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POLYTECHNIQUE MONTRÉAL

affiliée à l'Université de Montréal

***In vivo* experimental platform evaluating synchronously 3D bone growth,
(re)modeling, microdamage and mechanical properties**

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Thèse présentée en vue de l'obtention du diplôme de *Philosophiæ Doctor*

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Cette thèse intitulée :

***In vivo* experimental platform evaluating synchronously 3D bone growth,
(re)modeling, microdamage and mechanical properties**

présentée par **Bohao NING**

en vue de l'obtention du diplôme de *Philosophiæ Doctor*

a été dûment acceptée par le jury d'examen constitué de :

Carl-Éric AUBIN, président

Isabelle VILLEMURE, membre et directrice de recherche

Catherine LAPORTE, membre et codirectrice de recherche

Lama SÉOUD, membre

Annie LEVASSEUR, membre externe

DEDICATION

*To my parents and grandparents,
thank you for everything.*

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RÉSUMÉ

L'ostéoporose est un trouble métabolique majeur qui affecte des millions de personnes à travers le monde. La population féminine âgée est particulièrement sujette à une qualité osseuse compromise en raison de la ménopause. Les bisphosphonates, l'un des traitements courants de l'ostéoporose et de l'ostéopénie, peuvent augmenter la densité minérale osseuse en inhibant la résorption osseuse. Cependant, l'inhibition de la résorption pourrait réduire l'efficacité des réparations de microdommages, conduisant à une intégrité squelettique entravée. Pour surmonter cette limite thérapeutique, il est essentiel de mieux comprendre l'interaction entre les mécanismes clés reliés à l'accumulation et la réparation des microdommages osseux. Des modèles animaux de chargement des membres antérieurs ont été développés pour étudier les microdommages dans l'os cortical. Des modèles de rongeurs ovariectomisés ont également été largement utilisés pour représenter la perte osseuse post-ménopausique. La combinaison de ces deux modèles précliniques avec une évaluation simultanée des microdommages osseux, du remodelage et des propriétés mécaniques fournirait des connaissances fondamentales pour l'optimisation des traitements de l'ostéoporose et de l'ostéopénie.

Le pic de masse osseuse atteint au début de l'âge adulte influence la qualité osseuse plus tard dans la vie. Un développement osseux optimisé pendant l'enfance et la puberté contribue à l'amélioration du pic de masse osseuse. Les exercices physiques sont l'un des facteurs critiques pouvant affecter la croissance des os : des entraînements modérés peuvent améliorer le développement osseux, tandis que des exercices excessifs peuvent altérer le processus de croissance. Des modèles animaux précliniques ont été utilisés dans la littérature pour mieux comprendre les effets de différents niveaux d'exercice sur la croissance osseuse. Cependant, la méthode conventionnelle pour l'évaluation de la croissance osseuse est basée sur l'histologie et nécessite donc des sacrifices d'animaux, ce qui rend impossible le suivi du taux de croissance osseuse d'un animal donné dans le temps. En comparaison, l'imagerie micro-CT *in vivo* permet une évaluation non destructive de la microstructure osseuse de manière tridimensionnelle (3D). Cette approche offre un grand potentiel pour la mesure de la croissance osseuse *in vivo* en appliquant les algorithmes de recalage sur les images acquises à différents moments.

En combinant ces informations, l'objectif général de la présente thèse était de développer une plateforme expérimentale qui permet la quantification simultanée de la croissance osseuse, du (re)modelage, des microdommages et des propriétés mécaniques chez les rats de laboratoire soumis à un chargement mécanique *in vivo* engendrant du microdommage. Quatre objectifs spécifiques ont été établis afin de progresser étape par étape vers cet objectif général. Tout d'abord, une méthode *in vivo* basée sur le micro-CT a été développée pour la quantification de la croissance osseuse longitudinale, puis validée par rapport à la méthode conventionnelle basée sur l'histologie. Deuxièmement, une méthode *in vivo* basée sur le micro-CT pour l'évaluation du (re)modelage osseux a été adaptée et appliquée dans une étude rétrospective d'analyse de données. En outre, plusieurs expériences préliminaires ont été réalisées pour créer et quantifier les microdommages de type « sous-fracture » de manière consistante dans les membres antérieurs du rat. Pour finir, un modèle préclinique de microdommages a été réalisé et analysé en combinant les méthodologies développées et adaptées dans les objectifs spécifiques précédents.

Pour le premier objectif spécifique, des méthodes basées sur le micro-CT et sur l'histologie ont été appliquées sur la même cohorte de rats en croissance rapide (âgés de 35, 49 et 63 jours) pour évaluer le taux de croissance osseuse longitudinale de leur tibia proximal. Les rats ont été scannés deux fois sur leur membre postérieur droit par micro-CT avec un intervalle de cinq jours, et ils ont reçu des injections de calcéine dans un intervalle de trois jours. La méthode basée sur le micro-CT a utilisé la mesure de la distance de croissance à l'aide des scans recalés en 3D, par la suite divisée par l'intervalle de scan de cinq jours. La méthode basée sur l'histologie a utilisé une mesure de distance de croissance bidimensionnelle (2D) entre les bandes fluorescentes de calcéine consécutives sur les images microscopiques, avec cette distance divisée par l'intervalle d'injection de trois jours. La méthode basée sur le micro-CT a été validée en termes d'exactitude et de précision des algorithmes de recalage d'images 3D, et la répétabilité de la mesure de distance 2D a été évaluée pour la méthode basée sur l'histologie. Les deux méthodes ont montré une grande reproductibilité et l'erreur de précision globale était légèrement meilleure pour la méthode basée sur le micro-CT (2,50 %) par rapport à la méthode basée sur l'histologie (2,84 %). Les résultats des deux méthodes ont également été comparés directement pour chaque rat mesuré, montrant des erreurs relatives inférieures à 2,5 % et une corrélation de Pearson significativement positive de 0,998 ($p < 0,0001$). Selon ces résultats de validation, la méthode basée sur le micro-CT pourrait remplacer la méthode

basée sur l'histologie pour la quantification de la croissance osseuse, en particulier avec des études animales longitudinales nécessitant des analyses *in vivo* répétées.

