and ultrastructural properties may interact with each other and have a combined effect on bone fractures, however, the level of interaction is unknown [21]. Recently, it was suggested that bone microvasculature, osteocytes and osteocyte canalicular network, as major contributors to local tissue porosity, can also be directly linked to the mechanical failure properties of bone tissue [22-26].

Up to now, microdamage has mainly been analyzed qualitatively and quantitatively with 2D techniques such as histology and scanning electron microscopy. Confocal microscopy permits 3D visualization but only close to the sample surface [27-35]. In contrast, the whole sample thickness can be visualized in 3D with synchrotron radiation based micro-computed tomography (SRμCT) allowing for extensive 3D analyses. Due to its high resolution, the investigation of different mouse inbred strains by means of SRμCT will allow a better insight into microcrack behavior. Resolutions of 1 μm and better have made it possible to analyze tissue properties in the submicron domain [36]. Moreover, new features such as bone canalicular network and even single osteocytes within the bone tissue are now detectable and can be analyzed in a fully nondestructive and three-dimensional fashion [37]. Indeed, high resolution SRμCT uncovers ultrastructural void spaces and permits to relate their contribution to the failure behavior of bone, especially the initiation, propagation and accumulation of microcracks using time-lapsed imaging of the failure process.

Animal models are critical for experimentally defining the genetic regulation of bone density. In particular, inbred mice offer unlimited numbers of genetically identical "twins" whose environments can be strictly controlled. These inbred strains offer the opportunity to gain insight into the genetic basis of variation in bone density in phenotypically normal mice. Equally important, each inbred strain is typically genetically different from another inbred strain, allowing planned matings to study segregation of genes essential to bone density [38].
Inbred strains were used before to successfully identify chromosomal regions that control bone density and architecture [39]. Inbred strains of mice C57BL/6 (B6) and C3H/He (C3H) have very different microarchitectures and display profound differences in peak bone density [38, 40-42] and can therefore serve as models for studying the genetic regulation of bone architecture and strength in the context of osteoporosis.

Significant differences in mechanical properties such as bone strength and stiffness were previously reported for these two inbred strains. These inter-strain differences in strength and stiffness could be explained by differences in material and structural properties between the B6 and the C3H mice [6, 41, 43-51]. Significant changes in post-yield parameters, such as brittleness and toughness, of C3H and B6 bones under load were also shown [6, 41, 45, 50, 51], but the factors determining these post-yield differences remain poorly understood; bone morphometry failed in explaining these different behaviors. Some studies related differences in brittleness with the degree of bone mineralization [6, 52, 53], but mineralization could only explain the different post-yield behaviors of these different mice strains to a limited extent. In a previous study, Jepsen et al. suggested that damage accumulation in cortical bone is central to the strength, toughness, brittleness and fatigue resistance of bone [53].

In murine models, neither microcracks nor osteons are normally present in cortical bone [54]. The absence of pre-existing microcracks permits a better control of microcrack initiation and propagation for in vivo and in vitro experiments. Rodent bone is a simpler model than osteonal bone, since no osteonal structures are present and consequently one degree of complexity is removed. Therefore, rodent bone allows to directly relate microcrack initiation and propagation with bone porosity features such as bone canular network and osteocyte lacunae distribution. Bone canular network is a generic term comprising vasculature as well as resorption voids induced by bone remodeling.

In the present study, we therefore aimed to investigate microdamage initiation, propagation and accumulation in cortical bone of B6 and C3H mice and to relate microdamage accumulation to the cortical bone ultrastructure. We hypothesized that the difference in cortical porosity between the C3H and B6 strains would predict microdamage behavior.
Material and Methods

Sample preparation

Fourteen femora were dissected from two genetically distinct inbred strains of mice. Seven femora originated from 16-week old female C3H/He (C3h/He) and 7 from 16-week old female C57BL/6 (b6). Use of mice in this research project was reviewed and approved by the local authorities for all levels of investigation. The femora were embedded at both ends into polymethyl methacrylate cement (PMMA). We created a round notch in the proximal quadrant of the mid-diaphysis using a high-precision blade saw (Isomet 5000, Bühler LTD, Lake Bluff, Michigan, USA) (Fig. 4.11). The notch width was 750 μm. This notch created a weakened region in the cortical bone, where microcracks would initiate and propagate while the bone was loaded. Before imaging, the samples were stored in saline solution.

![Diagram of murine femur embedded into PMMA with a ball joint](image)

Figure 4.11: The murine femur embedded into PMMA is loaded within a setup with a ball joint in order to remove any frictional torsion.

