Doctoral Thesis

Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging

Author(s):
Scheuren, Ariane C.

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Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging

A thesis submitted to attain the degree of

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(Dr. sc. ETH Zurich)

presented by

Ariane C. Scheuren

M.Sc. Biomedical Engineering, ETH Zurich

born on 03.12.1989

citizen of Luxembourg

accepted on the recommendation of

Prof. Dr. Ralph Müller, examiner

Prof. Dr. Ilaria Bellantuono, co-examiner

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Ariane Scheuren
Summary

Frailty is a geriatric syndrome characterized by increased susceptibility to adverse health outcomes. One major determinant thereof is the gradual weakening of the musculoskeletal system and the associated osteosarcopenia. Although anabolic interventions such as mechanical stimuli are known to promote bone and muscle mass, it remains unclear whether the ability of the musculoskeletal system to sense mechanical signals is maintained with age. A better understanding of the pathophysiology of osteosarcopenia will help to identify interventions to strengthen the musculoskeletal system, which ultimately will be beneficial for the prevention and/or treatment of frailty. With the advent of longitudinal in vivo phenotyping techniques, animal models are of increasing interest in aging studies as disease progression can be monitored over time in multiple tissues of individual animals. This not only provides a more comprehensive analysis of multi-system dysfunctions but also reduces the number of required animals. However, a suitable animal model mimicking frailty and osteosarcopenia is still lacking.

Therefore, the presented thesis has been divided into three aims: (i) To develop an approach to study bone mechanobiology in vivo across multiple scales (ii) To develop an approach to study frailty and osteopenia in vivo in a model of premature aging (iii) To longitudinally assess frailty and osteosarcopenia in an in vivo model of premature aging.

To address the first aim, a previously developed methodological platform known as “Local in vivo Environment (LiVE) histochemistry” was optimized to investigate the relationship between loading frequency and bone adaptation across multiple scales. Specifically, the combination of longitudinal micro-CT imaging with micro-finite element (micro-FE) analysis of mouse caudal vertebrae subjected to either static or cyclic loading at varying frequencies showed that bone adaptation to load is controlled by local mechanical signals with net bone changes logarithmically dependent on loading frequency. In order to assess whether the link between bone remodeling and the local mechanical environments can also be observed at the cellular level, a rigid 2D-3D registration was used to map osteocytes, the orchestrators of bone remodeling, identified on 2D histology sections to the 3D micro-CT data. Following this, the expression of the anti-anabolic and anti-catabolic signaling factors Sclerostin and RANKL were evaluated both globally and locally in relation to the mechanical and remodeling environments.
Consistent with the anabolic responses observed at the tissue level, cyclic loading resulted in a down-regulation of osteocytic Sclerostin and RANKL expression on a global level. In line with this, the RANKL expression was lower in regions close to bone formation than in regions close to bone resorption, thus suggesting that alterations in protein levels are locally linked to the in vivo microenvironment.

In a second part of this thesis, a long-term in vivo micro-CT imaging approach was coupled with longitudinal assessments of the clinical mouse frailty index (FI), a tool to quantify the accumulation of health deficits, in order to evaluate the suitability of a mouse model of premature aging as a model for frailty and senile osteoporosis. Furthermore, as the proposed method requires repeated scanning over a long period, potential biasing effects of radiation, anesthesia and handling associated with in vivo micro-CT imaging were investigated. Although the long-term imaging approach can lead to small but significant changes in bone morphometric parameters, the comparison between genotypes was not impaired, and the overall health status of the animal (i.e., FI and body-weight) was not affected. Moreover, this study demonstrated that longitudinal designs including baseline measurements already at young age are more powerful at detecting age-related phenotypic changes than those including multiple groups with fewer imaging sessions.

Finally, in order to evaluate the suitability of the PolgA model as a model for frailty and osteosarcopenia, the thus established long-term FI and in vivo micro-CT approach was combined with extensive musculoskeletal phenotyping. Concomitant to a higher rate of deficit accumulation, PolgA mice displayed progressive musculoskeletal deterioration such as reduced bone and muscle mass as well as the functionality thereof. In addition to lower muscle weights and fiber area, PolgA showed impairments in grip-strength and concentric muscle forces. Longitudinal micro-CT imaging of the 6th caudal vertebrae showed that PolgA had reduced bone micro-architectural integrity as well as lower bone turnover, thus mimicking senile osteoporosis as observed in humans. Lastly, this study showed that PolgA mutation altered the response to various anabolic stimuli in skeletal muscle and bone, indicating that the mechanoregulation of the musculoskeletal system may indeed change with age.

In summary, the application of the multiscale bone mechanobiology approach was successful to improve our understanding of the relationship between loading frequency and trabecular bone adaptation in vivo. Secondly, the application of long-term in vivo micro-CT imaging combined with longitudinal FI assessments was shown to be pivotal for monitoring the
development of frailty and senile osteoporosis in PolgA mice. Lastly, the integration of comprehensive musculoskeletal phenotyping showed that prematurely aged PolgA mice mimic multiple signs of frailty and of osteosarcopenia and thus provide a powerful model to improve our understanding of frailty and the aging musculoskeletal system. Taken together, the development of a multiscale mechanobiology approach as well as the identification of a model of frailty and osteosarcopenia provide the groundwork to elucidate the pathophysiology of osteosarcopenia and to test potential interventions, which ultimately will be constructive towards the prevention and/or treatment of frailty.
Zusammenfassung


Um das erste Ziel zu erreichen, wurde eine zuvor entwickelte methodische Plattform, die als "Local in vivo Environment (LivE) histochemistry" bekannt ist, optimiert, um die Beziehung zwischen Belastungsfrequenz und Knochenanpassung über mehrere Skalen hinweg zu untersuchen. Insbesondere die Kombination der longitudinalen Mikro-CT-Bildgebung mit der Mikro-Finite-Elemente-Analyse (Mikro-FE) von Schwanzwirbeln einer Maus, die entweder statischen oder zyklischen Belastungen mit unterschiedlichen Frequenzen ausgesetzt worden sind, hat gezeigt, dass die Knochenanpassung an die Belastung durch lokale mechanische Signale gesteuert wird; wobei die Netto-Knochenveränderungen logarithmisch von der


Um schließlich die Eignung des PolgA-Modells als Modell für Gebrechlichkeit und Osteosarkopenie zu bewerten, wurde der so etablierte langfristige FI- und in vivo Mikro-CT-Ansatz mit einer umfangreichen muskulöskeletalen Phänotypisierung kombiniert. Parallel zu einer höheren Defizitakkumulationsrate zeigten PolgA-Mäuse eine progressive Verschlechterung des muskulöskeletalen Systems, wie z.B. reduzierte Knochen- und

Chapter 1

Introduction
1.1 Thesis motivation

Life expectancy has improved dramatically over recent decades with the proportion of people aged 65 or above expected to more than double in the next thirty years [1]. At the same time though, the number of people suffering from frailty will also rapidly increase. Although there is no universally accepted definition of frailty [2], it is considered as an age-related syndrome characterized by the decline of multiple physiological functions, leading to the accumulation of health deficits, and thus a higher vulnerability to adverse health outcomes such as morbidity and mortality [3, 4]. One of the most striking age-related physiological declines, in terms of function and structure, is that of the musculoskeletal system, resulting in diseases such as osteoporosis and sarcopenia. Recent evidence has shown that individuals suffering from both osteoporosis and sarcopenia, also known as “osteosarcopenia” [5, 6], are at higher risk of falls, fractures, disability, and frailty [7-9]. Although the exact mechanisms underlying age-related bone and muscle loss are not yet fully understood, it is known that the coupling between anabolic (i.e., bone formation and protein synthesis) and catabolic processes (i.e., bone resorption and protein degradation), which in healthy tissues are evenly balanced out, becomes less efficient with age [10]. This impaired remodeling capability can, at least in part, be explained by altered responses to anabolic stimuli such as mechanical signals [11-17]. In muscle tissue, this phenomenon, also termed “anabolic resistance”, has been linked to reduced protein synthesis due to diminished intracellular signaling through the mechanistic target of rapamycin complex 1 (mTORC1) pathway [12, 13] as well as to the reduction in the number and proliferation capacity of satellite cells [18-21]. The diminished response to mechanical stimuli in bone tissue on the other hand, most likely arises due to reduced mechanosensitivity of osteocytes, the main orchestrators of bone remodeling [22, 23]. However, the exact mechanisms by which mechanical stimuli are transduced into anabolic and catabolic signaling events remain unclear. A better understanding of the pathophysiology of osteosarcopenia will help to identify interventions that strengthen the musculoskeletal system, which ultimately will facilitate either the prevention or treatment of frailty or both.

Although several animal models have provided invaluable information on aspects of frailty and the aging musculoskeletal system [24-27], a suitable model that mimics both frailty and osteosarcopenia is currently still lacking. The PolgA(D257A/D257A) mouse model (referred to as PolgA) represents a desirable candidate. Due to the expression of an exonuclease-deficient
version of the mitochondrial DNA polymerase γ it develops multiple age-related phenotypes (including hearing loss, grey hair, kyphosis, enlarged heart, muscle loss) early in life and thus strongly mimics the multi-system morbidity observed during aging in humans [28, 29]. Intriguingly, subjecting PolgA mice to endurance exercise interventions resulted in a reduced multi-system pathology compared to their sedentary littermates [30, 31]. Even though PolgA mice have been shown to have lower muscle weights and reduced bone mineral density compared to their wild-type littermates (WT) [28-30, 32], little is known about the quality and functionality of their musculoskeletal system. Moreover, the frailty phenotype has not yet been assessed in these mice.

With the advent of tools such as the clinical mouse frailty index (FI), it is now possible to non-invasively quantify the accumulation of health deficits over time in individual animals [33, 34]. Indeed, the establishment of such standardized tools has been highly instrumental in the identification of rodent models of frailty and to test potential therapies to prevent, treat or even revert frailty [24, 25]. Similarly, longitudinal in vivo micro-CT imaging is widely used in pre-clinical studies to monitor changes in the three-dimensional bone micro-architecture over time in individual animals [35]. Furthermore, by registering consecutive images onto one-another, bone formation and resorption activities can be visualized and quantified over time [36], providing the possibility to detect differences in bone remodeling activities in response to various interventions such as ovariectomy [37] and mechanical loading [15-17, 38-40]. Although several animal models have been used to investigate changes in osteogenic responses to loading with age, contradicting results have been reported; while studies using a tibia-loading model have shown a reduced response of bone formation to mechanical loading both in cortical [16, 17] and in trabecular [15, 41] bone with age, trabecular bone adaptation in response to loading of the caudal vertebrae was maintained with age [39]. These differences could be explained by the different skeletal sites which were analyzed [42, 43] or by the differences in the applied loading waveforms (including for example loading frequency, peak strain, number of cycles), which are known to play a crucial role in determining osteogenic responses to load [43, 44]. Hence, an important prerequisite for the investigation of age-related changes in bone mechanosensitivity is a better understanding of how loads applied at the organ scale are sensed at the cellular scale, which ultimately leads to remodeling at the tissue scale. By combining in vivo micro-CT with advanced registration techniques and micro-finite element (micro-FE) analysis, previous studies have shown that bone remodeling activities at the tissue scale are
controlled by local mechanical signals [45, 46], enabling an improved understanding of bone mechanobiology. Moreover, by registering endpoint 2D histological data into the 3D bone volume obtained by micro-CT, an approach termed “Local in vivo Environment (LivE) histochemistry” has been established to link the protein expression of single osteocytes to their local mechanical microenvironment in vivo and to the bone remodeling history. Using this approach, a previous study has shown that the expression of Sclerostin, an inhibitor of bone formation, is correlated to the local remodeling and mechanical environment [47]. As such, investigating load-induced bone adaptation across multiple scales provides a promising approach to better understand changes in mechanosensitivity with age.

Taken together, the overall goal of this thesis was to longitudinally assess the development of frailty and of osteosarcopenia in prematurely aged PolgA mice. In addition, this study aimed to study the effects of various anabolic interventions such as eccentric contractions and mechanical loading on the musculoskeletal system of PolgA mice.

Therefore, prior to evaluating the PolgA mouse model, the first part of this thesis aimed at optimizing and further developing existing techniques to study bone adaptation in vivo across multiple scales. Specifically, the combination of in vivo micro-CT with micro-FE and immunohistochemistry was used to study the effects of loading frequency on bone adaptation at the tissue and molecular scale. The second aim of this thesis was to develop an approach to longitudinally monitor the development of frailty and senile osteoporosis in an in vivo model of premature aging. For this study, long-term in vivo micro-CT imaging was combined with longitudinal measurements of FI to monitor individual mice during the process of aging, providing the possibility to capture the onset of osteoporosis and to track other signs of aging. Furthermore, as aging is accompanied with increased sensitivity to external stimuli, the cumulative effects of radiation, anesthesia and handling associated with micro-CT imaging on bone morphometric parameters as well as on the overall well-being of the animal were assessed. Once established, the last aim of this thesis was to longitudinally assess frailty and osteosarcopenia in an in vivo model of premature aging. Therefore, in addition to the longitudinal micro-CT and FI measurements as developed in the previous aim, the quality and functionality of PolgA muscles was assessed using various in vivo and ex vivo phenotyping techniques such as the assessment of fore-limb grip-strength, concentric muscle forces and muscle masses, respectively. Lastly, the effects of various anabolic stimuli such as eccentric muscle contractions and mechanical loading in PolgA muscles and bones were investigated.
1.2 Specific aims

The immediate goal of this thesis was to longitudinally assess frailty and osteosarcopenia in an in vivo model of premature aging. Specifically, the following three aims were defined:

Aim 1: Development of an approach to study bone mechanobiology in vivo across multiple scales.

Aim 2: Development of an approach to study frailty and osteopenia in vivo in a model of premature aging.


1.3 Thesis outline

The thesis consists of five chapters. In addition to the current chapter outlining the motivation and specific aims, the content of the subsequent chapters is the following:

Chapter 2 provides a background chapter about bone mechanobiology in mice. After introducing existing models and methods to investigate load-induced bone remodeling at different length scales, more integrative approaches that allow to study bone mechanobiology across multiple scales – from the organ down to the molecular scale – are described. Lastly, an “in vivo mechanomics” approach is proposed to locally analyze the transcriptome of single cells with respect to their local 3D mechanical in vivo environment.

Chapter 3 describes the optimization and further development of existing techniques that ultimately will allow to study bone mechanobiology in vivo across multiple scales in aging mice. In the first part, a “Local in vivo Environment (LivE) histochemistry” approach combining micro-CT, micro-FE and immunohistochemistry was used to study the effects of varying loading frequencies on bone adaptation across multiple length scales. Specifically, load applied at the organ scale was linked to local mechanical and remodeling environments at the tissue and molecular scale. In the second part, in vivo micro-CT imaging was optimized for usage in a mouse model of premature aging. For this, long-term in vivo micro-CT imaging over 20 weeks was combined with longitudinal assessments of the clinical mouse frailty index to
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evaluate the suitability of the model as a model for frailty and senile osteoporosis. Furthermore, the cumulative effects of radiation, anesthesia and handling associated with long-term in vivo micro-CT imaging were investigated.

Chapter 4 describes the application of the techniques developed in the previous chapter to comprehensively evaluate the relevance of a mouse model of premature aging (PolgA(D257A/D257A)) as a model for frailty and osteosarcopenia. In addition to long-term in vivo micro-CT imaging and longitudinal assessments of the frailty index, we assessed the quantity and quality of bone and muscle tissue. Lastly, using a combination of in vivo, ex vivo and in vitro techniques, the effects of various anabolic stimuli such as mechanical loading, eccentric muscle contractions and leucine administration were assessed at various ages.

Chapter 5 is the synthesis of this thesis including the major findings, the limitations of the presented work, and an outlook for future research.
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Chapter 2

Background


2.1 Bone mechanobiology in mice: toward single-cell *in vivo* mechanomics

Ariane Scheuren\(^1\), Esther Wehrle\(^1\), Felicitas Flohr\(^1\), Ralph Müller\(^1\)

\(^1\)Institute for Biomechanics, ETH Zurich, Zurich, Switzerland

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**Abstract**

Mechanically driven bone (re)modeling is a multiscale process mediated through complex interactions between multiple cell types and their microenvironments. However, the underlying mechanisms of how cells respond to mechanical signals are still unclear and are at the focus of the field of bone mechanobiology. Traditionally, this complex process has been addressed by reducing the system to single scales and cell types. It is only recently that more integrative approaches have been established to study bone mechanobiology across multiple scales in which mechanical load at the organ level is related to molecular responses at the cellular level. The availability of mouse loading models and imaging techniques with improved spatial and temporal resolution has made it possible to track dynamic bone (re)modeling at the tissue and cellular level *in vivo*. Coupled with advanced computational models, the (re)modeling activities at the tissue scale can be associated with the mechanical microenvironment. However, methods are lacking to link the molecular responses of different cell types to their local mechanical microenvironment and bone (re)modeling activities occurring at the tissue scale. With recent improvements in “omics” technologies and single-cell molecular biology, it is now possible to sequence the complete genome and transcriptome of single cells. These technologies offer unique opportunities to comprehensively investigate the cellular transcriptional profiles within their specific microenvironment. By combining single-cell “omics” technologies with well-established tissue-scale models of bone mechanobiology, we propose a mechanomics approach to locally analyze the transcriptome of single cells with respect to their local 3D mechanical *in vivo* environment.
Keywords:
bone mechanobiology, mouse loading models, omics, single-cell biology, gene expression analysis, RNA-sequencing
2.1.1 Introduction

Mechanobiology is an interdisciplinary field at the interface of biology and mechanics that aims at understanding how physical forces are translated to biological signals contributing to tissue development, maintenance and disease [1]. In the field of bone mechanobiology, it is well established that bone is able to adapt its internal microstructure to changing mechanical demands by the coordinated actions of bone forming and resorbing cells. It is generally accepted that increased mechanical stimulation (e.g. exercise) leads to higher bone mass and strength whereas a lack of mechanical stimulation (e.g. chronic bed rest) is linked to bone loss [2-6]. Bone adaptation as a whole is achieved by two fundamental processes: modeling and remodeling (Fig. 2.1) [7].

![Diagram of bone structure](image)

**Fig. 2.1** Structure of trabecular and cortical bone, the process of bone remodelling, and cellular signals from coupling factors and the IL-6 cytokine family that regulate bone remodelling and periosteal modelling. The proximal and distal ends of the growing murine femur (epiphyses) contain a high proportion of trabecular (cancellous) bone. Trabecular bone is also prevalent in the metaphyses, which is separated from the epiphysis by the epiphyseal (growth) plate; this is the site of longitudinal bone growth. The midshaft of the femur, diaphysis, contains a high proportion of cortical (compact) bone that surrounds the inner marrow space and trabecular regions. Bone remodelling occurs on trabecular surfaces, and on the endocortical surface (both
surfaces together are termed the endosteum). Bone modelling occurs throughout life in murine bones on the outer periosteal surface (periosteum). During bone remodelling on the endosteum, (1) osteoclasts attach to the bone surface, resorb bone and release coupling factors that stimulate osteoblast differentiation on the endosteal surface. These coupling factors also signal to periosteal osteoblasts, perhaps through the osteocyte canalicular network. After the reversal phase, which remains poorly understood in murine bones, (2) pre-osteoblasts mature, attach to the bone surface and fill the cavity created by osteoclasts with bone matrix, termed osteoid. (3) Mature osteoblasts, when their task of producing osteoid is completed, become lining cells or (4) become embedded within the osteoid as it is mineralised. These osteoblasts become osteocytes and release factors that regulate mineralisation. IL-6 family cytokines are released by the osteoblast lineage and act to stimulate osteoblast differentiation and bone matrix production on endosteal surfaces, but limit osteoblast activity on the periosteum. Reprinted with permission by Sims et al. [7] and Elsevier.

Bone modeling describes the process where bone resorption and formation occur independently at different sites to sculpt bone (i.e. during growth and/or in response to mechanical loading). Bone remodeling, on the other hand, is the process of bone renewal throughout life during which small packets of bone are removed and subsequently replaced within so-called basic multicellular units (BMU) [6]. This coupling process is tightly controlled in space and time such that it can occur asynchronously at many different sites throughout the skeleton [8]. Both modeling and remodeling, collectively referred to as (re)modeling, are regulated by local strain distributions [6, 9] and ultimately rely on the ability of resident bone cells to sense mechanical signals (e.g. strain, fluid flow, pressure) [10-13].

Whereas experimental methods were still lacking, in silico models aiming at a better understanding of the relationship between global mechanical loads and the local stresses and strains influencing bone adaptation at the organ, tissue and cell level have been developed already years ago [14-17]. Since then, the integration of animal loading models [18] has improved the computational frameworks by providing in vivo data of bone responses to controlled loading conditions, which are crucial for the validation of simulations [19]. The distribution of the engendered strains can thus be quantified at selected surface locations and subsequently used to define the relationships between the administered loads and the structural responses that follow. Studies using mouse models have shown that mechanically-induced adaptation is controlled locally and varies between different bones of the skeleton [20] and between different sites (e.g. trabecular versus cortical bone) within the same bone [21-25]. It is to be noted though that different loading regimes have different efficiencies, which makes it challenging to determine the decisive factors that are necessary to promote bone formation [2, 26, 27]. Also, bone morphology and strain distributions vary depending on the mouse strain.
Chapter 2 Background

[20, 28, 29], their age [30-32] and gender [33], which leads to changes of the local (re)modeling characteristics. Among these factors, aging has been intensely studied for its potential implication in age-related bone loss. Although many studies have shown anabolic effects of mechanical loading on bone mass both in young and older mice [30, 34-38], there is a lack of consensus on bone’s mechanoresponsiveness with age. While studies using a tibia-loading model have shown a reduced response of bone formation to mechanical loading both in cortical [39, 40] and trabecular [30, 36] bone with age, trabecular bone adaptation in response to loading of the caudal vertebrae was maintained with age [37]. It therefore remains unclear whether and how age-related changes in bone’s response to mechanical loading occur and whether these changes are due to morphological and material property changes alone, or due to the loss of bone’s ability to sense and/or respond to mechanical signals. Hence, it is believed that a better understanding of the cellular activities involved in bone (re)modeling will provide some answers to ensure further progress in the field of bone mechanobiology.

Studies have identified several mechanoresponsive cell types and signaling pathways involved in the mechanotransduction of extracellular stimuli into intracellular biochemical responses thereby influencing bone (re)modeling. Herein, osteocytes, known as the major mechanosensors are able to sense and integrate mechanical and chemical signals from their environment, and in turn orchestrate appropriate responses such as recruitment, differentiation and activity of effector cells [10, 13, 41]. Embedded in the matrix in a connected system of voids, called lacunae, and slender canalicular channels, collectively known as the lacuno-canalicular network (LCN), they are ideally located to sense changes in their external mechanical environment and to communicate this information between each other and other bone and marrow cells. During mechanical loading, multiple mechanical signals such as bone matrix deformation [42], changes in hydrostatic pressure [43, 44], fluid flow into and out of the LCN and shear stresses along osteocyte membranes [45-47] arise. These signals lead to force-induced conformational changes in cellular structures (tethering elements, primary cilia, cell-cell adhesions, integrin complexes), which subsequently trigger the activation of mechanoresponsive signaling pathways (e.g. Wnt-, calcium- and estrogen-signaling) resulting in altered gene production [48]. Currently, fluid flow through the LCN seems to be the primary mechanical stimulus that is sensed by cells, however the exact mechanotransduction mechanisms are still under debate and have been reviewed elsewhere [10, 11, 41, 49, 50].
Many \textit{in vitro} models have been established to investigate biological responses of individual cell types to mechanical stimulation disconnected from their native environment (Wang and Thampatty 2006, Thompson, Rubin et al. 2012, Michael Delaine-Smith, Javaheri et al. 2015). Compared to \textit{in vivo} loading models, cell-culture experiments are extremely powerful in identifying relevant mechanical cues, candidate molecules and mechanisms of mechanosensing as the cellular adaptation (e.g. strain) can be directly measured in response to controlled mechanical cues (e.g. stresses). Furthermore, by combining experimental and computational techniques to correlate these experimental outcomes (strains, stresses) with cell-specific gene expression profiles, methods are currently arising to predict and investigate the “mechanome” of live cells [51, 52].

However, \textit{in vitro} studies in bone mechanobiology have shown that strains (1-10\%) that are necessary to cause signaling in two-dimensional cell cultures are much larger than strains applied to whole bones (0.04-0.3\%) [53, 54]. Bacabac et al. for example showed that osteocyte-like cells are more mechanosensitive to the same mechanical stimulus when they are rounded, i.e. with a more three-dimensional morphology, than when they are stretched out on a flat surface [55]. Indeed, it has also been shown \textit{in vivo} that the shape of osteocyte lacunae depends on local stress distributions and varies between different bones and different sites within the same bone [56, 57].

More recently, the importance of understanding bone (re)modeling in its entirety - as a result of the complex interactions among many contributing cells (osteocytes, osteoclasts, osteoblasts, marrow cells) – has been recognized [2, 7, 11, 12]. Furthermore, as strains are heterogeneously distributed throughout bone tissue, and as (re)modeling occurs at many different skeletal sites, (re)modeling activities should be assessed locally and considering signals from the local mechanical environment. Consequently, the understanding of load-induced bone (re)modeling requires a systems-level approach in which forces exerted at the organ scale can be linked to the responses at the cellular and molecular scale which lead to coordinated (re)modeling at the tissue scale (Fig. 2.2).
**Fig. 2.2** Multiscale process of load-induced bone remodeling. Mechanical forces exerted at the organ scale are distributed heterogeneously throughout the tissue. Cells sense these mechanical signals at the molecular level, which leads to coordinated remodeling at the tissue scale.

With increased computational power and recent experimental advances such as improved spatial and temporal resolution of imaging techniques [58, 59], dynamic bone (re)modeling can be tracked *in vivo* both at the tissue and the cellular level also allowing for the assessment of the micromechanical environment at each stage of development through multiscale simulation techniques [23, 60, 61].

On the molecular level, advances in “omics” technologies such as genomics, transcriptomics and proteomics are more and more enabling us to comprehensively map the cellular and molecular responses to biochemical and biomechanical stimuli [62]. In fact, improvements in sequencing technology and molecular biology are now leading to the emergence of genome-wide, quantitative analysis of single cells and, hence, to the convergence of genomics and single-cell biology (Fig. 2.3) [63-65]. Single-cell biology provides unique opportunities to dissect the complex spatiotemporal dynamics of biological processes and interactions, which are averaged out in bulk assays.
2.1 Bone mechanobiology in mice: toward single-cell in vivo mechanomics

Fig. 2.3 Convergence of “Omic” Biology and Single-Cell Biology. Technology that allows researchers to obtain genome-wide information from single cells is extending the boundaries of a field that has thus far been limited to the analyses of a select gene in eukaryotes. Reprinted with permission from Junker et al. [63] and from Elsevier.

Combining “omics” technologies with mechanobiology, the field of mechanomics is currently emerging, which aims to deepen our understanding of cellular responses to multiple types of mechanical stimuli (e.g. shear flow, tensile stretch etc.) [51, 62]. In vivo, these multiple types of mechanical stimuli are ubiquitous [10, 49] and can either act separately [66, 67] or – of higher physiological relevance - synergistically (often combined with other physical and chemical factors) on cells [68]. Furthermore, specific mechanical stimuli might cause cell type specific biological responses, which is difficult to account for when using individual cell lines [69, 70]. Extending the field of mechanomics to spatially resolved in vivo mechanomics in bone, we propose to combine single-cell “omics” techniques with established frameworks that locally link (re)modeling activities to mechanical environments at the tissue scale. The availability of this approach that allows to map the molecular profiles of single cells within their local 3D mechanical microenvironment will further our understanding of how bone cells interact and function as a biological system in response to mechanical loading in vivo.