Pour le deuxième objectif spécifique, une méthode basée sur le micro-CT *in vivo* a été adaptée et appliquée pour évaluer le (re)modelage osseux et la croissance osseuse 3D dans l'os long de rat. Pour ce faire, les données micro-CT d'une étude précédente ont été analysées rétrospectivement dans le temps. Les données provenaient de jeunes rats âgés de 28 à 77 jours, qui avaient reçu différents niveaux de chargement mécanique *in vivo* (impact faible, moyen ou élevé) 5 jours par semaine pour un total de 7 semaines. Les paramètres de (re)modelage comprenaient les ratios de volume osseux, les ratios de surface osseuse, les taux 3D d'épaisseur osseuse et les taux 3D de volume osseux normalisés. Ces paramètres ont été évalués pour la formation osseuse et la résorption osseuse, au niveau de l'endosteum (la surface interne) et du périosteum (la surface externe) de la diaphyse de l'os. La croissance osseuse proximale et de l'os complet ainsi que l'épaisseur des plaques de croissance distale et proximale ont également été mesurées à l'aide des images micro-CT. Les résultats ont révélé que le chargement d'impact élevé peut inhiber la croissance tibiale proximale tout en favorisant la croissance de l'os complet. Par ailleurs, les rats soumis à un impact élevé ont présenté des plaques de croissance plus épaisses que les autres rats en général. Sur la base de ces observations, il a été suggéré que l'inhibition de la croissance causée par un chargement d'impact élevé pourrait entraîner une ostéochondrose (ou anomalie de croissance) et une croissance de rattrapage. Les effets d'altération de la croissance peuvent être différents au niveau des plaques de croissance distales et proximales, car elles ont généralement des sections transversales et des volumes différents, entraînant des niveaux de contrainte potentiellement différents tout en supportant les mêmes forces de compression. En outre, le chargement d'impact était responsable de l'augmentation des taux de (re)modelage de l'os cortical avant et après la période de croissance maximale de l'os proximal, ce qui pourrait être expliqué par le passage du modelage au remodelage comme l'activité dominante vers la fin de la puberté. Enfin, il a été confirmé que l'endosteum est plus sensible mécaniquement que le périosteum du tibia de rat, tel qu'observé en comparant chaque paramètre de (re)modelage entre ces deux sites.

Pour le troisième objectif spécifique, plusieurs expériences préliminaires ont été réalisées afin d'adapter et d'appliquer une méthode à base de micro-CT à contraste amélioré utilisant la coloration au BaSO₄, qui permet d'évaluer l'accumulation de microdommages 3D dans l'os cortical

de rat. Tout d'abord, un protocole a été développé pour effectuer un chargement mécanique *in vivo* en compression cyclique (fréquence physiologique de 2 Hz) sur les membres antérieurs du rat à l'aide d'un appareil de microchargement « Mach-1 ». Ensuite, le point de fracture a été déterminé pour les membres antérieurs de 20 rats chargés *in vivo*, en termes de déplacement cumulatif de l'actionneur (1,0391 mm en moyenne), considéré comme un prédicteur du point de fracture. La majorité des rats n'ont pas subi de fracture à moins de 0,60 mm de déplacement cumulatif de l'actionneur. Par conséquent, un chargement mécanique avec un point d'arrêt de 0,60 mm a été considéré comme pertinent pour produire du microdommage tout en évitant une fracture osseuse. L'imagerie micro-CT à contraste amélioré pour la quantification des microdommages osseux a été optimisée sur des échantillons de 15 rats sacrifiés, au niveau de la procédure de coloration ainsi que des paramètres de scan de micro-CT. Subséquemment, deux niveaux de chargement de type « sous-fracture » ont été testés : 0,56 mm de déplacement, équivalent au 54 % du point de fracture; ou 0,60 mm de déplacement, équivalent au 58% du point de fracture. Les données de chargement et l'accumulation de microdommages ont été analysées dans les membres antérieurs du rat, et le niveau le plus élevé (58 % du point de fracture) a été choisi pour la suite des expériences *in vivo* sur la base d'une meilleure consistance du niveau de déplacement cumulatif de l'actionneur. Enfin, les effets de la coloration au BaSO₄ sur les propriétés mécaniques de l'os ont été étudiés pour vérifier si les mêmes échantillons d'os peuvent être réutilisés pour l'évaluation des propriétés mécaniques après la procédure de coloration. Les résultats ont montré qu'aucune propriété mécanique n'était pas altérée, à l'exception de la contrainte ultime du radius. Ces différentes expériences préliminaires ont mené au développement d'une méthode d'évaluation des microdommages osseux corticaux en 3D à l'aide de l'imagerie micro-CT à contraste amélioré par BaSO₄.

Pour le quatrième et le dernier objectif spécifique, une évaluation simultanée des microdommages osseux, du (re)modelage et des propriétés mécaniques a été menée chez des rats adultes sains, ovariectomisés, et traités au zoledronate. Tous les rats ont d'abord reçu une session de chargement mécanique sur leur membre antérieur droit pour générer des microdommages *in vivo*. Ils ont ensuite été sacrifiés pour la quantification des microdommages à l'aide de l'imagerie micro-CT à contraste amélioré par BaSO₄ et pour les tests mécaniques de flexion en trois points, soit 5 jours, soit 15 jours après le chargement, afin d'étudier deux périodes de réparation des dommages. Parallèlement

aux traitements, les rats ont reçu des scans de micro-CT *in vivo* pour suivre les changements de microstructure et les taux de (re)modelage sur une base hebdomadaire. Les résultats ont montré que le traitement au zoledronate peut réduire l'accumulation de microdommages corticaux chez les rats ovariectomisés, ce qui pourrait s'expliquer par l'amélioration de plusieurs propriétés structurelles osseuses telles que la force ultime, la force de rendement, la surface et le volume de l'os cortical. Les rats ont montré une augmentation du volume et de la surface de la formation osseuse après la génération de microdommages, et ce, de manière plus importante pour les rats normaux et ovariectomisés. La formation des os de type « woven » a également été observée dans les cubitus chargés, ce qui était le plus remarquable chez les rats ovariectomisés. Bien que tous les rats aient montré de fortes corrélations entre la formation d'os périosté et l'accumulation de microdommages, les niveaux de corrélation étaient plus faibles pour les rats traités au zoledronate, possiblement causés par ses niveaux de microdommage moins élevés. En résumé, le zoledronate s'est avéré bénéfique pour améliorer les conditions ostéopéniques de l'os cortical chez le rat. La plate-forme expérimentale a été appliquée avec succès pour l'évaluation 3D de plusieurs mécanismes clés reliés à l'accumulation et la réparation des microdommages osseux.