Sample loading

The samples were then placed into a custom-made loading device, which fitted into the positioning setup of the Materials Science (MS) Beamline of the Swiss Light Source (SLS). This loading device comprised an X-ray translucent loading chamber. To prevent any torsional constraint due to screwing, a ball bearing was included into the loading device (Fig. 4.11).

The samples were loaded in a stepwise manner. The loading process was deformation controlled from 0 to 4% overall compressive strain, with 1% overall strain steps. Since the notch removed 40% of the average cross sectional area in both C3H and B6, the local strain in
the notch region was, as long as no cracks initiated and the deformation remained elastic, 1.7 times higher than the overall strain.

**Sample imaging**

Between each step a high-resolution 3D image (700 nm nominal resolution) was acquired with the SRμCT. For each 3D image, in total 1001 projections were acquired over a range of 180 degrees at a photon energy of 17.5 keV. The data was reconstructed using filtered backprojection. One B6 sample was lost due to a long beam shutdown at the synchrotron facility. This resulted finally in populations of six B6 and seven C3H samples.

**Image post-processing**

For segmentation and further quantitative analysis of bone porosity and microcracks, component labeling and morphologic operations were applied to the data. The technique of negative imaging was previously described [55] and was applied in this study to assess the porosity within cortical bone. In this context negative imaging denotes the technique to first measure the matrix of a porous structure using CT, and subsequently, to extract the enclosed porosity as a negative imprint of the surrounding matrix. In this study, the extraction of the cortical void spaces included a combination of different image processing procedures (IPL, Scanco Medical AG, Bassersdorf, Switzerland), including morphological operators. They were optimized to extract the canal network, the osteocyte lacunar system and the microcracks within cortical bone as three separate phases. For each phase, a set of morphometrical parameters were measured. Lacunar volume density (Lc.V/Ct.V), lacunar number density (Lc.N/Ct.V), lacunar spacing (Lc.Sp), lacunar average volume (Lc.V/Lc.N) and average lacunar thickness (Lc.Th) were computed for the osteocytic lacunar system. Similarly, canular volume density (Ca.V/Ct.V), canular spacing (Ca.Sp), canular unit volume (Ca.V/Ca.N), canular average length (Ca.Le), average canular thickness (Ca.Th) and standard deviation of canular thickness (Ca.Th.SD) were measured from the canular network. Finally, microdamage was quantitatively determined by the following parameters: crack total volume (Cr.V), crack average thickness (Cr.Th), crack average length (Cr.Le), crack volume density (Cr.V/Ct.V) and crack number density (Cr.N/Ct.V).

**Statistical analyses**

Morphometric indices between both murine strains were compared using two-tailed Student t-test. Relationships between microdamage and other ultrastructure parameters were computed by single linear regression analyses. Significance level for all analyses was set to p < 0.05. All statistical analyses were performed with Excel 2003 (Microsoft, Redmond, USA) and the GNU statistical package R (Version 2.4.0, [http://www.r-project.org](http://www.r-project.org)).
4.2 Effects of bone structural and material properties on bone competence

Results

The amount of microcracks initiating in the notched region demonstrated that the notch created a region with high strains in the diaphysis. In the aforementioned loading setup, bone diaphysis in that region was mainly loaded under compression. Therefore, the planes of the observed cracks in both murine strains were always parallel to the loading axis. (Fig. 4.12).

Figure 4.12: Microcracks were oriented parallel to the loading axis in B6 (a) and C3H (b)

Morphometrical indices of the osteocytic lacunar phase were computed in both strains (Table 4.2). Lacunar number density (Lc.N/Ct.V) and lacunar volume density (Lc.V/Ct.V) as well as lacunar spacing (Lc.Sp) were not significantly different between both strains, while lacunar average volume (Lc.V/Lc.N) and average lacunar thickness (Lc.Th) were significantly different. Our observations revealed that osteocytic lacunae provided guidance to microcracks. Indeed, microcracked propagated following the lacunar distribution (Fig. 3). No difference was found between the two strains with respect to the involment of lacunar void spaces in microcrack behavior.