In this review, we will discuss advances in technologies and experimental methods that have enabled a better understanding of the complex interactions of mechanics and biology across different scales. More specifically, state-of-the-art technologies to quantify the mechanical environment and bone (re)modeling at tissue and cellular levels, and gene expression in mouse loading models will be discussed. Based on these methods, future, system-level strategies in the field of mechanomics are proposed, which will provide the possibility to comprehensively
map the mechanome of single cells *in vivo* (i.e. the responses of single cells to mechanical loading *in vivo*).

### 2.1.2 Bone mechanobiology in mice: models & methods to study load-induced bone surface (re)modeling at different length scales

*Mouse models to study load-induced bone (re)modeling (organ scale)*

In contrast to other animals, mouse models present numerous advantages such as robust breeding, short generation time and the potential to examine genetic factors using transgenic technology [71]. In computational applications, they furthermore provide invaluable benefits by reducing size, complexity and computation times of the calculations. Nevertheless, the differences in the physiology of human and murine skeletons as well as among different mouse strains must be considered before translating studies of murine to human bone [72]. Unlike in humans, longitudinal bone growth does not end at sexual maturity in mice, but continues at a slow rate. Also, mice lack a well-developed Haversian system and hence intracortical remodeling only rarely occurs. However, the resorption cavities that mice use for bone remodeling during fracture healing have been shown to be very similar to the Haversian remodeling in larger animals [73, 74]. Furthermore, they do exhibit cancellous and endocortical bone remodeling as well as age-related cancellous bone loss, which have been shown to be more strongly linked to postmenopausal osteoporosis than intracortical remodeling-induced bone loss [72, 75, 76]. Hence, as long as the inherent limitations are recognized, mouse models are considered suitable to mimic human skeletal physiology [77, 78] (e.g. for osteoporosis [72] and fracture healing [79]).

At the organ scale, a number of mouse loading models have been introduced to study load-induced bone (re)modeling *in vivo*. In most models, the loading is tightly controlled by directly loading a limb or bone by forces produced externally by a mechanical device. These include axial loading models of the ulna [80], fibula [81], tibia [21, 82, 83], and vertebra [84], four-point loading of the tibia [85] as well as knee loading of the tibia [86] or femur [87], and ankle loading of the tibia [25]. Alternatively, models such as treadmill running [22, 27] have been introduced which are more comparable to physiological exercise in humans. Complementarily, unloading models such as tail suspension, sciatic/tibial denervation or the injection of botulinum toxin have been used to study how bone adapts to a decrease or absence of
mechanical stimuli [18]. The effects of mechanical loading on bone tissue vary depending not only on the loading model but also on multiple parameters such as force (strain magnitude) [38, 88-92], the rate of strain (temporal change in strain magnitude within the tissue) [93-96], strain frequency (number of strain events per unit time) [97], strain gradients (spatial change in strain magnitude) [98-100], waveform, the number of loading cycles, duration of application and the inclusion of rest periods between the mechanical events [35, 101]. Moreover, the different combinations of parameters used in specific loading protocols [27, 102] together with other factors such as different animal strains [28, 29, 85], ages [21, 34] and sex [103] must also be considered when comparing results obtained by different research groups.

Methods to study load-induced bone (re)modeling (tissue scale)

The effects of loading on bone (re)modeling can be quantified at multiple scales. At the tissue scale, strain gauges and finite-element (FE) modeling can be used to characterize the strain values acting on whole bones for a specific loading model [18, 104]. Whereas strain gauges can only measure the strains at the surface to which they are attached to, FE analysis allows the computational estimation of the strain pattern throughout the entire bone.

In order to define the relationships between the administered loads and the structural responses that ensue, the outcomes of dynamic bone adaptation can be assessed. For this purpose, dynamic histomorphometry has traditionally been used, in which mice receive sequential injections of fluorescent dyes that adhere to regions of mineralizing bone, thus allowing the assessment of bone formation rates [105]. By identifying the sites of bone formation, it has been shown that responses to in vivo mechanical loading are determined locally and vary between different bones [20] and between different sites (e.g. periosteal vs. endocortical bone) within the same bone [21, 25, 92, 95]. However, the bone resorption rates cannot be assessed due to the lack of an equivalent method to label resorption over time. Furthermore, this type of analysis is limited to two-dimensional information of histological sections, which at the same time requires sample destruction. Nowadays, desktop micro-computed tomography (micro-CT) is considered a gold-standard technique to image and quantify bone as it provides a three-dimensional (3D) assessment of the net mineral response to a certain intervention or in a model of disease [58]. With information on bone mass, 3D microarchitecture and local mineralization levels, the exact amount of calcified tissue at a specific site can be measured in a standardized way, which eliminates subjectivity and inter-observer variability associated with histological methods.
Sugiyama et al. for example used both 2D fluorescent histomorphometry and micro-CT to analyze multiple bones per mouse (including loaded, adjacent and contra-lateral bones) and showed that structural changes in response to loading are not only confined to the loaded bones but also to specific sites within these bones [106]. Although, no significant differences were observed between the results obtained by histology and micro-CT, the latter is less time- and labor-intensive. Furthermore, with increasing resolution, high-resolution micro-CT scanners now allow the assessment of the 3D native environment of cells such as osteocyte lacunae and even canaliculi in mouse bones [59]. Other imaging techniques for the assessment of the osteocyte and LCN have recently been reviewed elsewhere [107]. The major limitation of both histology and ex-vivo micro-CT however, is that the measurement must be performed post-mortem, allowing only one end-point measurement per animal. In this respect, the availability of in vivo micro-CT scanners is highly beneficial, as changes in bone in response to loading can be tracked at multiple time points within a single animal [39, 61]. For example, using a cyclic mouse tail loading model in combination with time-lapsed in vivo micro-CT and image registration techniques, Lambers et al. were able to track dynamic bone formation and resorption in trabecular mouse bone [61]. In addition, Schulte et al. were able to quantify the formation and resorption rates in 3D, similar to 2D histological parameters [108]. Birkhold et al. further developed a computational approach to identify and quantify the frequency of specific sequences of (re)modeling in response to mechanical loading of the mouse tibia [109]. By tracking spatially correlated bone formation and resorption events over time, they were not only able to show temporal differences in the (re)modeling sequences upon mechanical loading, but also to distinguish between bone modeling and remodeling events. Although they found more modeling than remodeling sites, it remains unclear if this is due to the rarer occurrence of remodeling or due to the monitoring period, which would have to be longer in order to visualize a complete remodeling cycle.

Considering the tight coordination of (re)modeling in both space and time, it seems intuitive that the local stress distributions might also play an important role in the site-specific responses of bone to mechanical loading. In this regard, the combination of time-lapsed in vivo micro-CT imaging with the above mentioned computational FE analysis has been extremely valuable to not only characterize local mechanical environments within bone, but also to link mechanical influences to local (re)modeling activities at the bone surface. By using micro-finite element models (micro-FE) derived from in vivo micro-CT images prior to loading, Schulte et al. resolved the mechanical strains occurring throughout the trabecular micro-architecture at a
resolution of 10 µm [60]. The direct comparison of bone forming and resorbing surfaces with the micro-FE model showed that bone was formed in regions of high strain and resorbed in regions of low strain (Fig. 2.4) [60].

![Image](https://via.placeholder.com/150)

**Fig. 2.4** Comparison of local bone formation and resorption sites with the mechanical environment. (A) Three-dimensional trabecular bone formation and resorption sites measured with *in vivo* micro-CT over 4 weeks. The inset shows a magnified view of formation and resorption locations in individual trabeculae. (B) Corresponding SED computed with micro-FE in the basal scan. The same regions as in (A) are enlarged. A visual comparison reveals that high SED (red) matches with sites of bone formation (yellow), while low SED (blue) is found at locations of bone resorption (violet). Reprinted with permission from Schulte et al.[60] and *PLOS ONE*.

In this study, the (re)modeling activity at the bone surface was directly linked to the mechanical environment at the bone surface, defined as the strain energy density (SED). SED, which represents a scalar measure reflecting contributions from all stresses and strains acting at a given location [9, 14, 110], is often used to correlate local (re)modeling activities to the mechanical environment. However, theoretical and experimental studies in various species have linked other types of stimuli (e.g. strain magnitude [38, 88-92], strain frequency [97], strain history related parameters [111], strain rate [93-96] and strain energy gradients [98-100] to bone (re)modeling activities. Due to their relation to pressure differentials within bone, the latter parameters, namely strain rates and gradients, are thought to influence the magnitude of fluid
flow within bone, suggesting a greater physiological relevance of these parameters compared to others (e.g. SED) [95, 98, 99, 112]. In line with this hypothesis, Webster et al. [100] extended the model based on Schulte et al. further by incorporating not only the bone but also the marrow phases, which showed that mechanical signals derived from the bone marrow, namely the gradient in SED, are even more precise in predicting trabecular bone adaptation. These results suggest that many cell types - including bone marrow cells – are able to sense mechanical signals and influence bone formation and bone resorption by osteoblasts and osteoclasts respectively.

Using similar methods to Webster et al., Metzger et al. characterized the local micromechanical environment of bone marrow in trabecular compartments of porcine femora [113]. By combining micro-CT imaging, micro-scale computational fluid dynamics modeling and experimental assessments of pressure gradients during physiological loading, the authors demonstrated that deformation of whole bones induces motion and consequently shear stress in the bone marrow, which could be sensed by cells. Indeed, it has recently been shown that mechanical loading of mouse limbs in vivo induces mechanical signaling between marrow cells [114] and increased proliferation and osteogenic gene expression in marrow stromal cells [2, 11].

Recently, Birkhold et al. also used a similar approach based on time-lapsed in vivo micro-CT and FE modeling to investigate site-specific responses of cortical bone to in vivo tibial loading [23]. Compared to the periosteal surface, the response to loading was higher at the endocortical surface, which interestingly however was not linked to higher strains at that surface. Consistent with an important role of marrow cells, the authors speculate that these results may be due to the higher amount of vascularity or presence of bone marrow at the endocortical surface, which is thought to play a role in the amplification of mechanical strain.

Methods to study load-induced bone (re)modeling (cellular and molecular scale)

As is outlined above, organ- and tissue-scale information is not sufficient to validate and improve existing models aiming at understanding the underlying mechanisms of bone mechanobiology. The integration of information at the cellular and molecular level is thus required. To address this need, methods such as immunohistochemistry or in situ hybridization have been used to locally determine protein or gene transcript levels within the mechanical environment of loaded bone [115-118]. For example, a reduction in expression of Sclerostin
(SOST) protein has been observed both in axially loaded ulnae [115] and tibiae [118]. However, whereas Robling et al. [115] directly associated this reduction with local strain levels, Moustafa et al. [118] found that SOST expression was more closely related to sites of bone formation than to local strain levels. Possibly, these differences arise due to the different methods used for estimations of the mechanical environment within bone. Similarly, the latter approach was recently used by Lara-Castillo et al. to investigate the activation of the β-catenin pathway in osteocytes after ulna loading in mice [117]. Osteocyte gene expression patterns could not be associated to the uniform strain fields predicted by traditional FE models. Including the osteocyte lacunae (identified by histology) into the FE models increased the heterogeneity of the predicted strain field, with high strain concentrations observed around some osteocyte lacunae (Fig. 2.5 and 2.6) [117]. This strain field correlated much better with gene expression patterns.

Fig. 2.5 Kinetics of β-catenin activation after a single load session. (A) Representative images and close-up of cross-sections at the midshaft region of non-loaded and loaded ulnas (B) Graph showing counts of manually counted β-catenin positive cells. Graph represents mean ± standard error of the mean (n=4, *p<0.05) (C) Increased magnification view of a cross-section at the midshaft region of a loaded ulna (24 hour time point) illustrating activated cells at the bone surface. Reprinted with permission from Lara-Castillo et al. [117] and Elsevier.
Fig. 2.6 Finite Element models of ulna midshaft. Osteocyte lacunae locations based upon corresponding histological sections were matched to micro-CT images of the bone modeled. Seven serial sections were included in the loading simulation and the FE predicted Max Principal Strains from the middle section are shown. Reprinted with permission from Lara-Castillo et al. [117] and Elsevier.

These studies show that incorporation of cellular and molecular analysis into existing frameworks, which link the mechanical environment with (re)modeling activities at the tissue scale, is a powerful approach to better understand the multiscale process of load-induced bone (re)modeling. However, the major drawback of these approaches is that they are limited to cross-sectional studies in which measurements are only able to provide a snapshot of the dynamic molecular responses at the endpoint of the experiment. The ability to assess the effects longitudinally within the same animal over time could potentially lead to more consistent results.

In this direction, techniques have been established to track the dynamic signaling of intracellular calcium (Ca$^{2+}$), an important and ubiquitous second messenger regulating many downstream cellular activities, *ex vivo*, in cultured calvarial bone explants [42, 119, 120] and more recently, in a mechanical loading model of the mouse tibia [121]. By a combination of fluorescence recovery after photobleaching (FRAP) imaging and transport modeling [122], the magnitudes of applied mechanical loads, tissue strains, LCN fluid flow velocities and shear stresses could be linked to the spatiotemporal parameters of Ca$^{2+}$ responses in the osteocyte network [121]. Moreover, *in vivo* imaging technologies at the cellular scale such as confocal and two-photon excitation based microscopy now provide the possibility to image bone cells in living mice with high spatiotemporal resolution [123]. Coupled with the availability of
transgenic mouse models that express fluorescent reporter proteins in defined cell populations, the visualization and tracking at the molecular level of distinct cell types at specific stages of differentiation has been greatly facilitated [124]. Although still in their infancy, the incorporation of these in vivo live cell imaging approaches into existing frameworks would provide more detailed spatiotemporal information of biochemical processes involved in bone (re)modeling. So far, however, the number of markers that can be assessed simultaneously in a given sample is limited, and hence the throughput of experiments is very low. If the molecular networks involved in load-induced bone (re)modeling are to be fully characterized, experimental approaches are required that can enable similar investigations to be performed with a wider range of molecular targets.

Gene expression analyses using qPCR [22, 24, 28, 34, 116, 125] or DNA microarrays [25, 126-129] performed in in vivo mouse models of load-induced bone (re)modeling have identified a number of mechanically regulated genes and pathways. In these studies total RNA was isolated from whole bones, either including [25, 28] or excluding bone marrow tissue [22, 24, 125-128]. Only few studies limited their gene expression analysis to only cortical [34] or trabecular bone [129]. Indeed, the direct comparison between RNA from mouse cortical and trabecular bone revealed differences in gene expression in response to mechanical loading [130]. However, these bulk assays report gene expression averaged over all the different cell types present in bone, each of which have a specific role and their own corresponding expression profile. To overcome this limitation, several studies have used cell populations (osteoblasts and osteocytes) from mouse bone separately in order to investigate differential gene expression profiles and thus, several cell-type specific markers (e.g. Sost, Keratocan) have been identified [69, 70, 129, 131-134]. Wassermann et al. [129] for example isolated large populations of osteocytes from trabecular bone subjected either to one single or repeated sessions of in vivo vertebral loading. Microarray analysis revealed hundreds of genes differentially expressed between control and loaded animals. Similarly, differential gene expression has been observed between the different cell types both without additional loading and in response to unloading [69, 70]. However, for some markers (e.g. Rankl), inconsistent observations were made by different authors with regard to the cell-type specificity of gene expression [69, 131-134]. These results might be explained by the different purification protocols used – namely sequential collagenase digestion [69, 70, 129, 134] or fluorescent activated cell sorting (FACS) [131-133]. In addition, differences in protocols (such as the additional depletion of hematopoietic and endothelial cells)
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or the specific transgenic mouse models used can influence the results [131, 132], which makes the comparison of gene expression analyses between studies using differently grouped populations challenging.

Considering both the multitude of cell types present in bone and the different mechanical stimuli to which they are exposed, analysis at the single-cell level could overcome the difficulties of grouping. Studying individual cells would provide a more detailed understanding of cell-specific responses to mechanical stimuli, which ultimately would deepen our understanding of their specific roles and the multifaceted interactions between them.

2.1.3 Toward single-cell in vivo mechanomics

“Omics” technologies in bone biology

“Omics” technologies such as genomics, transcriptomics and proteomics have enabled researchers to study biological systems extensively and at unprecedented resolution. Thereupon the field of mechanomics is recently emerging which aims to understand the changes in gene expression in response to multiple types of mechanical stimuli by combining mechanobiology and high-throughput “omics” approaches [62]. In gene expression studies, RNA sequencing (RNA-seq) is now considered the method of choice to comprehensively examine transcriptional profiles of cells. Unlike qPCR or DNA microarrays, it does not introduce a bias by being limited to a specific set of genes and thereby provides the possibility to detect previously unknown regulatory mechanisms or lineage-specific markers. Not surprisingly, it is rapidly becoming an established technique in bone research as well. RNA-seq has for example been used both in vitro using osteocytic cell lines [135, 136] and in vivo [130, 137-140]. In a series of publications, Govey et al. evaluated the utility of three high-throughput approaches, namely DNA microarrays, RNA-seq and protein mass spectrometry, to investigate the response of osteocyte-like MLO-Y4 cells to oscillating fluid flow both at the transcript and protein level [135, 141]. This integrative approach of using the three different techniques on the same set of samples provides not only a direct comparison between the methods, but also the possibility to map the time-course of flow-induced fluctuations in both transcript and protein abundances. RNA-seq showed higher sensitivity in detecting differential gene expression profiles compared to microarray, which was also confirmed by others [137]. Moreover, the usage of these high-throughput approaches revealed both known and previously unknown mechanoresponsive
signaling molecules. However, considering the lack of a three-dimensional environment and the changes in gene expression that can occur during cell culture, care must be taken when comparing these results to responses observed in vivo.

Most in vivo RNA-seq studies performed so far consist of bulk measurements performed either on cortical diaphysis [138-140] or whole bone samples [137]. Kelly et al. observed differential transcriptomic profiles between cortical and trabecular bone samples of mouse tibiae subjected to one session of axial compression [130]. These results were slightly discordant with the results previously obtained from trabecular bone [129], which may be due to the differences between the used methods such as the type of bones, time points or the grouping of mixed cellular populations.

Single-cell analysis in bone

Nowadays, single-cell technologies are highly valued as they are able to detect biologically relevant cell-to-cell variability such as intrinsic stochastic variation or differences induced by extrinsic factors such as the local microenvironment [63]. However, single-cell approaches widely adopted in other fields are still in their infancy in the field of bone biology. This is not surprising considering the difficulty of extracting cells from bone tissue even as grouped populations. Previous studies have used imaging techniques to study single cells exposed to fluid flow in vitro [142, 143]. Single osteoblasts and osteocytes obtained from embryonic chick calvaria showed differential intracellular calcium concentrations in response to fluid flow [142]. Similarly, a quasi 3D microscopy approach has been used to image the complex 3D deformational behavior of single osteocyte-like MLY-O4 cells exposed to fluid flow [143, 144]. Using a different approach, Flynn et al. sorted 190 single osteoblast lineage cells from adult long mouse bones for subsequent gene expression profiling by nanofluidic PCR [145]. The single-cell data revealed information about the variance and co-expression of transcripts which was largely lost when analysing the mean gene expression of the combined population. Single-cell analysis therefore is important to gain further insights into specific gene regulatory mechanisms that control various aspects of cell function in vivo. The limitation of cell sorting however is that it requires either enzymatic or mechanical disaggregation of the entire tissue, which can not only influence gene expression [64], but more importantly unavoidably causes loss of spatial information. Considering that every bone cell resides in a different microenvironment characterized by a different mechanical strain and local (re)modeling...
activity, preserving the spatial information is of paramount importance for successfully deciphering of the molecular pathways involved in these processes.

Using laser-capture microdissection (LCM), several studies have isolated specific osteoblastic cell populations (ca. 200 cells) from morphologically intact cryosections of murine bone tissue with high spatial resolution for downstream gene expression analysis by either qPCR or microarrays [114, 146, 147]. Furthermore, in an approach termed mechanical systems biology, well-established and emerging techniques are combined to spatially map the gene expression profiles of osteocytes within their 3D mechanical microenvironment [148]. More specifically, small populations of osteocytes are harvested by LCM and subjected to downstream multiplexed gene expression analysis by qPCR. The mapping of these cells within the 3D time-lapsed micro-CT data allows for time tracking and grouping according to local in vivo histories of cells [148]. Furthermore, the correlation of these spatial gene expression maps with both the micro-CT images and the micro-FE model allows to characterize the relationship between molecular activity, the local (re)modeling activity and the associated mechanical microenvironment.

**Toward single-cell mechanomics in bone**

In the last decade, rapid advances in genomics made it possible to sequence the entire genome or transcriptome simultaneously in hundreds of dissociated single cells from various tissues [63-65]. Single-cell “omics” approaches, such as single-cell RNA-seq, provide an unbiased and complete description of transcriptional heterogeneity in cell populations and thereby are well suited to reveal the responses to a cell in a specific environment (Fig. 2.7) [65].

Additionally, many advances have more recently been made to perform single-cell “omics” approaches in situ [149]. Approaches such as fluorescent-in situ RNA-seq (FISSEQ) [150] or transcriptome in vivo analysis (TIVA) [151], in which omic data are collected from cells or tissues by means that preserve spatial information are now leading to the emergence of a new technology-driven field known as “spatially resolved omics” [149]. TIVA uses a photoactivatable mRNA capture molecule called the TIVA-tag to isolate mRNA of a single cell, which subsequently is sequenced. However, the throughput of this approach is limited in terms of the number of cells that can be labeled and then processed. FISSEQ allows for single cells to be sequenced in situ on tissue sections, with the limitation that only cells in close proximity to one another can be assayed in the same experiment. Alternatively, single-cell
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RNA-seq has been combined with LCM (referred to as “LCM-Seq”) to resolve transcriptome profiles of very low cell numbers and also single cells from both mouse and human brain tissues [152]. As is the case for most single-cell isolation techniques, there are drawbacks to consider when performing LCM. Firstly, it requires expert manual operation, which consequently reduces the throughput of the technique. Secondly, the steps needed for tissue preprocessing (e.g. tissue dissection, cryosectioning, staining) can lead to partial RNA degradation. Lastly, potential RNA contamination from neighboring cells to the cell of interest must be considered. However, the isolation of single cells by LCM has successfully been used in previous studies, albeit not in bone [152-155]. Nevertheless, Trüssel et al. have shown the possibility of isolating small populations of 9 cells from bone [156] and thus, future efforts in this direction might enable the isolation of single cells as well.

![Cell-type discovery](image)

**Fig. 2.7** Cell-type discovery by unbiased sampling and transcriptome profiling of single cells. (a) A sample of cells is taken from the tissue of interest, with the aim of obtaining a representative sample of the types of cells that are present in the tissue. (b) Each cell is profiled using single-cell RNA sequencing (RNA-seq). (c) Subsequently, the resulting expression profiles are clustered. The result is a map of ‘cell space’, in which similar cells are grouped close to each other. The strategy is shown here in cartoon form, but in practice it will be necessary to collect and analyze thousands of cells in each tissue (that is, millions of cells overall) to make a comprehensive cell space map of a whole organism. Reprinted with permission from Shapiro et al. [65] and *Nature Publishing Group.*

Bearing the above in mind, we propose a novel methodological framework within the field of mechanomics [62] utilizing established and emerging technologies: By combining RNA-seq of single cells isolated by LCM with micro-FE analysis based on time-lapsed *in vivo* micro-CT, we are able to link the transcriptome of single cells to their local 3D mechanical micro-environment *in vivo* and to the bone (re)modeling history, providing the opportunity to investigate the molecular responses of different cell types to changes in their native cellular network and mechanical environment (Fig. 2.8).
Fig. 2.8 The mechanical systems biology framework for investigating load-induced bone adaptation is a combined experimental and computational approach which can be separated into three different workflows. With the availability of “omics” technologies, the third workflow can now be updated to allow transcriptomic analysis of the laser-microdissected samples. Adapted from Trüssel et al. [148] with kind permission.

As the current practical resolution (10μm) of in vivo micro-CT is (and most likely always will be) insufficient for imaging single cells directly in the living mouse because of the radiation dose, the proposed single-cell mechanomics framework can be achieved by using a multimodal imaging approach: By registering end-point 2D histological slices into the 3D micro-CT volume, individual cells are made visible in the 3D micro-CT image without an increase in dose. In future, further advances such as “cryo-CT” could provide the possibility to image frozen samples by ex vivo high-resolution micro-CT allowing to image all osteocyte lacunae at single-cell resolution prior to cryosectioning and subsequent cell isolation by LCM. The availability of single-cell resolved data leads to a perspective allowing for the identification of previously unknown markers of different cell types and thereby paves the way to fill many gaps in the molecular and functional characterization of the heterogeneous cell populations involved.
in load-induced bone (re)modeling. Moreover, by integrating data from other “omics” fields such as proteomics [157, 158], and epigenomics [136, 159] that are currently on the rise in bone research, this mechanomics approach can yet be widely expanded to encompass further aspects of the molecular pathways while retaining information of the local mechanical environment in vivo.

2.1.4 Conclusion

Load-induced bone (re)modeling is a multiscale process which is mediated through complex interactions between multiple cell types and their microenvironments. By combining mouse loading models with advanced computational and experimental techniques, methods have been established to locally link (re)modeling activities at the tissue scale to local mechanical environments. At the molecular scale, “omics” technologies are powerful approaches to comprehensively characterize cellular responses to mechanical stimuli.

However, they have not yet been applied to understand bone (re)modeling in its entirety – as a process involving multiple, interacting cell types influenced by their local 3D mechanical microenvironment. The combination of spatially resolved single-cell “omics” with well-established loading models, imaging and multiscale computational frameworks will thereby provide the unique opportunity to reassociate the molecular pathways at the cellular scale to the local mechanical in vivo environment.

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Conflict of Interest

The authors declare no conflict of interest.
References


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Chapter 3

Development of an *in vivo* model of premature aging for the longitudinal assessment of bone and frailty
3.1 Optimization of an in vivo multiscale mechanobiology approach

Ariane C. Scheuren¹, Paul Vallaster¹, Gisela A. Kuhn¹, Graeme R. Paul¹, Bryant Schroeder¹, Duncan C. Tourolle né Betts¹, Angad Malhotra¹, Yoshitaka Kameo¹², Ralph Müller¹

¹ Institute for Biomechanics, ETH Zurich, Zurich, Switzerland
² Institute for Frontier Life and Medial Sciences Kyoto University, Kyoto, Japan

Abstract

It is well established that cyclic, but not static, mechanical loading has anabolic effects on bone. However, the function describing the relationship between the loading frequency and the amount of bone adaptation remains unclear. We hypothesized that load-induced bone adaptation at the tissue scale is controlled by local mechanical signals and that net bone changes will depend on loading frequency. By combining in vivo micro-computed tomography (micro-CT) imaging with micro-finite element (micro-FE) analysis, we monitored changes in microstructural as well as mechanical environments (strain energy density (SED) and SED gradient) of mouse caudal vertebrae over 4 weeks of either static or cyclic loading at varying frequencies of 2Hz, 5Hz, or 10Hz. Furthermore, using an approach termed “Local in vivo Environment histochemistry”, we linked protein level data obtained by immunohistochemistry to the corresponding local remodeling and mechanical environments in vivo.

Higher values of SED and SED gradient led to an increased probability of bone formation and a decreased probability of bone resorption. Compared to the sham- and static loading groups, cyclic loading induced an overall positive net remodeling rate, mainly due to an increase in the mineralizing surface and a decrease in the eroded surface. Consequently, the bone volume fraction (BV/TV) increased over time in 2Hz, 5Hz and 10Hz, while static loading led to a slight decrease in BV/TV. Furthermore, regression analysis revealed a logarithmic relationship between loading frequency and the relative change in BV/TV (∆BV/TVweek4/week0, R²=0.74). Compared to sham-loading, cyclic loading resulted in a global downregulation of Sclerostin and RANKL expression. Furthermore, osteocytes from the cyclic loading groups that were close to resorption surfaces had higher RANKL expression compared to those close to
formation surfaces. In conclusion, these results suggest that bone adaptation is regulated by the local mechanical environment and furthermore, there seems to be a logarithmic behavior with frequencies below a certain threshold having catabolic effects, and those above anabolic effects.