Il existe plusieurs limites concernant les approches expérimentales utilisées dans la présente thèse. Premièrement, les rats de laboratoire ne sont pas forcément les meilleurs modèles animaux pour étudier l'ostéoporose (ou l'ostéopénie) humaine en raison de l'absence de systèmes haversiens, qui sont essentiels pour le remodelage intracortical. Cependant, l'application du chargement mécanique externe est capable d'initier un tel remodelage dans les os de rat, ce qui contourne cette limite expérimentale. En outre, l'évaluation de la croissance osseuse et des microdommages s'est concentrée sur les os longs des membres postérieurs et antérieurs du rat, donc les résultats obtenus pourraient être limités à ces os. D'autres sites osseux, tels que les fémurs et les vertèbres qui sont aussi fréquemment ostéoporotiques (ou ostéopéniques), pourraient être explorés dans de futures études afin de confirmer les résultats provenant de la présente thèse. De plus, les méthodes d'évaluation étaient principalement basées sur l'imagerie micro-CT *in vivo*, pour laquelle des compromis doivent fréquemment être faits entre la dose de radiation et la qualité d'image. En lien avec la nature *in vivo* de l'imagerie longitudinale, l'anesthésie et l'immobilisation répétées pourraient entraîner des problèmes de santé chez les animaux testés. Bien que l'évaluation basée sur le micro-CT de la croissance osseuse, du (re)modelage et des microdommages soit effectuée

de manière non destructive en 3D, les niveaux de détail sont toujours inférieurs aux méthodes conventionnelles basées sur l'histologie. D'autres études pourraient se concentrer sur l'amélioration de la qualité de l'image micro-CT à l'aide d'algorithmes basés sur l'apprentissage avec des étapes de traitement d'image hautement automatisé. Enfin, les propriétés mécaniques osseuses n'ont été examinées qu'à l'aide du test de flexion trois-points, qui est destructif pour les échantillons. Des études futures pourraient explorer les possibilités de tests mécaniques *in vivo* à l'aide des dispositifs de nano-indentation, qui ont également des désavantages en termes de la nature locale des mesures, la limite de taille des échantillons, et la complexité d'interprétation des données.

Malgré les limites, cette thèse offre des avancées notables à la fois dans la méthodologie et les connaissances scientifiques. Une méthode basée sur le micro-CT *in vivo* a été développée et validée pour la quantification de la croissance osseuse longitudinale, qui peut être particulièrement utile pour suivre les profils de croissance dans les études animales longitudinales. En appliquant l'évaluation hebdomadaire basée sur le micro-CT dans un modèle de rat pubertaire soumis à un chargement mécanique, cette étude a été la première à illustrer l'évolution de la croissance osseuse et du (re)modelage tout au long de la puberté du rat. De plus, deux des modèles de rat couramment utilisés, d'une part pour les études sur le microdommage, et d'autre part pour les études sur l'ostéoporose et l'ostéopénie, ont été adaptés et combinés pour étudier l'accumulation de microdommages dans l'os cortical des rats ovariectomisés. La plate-forme expérimentale *in vivo* a été utilisée avec succès pour l'évaluation simultanée des microdommages osseux, du (re)modelage et des propriétés mécaniques. Les résultats ont fourni des connaissances pertinentes pour la gestion clinique de perte osseuse à l'aide du zoledronate. La plateforme expérimentale *in vivo* développée offre un grand potentiel pour de futures études précliniques sur le métabolisme et les pathologies osseuses.

ABSTRACT

Osteoporosis is a major metabolic disorder that affects millions of people across the globe. The elderly female population is especially prone to compromised bone quality because of menopause. Bisphosphonates, one of the common treatments for osteoporosis and osteopenia, can increase bone mineral density by inhibiting bone resorption. However, the resorption inhibition might reduce the effectiveness of microdamage repairs, leading to hindered skeletal integrity. To overcome this therapeutical limit, it is essential to better understand the interplay between the key mechanisms related to the bone microdamage accumulation and repair. Animal forelimb loading models have been developed to study microdamage in cortical bone. Ovariectomized rodent models have also been extensively used to represent post-menopausal bone loss. Combining these two preclinical models with concurrent evaluation of bone microdamage, remodeling, and mechanical properties would provide fundamental knowledge for optimization of osteoporosis and osteopenia treatments.

Peak bone mass achieved at early adulthood influences bone quality later in life. Optimized bone development during childhood and puberty contributes to improved peak bone mass. Physical exercises are one of the most critical factors that can affect young bone growth: moderate training can improve bone development, whereas excessive exercises might alter the growth process. Preclinical animal models have been used in the literature to better understand the effects of different exercise levels on bone growth. However, the conventional method for bone growth evaluation is based on histology and thus requires animal sacrifices, which makes it impossible to follow up the bone growth rate of a given animal across time. In comparison, the *in vivo* micro-CT imaging allows for non-destructive evaluation of bone microstructure in a three-dimensional (3D) manner. This approach shows great potential for *in vivo* bone growth measurement by applying the registration algorithms on the images acquired at different time.

Taken together, the general objective of the present dissertation was to develop an experimental platform which enables simultaneous quantification of bone growth, (re)modeling, microdamage and mechanical properties in laboratory rats undergoing *in vivo* microdamage-inducing mechanical loading. Four specific objectives were set to achieve this general objective. Firstly, an *in vivo* micro-CT-based method was developed for the quantification of longitudinal bone growth, then

validated against the conventional histology-based method. Secondly, an *in vivo* micro-CT-based method for bone (re)modeling evaluation was adapted and applied in a retrospective data analysis study. Furthermore, several preliminary experiments were performed to consistently create and further quantify “sub-fracture” type of microdamage in rat forelimbs. In the end, a preclinical microdamage model was carried out and analyzed by combining the methodologies developed and adapted in the previous specific objectives.