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<th>C3H</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lc.V/Ct.V (%)</td>
<td>1.27 ± 0.38</td>
<td>1.58 ± 0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Lc.N/Ct.V (mm³)</td>
<td>55708 ± 15507</td>
<td>50771 ± 16798</td>
<td>0.3</td>
</tr>
<tr>
<td>Lc.Sp (µm)</td>
<td>34 ± 4</td>
<td>34 ± 3</td>
<td>0.49</td>
</tr>
<tr>
<td>Lc.V/Lc.N (µm³)</td>
<td>231 ± 59</td>
<td>315 ± 53</td>
<td>0.01</td>
</tr>
<tr>
<td>Lc.Th (µm)</td>
<td>2.43 ± 0.18</td>
<td>2.69 ± 0.18</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Figure 4.13: Reconstructed cross-sectional slice of C3H cortical bone. The arrow indicates a microcrack whose path is determined by the lacunar distribution pattern.
4.2 Effects of bone structural and material properties on bone competence

Morphometric indices of the canular phase were computed in both strains (Table 4.3). Canular volume density (Ca.V/Ct.V) and canular unit volume (Ca.V/Ca.N) were significantly different between B6 and C3H, while canular spacing (Ca.Sp) was not. Canular unit volume was defined as the volume of a main canal with all its ramifications of lower orders. Typically, the canular units were bigger in C3H than in B6. In C3H, the canular network was characterized by main canals which diverged to canals with smaller thickness. In B6, the canular units were less ramified and more constant in thickness (Fig. 4.14). These observations were confirmed by the average canular thickness (Ca.Th) that was not significantly different between both strains and, on the other hand, by the standard deviation of the canular thickness which showed higher variability in C3H.

Table 4.3: Canular morphometric indices for B6 and C3H: canular volume density (Ca.V/Ct.V), canular spacing (Ca.Sp), canular unit volume (Ca.V/Ca.N), canular average length (Ca.Le), average canular thickness (Ca.Th) and standard deviation of canular thickness (Ca.Th.SD). Ct.V and Ca.N refer to cortical bone volume and number of canals, respectively.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>C3H</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca.V/Ct.V (%)</td>
<td>0.67 ± 0.5</td>
<td>1.4 ± 0.45</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Ca.Sp (μm)</td>
<td>110 ± 30</td>
<td>130 ± 20</td>
<td>0.08</td>
</tr>
<tr>
<td>Ca.V/Ca.N (10^3 μm^3)</td>
<td>7.5 ± 3.1</td>
<td>54.7 ± 32.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ca.Le (μm)</td>
<td>200 ± 160</td>
<td>800 ± 700</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ca.Th (μm)</td>
<td>8 ± 4</td>
<td>11 ± 4</td>
<td>0.1</td>
</tr>
<tr>
<td>Ca.Th.SD (μm)</td>
<td>3 ± 2</td>
<td>6 ± 3</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
Figure 4.14: (top) Semi-transparent portions of cortical bone with canular (red) and lacunar (yellow) phases. (bottom) Canular phase only. C3H cortical bone shows higher canal unit volume than B6. The thickness of the B6 canals is less variable than in C3H.

Morphometric indices of the microcracks accumulation before ultimate failure were computed in both strains (Table 4.4). Microdamage accumulated more in C3H than in B6; crack total volume (Cr.V), crack volume density (Cr.V/Ct.V) and crack number density (Cr.N/Ct.V) were significantly higher in C3H. On average, cracks were longer in C3H, while both types of cortical bone showed microcrack of similar thickness.

Our method also allowed to follow and quantify the progression of microcracks while increasing the overall deformation of the bone sample. One percent overall compressive strain, from 1 to 2%, in B6 represented, on average, 130, 105 and 115% increases in Cr.V/Ct.V, Cr.Th and Cr.Le, respectively. In C3H, 1% strain caused 300, 120 and 190% increases in Cr.V/Ct.V, Cr.Th and Cr.Le, respectively.

In B6, 100% of the cracks initiated at the endosteal or periosteal surface of the cortex (Fig. 4.15). In C3H, 48 ± 34 % of the cracks initiated at canular surface and 52 ± 34 % of the cracks at the endosteal surface of the cortex. Canular voids, where microcracks initiated, were in the 25th percentile of the canals with the largest thickness (Fig. 4.16).
4.2 Effects of bone structural and material properties on bone competence

Table 4.4: Microcrack morphometric indices for B6 and C3H: Crack total volume (Cr.V), crack average thickness (Cr.Th), crack average length (Cr.Le), crack volume density (Cr.V/Ct.V) and crack number density (Cr.N/Ct.V). Ct.V and Cr.N refer to cortical bone volume and number of cracks, respectively.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>C3H</th>
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<tbody>
<tr>
<td>Cr.V ($10^3 \mu m^3$)</td>
<td>119 ± 67</td>
<td>480 ± 120</td>
<td>$p &lt; 0.005$</td>
</tr>
<tr>
<td>Cr.Th (μm)</td>
<td>2.5 ± 0.9</td>
<td>2.6 ± 0.8</td>
<td>$p = 0.43$</td>
</tr>
<tr>
<td>Cr.Le (μm)</td>
<td>110 ± 50</td>
<td>290 ± 190</td>
<td>$p &lt; 0.05$</td>
</tr>
<tr>
<td>Cr.V/Ct.V (%)</td>
<td>0.3 ± 0.17</td>
<td>0.7 ± 0.27</td>
<td>$p &lt; 0.005$</td>
</tr>
<tr>
<td>Cr.N/Ct.V (mm$^{-3}$)</td>
<td>3.4 ± 1.3</td>
<td>7.6 ± 3</td>
<td>$p &lt; 0.005$</td>
</tr>
</tbody>
</table>