**Keywords:**

Bone adaptation, mechanical loading, *in vivo* micro-CT imaging
3.1.1 Introduction

It is well established that cyclic, but not static, loading has anabolic effects on bone [1-4]. This clear-cut discrepancy in osteogenic responses to both loading patterns highlights the key role of loading frequency in the regulation of bone mechano-adaptation. Yet, the exact relationship between loading frequency and bone adaptation remains unclear. While both experimental [5-7] and theoretical studies [8, 9] have suggested a dose-response relationship such that bone formation increases with higher loading frequencies, Warden and Turner have shown this relationship to rather be of non-linear nature [10]. Specifically, this study used an axial loading model of mouse ulnae and showed that cortical bone adaptation increased with frequencies up to 5 and 10Hz, but then plateaued thereafter. In line with these results, more recent in silico studies have found non-linear relationships between loading frequency and bone adaptation both in the cortical [11] as well as in trabecular [12] bone. In the latter study, a single trabecula was subjected to cyclic uniaxial loading at varying frequencies of either 1Hz, 3Hz, 5Hz, 10Hz, 20Hz, respectively. Similar to the study by Warden et al., the bone volume fraction increased up to 10Hz but then plateaued thereafter [12]. However, owing to the lack of in vivo studies investigating the effects of loading frequency on trabecular bone adaptation, the validity of such in silico studies remains unclear. Furthermore, as frequency effects have been shown to vary depending on the anatomical region investigated [13], the optimal frequency must be identified for every specific loading model.

One of the most widely suggested theories explaining the relationship between loading frequency and bone adaptation is the induction of fluid flow through the lacuna-canalicular network (LCN), which plays a major role in determining the local mechanical environment surrounding osteocytes [14-16]. Known as the main mechanosensors in bone, osteocytes sense shear stresses caused by fluid flow and subsequently release signaling molecules such as Sclerostin, RANKL and OPG, thereby orchestrating the recruitment and activity of osteoblasts and osteoclasts [17-22]. Although several studies have investigated the effects of frequency on signaling pathways in bone remodeling in vitro [23, 24] and in vivo [25], the availability of methods that enable an investigation of the effects of loading frequency across multiple scales – from the entire organ down to the molecular scale – remain scarce.

By combining time-lapsed micro-computed tomography (micro-CT) imaging with micro-finite element (micro-FE) analysis in a mouse model of load-induced bone adaptation [26], we have
previously shown that bone formation and resorption are controlled by local mechanical signals at the tissue level [27-29]. Furthermore, using a “Local in vivo Environment histochemistry” (LivE) approach, based on the registration of two-dimensional (2D) histological sections into the three-dimensional (3D) in vivo micro-CT volume, individual cells can be mapped into the 3D time-lapsed micro-CT data allowing time tracking and grouping of cells according to their local in vivo environment [30, 31]. In this respect, immunohistochemistry using anti-Sclerostin antibodies revealed that the expression of Sclerostin, an osteocyte-specific inhibitor of bone formation, was directly linked to the bone remodeling and mechanical microenvironments [32]. In this study, we therefore aimed to investigate the effect of varying loading frequencies on trabecular bone adaptation in mouse caudal vertebrae. Specifically, we used time-lapsed in vivo micro-CT imaging to monitor bone adaptation over time in individual animals in response to static as well as cyclic loading at frequencies of 2Hz, 5Hz and 10Hz. Compared to conventional 2D histomorphometric techniques, which have previously been used to investigate effects of varying frequencies on bone adaptation [1, 4, 7, 13], the registration of consecutive time-lapsed micro-CT images provides the possibility to directly quantify not only bone formation but also resorption [33], which could be pivotal in better understanding the contrasting effects observed after static and cyclic loading. Furthermore, combined with micro-FE analysis and immunohistochemistry, we sought to investigate whether bone adaptation can be linked to local mechanical environments at the tissue and molecular scale.

3.1.2 Materials and Methods

Study Design

To investigate the effect of loading frequency on mouse caudal vertebrae, 50 11-week old female C57BL/6J mice were purchased (Charles River Laboratories, France) and housed at the ETH Phenomics Center (12h:12h light-dark cycle, maintenance feed and water ad libitum, 3-5 animals/cage) for one week. To enable mechanical loading of the 6th caudal vertebrae (CV6), stainless steel pins (Fine Science Tools, Heidelberg, Germany) were inserted into the fifth and seventh caudal vertebrae of all mice at 12 weeks of age. After three weeks of recovery, the mice received either sham (0N), 8N static or 8N cyclic loading with frequencies of 2Hz, 5Hz, or 10Hz and were scanned weekly using in vivo micro-CT. All procedures were performed under isoflurane anaesthesia (induction/maintenance: 5%/1-2% isoflurane/oxygen). All mouse
experiments described in the present study were carried out in strict accordance with the recommendations and regulations in the Animal Welfare Ordinance (TSchV 455.1) of the Swiss Federal Food Safety and Veterinary Office (license number 262/2016).

**Mechanical loading**

The loading regime was performed for five minutes, three times per week over 4 weeks as described previously [26]. For the cyclic loading groups, forces varying sinusoidally forces (8N amplitude) were applied at 2Hz, 5Hz or 10Hz resulting in cycle numbers of 600, 1500 and 3000, respectively. For the static loading group, the force was maintained at 8.5N during the five minutes. For the sham-loaded group, the tails were fixed in the loading device for five minutes, but no loading was applied (0N).

**Micro-CT imaging and analysis**

*In vivo* micro-CT (vivaCT 40, Scanco Medical AG, isotropic nominal resolution: 10.5 μm; 55 kVp, 145 μA, 350 ms integration time, 500 projections per 180°, scan duration ca. 15 min, radiation dose per scan ca. 640 mGy) images of the CV6 were performed every week. Micro-CT data was processed and standard bone microstructural parameters were calculated in trabecular, cortical and whole bone by using automatically selected masks for these regions as described previously [34]. To calculate dynamic morphometric parameters, micro-CT images from consecutive time-points were registered onto one another. The voxels present only at the initial time point were considered resorbed whereas voxels present only at the later time point were considered formed. Voxels that were present at both time points were considered as quiescent bone. By overlaying the images, morphometrical analysis of bone formation and resorption sites within the trabecular region allowed calculations of bone formation rate (BFR), bone resorption rate (BRR), mineral apposition rate (MAR), mineral resorption rate (MRR), mineralizing surface (MS) and eroded surface (ES) [33].

**Micro-Finite Element (micro-FE) analysis**

For each mouse at each time point, segmented image data was converted to 3D micro-FE models, with additional voxels added to the proximal and distal ends of the vertebrae mimicking intervertebral discs. All voxels were converted to 8 node hexahedral elements and assigned a Young’s modulus of 14.8 GPa and a Poisson’s ratio of 0.3 [26]. The bone was assumed to have linear elastic behaviour, which allowed for static loading in the micro-FE analysis [35]. The top
was displaced by 1% of the length in z-direction (longitudinal axis), while the bottom was constrained in all directions. The micro-FE model was solved using a micro-FE solver (ParOSol). The results were then rescaled to an applied force of 8N for the loaded groups and 4N (physiological loading) for the sham-loaded group (0N) as described previously [36].

**Mechanical environment**

The mechanical stimuli which are hypothesized to drive load induced bone adaptation are deformation (direct cell strain) and interstitial fluid flow (shear stress) [37]. As a measure of the mechanical deformation, strain energy density (SED) magnitudes, defined as the increase in energy associated with the tissue deformation per unit volume, were analysed on the bone surface on the marrow-bone interface. Furthermore, based on the assumption that spatial differences in tissue deformation induce fluid flow, the spatial gradient of the SED was analyzed on the marrow side of the marrow-bone interface [38]. The spatial gradients in x, y and z-direction were calculated as follows:

$$\frac{D_{ena_i} - D_{ena_{i+1}}}{2\Delta z} \text{ for voxel } 1 < i < N_x$$

Where $D_{ena_i}$ is the SED of a voxel at $x, y, z$-position $i$, $N_{x,y,z}$ the number of voxels in the $x,y,z$-direction and $\Delta$ the nominal resolution. The norm of the gradient vector ($\nabla$SED) was used as a quantity for the fluid flow as described previously [28].

$$\nabla \text{SED} = \sqrt{\left(\frac{D_{ena_x}}{\Delta x}\right)^2 + \left(\frac{D_{ena_y}}{\Delta y}\right)^2 + \left(\frac{D_{ena_z}}{\Delta z}\right)^2}$$

The conditional probabilities for a certain remodeling event (formation, quiescence, resorption) to occur at a given value of SED and $\nabla$SED were calculated as described previously [27]. Briefly, the surface SED and $\nabla$SED values were normalized within each animal and measurement by the maximal SED or $\nabla$SED, respectively (chosen as the 99th percentile of the values present at the surface and in the volume of interest (VOI)) in order to remove the variance due to temporal bone adaptation, applied force in FE analysis and individual animals. For each region (formation, quiescence and resorption), a frequency density histogram with 50 bins and equal bin width was created. In order to rule out the dependence on the imbalance between bone formation and resorption, all remodeling events were assumed to have the same occurrence probability (i.e., formation, resorption and quiescent regions were rescaled to have the same...
amount of voxels). The remodeling probabilities were fitted by exponential functions using non-linear regression analysis.

To quantify the modeling performance of SED and ∇SED, the area under the curve (AUC) of a receiver operating characteristic (ROC) curve was used. The AUC can be defined as the probability that a randomly selected case (“true”) will have a higher test result than a randomly selected control (“false”) [39]. The ROC curve is a binary classifier, therefore the three different surface regions were analysed separately and only voxels and mechanical quantity values on the bone or marrow surface were used for the classification.

Histological processing and immunohistochemistry

Twenty-four hours after the last loading cycle, the CV6 were harvested and fixed in 4% neutrally buffered formalin for 24 hours followed by decalcification in 12.5% EDTA for 10 days. The samples were embedded in paraffin and 5 µm thick longitudinal sections were stained either with Hematoxylin and Eosin (H&E) or processed for immunohistochemistry (IHC) using primary antibodies against Sclerostin (1:100, AF1589, R&D Systems) and RANKL (1:100 abcam, ab 9957). The sections were deparaffinized with xylene and rehydrated through graded ethanol. The primary antibodies were incubated overnight at 4°C, rinsed with TBS-Tween and blocked for 10 minutes at room temperature (RT) using peroxidase-blocking buffer (S2023, Dako). The slides were incubated either with an anti-goat IgG HRP (for Sclerostin) or with Envision+ System HRP Labelled Polymer Anti-Rabbit (K4003, DAKO, for RANKL) for 60 minutes at RT. Diaminobenzidine (K3468, Dako) was used as detection substrate. Counterstaining was performed with FastGreen (F7258, Sigma-Aldrich, St. Louis, MO). Species-specific IgG was used as isotype control. Images were taken with Slide Scanner Pannoramic 250 (3D Histechn, Budapest, Hungary) at 20x magnification.

Image registration & evaluation of osteocytes

2D histology sections without visible artefacts were downsampled to the resolution of the in vivo micro-CT scanner, binarized by manual contouring and manually registered into the 3D micro-CT image using an in-house developed registration tool. To investigate the protein expression of individual osteocytes, one histology section per group was analysed as previously described [30]. Specifically, the signal of the counterstain staining (blue) was removed by splitting the RGB colour channels, and the images of the blue channel were used to threshold below the background value (thresholdSclerostin=180, thresholdRANKL=130 for RANKL) to separate the
stained area from the non-stained area. The number of stained pixels was measured on each osteocyte and served as a non-quantitative measure of the protein expression in individual osteocytes.

_Scale 3D visualization and qualitative analysis_

Osteocyte positions were mapped into the 3D micro-CT coordinates and the corresponding micro-FE simulation results using an in-house developed 2D/3D registration tool. A Python procedure was developed to visualize the cellular protein level data within the 3D bone volume in ParaView (Paraview 4.3.1. Kitware Inc.). Briefly, spheres were inserted into the _in vivo_ 3D model of the bone volume and the corresponding micro-FE models to indicate the location of individual osteocytes. These were then color-coded according to positive (black) or negative (red) SCL staining.

**Grouping of osteocytes**

To link the protein level data to the remodeling events, the osteocytes were grouped according to the activity on the closest bone surface i.e., either to formation, quiescence or resorption. Specifically, for each osteocyte the Euclidean distances to the closest formation, quiescence and resorption surfaces were measured and the smallest distance was used to classify the osteocytes to either formation, quiescence and resorption. Osteocytes with an equal distance to multiple surfaces were excluded from the analysis. To link the protein level data to the mechanical environments, the maximal SED (SED$_{\text{max}}$) surrounding each osteocyte in a cube with a length of 6 voxels (63 µm) was measured. The change in SED was calculated according to the following formula: $\Delta$SED$_{\text{max}}$ = SED$_{\text{max}}$ (t=week4) − SED$_{\text{max}}$(t=week2) and osteocytes were grouped according to the sign of $\Delta$SED$_{\text{max}}$.

**Statistical analysis**

Data are represented as mean±SD. For analysis of the longitudinal measurements of bone structural parameters, repeated measurements ANOVA implemented as a linear mixed model was used using the lmerTEST package [40] in R (R Core Team (2019), R Foundation for Statistical Computing, Vienna, Austria). The between subjects effect was allocated to the different groups (sham, static, 2Hz, 5Hz, 10Hz) while the within-subjects effects were allocated to time and time-group interactions. Random effects were allocated to the animal to account for the natural differences in bone morphometry in different mice. In cases where a significant
interaction effect (group*time) was found, a Tukey post-hoc multiple comparisons test was performed. For comparisons between groups one-way Anova analysis followed by Tukey’s or Dunnet’s multiple comparisons test were performed as stated in the corresponding figure legends. Significance was set at α<0.05 in all experiments.

3.1.3 Results

Bone adaptation to load is dependent on loading frequency

In order to investigate the effects of varying loading frequencies on bone adaptation, we used an in vivo micro-CT approach to monitor bone adaptation of the 6th caudal vertebrae of C57BL/6J mice subjected to a 4-week loading regime of either sham (0N), 8N static or 8N cyclic loading with frequencies of 2Hz, 5Hz, or 10 Hz, respectively. Table 3.1.1 shows the absolute difference between the first and last time point (i.e., bone parameter

The sham and static loading groups showed a net decrease in BV/TV and Tb.Th (absolute difference <0), while the cyclic loading groups at 2Hz, 5Hz and 10Hz had a net increase in BV/TV and Tb.Th, being significantly different to sham. In respect to the structural parameters of the cortical bone, the absolute difference between the first and last time point was significantly different between groups for cortical area fraction (Ct.Ar/Tt.Ar) and cortical thickness (Ct.Th), with the cyclic loading groups having a significantly greater increase compared to the static and sham-loaded groups.
Table 3.1.1 Absolute difference between week 0 and week 4 of the bone structural parameters in the trabecular and cortical region for the different groups. P-values denote a significant difference between groups determined by one-way ANOVA, while “a” denotes significant difference to control as assessed by multiple comparisons Dunnett’s test.

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>static</th>
<th>2Hz</th>
<th>5Hz</th>
<th>10Hz</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>-0.93</td>
<td>-1.408</td>
<td>2.333</td>
<td>3.240</td>
<td>3.680</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>±0.789</td>
<td>±1.392</td>
<td>±1.315a</td>
<td>±1.692a</td>
<td>±1.084a</td>
<td></td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.006</td>
<td>0.006</td>
<td>0.021</td>
<td>0.020</td>
<td>0.021</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>±0.005</td>
<td>±0.005</td>
<td>±0.01a</td>
<td>±0.007a</td>
<td>±0.006a</td>
<td></td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>0.263</td>
<td>-0.286</td>
<td>-0.234</td>
<td>-0.341</td>
<td>-0.295</td>
<td>0.750</td>
</tr>
<tr>
<td></td>
<td>±0.122</td>
<td>±0.076</td>
<td>±0.127</td>
<td>±0.082</td>
<td>±0.221</td>
<td></td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.034</td>
<td>0.037</td>
<td>0.031</td>
<td>0.043</td>
<td>0.034</td>
<td>0.883</td>
</tr>
<tr>
<td></td>
<td>±0.019</td>
<td>±0.009</td>
<td>±0.024</td>
<td>±0.013</td>
<td>±0.027</td>
<td></td>
</tr>
<tr>
<td>Ct.Ar/Tt.Ar (%)</td>
<td>0.507</td>
<td>0.524</td>
<td>2.746</td>
<td>3.838</td>
<td>3.496</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>±1.187</td>
<td>±1.931</td>
<td>±0.950a</td>
<td>±2.209a</td>
<td>±1.733a</td>
<td></td>
</tr>
<tr>
<td>Ct.Th (mm)</td>
<td>0.004</td>
<td>0.005</td>
<td>0.013</td>
<td>0.014</td>
<td>0.014</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>±0.005</td>
<td>±0.005</td>
<td>±0.005a</td>
<td>±0.013a</td>
<td>±0.009a</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.1.1 shows the relative changes in trabecular bone morphometric parameters over the 4-week loading period for the different loading groups. BV/TV developed differently over time between the loading groups (interaction effect, p<0.0001). Compared to the sham-loaded group, which showed no change in BV/TV over time (-6%, p>0.05), cyclic loading at all frequencies (2Hz, 5Hz and 10Hz) lead to an increase in BV/TV over time (Fig. 3.1.1A). Herein, the 5Hz and 10Hz groups showed a significant increase compared to baseline already 2 weeks after the start of loading (p=0.0002 and p<0.0001), while the 2Hz group showed a significant increase relative to baseline only after three weeks (p=0.0006). At the end of the 4-week loading regime, these groups showed a 15%, 21% and 24% higher BV/TV relative to baseline (p<0.0001 for 2Hz, 5Hz and 10Hz). Static loading on the other hand, had catabolic effects resulting in significantly lower BV/TV (-9%, p=0.0015) at the last time point relative to baseline. In line with the changes in BV/TV, Tb.Th developed differently over time between the loading groups (interaction effect, p<0.0001, Fig. 3.1.1B). By the end of the 4-week loading intervention, all cyclic loading groups showed significant increases in Tb.Th (p<0.0001), which was not observed in the static and sham-loaded groups (p>0.05). Although the number of trabeculae (Tb.N) as well as the separation in between trabeculae (Tb.Sp) changed over time (Fig. 3.1.1C,D, p<0.001), no differences were observed between the different groups (p>0.05). These results thus suggest that increases in BV/TV due to cyclic loading were mainly driven by thickening of the trabeculae rather than by the inhibition of the reduction in the number of trabeculae.
3.1 Optimization of an in vivo multiscale mechanobiology approach

- **Fig. 3.1.1** Relative changes of structural bone morphometric parameters in the trabecular compartment over the 4-week loading period as assessed by in vivo micro-CT. (A) bone volume fraction (BV/TV), (B) trabecular thickness (Tb.Th), (C) trabecular number (Tb.N) and (D) trabecular spacing (Tb.Sp). (Data represent mean±standard deviation (SD) for n=5-8/group, p-values for interaction effect between group and time are shown as determined by linear mixed effects model) (E) The relative change from week 4 relative to baseline (BV/TVweek4/BV/TV0) (F) was fitted with a logarithmic regression line. (Data represent mean±SD for n=5-8/group, p-value for main effect of group determined by one-way ANOVA, **** p<0.0001 denotes significant difference between groups determined by post hoc Tukey’s multiple comparisons test)
Chapter 3 Development of an in vivo model of premature aging for the longitudinal assessment of bone and frailty

By plotting the relative changes in BV/TV as a function of loading frequency, regression analysis revealed a logarithmic relationship between bone adaptation and loading frequency \((R^2=0.74, \text{Fig. 3.1.1F})\) with loading frequencies above 0.36Hz having anabolic effects, and frequencies below this threshold having catabolic effects. Although there were no significant differences between the cyclic loading groups, loading at 10Hz had the earliest and largest anabolic effects compared to the other frequencies.

Aside from providing information on changes in bone structural parameters over time, \textit{in vivo} micro-CT also provides the possibility to assess dynamic bone formation and resorption activities such as bone formation/resorption rate (BFR/BRR), mineral apposition/resorption rate (MAR/MRR) and mineralizing/eroded surface (MS/ES). The net remodeling rate (BFR-BRR), which gives an indication whether there was overall bone gain (i.e., BFR-BRR>0) or loss (i.e., BFR-BRR<0) occurring within the trabecular compartment, tended to develop differently between groups \((p=0.05569)\). Compared to the static and sham-loaded groups, which had an overall negative remodeling balance, the 2Hz, 5Hz and 10Hz had an overall positive remodeling balance \((p=0.0031, p=0.0009 \text{ and } p<0.0001)\) (Fig. 3.1.2A). The net remodeling rate did not significantly change over time. When the bone formation and resorption rates were analyzed separately, it is clear that the main differences lie in the reductions in BRR in the cyclic loading groups. While the BFR did not significantly differ between groups \((p>0.05, \text{Fig. 3.1.2B})\), the BRR was 35% \((p=0.0028)\), 50% \((p=0.0001)\) and 44% \((p=0.0001)\) lower in the 2Hz, 5Hz, 10Hz groups, respectively, compared to the sham-loaded group (Fig. 3.1.2C). The static group on the other hand had a similar BRR \((-2\%, p>0.05)\) as the sham-loaded group (Fig. 3.1.2C).

A difference between the cyclic and static loading groups was also apparent when investigating the surfaces of formation (mineralized surface, MS, interaction effect \(p=0.024\)) and resorption (eroded surface, ES, interaction effect \(p=0.389\)) sites with the cyclic loading groups having a higher MS and lower ES compared to the static and sham-loaded groups (Fig. 3.1.2D). On average, formation sites occupied 2, 2.5 and 2.6 more surface than resorption sites for the 2Hz, 5Hz and 10Hz groups, respectively, and only 1.4 times more for the control and static groups.
Fig. 3.1.2 Dynamic bone morphometric parameters in the trabecular compartment in the different loading groups as assessed by *in vivo* micro-CT. (A) Changes in the net remodeling rate shown as the difference between bone formation rate (BFR) and bone resorption rate (BRR) over the 4-week loading period. Overall difference between groups of (B) BFR and (C) BRR. (D) Mineralized surface (MS) and eroded surface (ES) over the 4-week loading period. Overall difference between groups of (E) MS and (F) ES. (G) Mineral apposition rate (MAR) and mineral resorption rate (MRR) over the 4-week loading period. Overall difference between groups of (H) MAR and (I) MRR. (Data represent mean±SD for n=5-8/group, p-values for interaction effect between group and time are shown as determined by linear mixed effects model (A,D,G), boxplots showing the differences between groups as determined by Tukey’s post hoc multiple comparisons test * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (B,C,E,F,H,I))

Furthermore, the 2Hz, 5Hz and 10Hz groups had a 18% (p=0.0078), 25% (p=0.0007) and 26% (p>0.0001) higher mineralized surface (MS) and a 22% (p<0.0001), 32% (p<0.0001) and 26% (p<0.0001) lower eroded surface (ES) compared to the sham-loaded group, while the static group had similar MS and ES compared to sham-loading (p>0.05, Fig. 3.1.2E-F). The mineral apposition and resorption rates (MAR and MRR), which represent the thicknesses of formation and resorption packages, respectively, did not develop differently between groups (interaction
effects \( p=0.586 \) and \( o=0.459 \). Furthermore, the MAR and MRR were similar between groups (\( p>0.05 \)), thus suggesting that they are not affected by varying loading frequencies (Fig. 3.1.2G-I). This indicates that cyclic loading had a greater effect on the surface area than on thickness of formation as well as resorption sites.

**Bone adaptation to load is controlled by local in vivo mechanical environments**

In order to assess whether bone remodeling events (formation, resorption, quiescence) can be linked to the corresponding mechanical environments *in vivo*, we performed micro-finite element (micro-FE) analysis to calculate the strain distribution within the tissue. As deformation (direct cell strain) and interstitial fluid flow (shear stress) are hypothesized to be the main mechanical stimuli that regulate load-induced bone adaptation [37], we quantified the strain energy density (SED) magnitudes as a measure of mechanical deformation and the spatial gradient thereof, as a measure of fluid flow, respectively [28, 38]. Figure 3.1.3 shows a representative visualization of a section of the vertebrae of the 10 Hz group showing sites of bone remodeling (Fig. 3.1.3A) as well as the corresponding maps of SED (Fig. 3.1.3B) and \( \nabla \text{SED} \) (Fig. 3.1.3C). From this qualitative analysis, it is apparent that bone resorption occurs at sites of lower SED and SED gradient, whereas bone formation occurs at sites of higher SED and SED gradient (Fig. 3.1.3).
3.1 Optimization of an in vivo multiscale mechanobiology approach

Fig. 3.1.3 Qualitative visualization linking bone (re)modeling sites (formation, quiescence, resorption) with the mechanical environments in vivo. (A) Overlay of time-lapsed micro-CT images showing sites of bone formation (orange), quiescence (grey) and resorption (purple). Corresponding map of the (B) strain energy density (SED) and (C) gradient thereof (VSED) showing sites of higher (red) and lower (blue) SED/SED gradient values obtained by micro-finite element (micro-FE) analysis.

To establish a quantitative description of the mechano-regulation of bone remodeling, we calculated the conditional probabilities for a given (re)modeling event to occur as a function of the mechanical stimuli, also known as remodeling rules [27]. Figure 3.1.4 shows the conditional probability curves for formation (orange), quiescence (grey) or resorption (purple) to occur at a given value of SED (Fig. 3.1.4A,C,E) or VSED (Fig. 3.1.4B,D,F) for the different groups, respectively averaged over all time points. For all groups, the conditional probability for bone formation to occur was higher at higher values of SED and VSED (SED/SED_{max} > 0.18) whereas bone resorption was more likely to occur at lower values (SED/SED_{max} < 0.18). The probability curves for all groups were fit by exponential functions (Table 3.1.S1), of which the coefficients provide information on the functioning of the mechanosensory system as described previously.
[27]. When comparing the slopes of the formation probability curves (parameter \( a \), Fig. 3.1.4A,B and Table 3.1.S1), which can be interpreted as the mechanical sensitivity of the system, there was a gradual increase of the mechanical sensitivity with the 10Hz group showing the highest mechanical sensitivity (\( a_{\text{SED}} = 0.217, a_{\text{SEDgrad}} = 0.316 \)). For the resorption probability curves (Fig. 3.1.4E,F and Table 3.1.S1), the 5Hz and 10Hz groups showed similar mechanical sensitivity to SED (\( a_{\text{SED}} = 0.284 \), while the 5Hz group showed highest sensitivity to \( \nabla \text{SED} \) (\( a_{\text{SEDgrad}} = 0.264 \) compared to \( a_{\text{SEDgrad}} = 0.252 \) in 10Hz group). The probability of the quiescence however, was not influenced by loading frequency (Fig. 3.1.4C,D). When comparing between SED and \( \nabla \text{SED} \) as mechanical stimuli driving bone remodeling, it seems that in all groups, formation was more sensitive to \( \nabla \text{SED} \) shown by the higher slopes (\( a_{\text{SED}} < a_{\text{gradSED}} \)) of the probability curves (Fig. 3.1.4A,B and Table 3.1.S1). In contrast, resorption seemed to be more sensitive to SED (\( a_{\text{SED}} > a_{\text{gradSED}} \), Fig.3.1.4 E,F and Table 3.1.S1).
Fig. 3.1.4 Conditional probabilities connecting the SED (left side) and SED gradient (VSED, right side) with the (A,B) formation (top row), (C,D) quiescence (middle row) and (E,F) resorption (bottom row) events. The plots show the exponential fitting functions for bone formation, resorption and quiescence in all the loading groups averaged over all time points.