For the first specific objective, both micro-CT-based and histology-based methods were applied on the same cohort of rapidly growing rats (35, 49, and 63 days old) to evaluate the longitudinal bone growth rate of their proximal tibia. The rats were scanned twice on their right hindlimb by micro-CT with a five-day interval, and they were given calcein injections in a three-day interval. The micro-CT-based method used a growth distance measured by the 3D-registered time-lapse scans, and then divided by the five-day scan interval. The histology-based method performed a two-dimensional (2D) growth distance measurement between the consecutive calcein fluorescent bands on the microscopic images, with this distance divided by the three-day injection gap. The micro-CT-based method was self-validated in terms of the accuracy and precision of the 3D image registration algorithms, and the repeatability of the 2D distance measurement has been evaluated for the histology-based method. Both methods showed great reproducibility, and the overall precision error was slightly better for the micro-CT-based method (2.50%) compared to the histology-based method (2.84%). The results from both methods were also compared directly for each measured rat, showing relative errors less than 2.5% and a significantly positive Pearson’s correlation of 0.998 ($p < 0.0001$). Based on these validation results, the micro-CT-based method could replace the histology-based method for bone growth quantification, especially with longitudinal animal studies requiring repeated *in vivo* scans.

For the second specific objective, an *in vivo* micro-CT-based method was adapted and applied to evaluate 3D bone (re)modeling rates and bone growth in rat long bone. To do so, micro-CT data from a previous study was retrospectively analyzed across time. The data originated from young rats of 28-77 days old, which had received different levels of *in vivo* compressive mechanical loading (low, medium, or high impact) 5 days per week for a total of 7 weeks. The (re)modeling parameters included bone volume ratios, bone surface ratios, 3D rates of bone thickness, and 3D rates of normalized bone volume. These parameters were evaluated for the bone formation and

resorption, at the endosteum (the internal surface) and the periosteum (the external surface) of the bone diaphysis. The proximal and total bone growth as well as the thickness of the distal and proximal growth plates were also measured using the micro-CT images. The results revealed that high impact loading can inhibit proximal tibial growth while promoting the total bone growth. On the other hand, high impact rats showed thicker growth plates than the other rats in general. Based on these observations, it was hypothesized that the growth inhibition caused by high impact loading might lead to osteochondrosis (or growth anomaly) and catch-up growth. The growth alteration effects might be different at distal and proximal growth plates as they generally have different cross-sectional areas and volumes, leading to potentially different strain levels while supporting the same compressive forces. Furthermore, the impact loading was responsible for the increase of cortical bone (re)modeling rates before and after the period of peak proximal bone growth, which could be explained by the transitions from modeling to remodeling as the predominant activity towards the end of puberty. Lastly, it was confirmed that the endosteum is more mechanically responsive than the periosteum in rat tibia, as observed by comparing each remodeling parameter between these two sites.

For the third specific objective, several preliminary experiments were performed to adapt and apply a contrast-enhanced micro-CT-based method using BaSO₄ staining, which allows evaluating 3D microdamage accumulation in rat cortical bone. Firstly, a loading protocol was developed to perform *in vivo* cyclic compressive mechanical loading (physiological frequency of 2 Hz) on rat forelimbs using a “Mach-1” micro-loading device. Then, the fracture point was determined for the forelimbs from 20 *in vivo* loaded rats, in terms of cumulative actuator displacement (1.0391 mm on average), considered as a predictor for the fracture point. The majority of the rats did not encounter a fracture within 0.60 mm of the cumulative actuator displacement. Therefore, mechanical loading with a stopping point at 0.60 mm was considered relevant to produce microdamage while avoiding bone fractures. The contrast-enhanced micro-CT imaging for bone microdamage quantification was optimized on test samples of 15 sacrificed rats, in terms of the staining procedure as well as some micro-CT scan settings. Subsequently, two loading levels of the “sub-fracture” type were tested: 0.56 mm of displacement, equivalent to 54% of the fracture point; or 0.60 mm displacement, equivalent to 58% of the fracture point. The loading outputs and microdamage accumulation in rat forelimbs were analyzed, and the higher level (58% of the

fracture point) was chosen for the following *in vivo* experiments based on a better consistency of the cumulative actuator displacement. Lastly, the effects of BaSO₄ staining on bone mechanical properties were investigated to verify if the same bone samples can be reused for the evaluation of mechanical properties after the staining procedure. Results showed that none of the mechanical properties was altered, except for the ultimate stress of the radius. These different preliminary experiments led to the development of an evaluation method of cortical bone microdamage in 3D using BaSO₄ contrast-enhanced micro-CT imaging.

For the fourth and last specific objective, a concurrent evaluation of bone microdamage, (re)modeling, and mechanical properties was conducted in healthy, ovariectomized, and zoledronate-treated adult rats. All the rats firstly received one single session of mechanical loading on their right forelimb to generate *in vivo* microdamage. They were then sacrificed for microdamage quantification using BaSO₄ contrast-enhanced micro-CT imaging, and for mechanical testing using three-point-bending, either 5 days or 15 days post-loading, to study two periods of damage repair. Along the treatments, the rats received *in vivo* micro-CT scans to follow the changes of microstructure and (re)modeling rates on a weekly basis. Results showed that the zoledronate treatment can reduce cortical microdamage accumulation in ovariectomized rats, which might be explained by the enhancement of several bone structural properties such as ultimate force, yield force, cortical bone area and volume. The rats showed increased bone formation volume and surface after the generation of microdamage, especially for the normal and the ovariectomized rats. Woven bone formation has also been observed in loaded ulnae, which was most significant in ovariectomized rats. Although all the rats showed strong correlations between periosteal bone formation and microdamage accumulation, the correlation levels were lower for the zoledronate-treated rats, potentially caused by their lower levels of microdamage. In summary, the zoledronate was shown to be beneficial to improve the osteopenic conditions of cortical bone in rats. The *in vivo* experimental platform has been successfully applied for 3D evaluation of several key mechanisms related to the bone microdamage accumulation and repair.