Figure 4.15: Reconstructed cross sectional slice of B6 cortical bone, where a microcrack initiated at the periosteal side of the cortex after 1% compression.
Figure 4.16: (a) Reconstructed cross-sectional slice of C3H cortical bone showing a crack which initiated at the surface of a large canal (black arrow) (b, left) 3D representation of C3H cortical bone. Bone is semi transparent, canals are in red, lacunae in yellow and the internal microcrack in green. (b, right) The same representation without lacunae. The three-dimensional imaging capability permits to visualize the crack entirely confined in bone, with no connection to periosteal or endosteal surface. Only in this configuration, a microcrack is qualified as internal.

Microcrack paths and orientations were different in both murine strains as a consequence of the differences in porosity. In B6, 84 ± 40 % of the microcracks were connected to at least one canal. Among them, 90 ± 22 % were oblique to the canal, while 10 ± 22% were parallel to the canal. In C3H, 100% of all the microcracks were connected to at least one canal. Only 3.5 ± 9.5 % were oblique, while 96.5 ± 9.5 % were parallel to the canals they were connected to (Fig. 4.17).

Linear regression analyses involving both murine strains showed that canular parameters explained, to a great extent, the microcrack behavior. Indeed, canular volume density and canular unit volume were good (r = 0.80, p < 0.005), and excellent (r = 0.87, p < 0.001), predictors for the number of microcracks per unit volume of cortical bone (Fig. 4.18). In contrast, the total volume of osteocytic lacunae did not correlate with the number of microcracks (r = -0.09). More classical morphometric parameters correlated only moderately to the number of microcarks. In that, cortical thickness (r = 0.67) showed the highest correlation (Fig. 4.19)
4.2 Effects of bone structural and material properties on bone competence

Figure 4.17: (a) In B6, microcracks (green) are most often oblique to the canals (red), while in C3H (b), microcracks are parallel to canals. The white arrows indicate microcracks which bridge canal unit ramifications.

Figure 4.18: (a) Correlation between canular volume density (Ca.V/Ct.V) and crack number density (Cr.N/Ct.V) ($r = 0.8$, $p < 0.005$). (b) Correlation between canular unit volume (Ca.V/Ca.N) and crack number density (Cr.N/Ct.V) ($r = 0.87$, $p < 0.001$).
Discussion

In this study, B6 and C3H notched femora were tested under axial compression in a time lapsed way. Between each micro-compression step, high-resolution three-dimensional images were acquired using SRμCT. This investigation permitted to assess the cortical bone ultrastructure and to relate directly, for the first time, cortical porosity with microdamage accumulation in a volumetric fashion.

All the cracks observed in this study were linear and parallel to the loading axis (Fig. 4.12). This was in accordance with previous studies claiming that in region subject to tensile strains, the bone showed predominantly focal regions of diffuse microdamage, while in compressive strain regions, the tissue developed linear microcracks. Further, it has also been hypothesized that under compression the linear crack wakes tend to be parallel to the loading direction [56-58].

Morphomeric analysis of the lacunae showed that single osteocytic lacunae of C3H were slightly larger and less numerous than in B6, but their volume density and their spacing was similar (Table 4.2). This indicated that for a given amount of cortical bone in each strain, the same amount and the same distribution of osteocytes is required to control processes such as bone modeling and remodeling [25]. Further, our data suggested that the osteocyte lacunae played a fundamental role in microcrack propagation. The microcracks did not initiate at the lacunae surface, but the cracks propagated through the lacunae. The lacunar voids, more exactly their distribution, guided the microcrack paths (Fig. 4.13). Some previous studies suggested that osteocyte lacunae are stress concentrators [20, 25, 59] with average

Figure 4.19: (a) Correlation between lacunar volume density (Lc.V/Ct.V) and crack number density (Cr.N/Ct.V) ($r = 0.098, p = 0.75$). (b) Correlation between cortical thickness (C.Th) and crack number density (Cr.N/Ct.V) ($r = 0.67, p = 0.88$).
4.2 Effects of bone structural and material properties on bone competence

surrounding strain 1.5-4.5 times greater than the strain applied on bone [24, 26, 60]. These higher stresses and strains could drive the microcracks paths across the cortical bone.