To better compare the modeling performance of SED versus VSED, an area under the receiver operator characteristic curve (AUC) approach was used (Fig. 3.1.5). For all groups, the AUC values for formation (for all groups p<0.0001, Fig. 3.1.5A) and resorption (sham p=0.0345, static p=0.0262, 2Hz p=0.0187, 5Hz p=0.0758, 10Hz p=0.0312, Fig. 3.1.6C) events were higher for the VSED compared to SED. No difference was observed for quiescence (Fig. 3.1.5B). These results suggest that VSED has a better modeling performance compared to SED for determining bone formation and resorption events.

Fig. 3.1.5 Area under the curve (AUC) for (A) formation (orange), (B) quiescence (grey) and (C) resorption (violet) for the different groups comparing modeling performance of SED (solid bars) and SED gradient (VSED, striped bars). (Boxplots for n=5-8/group, * p<0.05, **** p<0.0001 differences between groups determined by Tukey’s multiple comparisons test)

After having shown that bone adaptation to load is controlled by local mechanical stimuli at the tissue level, we assessed whether this relationship can also be observed at the protein level. Figure 3.1.6 shows representative histological sections of trabecular bone of caudal vertebrae stained with anti-Sclerostin (A-E) and anti-RANKL (G-K) antibodies, respectively. At the global level, cycling loading at 2Hz, 5Hz and 10Hz resulted in a down-regulation of Sclerostin compared to sham- (p<0.00001 for all groups) and static loading (p<0.05 for 2Hz, p<0.0001 for 5Hz and 10Hz, Fig. 3.1.6M). Likewise, RANKL expression was lower in 2Hz (p<0.0001), 5Hz (p<0.001) and 10Hz (p<0.001) compared to control, whereas only the 2Hz group showed a significant difference to static loading (p<0.05).
Fig. 3.1.6 Representative sections of caudal vertebrae showing osteocytes in trabecular bone stained with anti-Sclerostin (A-E), anti-RANKL (G-K) and isotype controls (F,L), respectively, scale bar = 50 µm. The area of the Sclerostin (M) and RANKL (N) staining in individual osteocytes served as a comparison between low and high protein expression. (Boxplots showing cells from one section per group with the number of analyzed cells being n=170/185 2Hz, n=152/258 5Hz, n=150/232 10Hz, n=256/119 sham, n=163/179 static for Sclerostin and RANKL staining, respectively. ** p<0.01, *** p<0.001, **** p <0.0001 compared to sham, # p<0.05, #### p<0.0001 compared to static determined by one-way Anova and Tukey’s multiple comparison’s test)

In order to assess whether such changes at the protein level can be linked to changes in their local in vivo environments, we used a previously developed “Local in vivo Environment (LivE) histochemistry” approach to register 2D histological sections into the 3D micro-CT and micro-FE volumes [30, 31]. We furthermore extended this approach to integrate the 3D structural, mechanical and molecular information into one single 3D visualization, which not only allows to explore the data interactively in 3D - from the entire organ down to the single-cell level - but also to qualitatively investigate the relationship between the diverse local in vivo environments and the fluctuations of protein levels in individual osteocytes (Fig. 3.1.7).
3.1 Optimization of an in vivo multiscale mechanobiology approach

**Fig. 3.1.7**: Overview describing how the LivE imaging approach allows to create spatial maps of osteocyte networks in diverse microenvironments. By registering 2D histology sections into the corresponding micro-CT and micro-FE volumes, the spatial fluctuations of Sclerostin expression of individual osteocytes can be assessed. Sclerostin positive and negative (displayed in black and red, respectively) are visualized in 2D (upper row) and 3D (bottom row) within their diverse microenvironments i.e., tissue remodeling, SED and SED gradient (∇SED) and change in SED over time (ΔSED).

Figure 3.1.7 shows an example of how individual cells can be visualized both in 2D (upper row) and in 3D (lower row) within the remodeling (showing sites of bone formation (orange), quiescence (grey) and resorption (purple)), SED and SED gradient at the end of the loading period (showing sites of high (red) and low (blue) strains) and the change in SED over time (ΔSED_max, showing an increase (red) or decrease (blue) in SED over time). Whereas the 2D sections can be informative in showing that Sclerostin negative cells (marked in red box) were located within regions with higher SED values (green) compared to the Sclerostin positive cells (Fig. 3.1.7), the corresponding 2D visualization of the tissue developmental changes suggested that all cells were in an area of quiescence (i.e., where neither formation nor resorption
occurred). However, looking at the same cells in 3D revealed that the Sclerostin negative cells were indeed surrounded by bone formation, whereas the Sclerostin positive cells were surrounded by bone resorption. Likewise, the 3D SED map was more clearly distinguishable showing a region of lower SED (blue) values surrounding the Sclerostin positive cells and higher SED values (green/yellow) surrounding the Sclerostin negative cells. When looking at the change in SED over time, Sclerostin negative cells were also located in regions in which the SED increased over time, whereas Sclerostin positive cells experienced a decrease in SED over time. Hence, this qualitative analysis highlights the benefits of mapping cells within their local 3D environment and suggests that spatial fluctuations of the osteocytic Sclerostin expression are linked to the 3D local tissue remodeling and mechanical environment surrounding individual cells in trabecular bone.

Finally, individual osteocytes of the sham, static and cyclic loading groups were grouped according to their local remodeling (Fig. 3.1.8A,B) and mechanical (Fig. 3.1.8C,D) environments and the Sclerostin and RANKL expression was compared. The Sclerostin expression did not significantly differ between cells close to resorption or formation regions (Fig. 3.1.8A). The RANKL expression was similar between regions for sham and static loading groups, whereas in the cyclic loading groups, the RANKL expression was significantly higher in cells close to resorption surfaces compared to cells close to formation surfaces (Fig. 3.1.8B). No significant differences were found when the cells were grouped according to areas of increasing or decreasing SED over time (Fig. 3.1.8C,D), nor to SED or gradient SED (data not shown).
Fig. 3.1.8 Sclerostin and RANKL expression of osteocytes grouped according to their local (A,B) remodeling and (C,D) mechanical environments in vivo. (A) Sclerostin and (B) RANKL expression in sham, static and cyclic loading groups were grouped according to the nearest remodeling surface (formation, quiescence, resorption displayed in orange, grey, purple, respectively). Osteocytes with resorption on the closest surface show a significantly higher RANKL expression compared to osteocytes near a formation surface. (Boxplots with total cells per group: n_sham,Sclerostin =159, n_stat,Sclerostin =137, n_cyclic,Sclerostin=363 and n_sham,RANKL =86, n_stat,RANKL =120, n_cyclic,RANKL=530, * p<0.05 determined by Kruskall Wallis test and post hoc Dunn’s multiple comparison’s test). (C) Sclerostin and (D) RANKL expression in osteocytes grouped according to areas of increasing or decreasing SED\textsubscript{max} from week 2 to week 4 (positive (red) vs negative (blue) ΔSED\textsubscript{max}). (Boxplots with total cells per group: n_sham,Sclerostin =252, n_stat,Sclerostin =260, n_cyclic,Sclerostin=483 and n_sham,RANKL =119, n_stat,RANKL =181, n_cyclic,RANKL=577)
3.1.4 Discussion

In this study, the effects of static as well as cyclic loading at varying frequencies on trabecular bone adaptation in mouse caudal vertebrae were investigated. Furthermore, using a combination of in vivo micro-CT, micro-FE analysis and end-point immunohistochemistry, we assessed whether local bone remodeling events can be linked to diverse mechanical environments in vivo.

While static loading had catabolic effects, cyclic loading at 2Hz, 5Hz and 10Hz had anabolic effects on trabecular bone. In line with previous studies using the tail loading model [29, 34], cyclic loading over four weeks lead to an increase in BV/TV, which was driven by an increase in the thickening of individual trabeculae rather than a prevention of loss in the trabecular number. Furthermore, by registering consecutive time-lapsed images onto one-another, we were able to quantify both formation as well as resorption activities in three dimensions [34], which to the best of our knowledge, has not yet been used to assess the effects of static loading regimes. Specifically, we showed that cyclic loading mainly affects the surfaces of bone formation and resorption sites (MS and ES) rather than the thickness of these sites (MAR and MRR). In agreement with previous studies [33, 34], these results thus suggest that cyclic loading promotes osteoblast recruitment, while simultaneously inhibiting the recruitment of osteoclasts. Ultimately, cyclic loading thus results in larger mineralized surfaces and smaller eroded surfaces.

Notably, this study showed a logarithmic relationship between loading frequency and load-induced bone adaptation with frequencies above a certain threshold having anabolic effects and those below having catabolic effects. The fact that cyclic but not static loading has anabolic effects on cortical bone has been shown in various animal models including rabbits [2], turkeys [1] and rats [3, 4]. However, to the best of our knowledge the effect of static loading has not yet been assessed in trabecular bone. In line with the existence of a frequency threshold to elicit anabolic responses, Turner et al. found that bone formation rate in rat tibiae only increased with frequencies above 0.5Hz, followed by a dose-response increase up to 2Hz [5]. Using a similar design as our study, Warden et al. showed increased cortical bone adaptation with increasing loading frequencies up to 5-10Hz with no additional benefits beyond 10Hz [10]. In a theoretical study, Kameo et al. furthermore showed similar results by subjecting individual trabeculae to uniaxial loading at frequencies ranging from 1 to 20Hz [12]. Although one would expect higher
loading frequencies to lead to higher cellular stimulation and a consequent greater anabolic response, it has been suggested that frequencies above a certain threshold (10Hz) reduce the efficiency of fluid flow through the LCN, thus resulting in inefficient mechanotransduction [10, 41]. More recently, by monitoring Ca\(^{2+}\) signaling in living animals, Lewis et al. have shown that osteocyte recruitment was strongly influenced by loading frequency [25]. Another physiological system, for which the relationship between frequency and mechanotransduction is widely studied, is the inner ear [42, 43]. Hair cells, the cells responsible for mechanotransduction in human hearing, are sensitive to frequency [42, 44]. Furthermore, in humans, the perception of sound is limited to a specific range of frequencies; below and above which no sound is perceived. Hence, drawing an analogy to the theory of sound pressure level, it is possible that bone’s response to frequency is similar to the logarithmic perception of sound in human hearing.

One limitation of this study was that loading at low (1 Hz) and higher (>10 Hz) frequencies was not assessed. Furthermore, as the strain magnitude and duration of individual loading bouts were the same for all loading groups, the number of cycles and strain rate differed between the different loading groups. From this study design, it therefore remains impossible to know whether it is the number of cycles or the loading frequency that are the main factors driving load-induced bone adaptation. Hence, whether bone’s osteogenic response to loading is indeed limited to a specific range of frequencies, below and above which bone becomes less osteogenic, requires further in vivo experiments.

Using the combined approach of time-lapsed in vivo micro-CT imaging and micro-FE analysis, we showed that bone remodeling activities were correlated to the local mechanical environment at the tissue level. In agreement with previous studies [27, 29], bone formation was more likely to occur at sites of higher SED whereas bone resorption was more likely to occur at sites of lower SED. Furthermore, compared to static loading, cyclic loading decreased the probability of non-targeted bone remodeling occurring, which led to an increase in bone formation and a decrease in bone resorption. In addition, we showed that the SED gradient could be better at predicting bone formation and resorption events compared to SED. As the SED gradient encompasses the neighboring SED voxels, it provides information of a broader mechanical environment, which could explain the higher modeling performance observed with the SED gradient compared to SED. That the SED gradient can improve predictions of remodeling events compared to SED has been suggested [28]. At this point, a further limitation of this study
was that the micro-FE analysis did not take into account the component of frequency. Although our approach enabled us to link bone remodeling events to mechanical environments in vivo at the local level, the addition of theoretical models that incorporate cellular mechanosensing and intercellular communication [12] will be highly useful to improve our understanding of the relationship between loading frequency and bone adaptation across multiple scales.

At the molecular scale, we observed significantly lower Sclerostin and receptor activator of nuclear factor-kB ligand (RANKL) expression in cyclic loading groups compared to sham. Furthermore, in line with the catabolic effects observed with static loading, the Sclerostin expression was lower in the cyclic loading groups compared to the static loading group with this difference being less pronounced in the RANKL expression. Load-induced downregulation of Sclerostin, an osteocyte-specific inhibitor of bone formation [22, 45-48], and of RANKL, an osteoclast stimulatory factor, have previously been reported [24, 49]. Furthermore, studies applying oscillating fluid flow (OFF) in vitro have shown a frequency dependency in RANKL/OPG gene expression in osteocyte-like cells [50] and in murine bone marrow stromal cells [24]. In one study, OFF both at frequencies of 1Hz and 10Hz resulted in a decrease in the mRNA expression of the RANKL/osteoprotegerin (OPG) ratio, whereas this decrease was not observed with OFF at 20Hz [24]. Interestingly though, the different loading frequencies affected the RANKL and OPG mRNA expression differently. Specifically, the downregulation of RANKL/OPG ratio in the cells treated with OFF at 1Hz was due to an increase in OPG mRNA, whereas at 10Hz, the decrease was caused by a decrease in RANKL mRNA [24], suggesting that there may be an optimal loading frequency where the synergistic effect between decrease in RANKL and increase in OPG is maximal. Hence, future studies should include OPG so that the relative ratio between RANKL/OPG can be assessed. By grouping osteocytes according to their nearest remodeling surface (i.e., either to formation, resorption or quiescence), we observed that RANKL expression was higher in regions close to resorption compared to regions close to formation, whereas no significant difference between regions was found for the Sclerostin expression. Furthermore, no differences were observed when the cells were grouped according to areas with increasing or decreasing SED. Using a similar study design, Trüssel et al. showed significant differences in the Sclerostin expression between the formation and resorption groups and between areas of increasing and decreasing SED [30]. Furthermore, Rankl mRNA tended to decrease with increasing mechanical stimuli, which was not the case for Sost mRNA [30]. That Sclerostin is more closely linked to remodeling than to
mechanical stimuli has also been reported in cortical bone [46]. In the current study, the number of analysed cells was low and hence, future studies investigating multiple sections per animal and from multiple animals would be required to investigate whether changes in protein expression can be directly linked to the mechanical and remodeling environments in vivo. Furthermore, the changes in SED at additional time-points should be assessed. Nevertheless, the LivE imaging approach has been further extended such that the data can be interactively and qualitatively explored in 3D, which will be useful in future studies investigating the link between protein level data and the local environments in vivo.

In conclusion, this study shows that the local mechanical environment regulates bone adaptation and furthermore, there seems to be a logarithmic behavior with frequencies below a certain threshold having catabolic effects, and those above anabolic effects on bone. Finally, we present an extended LivE histochemistry approach that allows to link protein level data to local remodeling and mechanical environments in vivo.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.
Supplementary Materials

Table 3.1.S1 Summary of non-linear regression functions and corresponding coefficients for the conditional probability (SED and SED gradient) in trabecular bone for the different groups averaged over all time points.

<table>
<thead>
<tr>
<th>Regression line</th>
<th>Formation</th>
<th>Resorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F = a \times (1 - \exp(-b \times \text{SED}/\text{SED}_{\text{max}})) + y_0$</td>
<td>$R = a \times \exp(-b \times \text{SED}/\text{SED}_{\text{max}}) + y_0$</td>
</tr>
<tr>
<td>Sham</td>
<td>SED</td>
<td>SED gradient</td>
</tr>
<tr>
<td>a</td>
<td>0.139</td>
<td>0.240</td>
</tr>
<tr>
<td>b</td>
<td>3.471</td>
<td>3.287</td>
</tr>
<tr>
<td>$y_0$</td>
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<td>0.248</td>
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<tr>
<td>$R^2$</td>
<td>0.979</td>
<td>0.993</td>
</tr>
<tr>
<td>Static</td>
<td>SED</td>
<td>SED gradient</td>
</tr>
<tr>
<td>a</td>
<td>0.141</td>
<td>0.249</td>
</tr>
<tr>
<td>b</td>
<td>3.742</td>
<td>3.244</td>
</tr>
<tr>
<td>$y_0$</td>
<td>0.274</td>
<td>0.240</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.973</td>
<td>0.990</td>
</tr>
<tr>
<td>2 Hz</td>
<td>SED</td>
<td>SED gradient</td>
</tr>
<tr>
<td>a</td>
<td>0.197</td>
<td>0.307</td>
</tr>
<tr>
<td>b</td>
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<td>3.775</td>
</tr>
<tr>
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<td>0.205</td>
</tr>
<tr>
<td>$R^2$</td>
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<td>0.995</td>
</tr>
<tr>
<td>5 Hz</td>
<td>SED</td>
<td>SED gradient</td>
</tr>
<tr>
<td>a</td>
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<td>0.311</td>
</tr>
<tr>
<td>b</td>
<td>5.664</td>
<td>4.045</td>
</tr>
<tr>
<td>$y_0$</td>
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<td>0.200</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.979</td>
<td>0.993</td>
</tr>
<tr>
<td>10 Hz</td>
<td>SED</td>
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<tr>
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</tr>
<tr>
<td>b</td>
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<tr>
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<tr>
<td>$R^2$</td>
<td>0.988</td>
<td>0.996</td>
</tr>
</tbody>
</table>
3.1 Optimization of an in vivo multiscale mechanobiology approach

References


3.1 Optimization of an in vivo multiscale mechanobiology approach


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Chapter 3 Development of an in vivo model of premature aging for the longitudinal assessment of bone and frailty


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3.2 Optimization of longitudinal in vivo phenotyping techniques

Ariane C. Scheuren¹, Gisela A. Kuhn¹, Ralph Müller¹

¹Institute for Biomechanics, ETH Zurich, Zurich, Switzerland

Abstract

Frail individuals are at higher risk of experiencing osteoporotic fractures, while at the same time, individuals experiencing a fracture are at higher risk of developing more severe frailty. Although the exact mechanisms linking frailty to senile osteoporosis remain unclear, studies measuring frailty as a predictor of osteoporosis are currently emerging. With the advent of longitudinal in vivo phenotyping techniques such as the clinical mouse frailty index and in vivo micro-computed tomography (micro-CT) imaging, animal models are of great value in aging-related studies as disease progression can be monitored over time in multiple tissues of individual animals. However, as frailty is accompanied by increased sensitivity to external stimuli such as radiation, handling and anesthesia, the extent to which long-term longitudinal micro-CT imaging can be applied without impairing the overall health status of the animals requires further optimization and standardization. In this study, we show how the combination of long-term in vivo micro-CT imaging with longitudinal assessments of the FI can be applied for comprehensive evaluation of the bone phenotype and the development of frailty in a model of accelerated aging (PolgA^{D257A/D257A}). Furthermore, the cumulative effects of radiation, anesthesia and handling associated with longitudinal micro-CT imaging on the bone phenotype as well as on the overall health status of individual mice were assessed.

Specifically, the 6th caudal vertebrae of four groups of mice (with n=12 PolgA^{D257A/D257A} and n=10 PolgA^{+/+} (WT) per group) were monitored by in vivo micro-CT every 2 weeks between the age of 20 and 46 weeks. The first group was evaluated between weeks 20-40 (11 scans), the second group between weeks 26-34, the third between weeks 32-40 and the last group between weeks 40-46 (5 scans each). Frailty was assessed using the FI. Both the bone phenotype as well as the FI developed differently over time between genotypes. Compared to WT, PolgA showed lower bone morphometric parameters (i.e., BV/TV, Tb.Th, Ct.Ar/Tt.Ar, Ct.Th) as well as higher
FI scores with age. Although the long-term monitoring approach in the first group showed small but significant changes in bone morphometric parameters compared to the other groups, no interaction effect between groups and genotype was found. Moreover, the overall health-status of the animals (i.e., body weight and FI) was not affected. Finally, we observed that longitudinal designs including baseline measurements already at young age are more powerful at detecting age-related phenotypic changes than those including multiple groups with fewer imaging sessions.

**Keywords:**

Aging, osteoporosis, bone, longitudinal micro-CT imaging, FI
3.2.1 Introduction

Senile osteoporosis is a widespread degenerative disease of the skeleton characterized by attenuated bone turnover, resulting in a reduction in bone mass and quality, and consequently, increased fracture risk [1, 2]. However, as the skeletal system is only one of many tissues that deteriorate with age, the co-morbidities associated with aging must be considered, rather than focusing only on single diseases at a time. In this respect, the relationship between frailty - defined as an age-related clinical syndrome characterized by the decline of multiple physiological functions, leading to the accumulation of health deficits, and thus a higher vulnerability to adverse health outcomes such as morbidity and mortality [3] - and osteoporosis is becoming of increasing interest in aging research. Indeed, several studies have shown that frailty, measured using tools such as the frailty phenotype [4] or frailty index (FI) [5], is predictive of osteoporotic fractures [6-9], and hence, the combined assessment of frailty and of skeletal health could be beneficial for the clinical diagnosis of osteoporosis and of frailty.

With the advent of longitudinal in vivo phenotyping techniques, animal models are of great value in aging-related studies as disease progression can be monitored over time in multiple tissues of individual animals as well as in response to treatments or interventions. This not only provides a more comprehensive analysis of multi-system dysfunctions but also reduces the number of required animals. In this respect, tools such as the clinical mouse frailty index (FI) [2], with which the accumulation of health deficits can be quantified over time, have been valuable to identify rodent models of frailty in a standardized way [10-12]. Likewise, longitudinal in vivo micro-CT imaging – allowing a non-invasive quantitative and qualitative assessment of the 3D bone micro-architecture over time in individual animals – has become of key importance to investigate time-dependent effects of pathologies and/or treatments in preclinical studies [13-15]. However, although a number of mouse strains have been proposed as potential models for the investigation of senile osteoporosis [16, 17], several recognised limitations have prevented the establishment of a gold-standard model. For example, the commonly used osteoporotic Samp6 mouse has an undefined genetic background [17], while the Klotho mouse, another commonly used model, is infertile and never becomes sexually mature [18]. Additionally, although cortical bone is reduced in Klotho-deficient mice, the model shows increases in trabecular bone in the vertebra and the metaphysis of tibia and femur [18]. Therefore despite the mutation providing relevant decreased bone mineral density in some
skeletal regions, the presence of additional phenotypes irrelevant to senile osteoporosis indicate that an investigation of the molecular pathways leading to these features may not be completely relevant to natural murine or human aging.

With this in mind, the PolgA(D257A/D257A) mutator mouse, which, due to a defect in the proofreading activity of its mitochondrial DNA polymerase gamma exhibits an accelerated aging phenotype, is particularly renowned as it develops multiple signs of aging (e.g., alopecia, graying, kyphosis, hearing loss, sarcopenia etc.) early in life [19, 20]. While frailty has not yet been assessed in this model, previous studies using either X-ray densitometry or as of recently, ex vivo micro-CT have shown reduced bone mineral density and bone volume in PolgA(D257A/D257A) mice (in the following referred to as PolgA) compared to their wild-type littersmates (PolgA(+/-), in the following referred to as WT) [19-21]. However, owing to the cross-sectional designs of these studies, it remains unclear how these differences in the bone phenotype between genotypes develop over time. Furthermore, the bone remodeling activities in PolgA mice have not yet been assessed, making it difficult to evaluate the suitability of this model as a model for senile osteoporosis. In this respect, longitudinal micro-CT imaging provides a major advantage as both bone formation as well as bone resorption activities can be directly quantified by registering consecutive time-lapsed images onto one another [22]. However, despite the numerous advantages of in vivo micro-CT imaging, the cumulative effects of increased radiation, anesthesia and handling on the tissue and organ functions as well as on the general well-being of the animals must be considered [23-26]. Several studies have investigated the effects of repeated in vivo micro-CT imaging on bone morphometric parameters in rodents, however the reports have been controversial [13-15, 27-32]. While radiation associated with time-lapsed micro-CT imaging has been shown to have dose-dependent effects in adolescent rats [31], studies in ovariektomized [13, 27] and adult rats [30] have shown no effects of radiation. Similarly, studies using mouse models have shown small but significant effects in the trabecular bone compartment [27, 29], whereas other studies reported no radiation associated effects on bone morphometric parameters [15, 28, 32]. Furthermore, the effects of radiation on bone morphometry have been shown to be dependent on the age of the animals, with older animals being less susceptible to radiation [29]. Conversely, aged mice are known to be more sensitive to external stimuli such as handling and anesthesia [33, 34], and hence, the extent to which long-term longitudinal micro-CT imaging can be applied in aging studies remains unclear. It is therefore of paramount importance to
3.2 Optimisation of longitudinal in vivo phenotyping techniques

develop optimized and standardized approaches that maximize the data obtained from individual animals without impairing the overall well-being of the animal. The combination of time-lapsed in vivo micro-CT imaging with longitudinal assessments of the FI would therefore not only allow to link age-related changes in bone micro-architecture to other signs of aging, but would also provide information on whether the cumulative effects of radiation, anesthesia and handling negatively impair the overall health status of individual mice.

Therefore, the aims of this study were 1) to analyse the suitability of PolgA mice as a model for frailty and senile osteoporosis and 2) to assess whether long-term longitudinal imaging of individual mice will have biasing effects on bone morphometric parameters and FI scores at various stages of the aging process.

3.2.2 Materials and Methods

Study Design

To investigate how differences in the bone phenotype between PolgA(D257A/D257A) (in the following referred to as PolgA) and PolgA(+/-) (referred to as WT) mice develop with age, longitudinal in vivo micro-CT imaging was used to monitor the 6th caudal vertebrae of PolgA and WT mice between 20 and 46 weeks of age. Furthermore, to assess potential effects of increased radiation, anesthesia and handling associated with micro-CT imaging, the mice (n=88 females) were aged in parallel and divided into four groups (with n=12 PolgA and n=10 WT per group). The first group was scanned over 20 weeks (11 scans between age 20-40 weeks), whereas the other groups were scanned over 8 weeks (5 scans between weeks 26-34, 32-40 and 40-46, respectively) as illustrated in Fig. 3.2.1. For each group, the two last scans overlapped with the two first scans of the subsequent group to allow comparison of the dynamic morphometric parameters between groups. All mouse experiments described in the present study were carried out in strict accordance with the recommendations and regulations in the Animal Welfare Ordinance (TSchV 455.1) of the Swiss Federal Food Safety and Veterinary Office and were approved by the local authorities (license numbers 262/2016, Verterinäramt des Kantons Zürich, Zurich, Switzerland).
**Fig. 3.2.1** Illustration of the study design showing the time-points and duration of *in vivo* micro-CT imaging for the four groups of mice (n=12 PolgA (red) and n=10 WT (blue) mice per group).

*Animals & breeding scheme*

A colony of the mouse strain expressing an exonuclease-deficient version of the mitochondrial DNA polymerase γ (PolgA<sup>D257A</sup>), B6.129S7(Cg)-Polg<sup>im1Prol</sup>/J, JAX stock 017341, The Jackson Laboratory, Farmington CT, USA) was bred and maintained at the ETH Phenomics Center (12h:12h light-dark cycle, maintenance feed and water ad libitum, 3-5 animals/cage). As both heterozygous (PolgA<sup>D257A/+</sup>) and homozygous (PolgA<sup>D257A/D257A</sup>) mice have progressive accumulation of mitochondrial DNA point mutations, specific breeding considerations were taken into account while expanding the colony. Specifically, as paternal mitochondrial DNA is actively eliminated following fertilization in mice, the (undesirable) accumulation of mutations in the germline was minimized by mating heterozygous male mice (PolgA<sup>D257A/+</sup>) with C57Bl/6J inbred females (Charles River Laboratories, Sulzfeld, Germany). The thus obtained heterozygous (PolgA<sup>D257A/+</sup>) females and males (originating from a wild type (WT) C57BL/6J mother) were crossed (age 7-9 weeks) to generate homozygous (PolgA<sup>D257A/D257A</sup>, referred to as PolgA) and WT littermates (PolgA<sup>+/+</sup>, referred to as WT) with only a single generation of mutation burden.