There are several limitations regarding the experimental approaches utilized in the present dissertation. Firstly, the laboratory rats may not be the best animal models to study human osteoporosis (or osteopenia) due to the lack of Haversian systems, which are essential for intracortical remodeling. However, the application of external mechanical loading is able to initiate

such remodeling in rat bones, which circumvents this experimental limitation. Besides, the evaluation of bone growth and microdamage was focused on the long bones of rat hindlimbs and forelimbs, hence the results obtained could be limited to these bones. Other bony sites, such as femora and vertebrae that are also frequently osteoporotic (or osteopenic), could be explored in future studies to confirm the results from this dissertation. Furthermore, the evaluation methods were primarily based on *in vivo* micro-CT imaging, for which there are always compromises to be frequently reached between radiation dose and image quality. In connection with the *in vivo* nature of the longitudinal imaging, the repeated anesthesia and immobilization might bring health issues on tested animals. Although the micro-CT-based evaluation of bone growth, (re)modeling, and microdamage are performed non-destructively in 3D, the levels of detail are still inferior to the conventional histology-based methods. Further studies could focus on improving the micro-CT image quality using learning-based algorithms with highly automated image processing pipelines. Lastly, the bone mechanical properties were only examined using the three-point-bending test, which is destructive to the samples. Future studies could explore the possibilities of *in vivo* mechanical testing using the nano-indentation devices, which also have disadvantages in terms of the local nature of the measurements, the sample size limit, and the complexity of interpreting the data.

Despite these limitations, this dissertation offers notable advancements in both methodology and scientific knowledge. An *in vivo* micro-CT-based method was developed and validated for quantification of longitudinal bone growth, which can be especially useful to track the growth profiles in longitudinal animal studies. By applying the weekly micro-CT-based evaluation in a pubertal rat model undergoing mechanical loading, this study was the first in the literature to illustrate the evolution of bone growth and (re)modeling across the entire rat puberty. Moreover, two of the commonly used rat models, one for the microdamage studies, and another for the osteoporosis and osteopenia studies, were adapted and combined to investigate the microdamage accumulation in cortical bone of ovariectomized rats. The *in vivo* experimental platform was successfully utilized for the concurrent evaluation of bone microdamage, (re)modeling, and mechanical properties. The results provide relevant knowledge for clinical management of bone loss using zoledronate. The developed *in vivo* experimental platform shows great potential for future preclinical studies on bone metabolism and pathologies.

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LIST OF SYMBOLS AND ABBREVIATIONS

1/mm	Per millimeter
1/mm ³	Per cubic millimeter
2D	Two-dimensional
3D	Three-dimensional
3R	Replacement, reduction, refinement
AFF	Atypical femur fracture
ALN	Alendronate
ANOVA	Analysis of variance
BFR/BS	Bone formation rate per bone surface
BMC	Bone mineral content
BMD	Bone mineral density
BMU	Bone (or basic) multicellular unit
BP	Bisphosphonate
BRR/BS	Bone resorption rate per bone surface
BV/TV	Bone volume fraction
<i>c</i>	Radius of the bone perpendicular to the neutral axis
CCAC	Canadian Council on Animal Care
CI	Confidence interval
cm	Centimetre
Conn.Dn	Connectivity density
Cr.Dn	Microcrack (microdamage) density
Ct.Ar	Average cross-sectional cortical area
Ct.BV	Cortical bone volume

Ct.Th	Average cortical thickness
CT	Computed tomography
d	Maximal diameter of the bending section
°	Degree
°C	Degree Celsius
δ	Displacement
DGPTh	Distal growth plate thickness
D_{post}	Post-yield displacement to failure
D_{pre}	Pre-yield displacement
DS/DV	Damaged surface per damaged volume
DV/BV	Damaged volume per bone volume
E	Young's modulus
ES/BS	Newly eroded surface per bone surface
EV/BV	Newly eroded bone volume per bone volume
ϵ	Strain
ϵ_{pre}	Pre-yield strain
ϵ_{post}	Post-yield strain
F	Force
F_f	Failure force
F_u	Ultimate force
F_y	Yield force
g	Gram
g/cm ³	Gram per cubic centimeter

GPa	Gigapascal
Gy	Gray
h	Hour
H _a	Alternative hypothesis
H ₀	Null hypothesis
HR-pQCT	High-resolution peripheral quantitative computed tomography
HSD	Honestly significant difference
Hz	Hertz
I_x	Second moment of area about the long axis
kg	Kilogram
kVp	Peak kilovoltage
L	Litre
L/min	Litre per minute
L	Span between the two sample supports
LER	Longitudinal erosion rate
m	Metre
M	Molar concentration
MAR	Mineral apposition rate
mg	Milligram
mg/kg	Milligram (of drug) per kilogram (of body weight)
Micro-CT	Micro-computed tomography (also called μ CT)
min	Minute
mJ	Millijoule
mL	Milliliter

mL/kg	Milliliter (of solution) per kilogram (of body weight)
mm	Millimetre
mm ²	Square millimetre
mm ³	Cubic millimetre
MMA	Methylmethacrylate
mmHg	Millimetre of mercury
MPa	Megapascal
MRR	Mineral resorption rate
ms	Millisecond
MS/BS	Newly mineralized bone surface per bone surface
MV/BV	Newly mineralized bone volume per bone volume
μA	Microampere
με	Microstrain
μg	Microgram
μg/kg	Microgram (of drug) per kilogram (of body weight)
μm	Micrometre (micron)
μm/day	Micrometre per day
N	Newton
N/g	Newton (of force) per gram (of body weight)
N/mm	Newton per millimetre
n	Number of animal per group, or number of measurements
nano-CT	Nano-computed tomography
n_i	Number of repeated measurements for rat i
NIH	National Institutes of Health