Canular volume density was higher in C3H than in B6. Specifically, C3H showed larger canular unit (Table 4.3). Indeed, the canular network in C3H was characterized by main canals which bifurcated to canals of smaller orders. A canular unit was defined as one main canal with all its ramifications. In B6, such ramifications were less numerous. There, canular unit were often composed of one single canal and each canal was of similar size. This resulted in higher variability in canal thickness for C3H than for B6 (Fig. 4.14). Nevertheless, like lacunar spacing, canular spacing was similar in both strains. These new canular parameters are fundamental to get a better insight into bone canular network of these two murine strains and thereby to better understand the canular contribution to bone metabolism, bone mechanics, and bone pathology [61, 62]. We specifically focused our investigations on the contribution of bone canular network to bone mechanics.

Our results showed that all the microcracks initiated at the endosteal or periosteal cortical surfaces in B6, while almost half of the microcracks initiated at a canal surface in C3H. These canals, where microcracks initiated, were characterized by large thickness. These bigger voids acted as local stress concentrators and therefore as microdamage starters [20]. In B6, no canal reached the critical thickness threshold to initiate microcracks. Furthermore, the size of the canular unit played a very important role in microcrack initiation and propagation, since the crack number was strongly dependent of the canular unit volume ($r = 0.87, p < 0.001$) (Fig. 4.18). This dependence suggested that microcracks propagated through the ramification of a canal unit (Fig. 4.17b). The microcracks bridged close regions of higher local strains which resulted from the canal unit ramifications. As a consequence of this bridging process, the microcracks oriented parallel to the canals in C3H. Indeed, the crack planes were determined in one direction by the loading configuration and in the other direction by the canals orientation (Fig. 4.17b). Unlike C3H, the smaller canal units in B6 did not determine the second direction, resulting in microcracks parallel to loading direction, but oblique to the canals (Fig. 4.17a).

This study not only demonstrated the strong effect of canals on microcrack initiation and propagation, but also showed that other porosity elements, such as osteocytic lacunae, did not affect the microdamage behavior in a quantitative manner (Fig. 4.19a). Lacunae only influenced the paths and the shapes of the microcracks but not their size, nor their number. We also investigated classical morphometrical parameters of the whole bone, with the cortex considered as a continuum No significant correlations between cortex morphometry and
microdamage indices were found (Fig. 4.19b). This could explain, to some extent, why bone morphometry failed in predicting post-yield behavior in mechanical studies on murine bone up to now [6, 41, 45, 50, 51].

In this study, microcracks were imaged and quantified in three-dimensions with a nominal resolution of 700nm. Moreover, increases in microcrack accumulation were also quantified in 3D in time-lapsed fashion. Other studies already showed increases in microdamage accumulation, but not in such an advanced manner. Diab and Vashishth quantified, in two-dimensions, microdamage accumulation using histology [63]. Tang and Vashishth used desktop micro-computed tomography and stained microdamage to follow the accumulation of microdamage in three-dimensions [64], but the limited resolution of the X-ray system as well as partial volume effects did not allow to compute very accurate values for the increase in damages. Relative, but not absolute, values of microdamage accumulation were reported.

Our investigation of three-dimensional microcrack morphometry and quantification of microdamage accumulation showed that microcracks accumulate more and faster in C3H than in B6. Indeed, there was on average more microdamage in C3H before ultimate failure and more microdamage accumulated in C3H while increasing overall strain of 1%. The amount of microdamage and the rate of accumulation were previously reported as being important factors that reduce bone post-yield properties. Especially, high accumulation of microdamage increases cortical bone brittleness significantly [6, 53]. Our results were in accordance with previous studies claiming higher brittleness of C3H in comparison with B6 and offered an explanation to it, relating the microcracking behavior to ultrastructural bone porosity. The results of the current study are key findings in the comprehension of the differences in post-yield behavior between B6 and C3H reported previously [6, 41, 45, 50, 51].

Murine cortical bone is different from human bone, since osteons and lamellar organizations are not present. It therefore offers a simpler model to relate microdamage to bone porosity. Previous studies showed that lamellar bone structures as well as bone cement lines significantly influence microcrack initiation and propagation [30, 65, 66]. Nevertheless, in certain cortical regions of human older bone, bone resorbs without remodeling and becomes therefore more porous and shows larger voids [53]. Intracortical porosity as well as canular volume and interconnections between canals are known to increase with advancing age, while canular spacing decrease [67, 68]. Our data indicates that these voids could potentially support initiation and propagation of microdamage. This hypothesis has to be
4.2 Effects of bone structural and material properties on bone competence

confirmed with future studies using the same techniques as presented here, but applied on human cortical bone samples.