*Mouse genotyping*

The presence of the PolgA knock-in mutation was confirmed by extracting DNA from ear clips (Sigma-Aldrich, KAPA Express Extract, KK7103) followed by qPCR (Bio-Rad, SsoAdvanced
Universal SYBR Green Supermix, 1725272) and melt curve analysis. The primers used for genotyping (5’ to 3’; Rev Common: AGT AGT CCT GCG CCA ACA CAG; Wild type forward: GCT TTG CTT GAT CTC TGC TC; Mutant forward: ACG AAG TTA TTA GGT CCC TCG AC) were recommended by the Jackson Laboratory.

**Micro-CT imaging and analysis**

*In vivo* micro-CT (vivaCT 40, Scanco Medical AG, isotropic nominal resolution: 10.5 µm; 55 kVp, 145 µA, 350 ms integration time, 500 projections per 180°, scan duration ca. 15 min, radiation dose per scan ca. 640 mGy) scans of the 6th caudal vertebrae were performed every 2 weeks. Animals were anesthetized with isoflurane (induction/maintenance: 5%/1-2% isoflurane/oxygen). Micro-CT data was processed and standard bone microstructural parameters were calculated in trabecular, cortical and whole bone by using automatically selected masks for these regions as described previously [35]. To calculate dynamic morphometric parameters, micro-CT images from consecutive time-points were registered onto one another. The voxels present only at the initial time point were considered resorbed whereas voxels present only at the later time point were considered formed. Voxels that were present at both time points were considered as quiescent bone. By overlaying the images, morphometrical analysis of bone formation and resorption sites within the trabecular region allowed calculations of bone formation rate (BFR), bone resorption rate (BRR), mineral apposition rate (MAR), mineral resorption rate (MRR), mineralizing surface (MS) and eroded surface (ES) [22].

**Quantification of the clinical mouse frailty index (FI)**

As recommended in the recently established toolbox for the longitudinal assessment of healthspan in aging mice [36], frailty was quantified using the clinical mouse FI [37], which includes the assessment of 31 non-invasive clinical items. For 29 of these items, mice were given a score of 0 if not present, 0.5 if there was a mild deficit, and 1 for a severe deficit. The final two items were weight and body surface temperature, which were scored based on the number of standard deviations from a reference mean in young adult mice as previously described [37].

**Statistical analysis**

Data are represented as mean±SD. For analysis of the longitudinal micro-CT images, frailty index and bodyweight measurements, linear mixed model analysis was performed using the
ImerTEST package [38] in R (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Fixed effects were allocated to age, genotype and group and a random effect was allocated to the individual mice to account for inherent variability between mice. Furthermore, an interaction effect between age and genotype as well as between genotype and groups were assessed. For the comparison of the bone morphometric parameters, frailty index and bodyweights at 40 weeks of age, values of the individual mice are shown. Effects of genotype and scanning group were analyzed via two-way ANOVA followed by Tukey’s multiple comparison test or one-way ANOVA, respectively using SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA). Power analysis was performed in G*Power (G*Power, Version 3.1.3., Düsseldorf, Germany [39]) and in R (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

3.2.3 Results

Longitudinal evaluation of static and dynamic bone morphometry and FI

Effect of genotype

Figure 3.2.2 shows the changes in static bone morphometric parameters over time in the different scanning groups obtained by longitudinal in vivo micro-CT imaging. Taking all the longitudinal micro-CT measurements of the different groups into account, PolgA had lower bone volume fraction (BV/TV, -10%, p<0.0001), trabecular thickness (Tb.Th, -6%, p<0.0001), cortical area fraction (Ct.Ar/Tt.Ar, -6%, p<0.0001) and cortical thickness (Ct.Th, -5%, p<0.0001) compared to WT (Fig. 3.2.2A-D). The trabecular number (Tb.N) and trabecular spacing (Tb.Sp) were not significantly different between genotypes (p>0.05). Furthermore, the age-related changes in bone morphometric parameters developed differently between genotypes with a significant interaction effect between age and genotype for BV/TV, Tb.Th, Ct.Ar/Tt.Ar and Ct.Th (p<0.0001, Fig. 3.2.2). While BV/TV, trabecular thickness (Tb.Th), Ct.Ar/Tt.Ar and cortical thickness (Ct.Th) initially increased in both genotypes, the increase in these parameters ceased to continue in PolgA mice from 30-32 weeks onwards (Fig. 3.2.2A-D).
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Fig. 3.2.2 Static bone morphometric parameters obtained by longitudinal in vivo monitoring of the 6th caudal vertebrae between 20 and 46 weeks of age. (A) Bone volume fraction (BV/TV), (B) trabecular thickness (Tb.Th), (C) cortical area fraction (Ct.Ar/Tt.Ar) and (D) cortical thickness (Ct.Th). (* p<0.05 PolgA (red lines) vs WT (blue lines) determined by linear mixed model. The different patterns represent different groups of mice scanned at different time-points)

By registering consecutive time-lapsed in vivo micro-CT images onto one-another [22], we furthermore assessed the dynamic remodeling activities in PolgA and WT mice. On average, PolgA mice had significantly lower bone formation rate (BFR, -27%, p=0.001) and bone resorption rate (BRR, -24%, p=0.001) compared to WT (Fig. 3.2.3A,B). BFR did not develop differently between genotypes, whereas there was a significant interaction effect between age and genotype for BRR (p<0.05). Mineral apposition (MAR) and resorption rate (MRR), which represent the thickness of formation and resorption packages, were lower (-9%, p<0.001 and -18%, p<0.001) in PolgA mice compared to WT (Fig. 3.2.3C,D). While MAR did not develop differently between genotypes, MRR increased in WT but remained constant in PolgA mice,
thus leading to a significant interaction effect between age and genotype for MRR (p<0.001). The mineralized surface (MS), which represents the surfaces of formation sites was lower (-13%, p<0.0001) in PolgA mice compared to WT whereas ES was similar between genotypes (p>0.05, Fig. 3.2.E,F). Neither MS nor ES showed an interaction effect between age and genotype (p>0.05). Overall, these results suggest that PolgA mice have lower bone remodeling activities compared to WT.

**Fig. 3.2.3** Dynamic bone morphometric parameters obtained by longitudinal *in vivo* monitoring of the 6th caudal vertebrae between 20 and 46 weeks of age. (A) Bone formation rate (BFR), (B) bone resorption rate (BRR), (C) mineral apposition rate (MAR), (D) mineral resorption rate (MRR), (E) mineralizing surface (MS) and (F) eroded surface (ES). (* p<0.05 PolgA (red lines) vs WT (blue lines) determined by linear mixed model. The different line patterns represent different groups of mice scanned at different time-points)
Figure 3.2.4 shows the changes in clinical mouse frailty index (FI) and body weights of PolgA and WT mice in the different scanning groups. In line with the known accelerated aging phenotype of PolgA mice [19, 20], longitudinal assessments of the FI showed that the mean FI averaged over all time-points was significantly higher in PolgA (+98%, p<0.0001) compared to WT (Fig. 3.2.4A). Furthermore, the FI developed differently with age in PolgA mice compared to WT (interaction effect between age and genotype, p<0.0001, Fig. 3.2.4A). While PolgA and WT mice had similar FI scores at 34 weeks, PolgA mice continuously accumulated health deficits (i.e., graying, ruffled fur, distended abdomen) with age leading to higher FI scores compared to WT from 38 weeks onwards. Conversely, the body weight continuously increased in both genotypes, with no differences detected between genotypes (p>0.05, Fig. 3.2.4B).

![Graph A](image1)

![Graph B](image2)

**Fig. 3.2.4** Longitudinal quantification of the (A) frailty index (FI) and (B) bodyweight in PolgA and WT mice between 20 and 46 weeks of age. (**** p<0.0001 PolgA (red lines) vs WT (blue lines) determined by linear mixed model. The different line patterns represent different groups of mice scanned at different time-points).

**Effect of combined radiation, anesthesia and handling**

In order to address whether the combined effects of radiation, anesthesia and handling associated with *in vivo* micro-CT imaging influenced the bone phenotype of PolgA and WT mice, linear regression analysis was performed on the static and dynamic parameters of all four groups (see study design illustrated in Fig. 3.2.1). As is already visually evident in Fig. 3.2.2, there was a considerable heterogeneity in static bone morphometric parameters between the different groups. Compared to the first group which was scanned over 20 weeks, the fourth group showed higher BV/TV (p<0.001) and Tb.Th (p<0.01, Fig. 3.2.2A,B), along with higher Tb.N (p<0.01) and lower Tb.Sp (p<0.0001). With respect to the cortical bone, the fourth group also showed higher Ct.Ar/Tt.Ar (p<0.001) and Ct.Th (p<0.01) compared to the first group. However, none of
the morphometric parameters showed a significant interaction effect between genotype and group.

The differences between groups observed in the dynamic morphometric parameters were less pronounced than in the static morphometric parameters. Averaged over all time-points, a significant effect of group was only found for BFR (p<0.01), BRR (p<0.05) and MRR (p<0.05, Fig. 3.2.3A,B,D). Post-hoc analysis between groups showed that WT mice of the fourth group had significantly lower BRR and MRR compared to WT of the first group (p<0.05), thus explaining the higher BV/TV and Tb.Th observed in that group. Similar to the static morphometric parameters, no significant interaction effects between genotype and age were found for any of the parameters. Linear regression analysis of the longitudinal measurements of FI and bodyweights did not show any differences between scanning groups (p>0.05, Fig. 3.2.4A,B).

Cross-sectional evaluation of static and dynamic bone morphometry and FI

In addition to evaluating the longitudinal micro-CT data, the bone morphometric parameters and FI of the different scanning groups were cross-sectionally compared at 40 weeks of age, i.e. the time-point at which the different groups had received either 11 scans, 5 scans or 2 scans, respectively (Fig. 3.2.5 & Fig. 3.2.6). Both genotype and scanning group significantly affected BV/TV, Tb.Th, Ct.Ar/Tt.Ar and Ct.Th (Fig. 3.2.5A-D and Table 3.2.1 for p-values and effect sizes $f$), however none of the parameters showed a significant interaction effect between genotype and group. On average, PolgA mice had lower BV/TV (-8.4%, p<0.01), Tb.Th (-8%, p<0.01), Ct.Ar/Tt.Ar (-7.9%, p<0.01) and Ct.Th (-8.1%, p<0.05) compared to WT. Furthermore, for Tb.Th, Ct.Ar/Tt.Ar and Ct.Th, the effect of genotype was 1.3, 1.75 and 2.5 fold stronger than the effect of scanning (group) as shown by the higher effect sizes $f$ determined by two-way ANOVA analysis (Table 3.2.1). Conversely, for BV/TV, the effect of scanning was 1.48 fold stronger compared to the effect of genotype. Post-hoc analysis within genotypes showed that WT mice of the 11-scan group had lower BV/TV (p<0.01) and Ct.Ar/Tt.Ar (p<0.05) compared to those of the 2-scan group (Fig. 3.2.5A,C). Similar to WT mice, PolgA of the 11-scan group showed lower BV/TV (p<0.0001) and lower Tb.Th (p<0.01) compared to all other groups (p<0.0001), with no differences between scanning groups detected in the cortical morphometric parameters (Fig. 3.2.5B-D).
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Fig. 3.2.5 Static bone morphometric parameters at 40 weeks of age for the different groups having received either 11 scans, 5 scans or 2 scans, respectively. (A) bone volume fraction (BV/TV), (B) trabecular thickness (Tb.Th), (C) cortical area fraction (Ct.Ar/Tt.Ar), (D) cortical thickness (Ct.Th). * p<0.05, ** p<0.01 and **** p<0.0001 between genotypes determined by two-way ANOVA, # p<0.05, ## p<0.01 and #### p<0.0001 post-hoc test within genotypes determined by one-way ANOVA and Tukey’s multiple comparisons test.

With respect to the dynamic parameters, both genotype and scanning group significantly affected BFR, BRR and MRR (Fig. 3.2.6A,B,D and Table 3.2.1 for p-values and effect sizes f), however none of the parameters showed a significant interaction effect between genotype and group. On average, PolgA mice had lower BFR (-30%, p<0.01), BRR (-41%, p<0.0001), MAR (-10%, p<0.05 and MRR (-30%, p<0.0001) compared to WT. Furthermore, for BRR and MRR, the effect of genotype was 1.52 and 2.16 fold stronger than the effect of scanning (group) as shown
by the higher effect sizes \( f \) determined by two-way ANOVA analysis (Table 3.2.1). Conversely, for BFR and MAR, the effect of scanning was 1.22 and 1.25 fold stronger compared to the effect of genotype. Furthermore, WT mice of the 11 scan group had higher MRR compared to WT mice of the 2-scan group (\( p<0.05 \), Fig. 3.2.6H). PolgA mice of the 11-scan group had higher BFR and BRR compared to PolgA mice of the 5 scan group (Fig. 3.2.6E,F), whereas no differences were observed between 11- and 2-scan group, respectively.

**Table 3.2.1** The effects of genotype and scanning (group) on bone morphometric parameters and frailty index (FI) at 40 weeks of age were compared via two-way ANOVA analysis. The p-values and effect sizes (\( f \)) of the main and interaction effects, respectively are listed below (“ns” denotes non-significance with \( p>0.05 \), significance level \( \alpha =0.05 \)).

<table>
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<tr>
<th>two-way ANOVA</th>
<th>Interaction p value</th>
<th>effect size ( f )</th>
<th>Group p value</th>
<th>effect size ( f )</th>
<th>Genotype p value</th>
<th>effect size ( f )</th>
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<td>Tb.Th</td>
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<td>&lt;0.0001</td>
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</tr>
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<td>Ct.Ar/Tt.Ar</td>
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<td>&lt;0.0001</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>&lt;0.01</td>
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</tr>
<tr>
<td>BRR</td>
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<td>0.46</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>MAR</td>
<td>ns</td>
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<td>ns</td>
<td>0.35</td>
<td>&lt;0.05</td>
<td>0.28</td>
</tr>
<tr>
<td>MRR</td>
<td>ns</td>
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<td>&lt;0.01</td>
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<td>&lt;0.0001</td>
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<tr>
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<td>ns</td>
<td>0.34</td>
<td>&lt;0.0001</td>
<td>1.19</td>
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</tbody>
</table>
3.2 Optimization of longitudinal in vivo phenotyping techniques

Fig. 3.2.6 Dynamic bone morphometric parameters at 40 weeks of age for the different groups having received either 11 scans, 5 scans or 2 scans, respectively. (A) bone formation rate (BFR), (B) bone resorption rate (BRR), (C) mineral apposition rate, (D) mineral resorption rate (MRR). * p<0.05, ** p<0.01 and **** p<0.0001 between genotypes determined by two-way ANOVA, # p<0.05, post-hoc test within genotypes determined by one-way ANOVA and Tukey’s multiple comparisons test.

At 40 weeks of age, PolgA mice had significantly higher FI compared to WT (+ 166%, p<0.0001, Fig. 3.2.7C). This clear difference between genotypes was also present when the mice were separated by scanning groups (p<0.001 for 11-scan group, p<0.0001 5-scan group and p<0.01 for 2-scan group. The comparison between scanning groups showed that PolgA mice in the 5-scan group had significantly higher FI scores compared to PolgA mice of the 2-
scan group (p<0.05). No significant differences between genotypes or scanning groups were detected for the bodyweight at 40 weeks of age (Fig. 3.2.7D).

![Comparison of (A) FI and (B) bodyweights at 40 weeks of age in PolgA (red bars) and WT (blue bars) mice.](image)

**Fig. 3.2.7** Comparison of (A) FI and (B) bodyweights at 40 weeks of age in PolgA (red bars) and WT (blue bars) mice (****p<0.0001 between genotypes and #p<0.05 determined by post-hoc one-way ANOVA).

Considering the small sample sizes (n=9 per group in WT and n=12 per group in PolgA) used for this cross-sectional comparison between scanning groups, the achieved power of the analysis was computed given the obtained effect sizes (f) and alpha level of 0.05 (Table 3.2.2). While sufficient power (≥ 0.8) was achieved to detect any effects that might have existed in the trabecular morphometric parameters, the power for detecting differences in the cortical as well as dynamic morphometric parameters was not sufficient, suggesting that a higher number of samples would be required to detect any differences between scanning groups. Conversely, by performing a longitudinal comparison (paired t-test between parameters of individual WT and PolgA mice measured both at 20 and 40 weeks of age), sufficient power (power ≥ 0.8) was obtained for all static morphometric parameters. Nevertheless, as the dynamic bone morphometric parameters in PolgA mice remained relatively constant between 20 and 40 weeks of age, the effect sizes were small and hence, higher samples sizes would be beneficial to detect differences in dynamic bone morphometric parameters over time in individual mice.
3.2 Optimization of longitudinal in vivo phenotyping techniques

Table 3.2.2 p-values, effect sizes ($f$) and achieved power obtained by cross-sectional (one-way ANOVA) and longitudinal analysis (paired t-test) respectively (“ns” denotes non-significance with $p>0.05$, significance level $\alpha=0.05$).

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<th>PolgA</th>
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<td>p value</td>
<td>effect size $f$</td>
<td>achieved power</td>
</tr>
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</tr>
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</tr>
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<tr>
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<td>p value</td>
<td>effect size $f$</td>
<td>achieved power</td>
</tr>
<tr>
<td>BV/TV</td>
<td>&lt;0.0001</td>
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<td>0.99</td>
</tr>
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<td>Tb.Th</td>
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<td>Ct.Ar/Tt.Ar</td>
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<td>1.00</td>
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<tr>
<td>MRR</td>
<td>&lt;0.05</td>
<td>0.52</td>
<td>0.99</td>
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3.2.4 Discussion

By coupling time-lapsed in vivo micro-CT imaging with longitudinal assessments of the clinical mouse frailty index (FI), this study comprehensively characterized the bone and frailty phenotype of a premature aging model, specifically the PolgA mouse. Furthermore, as longitudinal in vivo micro-CT imaging is associated with cumulative anesthesia, radiation and handling [23, 24], we assessed the impact of long-term monitoring of individual mice on bone morphometric and FI parameters. The unique study design, for which a large cohort of animals was aged in parallel up to 46 weeks of age, provided the possibility not only for cross-sectional comparisons between genotypes and between groups (i.e., that were scanned at different time-
points) but also for longitudinal comparisons within individual animals (i.e., that were scanned both at young and old age).

In agreement with previous studies based on X-ray densitometry and as of recently, *ex vivo* micro-CT [19-21], PolgA and WT had similar bone morphometric parameters at 20 weeks of age, which then diverged over time such that PolgA had significantly lower bone volume and quality at 40 weeks of age. Concomitantly, PolgA accumulated multiple health deficits over time (e.g., graying, ruffled fur, distended abdomen, kyphosis) leading to a significantly higher FI in PolgA mice from 38 weeks onwards. The clear difference in the bone morphometric parameters and FI between genotypes was observed both when groups were cross-sectionally compared and when individual mice were monitored over time. Interestingly though, *in vivo* micro-CT imaging over 20 weeks showed that this difference was not due to bone loss in PolgA mice, but rather in the inability to reach peak bone mass, of which a more comprehensive description has been provided elsewhere [40]. Interestingly, the registration of consecutive micro-CT images revealed that PolgA mice had lower bone remodeling activities compared to WT as shown by reduced bone formation and resorption rates, with no differences in the net remodeling rate. Similarly, senile osteoporosis in humans is characterized by low bone turnover, as opposed to the high bone turnover rates (higher resorption activities) observed during postmenopausal osteoporosis [1, 2, 41].

The comparison between different scanning groups revealed a considerable heterogeneity between groups with the fourth group having higher trabecular and cortical bone morphometric parameters compared to the other groups. However, we did not observe an interaction effect between genotype and scanning groups, suggesting that the PolgA mutation does not render bone more or less susceptible to cumulative effects of radiation, anesthesia and handling associated with *in vivo* micro-CT imaging. Hence, the comparison between two genotypes remains valid despite the potential confounding effects of radiation and associated handling. Furthermore, with the exception of BV/TV, the effect of genotype on static bone morphometric parameters was larger than the effect of *in vivo* micro-CT imaging. That radiation associated effects on bone morphometric parameters are stronger in trabecular bone compared to cortical bone has previously been shown [27]. In that study, the tibiae of sham- and ovariectomized-C57Bl/6J mice showed lower trabecular bone volume compared to the contralateral non-irradiated limbs, whereas this effect was not observed in healthy untreated 8- to 10-week-old control C57Bl/6J mice [27]. In line with our study, no interaction effect between ovariectomy
and radiation was found; this suggests that despite small but significant effects of radiation on bone morphometric parameters, the comparison between different study groups/treatments remains valid. Willie et al. have also reported lower BV/TV in 10-week-old C57BL/6J mice subjected to multiple in vivo micro-CT scans compared to age-matched mice subjected to only one in vivo micro-CT scan [29]; however, this effect was not observed in 26-week old mice, suggesting that radiation associated effects are stronger in younger mice. Conversely, other studies have reported no radiation associated effects on bone morphometric parameters in mice ranging from pre-pubertal to adult age [28] up to late adulthood (48 weeks) [15]. Using the same micro-CT settings as in the present study, we have previously shown that five scans did not have an effect on the bone microstructure or bone remodeling rates in the caudal vertebrae of 15-week-old C57BL/6 mice [14]. More recently, we have furthermore used a similar in vivo micro-CT approach to monitor specific healing phases after osteotomy and did not observe any significant imaging-associated changes in bone volume and turnover in the fracture callus of mice after 7 scans [32]. In the present study, the comparison between groups at 40 weeks of age showed that the PolgA mice of the first group had lower trabecular bone morphometric parameters compared to the third and fourth group, respectively, while no differences between any of the groups were detected in the cortical bone. For the WT mice, the fourth group (2 scans) showed higher BV/TV and Ct.Ar/Tt.Ar compared to the first group, while no significant differences between the first (11 scans) and third group (5 scans) were found. Hence, although effects associated with multiple time-lapsed micro-CT scans seem to be present when compared to a very low number of 1-2 scans, there does not seem to be major differences between performing 5 or 11 scans. Furthermore, although small but significant effects were observed between scanning groups in the static bone morphometric parameters, the differences between groups were less pronounced in the dynamic remodeling parameters. Taking all the longitudinal data into account, no differences between groups were observed for the parameters associated with bone formation. For parameters associated with bone resorption, the WT mice of the first group showed higher BRR and MRR compared to the fourth group. When the groups were compared at 40 weeks of age (having received either 11 or 2 scans, respectively), the 11-scan group showed higher MRR compared to the 2-scan group, suggesting a potential radiation-induced increase in osteoclast activity. An increased number and activity of osteoclasts has previously been reported in C57Bl/6 mice subjected to whole-body irradiation with an x-ray dosage of 2Gy [42]. Furthermore, one study using in vivo micro-CT showed dose-dependent effects on bone formation and resorption activities; while 3 consecutive scans at a high dose
(776 mGy) resulted in increased bone resorption but no differences in bone formation in the tibiae of 10-week-old C57Bl/6J mice, no effects were observed when scanning at a lower dose (434 mGy) [28]. Although the dose used in the current study (640 mGy) lies in between the two doses reported previously, caudal vertebrae – which predominantly contain yellow (fatty) bone marrow – should be less sensitive to radiation compared to long bones, which predominantly contain red bone marrow [43, 44]. Nevertheless, owing to the low statistical power achieved in the cross-sectional comparison between scanning groups, further studies with higher animal numbers would be necessary to detect cumulative effects of radiation, anesthesia and handling associated with in vivo micro-CT imaging.

Interestingly, the heterogeneity in bone morphometry observed between scanning groups was not present for the FI measurements. At 40 weeks of age, the PolgA mice of the 5-scan group had significantly higher FI scores compared to those of the 2-scan group; however, as no differences were detected between the 11- and 2-scan groups, we expect that this difference was related to variation between animals rather than to the cumulative effects of radiation and anesthesia associated with in vivo micro-CT imaging. The scanning groups did not show any differences in body weights, which increased over time both in PolgA and WT mice. The absence of weight loss further supports the fact that the well-being of the animals was not negatively affected by the higher number of in vivo micro-CT scans in the first group, compared to groups 2, 3 and 4, respectively.

One major limitation of our present study is that we do not have baseline measurements of all mice at 20 weeks of age, making it impossible to know whether the differences observed between scanning groups were due to cumulative effects of radiation, anesthesia, and handling associated with micro-CT imaging or due to initial variation between animals. In this respect, we observed that longitudinal designs including baseline measurements already at young age are more powerful at detecting age-related phenotypic changes compared to those including multiple groups with fewer imaging sessions. Hence, although sample sizes of 10-12 animals per group are sufficient for longitudinal studies, higher animal numbers would be beneficial for cross-sectional comparisons of aging mice. Furthermore, the parallel tracking of the FI as an addition to longitudinal in vivo micro-CT imaging not only allows to link age-related changes in bone morphometry to the development of frailty but also provides a useful tool to assess whether treatments/interventions have biasing effects on the overall health status of the animals.
3.2 Optimization of longitudinal in vivo phenotyping techniques

By maximizing the data obtained from individual animals, the total number of animals can be reduced.

In conclusion, the combination of the longitudinal assessments of the FI and time-lapsed *in vivo* micro-CT imaging allowed to detect not only the osteopenic but also the multi-system aging phenotype of PolgA mouse model. Although the long-term monitoring approach can potentially lead to small but significant changes in bone morphometric parameters, the comparison between study groups and/or treatments is not impaired. Moreover, the overall health-status of the animal (i.e., body weight and frailty index) was not affected. In line with the goal of “Reduction”, the second principle of the 3R’s, long-term *in vivo* micro-CT imaging allows to reduce the number of animals required for experiments while maintaining sufficient statistical power to reach a valid conclusion and thus, provides a powerful tool for usage in aging studies.

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**Conflict of Interest**

The authors declare no conflict of interest.
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References


3.2 Optimization of longitudinal in vivo phenotyping techniques


3.2 Optimization of longitudinal in vivo phenotyping techniques


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Chapter 4

Longitudinal assessment of frailty and osteosarcopenia in an *in vivo* model of premature aging
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA^{D257A/D257A} mice

Ariane C. Scheuren^{†}, Gommaar D’Hulst^{†}, Gisela A. Kuhn^{1}, Evi Masschelein^{2}, Esther Wehrle^{1}, Katrien De Bock^{2}, Ralph Müller^{†,*}

^{1}Institute for Biomechanics, ETH Zurich, Zurich, Switzerland

^{2}Laboratory of Exercise and Health, ETH Zurich, Zurich, Switzerland.

^{†} The first two authors contributed equally to this study

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Abstract

**Background:** Frailty is a geriatric syndrome characterized by increased susceptibility to adverse health outcomes. One major determinant thereof is the gradual weakening of the musculoskeletal system and the associated osteosarcopenia. To improve our understanding of the underlying pathophysiology and, more importantly, to test potential interventions aimed at counteracting frailty suitable animal models are needed.