NOM	Normal
NSAID	Nonsteroidal anti-inflammatory drug
NSERC	Natural Sciences and Engineering Research Council of Canada
OVX	Ovariectomy or ovariectomized
p	p -value
PBS	Phosphate buffered saline
PGD	Proximal growth distance
PGPTh	Proximal growth plate thickness
PGR	Proximal growth rate
PGRatio	Proportion of proximal growth over total growth
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
QMS	Quality Management System
Q–Q plot	Quantile-quantile plot
RAL	Raloxifene
RPI	Reference point indentation
ρ	Pearson correlation coefficient
S	Structural stiffness
s	Second
SD	Standard deviation
SD_i	Standard deviation of growth rate for rat i
SD_{method}	Overall precision error for the method
SERM	Selective estrogen receptor modulator
SMI	Structure model index

SR micro-CT	Synchrotron radiation micro-computed tomography
SV/BV	Stained volume per bone volume
Δ SV/BV	Absolute difference of SV/BV between the contralateral bones
σ	Stress, or standard deviation
σ_f	Failure stress
σ_u	Ultimate stress
σ_y	Yield stress
T_0	Initial translation on average
T_{z1}	Translation along the longitudinal z axis (first stage)
T_{z2}	Translation along the longitudinal z axis (second stage)
Tb.BS	Trabecular bone surface area
Tb.BV	Trabecular bone volume
Tb.BV/TV	Trabecular bone volume fraction
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TGD	Total growth distance
TMD	Tissue mineral density
TPTD	Teriparatide
TTL	Total tibial length
$\bar{\tau}_i$	Average longitudinal growth rate for rat i (micro-CT-based method)
$\bar{\tau}_i^*$	Average longitudinal growth rate for rat i (histology-based method)
u	Modulus of toughness

u_{pre}	Pre-yield toughness
u_{post}	Post-yield toughness
U_{post}	Post-yield energy to failure
U_{pre}	Pre-yield energy
VOI	Volume of interest
WHO	World Health Organization
ZOL	Zoledronate (zoledronic acid)

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CHAPTER 1 INTRODUCTION

1.1 Motivation and goals

Osteoporosis is a major metabolic disorder that affects more than 2.3 million Canadians and over 200 million people worldwide (Osteoporosis Canada, 2022; Reginster & Burlet, 2006), it brings heavy societal and financial burdens to the global healthcare systems (Becker et al., 2010; Sözen et al., 2017). Osteoporotic and osteopenic patients encounter increased risk of fracture due to compromised bone qualities (Dempster et al., 2021; Föger-Samwald et al., 2020). Several factors can lead to significant bone loss, including aging (Burr, 2022; Li & Xu, 2020), menopause (Cohen, 2013; Khosla & Pacifici, 2013, 2021), long-term bed rest (Bauman & Cardozo, 2013; Cardozo & Bauman, 2021), and spinal cord injury (Garland et al., 1992; Jiang et al., 2006).

One of the most common treatments for osteoporosis and osteopenia is bisphosphonates (BPs), which can act to increase bone mineral density (BMD) by inhibiting bone resorption (Allen & Burr, 2011; Russell et al., 2007). However, bone resorption is also part of a daily process for the repair of microdamage (Burr, 1993; Burr, 2014), the inhibition of which can contribute to excessive microdamage accumulation and consequently increased skeletal fragility (Burr et al., 1997; Leali et al., 2011). To optimize BP treatments, it is therefore important to understand the interactions of the underlying mechanisms, which can determine the balance between microdamage accumulation and repair (Donahue & Galley, 2006; Seref-Ferlengez et al., 2015).

Experimental studies in the literature have investigated how microdamage is initiated (O'Brien et al., 2005; Wu et al., 2013), accumulated (Diab & Vashishth, 2007; Zioupos, 2001), propagated (Vashishth, Tanner, et al., 2000; Wang & Niebur, 2006), and repaired (Burr, 2014; Seref-Ferlengez et al., 2014). Bone microdamage was initially quantified using histology (Burr & Hooser, 1995; Burr et al., 1985), and subsequently characterized by contrast-enhanced micro-CT (Tang & Vashishth, 2007; Wang et al., 2007). Bone remodeling can also be evaluated using both histology (Dempster et al., 2013; Ma, 2022) and micro-CT (Birkhold et al., 2015; Schulte et al., 2011), of which the later method showed advantages for non-destructive study designs. Additionally, bone mechanical properties can reflect bone quality of animals under different pharmaceutical treatments (Allen, 2018; Allen & Burr, 2011; Burr, 2016).

Peak bone mass at early adulthood is also an important factor for bone quality later in life (NIH, 2001; Weaver et al., 2016). Optimized pubertal bone growth can increase the peak bone mass achievable upon reaching skeletal maturity (Luiza Loro et al., 2000; Rosengren et al., 2022). The most important factors that can influence pubertal bone growth include calcium intake (Welch & Weaver, 2005; Yadav et al., 2021) and physical exercise (Pitts & Bull, 1977; Welch et al., 2008), where the latter is considered a double-edged sword: moderate exercise levels can be beneficial to skeletal development (Welch & Weaver, 2005; Yan et al., 2020) while excessive training might lead to bone growth alteration (Caine et al., 2001; Theintz et al., 1993). Proper optimization of adolescent physical training is essential for bone growth during childhood (Weaver, 2002).

Past studies in the literature have utilized animal models to study various types of physical training such as mechanical loading (Ménard et al., 2014; Mustafy, Londono, Moldovan, et al., 2019), running (Kutsenko et al., 2021; Niehoff et al., 2004), tower climbing (Notomi et al., 2001), and swimming (Huang et al., 2003). Alongside, bone growth and bone remodeling parameters can be measured using histology to reflect the effects of training. However, the histology-based method is destructive in nature, and can only measure end-point data after animal sacrifice (Álvarez et al., 2000; Cancel et al., 2009; Ménard et al., 2014; Valteau et al., 2011). With the wide adoption of *in vivo* micro-CT imaging, it would be ideal to perform longitudinal evaluation of bone growth and remodeling in a non-destructive manner (Altman et al., 2015; Stern et al., 2015).