Acknowledgements

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References


4.2 Effects of bone structural and material properties on bone competence


4.2 Effects of bone structural and material properties on bone competence


The demographic changes in the last century lead to a large increase in the number of elderly people in most developed countries. With this, osteoporosis became a significant health problem and it was estimated that by 2050 over 6 million people will be affected by the disease [1]. Osteoporosis is a disease that is characterized by low bone mass and deterioration of bone structure that causes bone fragility and increases the risk of fracture. For this reason, efforts in finding independent measures predicting bone strength gained in significance over the last decades. The most obvious factor determining bone strength is bone mass, wherefore the world health organization [2] defined for practical reasons osteoporosis to be a value for bone mineral density or bone mineral content that was more than 2.5 standard deviations below the young adult mean value [3]. Nevertheless, since it was also recognized that bone density leaves a rather large variation in bone strength unexplained, its use to assess fracture risk in all patients has been limited. The potential addition of geometric and material properties, at multiple hierarchical levels, may improve fracture risk predictions significantly.

In the last few decades, the mouse has become one of the most important laboratory animals. From the early breeding studies and the development of individual inbred strains, to the first successful application of recombinant DNA technology and continuing on to countless models with specific genetic alteration (Nobel Prize in Medicine, 2007), it has contributed tremendously to the understanding of human physiology and pathology at the molecular level. Although mice and men are genetically not perfectly identical and findings can not be extrapolated directly from mice to men and vice versa, a large homology has been identified based on the findings of the recently completed genome sequencing project. In the
field of skeletal biology, various mouse models have also been developed that have provided a deeper understanding of the genetic control of, for instance, cellular activities, cell signaling, cell lineage, and cellular abnormalities [4-8] or bone material and structural properties [9-13]. All of these models help to link the specific genotype of an organism to its phenotype, thereby providing an opportunity to identify possible causes for skeletal diseases, such as osteoporosis. Many sophisticated analysis tools and procedures have been established to characterize the skeletal phenotype at the cellular, molecular and structural levels in great detail. However, the major limiting factor preventing the full potential benefits of genetically modified mouse models from being realized is an insufficient ability of being able to reliably evaluate the functional phenotype of the murine skeleton in a quantitative fashion.

Accurate mechanical and material data on bone strength are still lacking for the mouse. That is why, in this doctoral thesis, we proposed to focus on the advanced investigation of bone mechanical properties in a murine model for osteoporosis and bone fracture. We experimentally assessed bone properties such as absolute strength of whole bones and cortical samples as well as bone stiffness and post-yield behavior. Bone morphological indices at the microstructural and ultrastructural levels were related to bone mechanical parameters and failure behavior. To further unravel bone failure initiation, we investigated the effects of the known, but barely understood process of bone microcracking. We looked into the porosity of bone and examined how this ultrastructure influences bone microdamage.

This work proposes a new integrative approach of murine bone biomechanical testing ranging from the organ to the ultrastructural level. This novel approach provides useful mechanical data in order to better understand bone failure behavior and will also help in the future to develop new efficient FE models allowing high-throughput analyses and thereby powerful phenotyping of murine bones.

The major aims of Chapter 2 were to determine exact mechanical properties of the whole femur in two distinct murine inbred strains, the B6 and the C3H. The contribution of cortical and trabecular bone morphometry to whole bone competence was also investigated.

First, in order to optimize our testing setup and reduce the effect of uncontrolled boundaries conditions on the test results, a first study aimed to quantify the influence of sample positioning on stiffness and strength in the proximal femur. Femora from B6 inbred mice were tested under compression with different loading directions. Very low variability in bone properties between each animal can be expected. In order to accurately position the femora for biomechanical testing, an image-based alignment protocol was developed. The
results showed that accurate alignment was of utmost importance for accurate and precise
determination of bone stiffness and strength; a 5° deviation from perfect vertical alignment
led to a 28.5% deviation in estimated stiffness. The effects on strength were less dramatic, but
with a change of 8.5% per 5° remained still considerable. In order to limit alignment errors,
we developed and designed a new sample alignment protocol which was able to position
murine femora with an absolute error (RMS) of only 1.45°.