**Methods:** To evaluate the relevance of prematurely aged PolgA^{D257A/D257A} mice as a model for frailty and osteosarcopenia, we quantified the clinical mouse frailty index in PolgA^{D257A/D257A} and wild type littermates (PolgA^{+/+}, WT) with age and concordedly assessed the quantity and quality of bone and muscle tissue. Lastly, the anabolic responsiveness of skeletal muscle, muscle progenitors and bone was assessed.
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA<sup>D257A/D257A</sup> mice

**Results:** PolgA<sup>D257A/D257A</sup> accumulated health deficits at a higher rate compared to WT, resulting in a higher frailty index at 40 and 46 weeks of age (+166%, +278%, p<0.0001), respectively, with no differences between genotypes at 34 weeks. Concomitantly, PolgA<sup>D257A/D257A</sup> displayed progressive musculoskeletal deterioration such as reduced bone and muscle mass as well as impaired functionality thereof. In addition to lower muscle weights (-14%, p<0.05, -23%, p<0.0001) and fiber area (-20%, p<0.05, -22%, p<0.0001) at 40 and 46 weeks, respectively, PolgA<sup>D257A/D257A</sup> showed impairments in grip-strength and concentric muscle forces (p<0.05). PolgA<sup>D257A/D257A</sup> mutation altered the acute response to various anabolic stimuli in skeletal muscle and muscle progenitors. While PolgA<sup>D257A/D257A</sup> muscles were hypersensitive to eccentric contractions as well as leucine administration, shown by larger downstream signaling response of the mechanistic target of rapamycin complex 1 (mTORC1), myogenic progenitors cultured in vitro showed severe anabolic resistance to leucine and robust impairments in cell proliferation. Longitudinal micro-CT analysis of the 6th caudal vertebrae showed that PolgA<sup>D257A/D257A</sup> had lower bone morphometric parameters (e.g. bone volume fraction, trabecular and cortical thickness, p<0.05) as well as reduced remodeling activities (e.g. bone formation and resorption rate, p<0.05) compared to WT. When subjected to 4 weeks of cyclic loading, young but not aged PolgA<sup>D257A/D257A</sup> caudal vertebrae showed load-induced bone adaptation suggesting reduced mechanosensitivity with age.

**Conclusions:** PolgA<sup>D257A/D257A</sup> mutation leads to hallmarks of age-related frailty and osteosarcopenia and provides a powerful model to better understand the relationship between frailty and the aging musculoskeletal system.

**Keywords:** aging, osteopenia, sarcopenia, osteosarcopenia, mTORC1, in vivo micro-CT
4.1.1 Introduction

Although there is no universally accepted definition of frailty [1], it is considered as an age-related syndrome characterized by the decline of multiple physiological functions, leading to the accumulation of health deficits, and thus a higher vulnerability to adverse health outcomes such as morbidity and mortality [2]. One of the most prominent components of frailty is the progressive weakening of the musculoskeletal system [3-5], leading to common age-related diseases such as osteopenia and sarcopenia. There is growing evidence that both diseases often co-exist in frail older individuals (also termed osteosarcopenia [6, 7]), thereby further increasing the risk for negative outcomes such as falls and fractures [8, 9]. Although several anabolic interventions such as dietary protein supplementation and mechanical stimulation are known to promote muscle and bone formation in young individuals, the molecular insights behind osteopenia and sarcopenia in the elderly population are lacking.

In the field of muscle physiology, studies in humans and rodents have shown that aged muscles are less responsive to well-known anabolic stimuli such as amino acids [10-12] and muscle contractions [13]; this phenomenon, termed “anabolic resistance”, likely results from reduced protein synthesis due to diminished intracellular signaling through the mechanistic target of rapamycin complex 1 (mTORC1) pathway [14-16]. Next to impairments in intra-muscular mTORC1 signaling, age-related sarcopenia has been associated with a decrease in number [17, 18] and proliferation capacity [19, 20] of myogenic progenitors or satellite cells. These are not only instrumental for the maintenance of muscle fibers, but also for the adaptive responses to exercise and regeneration upon injury [21]. In the field of bone physiology, evidence pointing towards altered mechanosensitivity with age has also been shown in humans [22] and in mice [23-26]. However, this effect might be site-specific as studies using a tibia-loading model showed a reduced response of trabecular [23, 24] and cortical [25, 26] bone formation with age, while bone adaptation in response to loading of the caudal vertebrae was maintained with age [27].

Therefore, whether and how age-related changes in the responsiveness to anabolic stimuli occur remains unclear. A better understanding of the pathophysiology of osteosarcopenia will help to identify interventions to strengthen the musculoskeletal system, which ultimately will be beneficial for the prevention and/or treatment of frailty.
In order to address this, tools such as the frailty index (FI) have been established to quantify the accumulation of age-related health deficits (e.g., loss of hearing, tremoring, comorbid diseases) in humans [28] and more recently, also in mice [29]. Indeed, the striking similarities between key features of the FI scores in humans and in mice [30] have highlighted the potential of rodent frailty models to not only improve our understanding of frailty but also serve as a tool to test responses to interventions designed to modify (or even prevent) frailty [31-33]. In this study, we aimed to evaluate the PolgA(D257A/D257A) mouse (referred to as PolgA), which due to elevated mitochondrial DNA point mutations and systemic mitochondrial dysfunction, exhibits an accelerated aging phenotype [34, 35], as a model of frailty and osteosarcopenia. While these mice are known to develop multiple signs of aging (e.g., hair loss, greying, hearing loss) earlier (around 40 weeks of age) than their wild type littermates (PolgA+/+, referred to as WT), the frailty phenotype has to the best of our knowledge not yet been assessed in these mice. Furthermore, although several studies have reported lower muscle weights in PolgA mice compared to their WT littermates [34, 36, 37], little is known about their muscle quality and functionality. To address this, forelimb grip-strength and concentric muscle forces were measured in vivo and ex vivo, respectively, in addition to the evaluation of hind limb muscle masses. Furthermore, the response to acute anabolic stimuli such as eccentric contractions and the leucine administration were assessed. With respect to the bone phenotype, only two studies have reported reduced femoral bone density using X-ray densitometry [34, 35]. Although this technique is still the gold-standard to assess bone mineral density (i.e. bone quantity) clinically in humans, it does not provide insight regarding the quality of bone tissue. Therefore, bone phenotyping in preclinical studies is commonly performed using high-resolution micro-computed tomography (micro-CT) as it allows additional standardized evaluation of the three-dimensional bone microarchitecture [38]. Furthermore, using in vivo micro-CT, dynamic bone remodeling activities can be tracked longitudinally providing information both on bone formation and bone resorption [25, 39]. These markers are important for better understanding of age-related changes in bone microarchitecture due to osteopenia. Coupled with longitudinal measurements of FI, we aimed to monitor individual mice during the process of aging, allowing us to capture the onset of osteosarcopenia and to track other signs of aging. Lastly, by longitudinally monitoring bone adaptation in response to a long-term mechanical loading intervention, we aimed to investigate whether PolgA bones show altered mechanosensitivity with age.
4.1.2 Materials and Methods

Study Design

The study consisted of three parts. For parts 1 and 2, female mice were aged up to 46 weeks and divided into four groups. Three of the groups were used to cross-sectionally compare the musculoskeletal and frailty phenotype of PolgA and WT mice at 34, 40 and 46 weeks, respectively (in vivo and ex vivo, part 1). The fourth group was longitudinally monitored between the ages of 20 and 40 weeks to investigate the changes in bone microarchitecture and frailty over time (in vivo, part 2). Lastly, the effects of various anabolic interventions on bone and muscle tissue were assessed (in vivo, ex vivo, in vitro). The number of mice and sample sizes in various animal studies are provided directly in the figure legends and in Table 4.S1. The sample sizes for in vivo experiments were selected based on previous experiments and power analysis (power set at 0.80) was used to determine the sample size required for ex vivo experiments. All mouse experiments described in the present study were carried out in strict accordance with the recommendations and regulations in the Animal Welfare Ordinance (TSchV 455.1) of the Swiss Federal Food Safety and Veterinary Office and results are reported following the principles of the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines).

Animals

All animal procedures were approved by the local authorities (licence numbers 262/2016 and ZH255-16, Verterinäramt des Kantons Zürich, Zurich, Switzerland). A colony of the mouse strain expressing an exonuclease-deficient version of the mitochondrial DNA polymerase γ (PolgA<sup>D257A</sup>, B6.129S7(Cg)-Polg<sup>tm1Prol/J</sup>, JAX stock 017341, The Jackson Laboratory) was bred and maintained at the ETH Phenomics Center (12h:12h light-dark cycle, maintenance feed and water ad libitum, 3-5 animals/cage). The mice were bred and genotyped as described in Supplementary Materials (SM). In order to confirm that the premature aging phenotypes were associated with mitochondrial dysfunction, the activity of complex IV enzyme (COX IV) in m. gastrocnemius (GAS) was measured (SM). Compared to WT, PolgA muscles had lower COX IV activity both at 40 and 46 weeks (-19% and -24%, p<0.0001, respectively), thus confirming that the mice used in this study had the same phenotype as those previously reported [36, 37, 40].
Quantification of Frailty Index (FI)

As recommended in the recently established toolbox for the longitudinal assessment of healthspan in aging mice [41], frailty was quantified using the Mouse Clinical FI [29], which includes the assessment of 31 non-invasive clinical items. For 29 of these items, mice were given a score of 0 if not present, 0.5 if there was a mild deficit, and 1 for a severe deficit. The final two items were weight and body surface temperature, which were scored based on the number of standard deviations from a reference mean in young adult mice as previously described [29]. To compare rates of deficit accumulation between PolgA and WT, the natural log of the FI was plotted against age. The slope of this line provides an estimate of the rate of deficit accumulation, as shown in previous studies [29, 42, 43].

Forelimb Grip-Strength

Forelimb grip-strength was measured using a force tension apparatus (Grip Strength Meter, 47200, Ugo Basile) at 40 and 46 weeks of age. Once mice gripped the stationary bar with their forepaws, they were pulled horizontally at the base of their tail until they let go of the bar. The process was repeated 5 times to determine the average peak grip force value (gram-force) used for analysis. All measurements were performed by the same experienced user.

Muscle harvesting and force measurements

PolgA and WT GAS, TA and m. soleus (SOL) were excised under anesthesia (5% isoflurane/oxygen) and snap frozen in liquid nitrogen. Furthermore, EDL from both legs were dissected and maintained in 4°C Krebs–Henseleit buffer supplemented with 1×MEM amino acid mixture (Invitrogen) and 25mM glucose. For assessment of ex vivo force production, the EDL of the right leg was attached to the lever arms of an Aurora system (Aurora Scientific) and submerged in continuously gassed Krebs–Henseleit buffer maintained at 37°C. After 5 min of temperature acclimation, muscle length was adjusted until a single stimulus pulse elicited maximum force during a twitch (Lo) under isometric conditions. After 5 min rest, a force frequency protocol was initiated by subsequently providing a pulse train (lasting 250 ms) of 1-30-50-80-150-250 and 300Hz with 1 min rest between every intensity.

Eccentric contractions (ECC) protocol

5 min after the force frequency protocol, EDL from the right legs were subjected to an eccentric training protocol according to O’Neil et al. [44] consisting of 60 contractions in a 22 min time
window. In this study, the pulse train was changed to 200Hz (compared to 100Hz in O’Neil et al.), because pilot studies suggested that 100Hz was not sufficient to induce maximal eccentric force in older muscle. After the contractions, the muscle was maintained in 37°C Krebs–Henseleit buffer + 1xMEM amino acid mixture and 25mM glucose before snap-freezing 1h after the last contraction. The control EDL from the contralateral leg was kept in the same 37°C Krebs–Henseleit buffer during the complete ECC period.

**Leucine administration**

46-week-old mice were fasted for 5h in the beginning of their light cycle, after which saline (0.9% NaCl, CTL) or leucine (0.4g/kg, LEU) was administered via oral gavage. L-Leucine (Sigma Aldrich) was dissolved in a stock solution of 40g/L, heated (40-50°C) and acidified as described previously [45]. 30 min later, hind limb muscles were weighted and snap frozen for further analysis.

**Leucine stimulation in vitro**

Primary muscle progenitor cells (MPs) were isolated from muscle tissue as described in SM. 200’000 cells were seeded in 6-well-plates. Myogenic differentiation medium containing low-glucose DMEM, 2% Horse-serum (HS) (Invitrogen) and 1% P/S was added 24h later. 72h later, fully differentiated MPs were treated according to one of the following conditions: (1) STV; 1h DMEM w/o amino acids (Biomol GmbH) + 10% dialyzed FBS (Invitrogen) + 1% P/S, (2) LEU 0.8; 1h STV + 1h 0.8mM L-Leucine (Sigma Aldrich) and (3) LEU 5; 1h STV + 1h 5mM L-Leucine. Experiments were performed in biological triplicate and technical duplicate. Edu analysis was performed according to manufacturer’s protocol (Thermo Fischer Scientific) after a 4h pulse with 1µg/ml Edu. For proliferation analysis, 20’000 cells were plated on 12-well-plates, and 3 plates were counted per time point over 5 days.

**Immunoblotting**

Details have been described previously [46]. Briefly, bone tissue (20-25mg) was first pulverized with mortar and pestle and subsequently homogenized with a tissue homogenizer (Omni THq). Muscle tissue (10-15 mg) was homogenized directly with a tissue homogenizer. Lysis was performed in ice-cold lysis buffer (for specifications see SM, Table 4.3.2). Homogenates were centrifuged at 10’000 g for 10 min at 4°C. Supernatant was collected and protein concentration was measured using the DC protein assay kit. 10-25 µg of total protein was loaded in a 15-well pre-casted gradient gel (Bio-rad, 456-8086). After electrophoresis, a picture of the gel was taken
under UV-light to determine protein loading using strain-free technology. Proteins were transferred via semi-dry transfer onto a PVDF membrane (Bio-rad, 170-4156) and subsequently blocked for 1 h at room temperature with 5% milk in TBS-Tween. Membranes were incubated overnight at 4 °C with primary antibodies (listed in SM, Table S2). The appropriate secondary antibodies for anti-rabbit and anti-mouse IgG HRP-linked antibodies (SM, Table 4.S2) were used for chemiluminescent detection of proteins. Membranes were scanned with a chemidoc imaging system (Bio-rad) and quantified using Image lab software (Bio-rad).

**Micro-CT imaging and analysis**

For analysis of femoral, the right femora were harvested, placed in 70% Ethanol and scanned with micro-CT (micro-CT40, Scanco Medical AG, isotropic nominal resolution: 10 µm; 55 kVp, 145 µA, 200 ms integration time). A 3D constrained Gaussian filter (sigma 1.2, support 1) was applied to the image data, after which images were segmented by a global thresholding procedure [47]. Standard bone microstructural parameters were calculated in trabecular (BV/TV, Tb.Th, Tb.N, Tb.Sp), cortical (Ct.Ar/Tt.Ar, Ct.BV, Ct.MV, Ct.Ar, Tt.Ar, Ct.Th, Ps.Pm, Ec.Pm) and whole (AVD, length) bone using automatically selected masks for these regions [48].

For analysis of the 6th caudal vertebra (CV6), *in vivo* micro-CT (vivaCT 40, Scanco Medical AG, isotropic nominal resolution: 10.5 µm; 55 kVp, 145 µA, 350 ms integration time, 500 projections per 180°) was performed every 2 weeks between 20 and 40 weeks of age. Animals were anesthetized with isoflurane (induction/maintenance: 5%/1-2% isoflurane/oxygen). Micro-CT data was processed and standard bone microstructural parameters were calculated in trabecular, cortical and whole bone by using automatically selected masks for these regions as described previously [49].

To calculate dynamic morphometric parameters, micro-CT images from consecutive time-points were registered onto one another. The voxels present only at the initial time point were considered resorbed whereas voxels present only at the later time point were considered formed. Voxels that were present at both time points were considered as quiescent bone. By overlaying the images, morphometrical analysis of bone formation and resorption sites within the trabecular region allowed calculations of bone formation rate (BFR), bone resorption rate (BRR), mineral apposition rate (MAR), mineral resorption rate (MRR), mineralizing surface (MS) and eroded surface (ES) [39].
Chapter 4 Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging

Analysis of bone turnover markers

Immediately following euthanasia, blood samples from 40-week-old PolgA and WT mice were obtained by cardiac puncture. Serum was separated by centrifugation and stored at -80°C until further analysis. Markers for bone formation and resorption were measured in technical duplicates using ELISA kits for N-terminal propeptide of type I procollagen (PINP, AC-33F, Immunodiagnostics) and for C-terminal cross-linked telopeptides of type I collagen (CTX-I, RatLaps, AC-06F1, Immunodiagnostics) according to manufacturer’s instructions.

Cyclic mechanical loading of CV6

CV6 were subjected to a cyclic loading regime, which has previously been shown to have anabolic effects in 15-week-old female C57BL/6J mice [50]. Briefly, stainless steel pins (Fine Science Tools) were inserted into the 5th and 7th caudal vertebrae of 35- and 12-week-old female mice. Three weeks after surgery, the mice received either sham (0N control) or 8N cyclic (10 Hz) loading for 5 minutes, 3x/week over 4 weeks. Weekly in vivo micro-CT images (vivaCT 40 or vivaCT80, Scanco Medical AG) were acquired and analyzed as described above.

Statistical analysis

Data is reported as mean and standard error of the mean (±SEM), unless otherwise stated. All analysis, with the exception of the longitudinal micro-CT data, was performed using GraphPadPrism (8.0.0). Unpaired or paired Student’s t-test, Mann Whitney test, one- or two-way ANOVA with post hoc multiple comparison testing were performed as indicated in figure legends. For the analysis of the longitudinal micro-CT data, linear mixed-effects modelling was used with Tukey’s post hoc multiple comparison testing corrected with Bonferroni criteria (SPSS 24.0.0.0). Fixed-effects were allocated to the age and genotype. Random-effects were allocated to the animal to account for the natural differences in bone morphometry in different mice. Significance was set at α<0.05 in all experiments.
4.1.3 Results

*With age, PolgA mice become frailer and display signs of co-existing osteopenia and sarcopenia*

To characterize the frailty and the musculoskeletal phenotypes of the PolgA model, female PolgA<sup>D257A/D257A</sup> (referred to as PolgA) and wild type littermates (PolgA<sup>+/+</sup>), referred to as WT) were aged in parallel and sacrificed at 34, 40 and 46 weeks, respectively. Using the clinical mouse frailty index (FI) as a tool to quantify the accumulation of health deficits [29], we observed that older (40 and 46 weeks), but not younger (34 weeks) PolgA mice had higher FI scores compared to WT (Fig. 4.1A). This divergence between genotypes at later time-points was also observed when FI was assessed longitudinally in individual mice at 34, 38 and 40 weeks, respectively. Specifically, between 34 and 40 weeks, the mean FI in PolgA mice increased from 0.05±0.02 to 0.15±0.02 (mean±SD, p<0.0001), whereas the increase in WT from 0.04±0.03 to 0.06±0.04 was less pronounced (p<0.05, Fig. 4.1B). Thus, compared to WT, PolgA mice had 73% (p<0.05) and 128% (p<0.001) higher FI at 38 and 40 weeks, respectively. Furthermore, the slope of the natural logarithm of the FI versus age curve, which has been shown to correspond to the rate of deficit accumulation in humans [43], was 0.025 in PolgA mice and 0.012 in WT, suggesting that PolgA mice became frailer faster than WT (p<0.05, Fig. 4.1C).
Fig. 4.1 Comparison of the frailty and musculoskeletal phenotypes at different ages. (A) Frailty Index (FI) assessed in PolgA and WT at 34, 40 and 46 weeks (n=9-35/group). (B) Longitudinal monitoring of FI in individual mice (n=9-12/group) at 34 (solid bars) and 40 weeks (striped bars). (C) The natural logarithm FI was plotted as a function of age. The slope of the regression lines through these data, which represent the rate of deficit accumulation, was higher in PolgA mice. (D-I) PolgA mice displayed lower bone and muscle mass and cross-sectional area compared to WT. (D) Femoral cortical bone volume (Ct.BV) and (E) cortical marrow volume (Ct.MV) (n=8-12/group). (G) Muscle weight and (H) fiber area (n=5-7/group). Representative cross-sections of (C) femoral bone and (F) m. tibialis anterior (TA) muscle of PolgA and WT mice at 46 weeks. (Data represent mean±SEM; * p<0.05; **p<0.01, ***p<0.0001 WT (blue bars) vs PolgA (red bars) determined by unpaired t-test, corrected for multiple comparisons by Bonferroni; for (B), ***p<0.0001 WT vs PolgA and #p<0.05, ###p<0.0001 over time determined by two-way ANOVA)

Concomitant to the increase in FI with age, older PolgA mice displayed deteriorations of the musculoskeletal system. Specifically, micro-CT analysis of the femora showed that the cortical bone volume (Ct.BV) was similar between genotypes at 34 weeks of age (p>0.05), but then diverged over time such that PolgA had lower Ct.BV at 40 and 46 weeks, respectively (p<0.0001, Fig. 4.1D). In line with the decline in Ct.BV, the cortical area (Ct.Ar) and thickness (Ct.Th) were lower in PolgA femora compared to WT at 40 (-13% and -11%, p<0.0001) and
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA<sup>D257A/D257A</sup> mice

46 weeks (-17% and -15%, p<0.0001), respectively (Table 4.S3). The cortical marrow volume (Ct.MV) increased both in WT and PolgA with age with no differences between genotypes at any of the ages (p>0.05, Fig. 4.1E). The total cross-sectional area within the periosteal envelope (Tt.Ar) was similar between genotypes at 34 weeks of age, but significantly lower in PolgA compared to WT at 40 and 46 weeks (-5% and -7%, p<0.01), respectively (Table 4.S3). Hence, while PolgA and WT showed similar endocortical expansion with age, PolgA showed reduced periosteal expansion at 40 and 46 weeks, respectively. Furthermore, PolgA had slightly but significantly shorter femora compared to WT at 40 and 46 weeks (-2% and -1%, p<0.001, Table 4.S3), respectively. Regarding the muscle tissue, the weights of m. extensor digitorum longus (EDL) and fiber cross-sectional area of m. tibialis anterior (TA) were similar between genotypes at 34 weeks (p>0.05), but lower in PolgA mice at 40 (-14% and -20%, p<0.01) and 46 weeks (-23% p<0.0001, -22% p<0.01), respectively (p<0.05, Fig. 4.1G-I). Similarly, lower weights of TA and m. gastrocnemius (GAS) in PolgA mice as compared to WT were observed at 40 (-20%, p<0.0001 and -23%, p<0.0001) and 46 weeks (-22%, p<0.0001 and -16%, p<0.05), respectively.

**Decreased forelimb grip-strength and concentric force in PolgA mice**

Considering that PolgA mice showed clear reductions in muscle mass and cross-sectional area at 40 and 46 weeks, we aimed to comprehensively characterize the muscular functionality at these time-points. At 40 weeks, *in vivo* assessment of the forelimb grip-strength, a widely used method to assess muscle function in rodents, showed that PolgA mice had lower grip-strength relative to bodyweight (p<0.05, Fig. 4.2A). Furthermore, when the EDL was subjected to a force-frequency protocol, PolgA showed a tendency towards decreased absolute force at 250 and 300Hz (p<0.10), while relative force was not affected (Fig. 4.2B,C). At 46 weeks, the grip-strength was 11% lower in PolgA mice compared to WT, but did not reach significance (Fig. 4.2D). The differences in forces however, were more exacerbated with a lower absolute and relative force at 80 to 300Hz (absolute) and 250 to 300Hz (relative) in the PolgA (p<0.05, Fig. 4.2E,F).
PolgA mutation induces higher basal and eccentric contraction (ECC) evoked mTORC1 and mechanotransduction signaling

Because PolgA muscles were atrophied and weaker than those of their WT littermates of the same chronological age, we used an isolated ex vivo model to assess the acute response to ECC, which have been shown to effectively activate mTORC1, the main regulator of skeletal muscle protein synthesis [51, 52]. Data of muscle forces evoked by ECC are presented in Table 4.1. PolgA had similar average force during the 60 ECC contractions, but tended to have a lower peak force during the first set (Table 4.1).
Table 4.1. Forces eccentric muscle contractions at 40 and 46 weeks
((*)p<0.10, **p<0.001, ***p<0.0001 WT vs PolgA at 40 and 46 weeks, respectively determined by unpaired t-test, corrected for multiple comparisons by Bonferroni)

<table>
<thead>
<tr>
<th></th>
<th>Average peak force (mV)</th>
<th>WT</th>
<th>PolgA</th>
<th>WT</th>
<th>PolgA</th>
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<tr>
<td>all contractions</td>
<td>149.9±9.7</td>
<td>142.8±15.7</td>
<td>141.6±9.6</td>
<td>137.3±8.8</td>
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<tr>
<td>1st set</td>
<td>193.3±12.4</td>
<td>158.8±17.5</td>
<td>188.5±6.6</td>
<td>164.6±8.6(*)</td>
<td></td>
</tr>
<tr>
<td>10th set</td>
<td>108.8±8.0</td>
<td>107.5±11.2</td>
<td>98.6±8.2</td>
<td>111.8±5.8</td>
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At 40 weeks, ECC evoked increased phosphorylation of the downstream mTORC1 kinase Ribosomal protein S6 Kinase 1(S6K1) at Thr389 compared to the contralateral control leg in both WT (+147±50%, p<0.05) and PolgA (406±161%, p<0.05), but the increase was more pronounced in PolgA resulting in a higher pS6K1 upon ECC in the PolgA (ΔECC-CTL, p<0.05) (Fig. 4.3A). The direct downstream kinase of pS6K1, S6 Ribosomal Protein (RPS6) showed similar increases in phosphorylation at Ser235/236 upon ECC in WT and PolgA (64±24% and 352±146%, p<0.05), whereas there was only a trend towards higher activation in PolgA compared to WT (ΔECC-CTL, p=0.07) (Fig. 4.3B). At 46 weeks, downstream mTORC1 targets showed similar effects with a trend towards hyper-phosphorylated pS6K1 and pRPS6 after ECC contractions in the PolgA compared to WT (p=0.10, Fig. 4.3D,E). Moreover, basal pRPS6 was higher in PolgA (1257±427%, p<0.05) compared to WT (Fig. 4.3E), suggesting both basal and contraction-induced hyper activation of mTORC1 in PolgA mutated muscle.
Fig. 4.3 Downstream mTORC1 signaling upon eccentric contraction (ECC) in WT and PolgA EDL. Downstream mTORC1 targets pS6K1 and pRPS6 increased upon ECC both at 40 wk (A,B) and 46 wk (D,E). (C,F) representative blots. (Data represent mean±SEM, n=6-7/group, within genotypes: *p<0.05, **p<0.001 ECC (squares/striped bars) vs. CTL (circles/solid bars) by paired student’s t-test, between genotypes: (#)p<0.10, #p<0.05 by unpaired students t-test ΔECC-CTL and basal values)

One of the proposed pathways for high-load contractions to regulate mTORC1 is via activation of the stress responsive mitogen-activated protein kinase (MAPK) pathway [53]. To investigate whether the hyperactive mTORC1 signaling in the PolgA muscle was related to increased MAPK response, we measured activation of (Stress-Activated Protein Kinase/Jun-amino-terminal Kinase) pSAPK/JNK upon ECC. At 40 weeks, the stress-responsive pSAPK/JNK at Thr138/Tyr185 after ECC increased by 113±35.8% (p<0.05) in WT and by 156±26% (p<0.001) in PolgA (Fig. 4.4A), with a tendency towards hyperactivation in PolgA compared to WT (ΔECC-CTL, p=0.09). At 46 weeks, the increase in pSAPK/JNK upon ECC was more pronounced in the PolgA (2410±1035%, p<0.05) than in the WT (1410±179%, p<0.05) resulting in a higher phosphorylation in PolgA muscle after ECC (ΔECC-CTL, p<0.05) and a tendency towards higher basal pSAPK/JNK (p=0.07, Fig. 4.4D,J). Interestingly, another arm of the stress- responsive MAPK pathway, p42-44 MAPK at Thr202/Tyr204, was not affected by
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA<sup>D257A/D257A</sup> mice

ECC; however, basal p44/42 signaling was higher in PolgA muscle compared to WT at 46 weeks (Fig. 4.4B,D). These data show that hyperactive mTORC1 signaling mirrors increased basal and contraction-induced MAPK signaling in PolgA mutated muscle.