Taken together, bone growth at early ages can determine the bone quality later in life, where bone (re)modeling¹, microdamage accumulation and their mechanical responses are critical mechanisms in the context of bone loss management. A concurrent evaluation of bone growth, (re)modeling, microdamage and mechanical properties in animal models can provide insights to optimization of pubertal bone growth and clinical treatments by providing the correlations between these key mechanisms. To achieve these goals, several preliminary experiments were conducted to develop

¹ The term “(re)modeling” refers to both modeling and remodeling combined. Detailed explanations are provided in the section 2.2.3 of Chapter 2.

and adapt the necessary methodologies, followed by primary studies that were focused on applying these methods in preclinical rat models.

1.2 Dissertation structure

The present dissertation is article-based and is carried out within the program of Integrated Master's-Doctorate (Maîtrise Doctorat Intégré). Followed by the present introduction (Chapter 1), Chapter 2 conducts a thorough literature review on the relevant fundamental mechanisms and experimental concepts. Chapter 3 introduces the rationale, research questions and objectives of the thesis. Chapter 4 presents the first article entitled “Validation of an *in vivo* micro-CT-based method to quantify longitudinal bone growth of pubertal rats”, published in *Bone* in January 2022. Chapter 5 presents the second article entitled “Impact loading intensifies cortical bone (re)modeling and alters longitudinal bone growth of pubertal rats”, published in *Biomechanics and Modeling in Mechanobiology* in March 2023. Chapter 6 showcases the preliminary experiments for the quantification of bone microdamage. Chapter 7 presents the third article entitled “Zoledronate reduces loading-induced microdamage in cortical ulna of ovariectomized rats”, submitted to *Journal of the Mechanical Behavior of Biomedical Materials* in June 2023. Chapter 8 discusses the key experimental concepts explored in the previous chapters and highlights their advantages and limitations. Chapter 9 concludes the dissertation and provides recommendations for future studies. The Appendices include two preliminary experiments that were performed for the main studies in Chapter 4 and Chapter 7 respectively, as well as some complementary data for the study in Chapter 7.

CHAPTER 2 LITERATURE REVIEW

2.1 Bone structure and composition

2.1.1 The skeletal system

The adult skeleton system is composed of bone and cartilage. Bone, also called osseous tissue, is a hard type of dense connective tissue that acts as a system of lever and provides support for human body (Wallace, 2019). Cartilage is another form of connective tissue with semi-rigidity, which facilitates flexible movements with smooth surfaces. The human skeletal system ensures several vital functions: the structural support of the body and its movements, the protection of organs, the production of blood cells, and the storage and release of fat and minerals (Jerome et al., 2018; OpenStax, 2013).

As shown in Figure 2.1, bones are classified by shape (Jerome et al., 2018; OpenStax, 2013). Long bones have a cylindrical shape and can be found in the upper limbs (ulna, radius, humerus), lower limbs (tibia, fibula, femur), hands (metacarpals, phalanges) and feet (metatarsals, phalanges). They move when muscles contract, serving as levers. Short bones, such as tarsals and carpals, have even sizes in length, width, and thickness. They allow for some motion while providing support and stability. Flat bones are thin and curved in shape, including cranial bones, ribs, sternum, and scapulae. Muscles attach on flat bones and protect the internal organs. Irregular bones, such as vertebrae and facial bones, have complex shapes, and also serve as protection for internal organs and attachment sites for muscles and associated movements. Patellae are sesamoid bones that protect tendons against compression. They are embedded in tendons, in small and round shapes.

Rodents are among the most popular animal models in pre-clinical research. There are some notable anatomical differences between human and rodent skeletal systems (Jerome et al., 2018). Rodents have long tails, which humans do not have. Compared to humans, the brain of rodent accounts for a smaller proportion of their body, while their incisors and olfactory systems are relatively bigger. Humans have deep orbits, which rodents do not have. Although both humans and rodents are plantigrade animal, rodents are quadrupedal with adapted pelvic and shoulder girdles. Rats and mice have very similar skeletal structure besides their difference in size. Similar to

humans, rodents have long bones, short bones, flat bones, and irregular bones (Maynard & Downes, 2019).

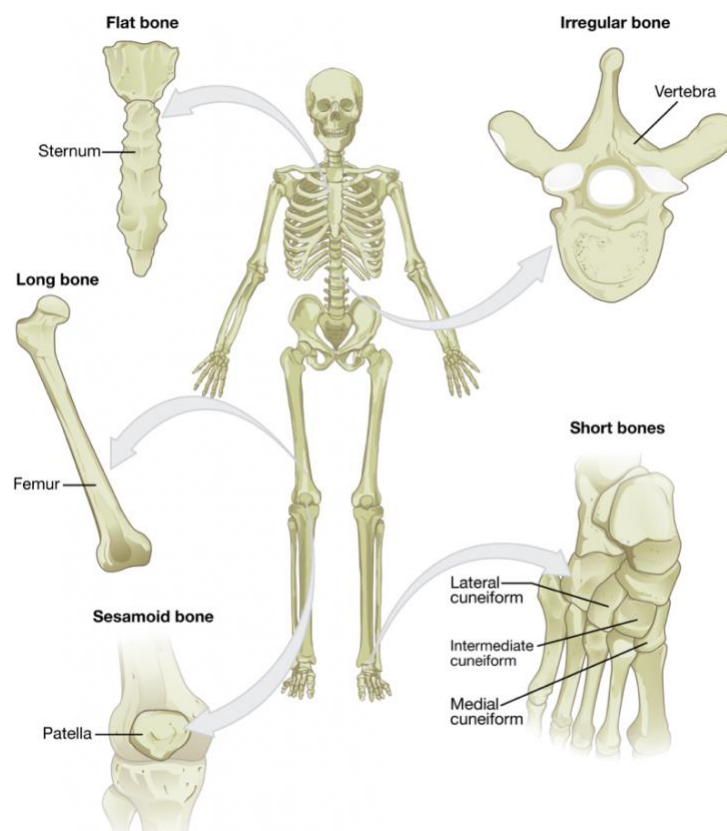


Figure 2.1 Bone classification by shape. Reproduced from (OpenStax, 2019) under the CC BY-SA 4.0 licence.