A second study investigated B6 and C3H femora under three-point bending. The
tested bones were previously imaged in three dimensions using micro-computed tomography
and morphometric parameters were computed from the scans. The specific aim was to
compare bone competence and cortical morphometric parameters of both strains in order to
better determine the role of bone structure and geometry in the process of bone failure
behavior. To better understand the bone failure behavior, linear regression analyses were
conducted combining mechanical and morphometric results. Furthermore, mechanical tests
were imaged with a high-speed and high-resolution camera in order to closely observe the
fracture initiation and overall failure behavior. This work showed that the C3H strain was a
more reproducible model regarding bone morphometrical and mechanical phenotypes than the
B6 strain. Strength, stiffness, yield force, toughness and morphometric traits were
significantly different between each strain, while post-yield displacement was not. Intra-strain
mechanical predictions from the morphometry were not possible due to the genetic identity of
the inbred strain and the resulting very low variance in the bone traits of these mice. Inter-
strain analyses revealed that the morphometric parameters bone volume, cortical thickness
and cross-sectional area predicted almost 80% of bone stiffness, strength and yield force.
Bone post-yield behavior could not be explained by morphometry. This result in combination
with post-yield whitening observed in cortical bone confirmed the importance of
ultrastructure and microdamage investigations in order to better understand the overall bone
failure behavior, especially in the post-yield phase.

The femoral neck was also closely investigated, since it is one of the sites most likely
to fracture when elderly suffer from osteoporosis. In this study, we evaluated the
microstructure and mechanical properties in the femoral neck of the two inbred strains B6 and
C3H. The aim was to compare femoral neck competence and morphometric parameters of
both strains in order to better determine the role of bone structure and geometry in the process
of bone failure at this precise location. The murine femora were loaded in the axial direction
and a compartmental morphometric analysis of the femoral neck was performed. This
investigation included for the first time a morphometric evaluation of the femoral neck
compartment by mean of μCT. The results revealed that B6 femora became stiffer, stronger, and tougher from 12 to 16 weeks, whereas bone brittleness stayed constant. C3H behaved differently during this time: stiffness increased, but strength remained constant, toughness decreased and bone became more brittle. Using μCT to assess structural bone parameters, including those at the femoral neck, it was shown that mechanical strength of the femoral neck in the B6 strain was explained to 83% by the cortical thickness at this location; in contrast, none of the mechanical properties were explained by bone morphometry in the C3H strain. The brittleness of C3H was higher compared to B6. This can be explained, to some extent, by higher bone mineral density, but also suggests that C3H bone is more porous and accumulates more microdamage. We therefore hypothesized that investigation of cortical porosity and microdamage initiation and propagation in mouse inbred strains may help uncovering the processes at ultrastructural scales which lead to bone failure, and consequently may further increase our understanding of bone failure behaviour.

The accurate mechanical properties of both C3H and B6, obtained in the previous studies, were then included in a μFE simulation of femora loaded under three-point bending. The last study of Chapter 2 investigated the effect of the femur aspect ratio on the computation of bone Young’s modulus when using the Euler-Bernoulli beam theory. The specific aim was to determine the precise effects of bone geometry on the outcomes of a three-point bending test. Specifically, we assessed those effects for two commonly used inbred strain of mice B6 and C3H. This study showed that tissue moduli as derived from three-point bending tests are strongly underestimated. We also showed that the extent of underestimation depends on bone size and shape, and that this is different for different inbred strains; Young's moduli for B6 were underestimated more than for C3H. Interestingly, we also found a sex effect, where moduli for male are underestimated more than for female. The reason for this underestimation is clear: murine bones are not perfect beams; their geometries deviate so much from the assumptions underlying beam theory that erroneous results are obtained from beam theory. Our results strongly suggested that bone tissue modulus in mice varies to a lot lesser extent than reported in literature, and that those reported differences are largely an effect of geometric differences, not accounted for by beam theory. These findings also indicated that genetic effects on bone tissue modulus are much less strong than previously suggested.

Direct mechanical testing provides detailed information on overall bone mechanical and material properties, but fails in revealing local properties such as local deformations and strains or quantification of fracture progression. Therefore, in Chapter 3 and 4, we
incorporated several imaging methods in our mechanical setups in order to get a better insight into bone deformation and failure characteristics. We developed an integrative approach for hierarchical investigation of bone. A particular focus was then applied on bone microdamage and on the influence of bone ultrastructure on microcracks initiation and propagation.

The major aim of Chapter 3 was to develop and implement an integrative approach to hierarchically investigate bone and its mechanical properties, working at different scales of resolution ranging from the whole bone to its ultrastructure. We also hypothesized that there would be differences in bone mechanics between the two strains, B6 and C3H, on all levels. In this study, we were able to observe at the whole bone level how overall fractures initiate and propagate. On the microscopic scale, we examined how the bone internal microstructure behaves when bone is under load. Local deformations and microarchitectural changes were observed and analyzed. Submicrometer tomography permitted to look into the ultrastructure of bone. The difference of failure behavior between two inbred strains, observed macroscopically, could be explained, to some extent, investigating bone properties at smaller scales. Especially, the different post-yield behaviors of B6 and C3H were related to differences in cortical porosity and microdamage accumulation.