Fig. 4.4 Downstream MAPK signaling upon eccentric contraction (ECC) in WT and PolgA EDL. pSAPK/JNK, but not p44/42 MAPK ERK1/2 increased upon ECC both at 40 wk (A,B) and 46 wk (D,E). (C,F) representative blots. (Data represent mean±SEM, n=6-7/group, within genotypes: *p<0.05, **p<0.001 ECC (squares/striped bars) vs. CTL (circles/solid bars) by paired student’s t-test, between genotypes: (#)p<0.10, #p<0.05 by unpaired students t-test ΔECC-CTL and basal values)

PolgA whole muscles are hypersensitive to leucine in vivo, while their primary myotubes are resistant to leucine in vitro

To examine whether other anabolic stimuli also hyper activate mTORC1 signaling in PolgA muscle, we administered a submaximal (0.4g/kg) dose of leucine via gavage to 46-week-old WT and PolgA littermates. Leucine induced an increase in pRPS6 in both WT (p<0.05) and PolgA (p<0.0001) TA, whereas pS6K1 was also increased in PolgA (p<0.001). The increase was 3.3±0.8 and 11.4±5.3 fold higher in PolgA for both pRPS6 and pS6K1, respectively
(p<0.001, Fig. 4.5A-C). These data demonstrate that the amino acid leucine increases downstream mTORC1 signaling to a greater extent in PolgA mutated compared to WT muscle.

In vivo, leucine sensing towards mTORC1 might be altered by the presence of other amino acids or growth factors. To rule out such interference, we cultured myogenic progenitor (MPs) cells from WT and PolgA hind limb muscle to verify proliferation capacity and leucine sensing without the availability of other amino acids. Strikingly, both 5-ethynyl-2’-deoxyuridine (Edu) incorporation and proliferation (Fig. 4.5D-F) were reduced in PolgA derived MPs. Furthermore, after differentiation, which was unaffected by PolgA mutation (Fig. 4.5G), we subjected myotubes to varying doses of leucine after 60 min of starvation. In contrast to our in vivo data, PolgA myotubes showed a strong anabolic resistance to leucine, as indicated by lower pS6K1 at 0.8mM and 5mM leucine, respectively (Fig. 4.5H,I). These data show that PolgA muscle progenitors have reduced cell proliferation capacity in vitro, potentially due to lower sensitivity to leucine.
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA^D257A/D257A mice

**in vivo**

(A) pRPS6 and pS6K1 signaling 30 min after submaximal dose of leucine gavage *in vivo* in 46-week-old WT and PolgA (Data represent mean±SEM, n=3-6/group, within genotypes: *p<0.05, **p<0.001, ***p<0.0001 LEU (squares/striped bars) vs. CTL (circles/solid bars), between genotypes: (#)p<0.10, #p<0.05, ##p<0.001 determined by two-way ANOVA). (C) Representative blots. (D-I) Proliferation and downstream mTORC1 signaling in primary myotubes from WT and PolgA muscle. (D,E) Cell proliferation was analyzed by EdU labelling and (F) cell count. (G) Brightfield of 2-d differentiated myotubes. (H) Representative blots of dose-response leucine experiment in fully differentiated WT and PolgA myoblasts. (I) Quantification of pS6K1 from three independent experiments. (Data represent mean±SEM, *p<0.05 WT (blue line/bars) vs PolgA (red line/bars), #p<0.05 over time determined by two-way ANOVA)
Reduced bone quantity and quality in PolgA mice

As the results of the cross-sectional experiments described above suggest that PolgA mice display clear signs of co-existing osteopenia and sarcopenia from 40 weeks onwards, we aimed to longitudinally track individual mice during the transition from young to frail status in order to gain a better understanding of when exactly and to what extent bone loss (osteopenia) occurs in individual mice. Therefore, we used an in vivo micro-CT approach to monitor the dynamic changes in the bone microarchitecture of the 6th caudal vertebrae (CV6) in individual mice between the ages of 20 and 40 weeks (Fig. 4.6). The comparison between genotypes showed that at 20 weeks, PolgA and WT had similar trabecular morphometric parameters. Initially, bone volume fraction (BV/TV) and trabecular thickness (Tb.Th) increased in both genotypes, but then started to diverge such that BV/TV and Tb.Th were higher in WT mice from 30 weeks onwards (p<0.05, Fig. 4.6A,B). Furthermore, from 30 weeks onwards, Tb.Th continuously increased in WT (+5%, p<0.0001), whereas it did not change in PolgA (-1.3%, p>0.5, Fig. 4.6B). No significant differences between genotypes were detected for the trabecular number (Tb.N) and separation (Tb.Sp, Fig. 4.6C,D). Nevertheless, Tb.N in WT decreased between 30 and 40 weeks (-2.5%, p<0.05), whereas Tb.N did not change over time in PolgA mice (-0.3%, p>0.05, Fig. 4.6C). The reduction in Tb.N together with the increase in Tb.Sp in WT explain the slight decrease in BV/TV in WT mice from 36 weeks onwards. A significant interaction between age and genotype, indicating that the time course developed differently between genotypes, was found for Tb.Th, Tb.N and Tb.Sp.

Similar results were obtained when the cortical bone morphometric parameters were analyzed. At 20 weeks, there were no differences between genotypes in cortical area fraction (Ct.Ar/Tt.Ar), Ct.Th and Ct.MV (Fig. 4.6F,G,I). Ct.BV was already lower (-6%, p<0.05) in PolgA compared to WT at 20 weeks (Fig. 4.6H) with this difference becoming more accentuated up to 40 weeks (-11%, p<0.001). Ct.Th increased over time, but then diverged such that Ct.Th was higher than that of PolgA mice from 32 weeks onwards (p<0.05). This resulted in a higher Ct.Ar/Tt.Ar in PolgA mice from 36 weeks onwards (p<0.05). No differences in Ct.MV were detected between genotypes. A significant interaction between age and genotype was found for Ct.Ar/Tt.Ar, Ct.Th, Ct.BV and Ct.MV. The thickening of the trabecular and cortical structure was also visually apparent from the micro-CT images and can be appreciated when the same cross-section is observed at 20 and 40 weeks (Fig. 4.6E,J).
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA257A/257A mice

Fig. 4.6 Longitudinal monitoring of trabecular and cortical bone morphometric parameters over 20 weeks: (A) bone volume fraction (BV/TV), (B) trabecular thickness (Tb.Th), (C) trabecular number (Tb.N), (D) trabecular separation (Tb.Sp), (F) cortical area fraction (Ct.Ar/Tt.Ar), (G) cortical thickness (Ct.Th), (H) cortical bone volume (Ct.BV) and (I) cortical marrow volume (Ct.MV). (E,J) Bone microstructure (cross-sections) of representative (median) WT (top row) and PolgA (bottom row) mice at 20 (left) and 40 (right) weeks of age. In WT mice, thickening of trabeculae and cortex can be observed, while in PolgA mice, little change can be seen between time points. (Data represent mean±SEM; n=9 WT and n=12 PolgA, *p<0.05 WT (blue line) vs PolgA (red line); #p<0.05 over time determined by linear mixed model and Tukey’s post hoc)
Reduced bone remodeling in PolgA mice

In addition to monitoring changes in bone morphometry over time, we used *in vivo* micro-CT to quantify dynamic bone formation and resorption parameters, respectively in PolgA and WT caudal vertebrae (Fig. 4.7). On average, PolgA mice had lower bone formation rate (BFR, -23.2%, p<0.05) and bone resorption rate (BRR, -28.8%, p<0.05) compared to WT (Fig. 4.7A,B). BFR and BRR changed over time in both genotypes (p<0.001), however the time course did not develop differently between genotypes (interaction effect, p>0.05). Hence, the net remodeling rate (BFR-BRR) was not different between genotypes. The mineral apposition and resorption rate (MAR and MRR, respectively), which represent the thickness of formation and resorption packages were lower (-8.9%, p<0.05 and -20.1%, p<0.001) in PolgA compared to WT (Fig. 4.7E,F). MAR did not change over time and showed no significant interaction between age and genotype (Fig. 4.7E). On the other hand, there was a significant interaction effect of age and genotype on MRR (p<0.05). Over time, MRR increased in WT, while MRR remained constant in PolgA (Fig. 4.7F). The mineralized surface (MS), which represents the surface of formation sites, was lower in PolgA (-11.8%, p<0.0001), whereas the eroded surface (ES) was similar between genotypes (p>0.05) (Fig. 4.7C,D). MS and ES changed over time in both genotypes, however the time course did not develop differently between genotypes (interaction effect, p>0.05). In line with lower BFR and BRR observed by micro-CT, bone turnover markers for formation (N-terminal propeptide of type I procollagen (PINP) and resorption (C-terminal telopeptides of type I collagen, CTX-I) were lower in serum from PolgA (-56% and -49%, p<0.05) compared to WT at 40 weeks of age (Fig. 4.7H,I). Overall, these results suggest that PolgA mice have reduced bone turnover compared to WT.
Fig. 4.7 Longitudinal monitoring of dynamic bone morphometry parameters over 20 weeks: (A) Bone Formation Rate (BFR), (B) Bone Resorption Rate (BRR), (C) Mineral Apposition Rate (MAR), (D) Mineral Resorption Rate (MRR), (E) Mineralizing Surface (MS), and (F) Eroding Surface (ES). (Data represent mean±SEM; n=9 WT and n=12 PolgA; *p<0.05 WT (blue line) vs PolgA (red line); #p<0.05 over time determined by linear mixed model and Tukey’s post hoc). (G) Overlay of thresholded micro-CT images from 20 and 40 weeks showing sites of formation (orange), quiescence (grey) and resorption (blue) in representative WT and PolgA mouse. Serum bone turnover markers for (H) formation (PINP) and (I) resorption (CTX-1), respectively at 40 weeks of age. (Data represent mean±SEM; n=8 WT and n=3 PolgA; *p<0.05 WT (blue bar) vs PolgA (red bar) determined by Mann Whitney test)
To assess whether the osteopenic phenotype in PolgA mice could be linked to alterations in mTORC1 signaling, we assessed basal mTORC1 and MAPK signaling in caudal vertebrae at 40 weeks of age. Overall, the expression of downstream mTORC1 and MAPK was lower in vertebrae compared to muscle tissue. Therefore, the bone immunoblots were overexposed in order to observe a signal. Compared to WT, PolgA mice tended to have lower pS6K1 at Thr389 (-69%, p≤0.1) while no significant difference was observed for the direct downstream kinase thereof (RPS6) at Ser235/236 (p>0.05) (Fig. 4.8A,B). Basal pSAPK/JNK at Thr183/Tyr185 was lower (-60%, p<0.001) in PolgA compared to WT, while no difference was observed in p44/42 at Thr202/Tyr204 (p>0.05) (Fig. 4.8C,D).

![Fig. 4.8 Basal mTORC1 and MAPK signaling in WT and PolgA caudal vertebrae at 40 weeks. PolgA mice showed a tendency towards reduced downstream mTORC1 targets pS6K1 (A) and pRPS6 (B). Downstream MAPK target pSAPK/JNK (C), but not p44/42 MAPK (D) was lower in PolgA compared to WT. (E) Representative blots with positive control (muscle tissue). Overall signal was low in bone lysates, so pS6K1 and pRPS6 are overexposed. (Data represent mean±SEM, n=5–6/genotype, (*)p≤0.10, *p<0.05 PolgA (red bars) vs WT (blue) by unpaired students t-test) Reduced mechanosensitivity of PolgA caudal vertebrae with age

To investigate whether prematurely aged PolgA bones are mechanosensitive, the 6th caudal vertebrae were cyclically loaded 3x/week over 4 weeks using a previously developed loading device [50]. Fig. 4.9 shows the absolute values of trabecular bone morphometric parameters at the end of the mechanical loading intervention in young (A,B) and aged (C,D) mice. At young
age, loading elicited an anabolic response both in WT and PolgA mice such that BV/TV and Tb.Th were higher (p<0.0001) in the loaded compared to the sham-loaded controls (Fig. 4.9A,B). Strikingly, the anabolic effect was maintained with age in WT (p<0.05), whereas PolgA mice did not respond to the mechanical loading intervention, with no differences detected between loaded and non-loaded controls (Fig. 4.9C,D).
Fig. 4.9 Effect of cyclic mechanical loading on trabecular bone morphometric parameters in PolgA and WT mice at young (top) and old age (bottom): (A,C) bone volume fraction (BV/TV) and (B,D) trabecular thickness (Tb.Th) at the end of the loading intervention. (Data represent mean±SEM, n=8-11/group; *p<0.05, ***p<0.0001 CTL (circles/solid bars) vs loading (squares/striped bars))

4.1.4 Discussion

Due to the accumulation of mitochondrial DNA mutations with age, PolgA mice are known to develop multiple signs of aging (alopecia, greying, hearing loss, kyphosis, reduced bone and muscle mass) early in life [34, 35]. Here, we used the clinical mouse FI [29], which was recently reverse translated from the FI in humans [28], to quantify the accumulation of these health deficits over time in individual mice. Furthermore, as the weakening of the musculoskeletal
system is a major component of age-related frailty, we used a combination of in vivo, ex vivo and in vitro techniques to comprehensively characterize the musculoskeletal phenotype of this model. Lastly, the effects of various anabolic interventions on the musculoskeletal system of PolgA mice were assessed.

This study demonstrated that in comparison to their chronologically aged WT littermates, PolgA mice developed many hallmarks of aging over time which collectively resulted in PolgA mice becoming frailer with age. Of the 31 items included in the FI scoring, changes in the integument category were the most striking. Specifically, PolgA mice showed a loss in fur color (from black to grey/brown) and a decline in the coat condition (ruffled fur, ungroomed appearance). Furthermore, they showed signs of enlarged abdomen and kyphosis. At 40 weeks of age (280 days), PolgA mice had mean frailty scores of 0.15±0.02 (mean±SD), whereas WT mice had 0.06±0.04, respectively. A previous study using male C57BL/6J mice reported mean FI values of 0.05±0.05 in young (30-299 days), 0.21±0.04 in middle-aged (300-599 days) and 0.29±0.07 in older mice (above 600 days) [30]. Comparing these values to the ones observed in our study, the (female) WT mice (at 40 weeks < 299 days) fall in the category of young mice, whereas the PolgA mice fall into the category of “middle-aged” mice. Although some studies have reported higher FI scores in female compared to male mice [29, 51], others showed no differences in frailty between sexes [52, 53]; furthermore, it seems that the rate of deficit accumulation is similar between sexes [51]. Nevertheless, future studies in which PolgA mice are aged for a longer period of time (>300 days) would be necessary to investigate the degree of frailty at later stages in life. Our ethical permit for breeding was constrained to a maximum of 46 weeks as previous studies reported median lifespans of PolgA mice ranging from ca. 59 weeks (416 days) [34], 48 weeks (336 days) [35], down to only 36 weeks (250 days) [37]. The discrepancies between studies regarding the lifespan is also apparent for the age at which symptoms of aging first appeared (ranging from 6- [37] to 9 months [34, 35]). As not only homozygous (PolgA^{D257A/D257A}), but also heterozygous (PolgA^{D257A/+}) mice progressively accumulate mitochondrial DNA point mutations with age, we suspect that the differences between studies resulted from differences in breeding schemes. Since all the mice used in our study were bred according to a standardized breeding scheme, thereby displaying only a single generation of mutation burden, the onset of the accelerated aging phenotype may have been delayed in the present work as compared to other studies. Nevertheless, we observed that PolgA mice became frailer faster than WT, as shown by plotting the natural logarithm of the FI as a
function of age and fitting the resulting relationship with a linear function. To our knowledge, we are the first to report the frailty phenotyping in the PolgA mouse model.

In line with increased FI with age, the musculoskeletal phenotype clearly diverged over time between genotypes, with PolgA mice developing multiple signs of osteosarcopenia with age. In agreement with previous reports showing lower muscle weights [34, 36, 37] and reduced femoral bone mineral density assessed by X-ray densitometry [34, 35], phenotyping of the femora and hind limb muscles revealed lower bone and muscle mass as well as cross-sectional area in PolgA mice compared to their WT littermates at 40 and 46 weeks of age. These reductions were not observed at 34 weeks, thus indicating a progressive weakening of the musculoskeletal system in the PolgA mice. As the clinical diagnosis of sarcopenia is not only characterized by reduced muscle mass but also by reduced muscle function and quality, we assessed the forelimb grip-strength, muscle force and intramuscular signaling upon anabolic stimuli. In agreement with clinically diagnosed sarcopenia, PolgA displayed lowered muscle strength, indicated by reduced grip-strength and lower concentric contraction force at different intensities.

One mechanism commonly thought of as a contributor to sarcopenia is the age-related lowered sensitivity to anabolic stimuli. This phenomenon, known as anabolic resistance, has been associated with the reduced acute activation of mTORC1 [13-15], the central protein complex that integrates external stimuli to regulate cell growth [54-57]. Note that the interpretation of the data described in previous studies, which showed increased anabolic resistance upon heavy-load contractions, might be confounded by the fact that the same relative load (% 1 repetition max) was used in old vs. young humans [13]. The lower absolute load in the aged subjects might have caused lower mechanical stress on the muscle, as demonstrated by lower MAPK signaling [58], and thus a dampened increase in mTORC1 signaling. By using the protocol described by O’Neil et al. [44], we could overcome this confounding factor by controlling the load applied to the muscles. Interestingly, although the average force produced during the 60 contractions was similar between WT and PolgA, both MAPK and downstream mTORC1 signaling was remarkably elevated in PolgA mutated muscle. Notably, leucine also activated mTORC1 to a greater extent in 46-week-old PolgA muscle compared to their chronologically aged WT littermates. This is intriguing as eccentric contractions and leucine activate mTORC1 signaling via independent mechanisms [59]. Potentially, increased contraction-induced mechanical signaling is responsible for increased mTORC1 activity in response to contractions,
but further research is needed to fully elucidate the mechanisms behind mTORC1 hyperactivity in PolgA muscle. Next to stimulus-induced mTORC1 activity, we also found hyperactive basal mTORC1 in PolgA at 46 weeks of age. In fact, basal mTORC1 hyperactivity has previously also been shown in other models of progeria [60], aging [61] and progeria-like syndromes (i.e., laminopathies) [62, 63]. Furthermore, chronically increased mTORC1 activity has been shown to be sufficient to cause progressive muscle atrophy, fiber damage, fiber death, and muscle weakness in humans and in mice [64]. Therefore, treatment with mTORC1 inhibitors has been proposed as a potential therapeutic approach to rescue elevated mTOR signaling in these models [62-64].

In vivo, the systemic availability of amino acids/nutrients and differences in the local niche might have interfered with hyperactive mTORC1 signaling in PolgA. To rule this out, we used an in vitro approach to study the activation of mTORC1 in MPs that were stimulated with identical concentrations of leucine, without the availability of other amino acids. We were able to demonstrate that PolgA primary MPs had substantial defects in proliferative capacity and were completely insensitive to leucine with respect to mTORC1 anabolic signaling. Thus, it seems that mitochondrial dysfunction per se is sufficient to induce impairments in muscle stem cell mTORC1 signaling, regardless of exposure to the local niche. The differences in downstream mTORC1 signaling upon anabolic signals between whole muscle and their respective stem cells are puzzling, but can potentially be induced by the higher accumulation of mitochondrial damage under proliferative in vitro conditions when compared to post mitotic differentiated muscle tissue that develops apoptosis at a later stage [34]. Nonetheless, these data warrant caution in the translation of MP-derived experimental data to the in vivo muscle setting and suggest that the accumulation of mitochondrial damage is tissue specific and can profoundly change the mTORC1 response to leucine.

With respect to the evaluation of the bone phenotype, this study is to the best of our knowledge, the first to use standardized micro-CT approaches to comprehensively characterize the bone phenotype of the PolgA mouse model [38, 39, 48]. Albeit still considered the clinical gold standard for assessing bone mineral density in humans, X-ray densitometry, which was previously used to evaluate the PolgA bone phenotype [34, 35], is confounded by the size and positioning of the bone, and furthermore, does not provide any information on the bone microarchitecture [65, 66]. Nevertheless, the results of this study did recapitulate the clear reductions measured in femoral bone mineral density in PolgA mice with age [34, 35]. Note
that the accuracy of the aforementioned phenotyping of the femora, both in the previous as well as in the current study, is limited by the cross-sectional study design. This type of study design cannot account for the intrinsic initial variability between animals at baseline [67, 68], thereby making it impossible to determine the amount of bone lost in individual mice. We therefore used an established in vivo micro-CT approach to monitor the spatio-temporal changes in bone micro-architecture in the caudal vertebrae of individual mice during the transition from healthy to frail state. Multiple bone morphometric parameters diverged over time, from being similar at 20 weeks to lower in PolgA mice with age. Interestingly though, this difference did not arise due to significant bone loss in PolgA mice, but rather in the failure to achieve normal peak bone mass. We and others have previously reported the absence of age-related bone loss in caudal vertebrae [69, 70]. Hence, mouse caudal vertebrae may not be optimal for investigating age-related bone loss. As the bone morphometric parameters in the femora declined with age in PolgA mice, this study suggests that age-related changes in bone micro-architecture differ depending on the skeletal site that is analyzed. Indeed, a more severe age-related deterioration of bone microarchitecture in long bones compared to lumbar vertebrae has previously been reported [68, 69, 71]. In a cross-sectional study performed by Glatt et al., the cross-sectional area of the lumbar and caudal vertebrae of female C57BL/6 mice increased by 25% between the age of 2 and 20 months, while the femoral cross-sectional area declined by 3% [68]. Furthermore, using in vivo micro-CT, we and others have previously shown differences in age-related changes in the lumbar and caudal vertebrae as compared to the tibiae of C57BL/6 mice [70, 71]. Both studies showed continuous declines in cortical bone of the tibiae, while no changes or even increases were observed in the lumbar and caudal vertebrae, respectively [70, 71]. We therefore assume that the osteopenic phenotype in PolgA mice is stronger in long bones compared to vertebrae. Hence, monitoring the long bones of PolgA mice by in vivo micro-CT may be beneficial in future studies. Beyond enhanced phenotyping, the current study capitalized on the ability of in vivo micro-CT to provide information on the dynamic coupling between bone resorption and formation. Compared to WT littermates, PolgA had lower bone remodeling activities as shown by reduced bone formation and resorption rates, with no differences in the net remodeling rate. These results were confirmed by lower bone turnover markers measured in PolgA serum compared to WT. Similarly, senile osteoporosis in humans is characterized by low bone turnover, as opposed to the high bone turnover rates (higher resorption activities) observed during postmenopausal osteoporosis. Despite the advantages of in vivo micro-CT, the anesthesia, cumulative radiation, and stress associated with repeated CT measurements could
have an effect on the bone morphometry and the well-being of the animals [72-76]. However, as effects of radiation are of greater concern in young, growing mice, and furthermore, have been shown to be independent of surgical treatments (e.g., ovariectomy) [72], we suspect that any effects of radiation would be similar in WT and PolgA mice. Nevertheless, we compared the bone remodeling activities of PolgA and WT mice used in this study (subjected to 11 in vivo measurements) with those of corresponding age-matched controls that received only 2 in vivo scans at 38 and 40 weeks, respectively, and saw no statistical differences between the groups. Furthermore, we did not observe any negative effects of any of these variables on the well-being of the animals.

Finally, after having established that PolgA mutation altered the acute sensitivity to anabolic stimuli in muscles and their progenitors, we investigated the responsiveness of PolgA bones to a previously established 4-week mechanical loading regime [49]. Interestingly, prematurely aged PolgA mice were not responsive to cyclic loading of the caudal vertebrae over 4 weeks, whereas loading had beneficial effects in WT mice. Although there are controversial reports on the maintenance of bone mechanosensitivity with age in mice [23-26], these results conflicted with our previous observation that caudal vertebrae remain mechanosensitive to cyclic mechanical loading with age [27]. Interestingly, in that study, 82-week-old C57BL/6J mice showed a greater anabolic response compared to the 52-week-old mice; hence, it is possible that even older PolgA mice would respond differently than the ones used in this study. Furthermore, the load in this study was not adjusted to account for differences in the initial bone volume fraction between PolgA and WT mice. An individualized loading approach, which ensures that all bones are subjected to the same mechanical strain, could yield differences in the mechanosensitivity of the PolgA and WT mice. Nevertheless, adapting the loading for the lower bone mass in the PolgA would decrease the stimulus and therefore, we do not expect a positive response at this even lower stimulus. Nonetheless, further research is therefore needed to elucidate the mechanosensitivity of PolgA bones. Of further notice was that BV/TV and Tb.Th declined over time in the aged sham-loaded WT mice, whereas loading was able to reduce this bone loss. As our in vivo micro-CT monitoring of the caudal vertebrae did not show any bone loss up to 40 weeks of age (Fig. 4.6 and 4.7), we suspect that the bone loss occurring in the loaded WT mice was due to the surgical insertion of the pins required for loading. Interestingly, this decline in trabecular bone was not observed in the PolgA mice, thus explaining the high BV/TV and Tb.Th in the sham-loaded PolgA mice at end-point. We have
previously observed similar bone loss in 52-week-old mice with loading being able to reduce this bone loss [27]. Similar to our current study though, the 15- and 82-week-old mice did not show any bone loss. It is possible that the prematurely aged PolgA mice behave similarly to the 82-week-old mice of that study. Nevertheless, the fact that PolgA bones lose mechanorresponsiveness to loading with age, while PolgA muscles show increased acute response to mechanical stimuli is intriguing. Along the same lines were the contrasting results in terms of basal mTORC1 and MAPK signaling, where basal downstream mTORC1 and MAPK signaling was almost absent in PolgA mutated bone, while it was hyperactive in PolgA muscle. Tissue-specific differences in mTORC1 signaling have been shown previously [77]. Genetic reduction of mTOR expression had beneficial effects on neurological and muscle function (e.g., rotarod, grip-strength) in aging mice, whereas declines in bone volume and immune function were accelerated [77]. Moreover, the pharmacological inhibition of mTORC1 signaling has been shown to impair osteogenic [78, 79] and osteoclast [80] differentiation in vitro as well as induce trabecular bone loss in vivo [81], while rapamycin treatment reduced muscle fiber loss in aging mice [64]. Hence, although treatments with mTOR inhibitors could be beneficial for long-term muscle health, this might not be the case for bone health. Future studies are required to better understand discrepancies and potential interactions in mTORC1 signaling between muscle and bone, and whether such differences can be linked to altered mechanosensitivity with age.

There are a number of limitations to our study that should be mentioned. Firstly, the acute response to anabolic stimuli in the muscle tissue cannot be directly compared to the long-term loading regime (over 4 weeks) to which the bone tissue was subjected. Physiological resistance training protocols leading to skeletal muscle hypertrophy in mice are difficult to achieve. The protocols, which are available such as voluntary resistance running [82] and high intensity interval running [83] might be cumbersome due to a reduced exercise capacity in PolgA [84]. Nevertheless, both voluntary [84] and forced [37] long-term endurance exercise regimes have been shown to improve the progeroid phenotype and locomotor behavior in PolgA mice. Hence, future studies should investigate the potential of longer-term resistance exercise training in alleviation of mTORC1 hyperactivity in (mouse models of) progeria. To be noted though is that the above mentioned exercise regimes were applied already at young (10-12 weeks) up to old age thereby making it unknown whether they would have the same effect in older PolgA mice already displaying signs of aging. In this respect, future studies subjecting PolgA caudal vertebrae to cyclic loading regimes throughout life (i.e, from young until old age) would be
interesting to assess whether the reduction in bone mechanosensitivity with age could be prevented.