In Chapter 4, microdamage initiation and accumulation were investigated in more details by means of SRµCT. The aims of the first study were to describe this new 3D method and to discuss in details the advantages of this technique over the classical methods used so far. Some preliminary results obtained with SRµCT in the B6 and C3H murine models were also presented. In summary, this study showed the great power of three-dimensional visualization and quantification of cortical bone porosity and microdamage at high resolution. Furthermore, it was shown how microcracks initiated and propagated within bone ultrastructure under time-lapsed loading. SRµCT was able to uncover in 2D and in 3D very small features such as uncracked ligament bridging and mineralized collagen fibrils which are thought to play important roles in bone toughening mechanisms. SRµCT allowed to visualize the volumetric extent of microcracks. We observed that a microcrack can appear as a linear crack in one plane, but as a diffuse crack in a perpendicular plane. Hence, we postulate the necessity of 3D imaging techniques to unravel microcrack initiation and propagation and their effects on bone mechanics. Finally we developed a semi-automatic tool to separate bone porosity and microdamage in three different phases and to morphometrically quantify each of them. This tool was then successfully used in the next and final study to compare different cortical bone samples.
The last study of this thesis concentrated on differences in the ultrastructure in B6 and C3H and related them to microdamage initiation and propagation in cortical bone. We hypothesized that the difference in cortical porosity between the C3H and B6 strains would influence the microdamage behavior. We strongly believe that understanding how and why microdamage accumulates is central to better explain bone failure behavior. Our data suggested that the osteocyte lacunae played a fundamental role in microcrack propagation. The microcracks did not seem to initiate at the lacunae surface, but our investigation showed that cracks propagated through the lacunae. The lacunar voids, more exactly their distribution, guided the microcrack paths. Furthermore, C3H showed larger canal unit than B6. The size of the canal unit played a very important role in microcrack initiation and propagation, since the crack number was strongly dependent of the canal unit volume \( r = 0.87, p < 0.001 \). This dependence suggested that microcracks propagated through the ramification of a canal unit. Indeed, the microcracks bridged close regions of higher local strains which resulted from the canal unit ramifications. Our investigation showed that microcracks accumulated more and faster in C3H than in B6. Indeed, there was on average more microdamage in C3H before failure and more microdamage accumulated in C3H while increasing overall strain by 1%. The amount of microdamage and the rate of accumulation were previously reported as being important factors that reduce bone post-yield properties. Especially, high accumulation of microdamage increases cortical bone brittleness significantly [14, 15]. Our results were in accordance with previous studies claiming higher brittleness of C3H in comparison with B6 and offered an explanation to it, relating the microcracking behavior to bone porosity. The results of the current study are key findings in the comprehension of the differences in post-yield behavior between B6 and C3H reported previously [15-19].

In this thesis, a number of limitations can be identified. SR\(\mu\)CT was time consuming and did not allow to perform a large study with many samples, such as high-throughput phenotypic studies. Furthermore, the resolution achieved with this technique was not as high as with scanning electron microscopy [20]. A better resolution would permit to visualize microcracks at earlier stages, as well as the interaction between cracks and the canalicular network. Another limitation of this study was the loading regime. We could only perform static loading. Performing fatigue test would be closer to physiological loading modes. Finally, our model showed rodent bone, which was described above as an advantage for looking at individual interactions between microdamage and bone ultrastructure, but murine cortical bone is different from human bone, since osteons and lamellar organizations are not present. It therefore offers a simpler model to relate microdamage to bone porosity. Previous
studies showed that lamellar bone structures as well as bone cement lines significantly influence microcrack initiation and propagation [21-23]. Nevertheless, in certain cortical regions of human older bone, bone resorbs without remodeling and become therefore more porous and shows larger empty voids [14]. Our model, stating that large voids encourage microcrack initiation and propagation, indicates that these resorbed voids could potentially support initiation and propagation of microdamage. Of course this hypothesis has to be confirmed with future studies using the same techniques as presented here, but applied on human cortical bone samples.

In conclusion, the integrative approach to functionally and hierarchically determine bone competence, developed in this PhD thesis, proved to be very powerful. The time-lapsed high resolution 3D imaging of bone under load provided better understanding of the bone failure processes by non-invasive assessment of local deformation, fracture initiation and fracture propagation, which cannot be assessed with other techniques in an integrative fashion. Future studies on bone competence, specifically focusing on cortical porosity contribution to bone strength, should therefore repeat the aforementioned investigations with human bones. The results of these studies would then lead to the development of better tools and measures for clinically predicting bone strength.
References


Curriculum Vitae

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