A further limitation of our study was that the evaluation of the bone remodeling activities was mainly based on time-lapsed micro-CT analysis, whereas two-dimensional (2D) histomorphometry using fluorescent labels would allow for the assessment of bone formation and mineralization at an even higher resolution [85]. However, owing to the lack of an appropriate marker, bone resorption can only be estimated from the number of osteoclasts. In this regard, the non-destructive nature of time-lapsed micro-CT imaging provides a major advantage as both bone formation as well as bone resorption can be assessed longitudinally in individual mice [25, 39], thereby reducing the number of animals needed for experiments. Nevertheless, we and others have previously shown high correlations between dynamic histomorphometric parameters quantified by micro-CT and conventional histomorphometry, respectively [25, 39, 86]. A further advantage of micro-CT, however, is that bone histomorphometric parameters can be assessed in the entire 3D compartment rather than in a limited number of 2D histological sections. This not only reduces labor-intensive processing of the samples but also reduces inter-and intra-observer errors associated with the analysis thereof [87, 88]. The final determining advantage for using in vivo micro-CT in this particular study though was the long-term period of 20 weeks over which bone remodeling activities were assessed. Due to the resorption of fluorochrome labels, 2D histomorphometry requires very short intervals (2-3 days) between marker injections [89], and hence, is not well suited for long-term studies.

In conclusion, we show that PolgA mice develop multiple hallmarks of aging, such as reduced bone remodeling and muscle mass early in life, which collectively can be quantified using the mouse FI. We further demonstrate acute mTORC1 hyperactivity in PolgA muscle upon anabolic signals, which is related to diminished satellite cell proliferation. By mimicking many aspects of osteosarcopenia, the PolgA mouse provides a powerful model that facilitates our understanding of the relationship between muscles and bones, and also between the aging musculoskeletal system and frailty.
Acknowledgements: We gratefully acknowledge Dr. Ilaria Bellantuono for her guidance and inputs for the quantification of the clinical mouse frailty index. We acknowledge Susanne Friedrich for performing the force-grip assessments. The authors certify that they comply with the ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2017 [90]. Funding: This manuscript is based upon work supported by the Swiss National Science Foundation (SNF IZCNZ0-174586), the European Cooperation in Science and Technology (COST Action BM1402: MouseAGE) and the European Research Council (ERC Advanced MechAGE ERC-2016-ADG-741883). Author contributions: ACS and GDH designed the experiments, collected the data and wrote the manuscript, GAK designed the experiments, collected the data and reviewed the manuscript, ME collected the data and reviewed the manuscript, ESW provided support establishing methods and reviewed the manuscript, RM and KDB designed the experiments and wrote the manuscript. Competing Interests: All authors declare that they have no conflicts of interest.
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA^{D257A/D257A} mice

Supplementary Materials and Methods

**Breeding scheme**

As both heterozygous (PolgA^{D257A/+}) and homozygous (PolgA^{D257A/D257A}) mice have progressive accumulation of mitochondrial DNA point mutations, specific breeding considerations were taken into account while expanding the colony. Specifically, as paternal mitochondrial DNA is actively eliminated following fertilization in mice, the (undesirable) accumulation of mutations in the germline was minimized by mating heterozygous male mice (PolgA^{D257A/+}) with C57Bl/6J inbred females (Charles River Laboratories, Sulzfeld, Germany). The thus obtained heterozygous (PolgA^{D257A/+}) females and males (originating from a wild type (WT) C57BL/6J mother) were crossed (age 7-9 weeks) to generate homozygous (PolgA^{D257A/D257A}, referred to as PolgA) and WT littersmates (PolgA^{+/+}, referred to as WT) with only a single generation of mutation burden.

**Mouse genotyping**

The presence of the PolgA knock-in mutation was confirmed by extracting DNA from ear clips (Sigma-Aldrich, KAPA Express Extract, KK7103) followed by qPCR (Bio-Rad, SsoAdvanced Universal SYBR Green Supermix, 1725272) and melt curve analysis. The primers used for genotyping (5’ to 3’; Rev Common: AGT AGT CCT GCG CCA ACA CAG; Wild type forward: GCT TTG CTT GAT CTC TGC TC; Mutant forward: ACG AAG TTA TTA GGT CCC TCG AC) were recommended by the Jackson Laboratory.

**Complex IV Enzyme Activity**

In order to confirm that the premature aging phenotypes were associated with mitochondrial dysfunction, we measured the activity of complex IV enzyme in *m. gastrocnemius* (GAS) using the Complex IV Rodent Enzyme Activity Microplate Assay Kit (ab109911, Abcam). Briefly, GAS was homogenized using a tissue homogenizer (Omni THg, Omni International) and protein concentration was determined using the DC assay protein method (Bio-Rad). 50 μg of proteins in 200 μL assay solution were loaded into each well of 96 well plates coated with COX specific antibodies, and incubated at room temperature for 3 hours. The oxidative capacity of COX in the presence of cytochrome C was monitored by absorbance at 550 nm at 30°C for 120 minutes using a photospectrometer (Spark, TECAN), with a measurement interval of 1 minute. Rates of oxidation were calculated by using absorbance values during the time when the
Chapter 4 Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging

decrease of OD values was linear. We found that COX IV activity in PolgA muscles was lower compared to WT muscles both at 40 (-19%, p<0.0001) and 46 weeks (-24%, p<0.0001), thus confirming that the mice used in this study had the same phenotype as those previously reported [36, 37, 40].

Muscle progenitor isolation

Primary muscle progenitor cells (MPs) were extracted according to an adapted protocol described by Gorski et al. [91]. In brief, muscle tissue was digested with 2mg/mL collagenase type II (Thermo Scientific Scientific) in collagenase buffer 2% Fetal Bovine Serum (FBS) (Invitrogen) in Hanks Balance Salt Solution (HBSS) (Invitrogen) for 1h at 37°C. The cell pellet was filtered using 40 and 100µm cell strainers. A heterogeneous cell population was purified from fibroblasts by serial pre-plating (1.5h) at day 0, 4 and 10 after cell extraction. Muscle progenitors were cultured on matrigel-coated dishes (Corning, 1/25 dilution) in medium containing 40% F10 nutrient mixture (Invitrogen, 40% low-glucose DMEM (Invitrogen), 20% FBS, 1% Penicillin-Streptomycin (P/S) (Invitrogen) and 5 ng/mL basic fibroblast growth factor (Thermo Fischer Scientific).

Muscle fiber cross-sectional area determination

For histology and morphometry of skeletal muscle, 10 µm thick cryosections of TA muscles were dried and washed for 5 min in PBS supplemented with 0.05% triton and subsequently incubated with wheat germ agglutinin Alexa Fluor 647 (1:250, Thermo Fischer Scientific) for 1h. Slides were mounted after a 3x 5min wash in PBS, sealed with glass cover slips and imaged with an epifluorescent microscope (Zeiss Axio observer Z.1) at 10x. Fiber cross-sectional area was automatically determined with a Muscle J plugin for Image J software [92].
### Table 4.S1 Animal experiments: Overview, setup, and analysis details

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Experiment details</th>
</tr>
</thead>
</table>
| 1. Frailty and musculoskeletal phenotyping in PolgA vs. WT:                | Total sample size: n=88 female mice  
4 groups with each group:  
WT: n=10  
PolgA: n=12  
3 groups  
in vivo measurements:  
- Frailty Index (Fig. 4.1)  
- Forelimb Grip-Strength (Fig. 4.2)  
ex vivo measurements:  
- Micro-CT of femora (Fig. 4.1)  
- Muscle weights & forces (Fig. 4.1 & 4.2)  
- Eccentric Contraction (Fig. 4.3 & 4.4) |
| - Cross-sectional comparisons at 34, 40 and 46 weeks                       |                                                                                                                                                                                                                      |
| - Longitudinal monitoring of frailty and bone morphometry                 | 1 group  
- Frailty Index (Fig. 4.1) measured at 34, 38 and 40 weeks  
- Bi-weekly in vivo micro-CT of caudal vertebrae (Fig. 4.6 & 4.7)  
- Western blots from caudal vertebrae (Fig. 4.8)                                                                                                         |
| - Bone turnover markers from serum at 40 weeks                            | Total sample size: n=11 female mice  
WT: n=8  
PolgA: n=3  
(Fig. 4.7)                                                                                                                                                 |
| 2. Leucine administration                                                 | Total sample size: n=18 female mice  
(intervention at 46 weeks)  
WT: n=3 CTL and n=6 LEU  
PolgA: n=3 CTL and n=6 LEU  
- Leucine administration (Fig. 4.5)                                                                                                                     |
| 3. Cyclic mechanical loading of caudal vertebrae                          | Total sample size: n=80 female mice  
Young mice (intervention start at 15 weeks):  
WT: n=10 CTL and n=10 Loaded  
PolgA: n=10 CTL and n=9 Loaded  
Aged mice (intervention start at 38 weeks):  
WT: n=11 CTL and n=8 Loaded  
PolgA: n=9 CTL and n=9 Loaded                                                                                                                               |
Chapter 4 Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging

- Cyclic mechanical loading of 6th caudal vertebrae (over 4 weeks)
- *In vivo* micro-CT of caudal vertebrae (Fig. 4.9)

<table>
<thead>
<tr>
<th>Table 4.52 Antibodies used for Immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagents</strong></td>
</tr>
<tr>
<td>Lysis buffer</td>
</tr>
<tr>
<td>TBST</td>
</tr>
<tr>
<td><strong>Primary antibodies 1:1000</strong></td>
</tr>
<tr>
<td>p-p70S6K&lt;sup&gt;Thr389&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-RPS6&lt;sup&gt;Ser235/236&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-SAPK/JNK&lt;sup&gt;Thr183/Tyr185&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-p44/42 MAPK (Erk1/2)&lt;sup&gt;Thr202/Tyr204&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-ULK&lt;sup&gt;Ser757&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-mTOR&lt;sup&gt;Ser2448&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Secondary antibody 1:5000</strong></td>
</tr>
<tr>
<td>Anti-Rabbit IgG, HRP-linked Antibody</td>
</tr>
</tbody>
</table>
Table 4.S3 Cortical bone morphometry of femora. Two-dimensional cortical bone morphometric parameters in WT and PolgA mice at 34, 40 and 46 weeks, respectively. (Data represent mean±SD, ** p<0.01, *** p<0.0001 WT vs PolgA at given age)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>34 weeks</th>
<th>40 weeks</th>
<th>46 weeks</th>
<th>34 weeks</th>
<th>40 weeks</th>
<th>46 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>PolgA</td>
<td>WT</td>
<td>PolgA</td>
<td>WT</td>
<td>PolgA</td>
</tr>
<tr>
<td>Ct.Ar (mm²)</td>
<td>0.778±0.027</td>
<td>0.755±0.031</td>
<td>0.780±0.038</td>
<td>0.679±0.054***</td>
<td>0.796±0.066</td>
<td>0.664±0.053***</td>
</tr>
<tr>
<td>Tt.Ar (mm²)</td>
<td>1.684±0.05</td>
<td>1.633±0.075</td>
<td>1.702±0.066</td>
<td>1.615±0.084**</td>
<td>1.820±0.061</td>
<td>1.694±0.074**</td>
</tr>
<tr>
<td>Ct.Ar/Tt.Ar (%)</td>
<td>46.208±1.686</td>
<td>46.316±2.099</td>
<td>45.802±1.018</td>
<td>42.015±2.336**</td>
<td>43.755±2.958</td>
<td>39.064±2.576***</td>
</tr>
<tr>
<td>Ct.Th (mm)</td>
<td>0.196±0.008</td>
<td>0.197±0.008</td>
<td>0.199±0.006</td>
<td>0.177±0.013***</td>
<td>0.195±0.016</td>
<td>0.166±0.012***</td>
</tr>
<tr>
<td>Ps.Pm (mm)</td>
<td>4.680±0.092</td>
<td>4.581±0.118</td>
<td>4.701±0.100</td>
<td>4.564±0.134**</td>
<td>4.847±0.086</td>
<td>4.654±0.105***</td>
</tr>
<tr>
<td>Ec.Pm (mm)</td>
<td>3.437±0.109</td>
<td>3.382±0.142</td>
<td>3.441±0.089</td>
<td>3.482±0.126</td>
<td>3.638±0.13</td>
<td>3.648±0.1</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>15.996±0.227</td>
<td>15.922±0.283</td>
<td>16.29±0.122</td>
<td>15.916±0.298**</td>
<td>16.269±0.206</td>
<td>16.085±0.222**</td>
</tr>
</tbody>
</table>
References


Chapter 4 Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging


4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA<sup>D257A/D257A</sup> mice


Chapter 4 Longitudinal assessment of frailty and osteosarcopenia in an in vivo model of premature aging


[61] Joseph GA, Wang SX, Jacobs CE, Zhou W, Kimble GC, Tse HW, Eash JK, Shavlakadze T, Glass DJ. Partial inhibition of mTORC1 in aged rats counteracts the decline in muscle mass and
4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA<sup>D257A/D257A</sup> mice


4.1 Hallmarks of frailty and osteosarcopenia in prematurely aged PolgA<sup>D257A/D257A</sup> mice


Chapter 5

Synthesis
5.1 Background

The proportion of people above 65 years of age is expected to double in the next 30 years [1]. As a result, the number of people suffering from the age-related syndrome of frailty will also substantially increase [2]. Although a universally accepted definition is still lacking [3], frailty is characterized by the decline of multiple physiological functions, leading to the accumulation of health deficits, the loss of independence and ultimately, mortality [4]. Considering the crucial role of musculoskeletal health for independence and mobility, diseases such as osteoporosis and sarcopenia are often associated with frailty [5, 6]. Indeed, the co-existence of both osteoporosis and sarcopenia, also termed “osteosarcopenia” [7, 8], in older individuals has been shown to increase the likelihood of frailty [9]. A better understanding of the pathophysiology of osteosarcopenia will help to identify interventions to strengthen the musculoskeletal system, which ultimately will be beneficial for the prevention and/or treatment of frailty. With the advent of longitudinal in vivo phenotyping techniques, animal models are of increasing interest in aging studies as disease progression can be monitored over time in multiple tissues of individual animals. However, a suitable animal model mimicking frailty and osteosarcopenia is still lacking.

Herein, the overall objective of this study was to longitudinally evaluate the suitability of the PolgA<sup>(D257A/D257A)</sup> mouse (PolgA), which due to the expression of an exonuclease-deficient version of the mitochondrial DNA polymerase γ, exhibits a premature aging phenotype [10, 11] as a model of frailty and osteosarcopenia. Based on the assessment of frailty in humans [12], tools such as the clinical mouse frailty index (FI) have been established to quantify the accumulation of health deficits non-invasively in pre-clinical studies [13-16]. Likewise, the ability to perform longitudinal measurements of the bone microstructure by means of in vivo micro-computed tomography (micro-CT) imaging has provided invaluable information on skeletal responses to diseases such as osteoporosis and osteosarcopenia [17-20] as well as to therapeutic interventions such as mechanical loading [21-23]. Although the anabolic effects of mechanical loading on healthy bone are well described, several studies have reported reduced mechano-responses with age [22-24], which in turn, could be linked to reduced mechanosensitivity of osteocytes, the main orchestrators of bone remodeling [25]. However, the mechanisms by which osteocytes – embedded deep within the bone matrix - sense mechanical loading exerted at the organ scale remain poorly understood.
Therefore, the first part of this thesis aimed at developing an approach to study bone mechanobiology \textit{in vivo} across multiple scales. Firstly, a previously established mouse tail loading model \cite{26} was used to assess the effects of varying loading frequencies on trabecular bone adaptation \textit{in vivo}. By combining \textit{in vivo} micro-CT imaging with micro-finite element (micro-FE) analysis, bone adaptation was monitored over time in individual animals and the link between bone remodeling activities and mechanical environments at the tissue scale were assessed. In addition, using an approach termed “Local \textit{in vivo} Environment (LivE) histochemistry”, the 3D structural and mechanical information revealed by micro-CT and micro-FE analysis, were linked to molecular information revealed by immunohistochemistry thereby allowing us to associate protein level data to the local mechanical and bone adaptation environments \cite{27,28}.

Once established, the second part of this study aimed at developing an optimized long-term \textit{in vivo} micro-CT imaging approach, which combined with longitudinal assessments of the FI, would allow us to monitor changes in the bone micro-architecture and the development of frailty in individual mice during the process of aging. As \textit{in vivo} micro-CT is associated with cumulative radiation dosage, anesthesia and stress, which could affect the bone morphometric and overall well-being of aged animals \cite{22,29-33}, it was imperative to ensure the applicability of long-term \textit{in vivo} micro-CT imaging for usage in aging mouse studies. Therefore, an additional aim of this chapter was to assess the cumulative effects of radiation, anesthesia and handling associated with long-term micro-CT imaging on bone morphometric parameters as well as the FI.

Finally, the thus established long-term micro-CT and FI approach was combined with comprehensive musculoskeletal phenotyping tools to longitudinally assess the development of frailty and osteosarcopenia in the PolgA model. Specifically, alongside the longitudinal measurements of the bone micro-architecture and FI, the quantity and quality as well as the anabolic responsiveness of bone and muscle tissue were evaluated.

\textbf{5.2 Main findings and implications}

This study is, to the best of our knowledge, the first \textit{in vivo} study showing that trabecular bone adaptation logarithmically depends on loading frequency. These results are in line with an experimental study performed on cortical bone \cite{34} as well as with a theoretical prediction performed on trabecular bone \cite{35}. Furthermore, by combining \textit{in vivo} micro-CT imaging with
Chapter 5 Synthesis

micro-FE analysis, this study showed that bone remodeling events (i.e., formation, resorption and quiescence) can be linked to local mechanical environments in vivo, in agreement with previous studies [21, 27, 28, 36]. Consistent with the anabolic responses observed at the tissue scale, cyclic, but not static loading resulted in an overall down-regulation of Sclerostin and RANKL expression. A load-induced down-regulation of Sclerostin, an osteocyte-specific inhibitor of bone formation, and of RANKL, an osteoclast stimulatory factor, have previously been reported [37-41]. Furthermore, the RANKL expression in osteocytes subjected to cyclic loading was lower in regions close to bone formation than in regions close to bone resorption, thus suggesting that alterations in protein levels are locally linked to the in vivo microenvironment. This study thereby showed that loading frequency affects bone adaptation not only at the tissue scale, both also at the cellular scale, consistent with previous studies [42-44]. That frequency influences the process of mechanotransduction has also been widely studied in other physiological systems such as the inner ear [45, 46]. Hence, drawing an analogy to the theory of sound pressure level, it is possible that bone’s response to frequency is similar to the logarithmic perception of sound in human hearing. Finally, this study demonstrates how the combination of a well-established loading model, in vivo micro-CT imaging, computational modeling and immunohistochemistry provides the possibility to investigate how forces exerted at the organ scale can be linked to responses at the cellular and molecular scale, which ultimately lead to coordinated remodeling at the tissue scale. By integrating the investigation of multiple cell types and molecular markers in future studies, this approach will allow to understand bone mechanobiology in its entirety - as a process involving multiple, interacting cell types influenced by their local 3D microenvironments.

The next achievement in this chapter was the development of a long-term in vivo micro-CT imaging approach, which, coupled with longitudinal assessments of the FI, allowed us to track dynamic bone remodeling activities as well as the onset of osteopenia and frailty in PolgA mice. While PolgA mice have been shown to develop multiple signs of aging early in life, including reduced bone mineral density as assessed by X-ray densitometry [10, 11] and ex vivo micro-CT [47], this study was the first to evaluate frailty as well as the temporal development of the bone micro-architecture and turnover in the PolgA model. Specifically, this study showed that the FI and bone morphometric parameters were initially similar between PolgA and wild-type (WT) littermates, but then diverged over time such that PolgA had higher FI as well as lower trabecular and cortical bone volume and thickness, respectively. In addition, the bone remodeling activities were lower in PolgA mice compared to WT. The PolgA mouse thus
mimics the attenuated bone turnover, which is characteristic of senile osteoporosis in humans [48].

Whereas in vivo micro-CT imaging has already been used to monitor the dynamic changes in the micro-architecture of mouse caudal vertebrae at various ages [19, 21], the approach was not optimized to longitudinally monitor individual mice during the entire process of aging. Although the cumulative effects of radiation, anesthesia and handling associated with in vivo micro-CT imaging resulted in small effects on static bone morphometric parameters, the observed effects were less than the observed effects of genotype, and no interactions between more frequent scanning and genotype were found, suggesting that the comparison between genotypes remained valid. Furthermore, the overall well-being of the animal was not affected by more frequent micro-CT scanning, as shown by similar FI and body-weights between the different scanning groups. An additional important finding of this study was that longitudinal designs including baseline measurements already at young age were more powerful at detecting age-related phenotypic changes than those including multiple groups with fewer imaging sessions. Coupled with frailty assessments, the data obtained from individual mice can be maximized in longitudinal studies, providing not only a more comprehensive evaluation of multi-system dysfunction but also the reduction in the number of animals needed for experiments. Indeed, the FI has already been shown to be a promising measure to predict osteoporotic fractures in clinical studies [49, 50]. In the future, the combination of FI measurements with novel time-lapsed in vivo High-Resolution peripheral Quantitative CT (HR-pQCT) approaches [51, 52] could therefore be a valuable tool improve clinical diagnostics for and management of osteoporosis.

In the last chapter, the combined in vivo micro-CT and FI approach developed in previous chapters were extended to include a comprehensive analysis of the musculoskeletal system of prematurely aged PolgA mice. Concomitant to an increase in FI over time, PolgA mice displayed progressive musculoskeletal deterioration such as reduced bone and muscle mass as well as impaired functionality thereof; In addition to lower muscle weights and fiber area compared to WT littermates, PolgA mice showed lower grip-strength and concentric muscle forces as well as lower bone remodeling and turnover. Furthermore, PolgA mutation altered the response to various anabolic stimuli in skeletal muscle, muscle progenitors and bone. While PolgA bones showed reduced mechano-responsiveness to mechanical loading with age, PolgA muscles showed increased acute response to mechanical stimuli such as eccentric contractions.
and protein administration. Interestingly, contrasting results between bone and muscle tissues in PolgA mice were also observed in the basal signaling of the mammalian target of rapamycin (mTORC1) and the stress responsive mitogen-activated protein kinase (MAPK) pathways; while basal downstream mTORC1 and MAPK signaling was almost absent in PolgA mutated bone, it was hyperactive in PolgA muscle. Tissue-specific differences in mTORC1 signaling have been shown previously [53] and could be linked to the controversial effects of pharmacological inhibition of mTORC1 signaling observed in bone and muscle, respectively [54-57]. However, future studies are required to better understand differences and potential interactions in mTORC1 signaling between muscle and bone, and whether such discrepancies can be linked to altered mechanosensitivity with age. Taken together, this study showed that PolgA mutation leads to many hallmarks of age-related frailty and osteosarcopenia and provides a powerful model to better understand the relationship between frailty and the aging musculoskeletal system. Furthermore, aside from testing the effects of non-pharmaceutical interventions such as exercise, the PolgA mouse provides a valuable model to investigate the effects of pharmaceutical interventions on multiple organs and systems [58].

5.3 Limitations and future research

With respect to Chapter 3, a general limitation of the study was that the strain magnitude and duration of the individual loading bouts were the same for all loading groups, resulting in a different number of loading cycles as well as a different strain rate between groups loaded at various frequencies. Although the number of loading cycles has not been shown to largely influence bone adaptation [59], the strain rate is known to be a major determinant thereof [60-62]. Hence, whether bone’s osteogenic response to loading is limited to a specific range of frequencies, below and above which bone becomes less osteogenic, requires further in vivo experiments that control for the number of loading cycles as well as the strain rate.

A further limitation in Chapter 3 concerns the “Local in vivo Environment histochemistry” approach. Owing to the low throughput with respect to the number of bone samples as well as the number of analyzed cells, the links between protein level data and the corresponding local in vivo mechanical and remodeling environments should be interpreted with caution. Similarly to what has been shown in previous studies [28, 63], a higher number of histological sections as well as of the number of animals are required to find clear evidence on the relationship between the local mechanical environment and variations in protein levels. Furthermore, owing
to unavoidable morphological distortions of the tissue sections that can arise during histological processing such as decalcification, it was difficult to ensure a fully accurate registration of the 2D histological section into the 3D micro-CT volume. As the 2D/3D registrations in this study were performed manually using an in-house developed graphical user interface, further studies should compare the results using an automatic registration tool to increase throughput and minimize operator error. Another shortcoming of the study was the limitation of immunohistochemistry, which did not allow a simultaneous investigation of multiple biochemical markers per osteocyte. In future, these limitations could be overcome by combining the LivE approach with other emerging techniques to analyze single cells such as laser micro-dissection combined with transcriptomic [64, 65] and proteomic [66] profiling, or imaging based methods such as fluorescence in situ hybridization [67, 68].

With respect to the last chapter, a large limitation of the study was the differences in methods used to evaluate the bone and muscle tissue. While in vivo micro-CT imaging allowed us to longitudinally track changes in bone architecture in individual mice, the analysis of the muscle tissue was performed ex vivo, thus cross-sectionally. Novel applications of in vivo micro-CT imaging such as the monitoring of whole body composition, including skeletal, lean and fat mass, during the process of aging should be considered for future studies using aging models to better capture concurrent changes in the bone and soft tissue [69]. Nevertheless, despite the numerous advantages of in vivo micro-CT imaging, the limited resolution does not provide any information on the cellular and molecular scale. Therefore, the incorporation of immunohistochemistry and/or gene expression analysis would be useful to gain a deeper understanding of the mechanisms underlying the osteopenic phenotype in PolgA mice. In the future, the application of the “LivE histochemistry” approach, as described in Chapter 3, could be valuable on investigating the mechanisms underlying alterations in mechanosensitivity in prematurely aged PolgA mice.

Lastly, although the PolgA model mimics multiple age-related phenotypes, the relevance of mitochondrial DNA mutations in natural human aging remains unclear. While increases in mitochondrial dysfunction and in mutational load have been linked to natural aging [70-72], the type and magnitude of mitochondrial DNA mutations observed in PolgA mice may not fully recapitulate natural aging [73]. Therefore, future studies comparing PolgA mice to naturally aged mice could be helpful to better understand the links between mitochondrial DNA and natural aging. Nevertheless, as “no model is perfect” and provided the differences to humans
are kept in mind, the PolgA model can provide insight into the mechanisms of age-related frailty and multi-system deterioration observed in normal aging [74]. In this respect, the advantages of using accelerated aging models should also be considered; these include reduction of time and costs required for experiments, as well as the opportunity of using age-matched WT littermates as a control, which ensures that both the genetic background as well as the environment are comparable between groups.

5.4 Conclusion

In conclusion, the optimization of an in vivo multiscale mechanobiology approach to study the effects of loading frequency on bone adaptation across multiple scales was successful. This study firstly highlighted that loading frequency affects bone adaptation both at the tissue and cellular scale and, that the integration of protein level data into the 3D structural and mechanical data, provides the possibility to link bone adaptation at the tissue and cellular scale to the local in vivo microenvironments. The investigation of bone mechanobiology across multiple scales has thereby been useful in improving our understanding of osteogenic responses to mechanical loading in vivo, and thus, provides the groundwork for elucidating age-related alterations in bone mechanosensitivity. Secondly, the combination of long-term in vivo micro-CT imaging together with longitudinal FI assessments, as developed in this thesis, was successfully applied to evaluate the development of frailty and senile osteoporosis in an in vivo model of premature aging. Finally, by extending the established approach with comprehensive musculoskeletal phenotyping techniques, this study showed that PolgA mice develop many hallmarks of frailty and osteosarcopenia including reduced bone and muscle mass, reduced functionality thereof as well as reduced sensitivity to various anabolic stimuli. The PolgA mouse thus provides a powerful model to improve our understanding of the relationship between muscles and bones, and between the aging musculoskeletal system and frailty. In future, the application of the multiscale mechanobiology approach in PolgA mice could provide valuable insight on the mechanisms underlying reduced mechanosensitivity in age-related diseases of the musculoskeletal system.
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