2.1.2 A hierarchical organization

Under a hierarchical organization (Figure 2.2), bone is formed by an organic matrix (25% to 35% of weight), minerals (65% of weight) and water (10% of weight) (Burr, 2019; Jee, 2001). At the macroscopic level, bone is a composite material including cortical and trabecular bone. Cortical bone (cortex, compact bone) is the exterior shell that supports and protects the inner structure. About 80% of bone mass in the skeleton is cortical bone (Hadjidakis & Androulakis, 2006; Morgan & Gerstenfeld, 2021). Trabecular bone (cancellous, spongy bone) fills the marrow cavity inside and ensures structural support with a light-weight design (Burr, 2019; Jerome et al., 2018; Maynard & Downes, 2019).

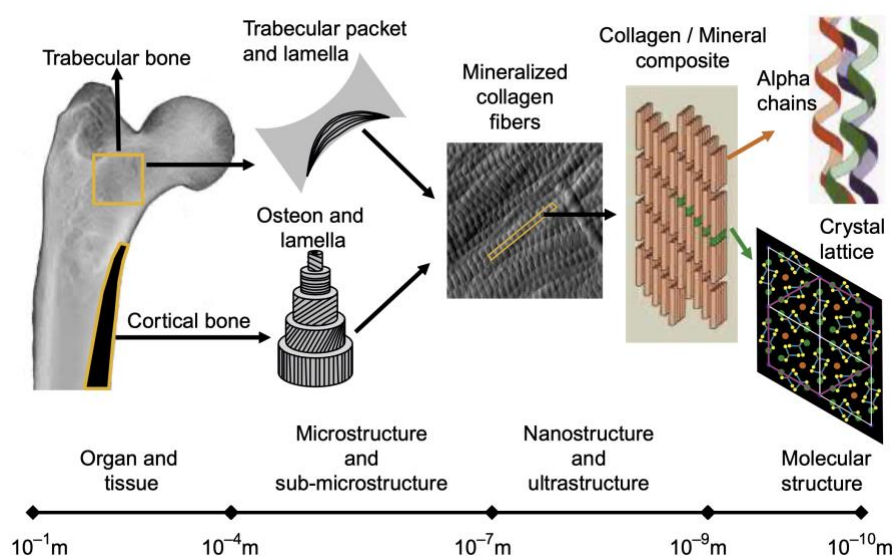


Figure 2.2 The hierarchical organization of bone from 10^{-1} m to 10^{-10} m. Reproduced from (Burr, 2019) with permission of the copyright owner.

At the microscopic level, cortical bone is formed by osteons (Haversian systems), as shown in Figure 2.3. Bone formation and resorption occur in osteons, which are surrounded by concentric lamellae containing collagen and calcified matrix. Cement lines separate osteons from interstitial bone (Burr et al., 1988; Schaffler et al., 1987), which can help to prevent the propagation of fatigue damage (Donahue & Galley, 2006). In the middle of each osteon, a central canal (Haversian canal) carries nerves, lymphatics, and blood vessels (Burr, 2019; OpenStax, 2019). It is believed that this cortical microstructure is adapted for transverse fracture resistance since the osteons and the diaphysis share approximately parallel long axes (Donahue & Galley, 2006).

As for the microstructure of the trabecular bone (Figure 2.4), the lamellae form in a parallel fashion to the trabecular surface (Burr, 2019). Within the layers of lamellae, the lacunae contain osteocytes, forming a network of matrix named trabeculae. The trabeculae are about 100 to 200 μ m thick, in plate- or rod-like shapes, separated by spaces (Donahue & Galley, 2006). Red marrow responsible for hematopoiesis can be found in the spaces within some trabecular bones. Trabecular bone is less stiff than cortical bone for about 20% to 30%, because of their different microstructures (Guo, 2001). However, the trabecular network counterbalances the heavy and dense cortical bone to provide strength and to make muscle movements easier (OpenStax, 2013, 2019).

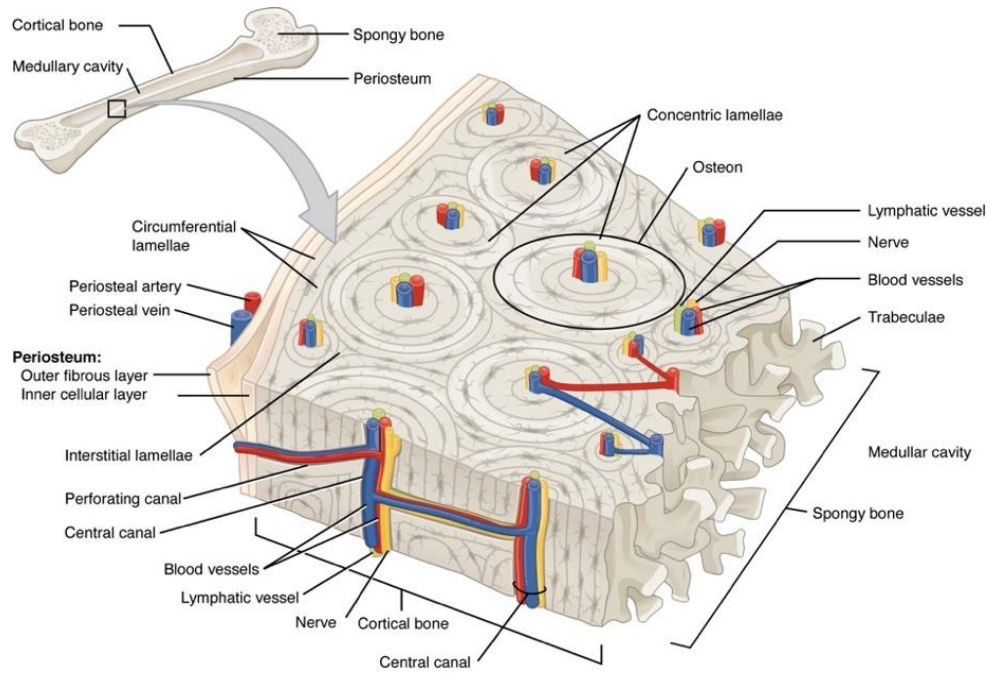


Figure 2.3 A cross-sectional view of the microstructure of cortical bone. Adapted from (OpenStax, 2019) under the CC BY-SA 4.0 licence.

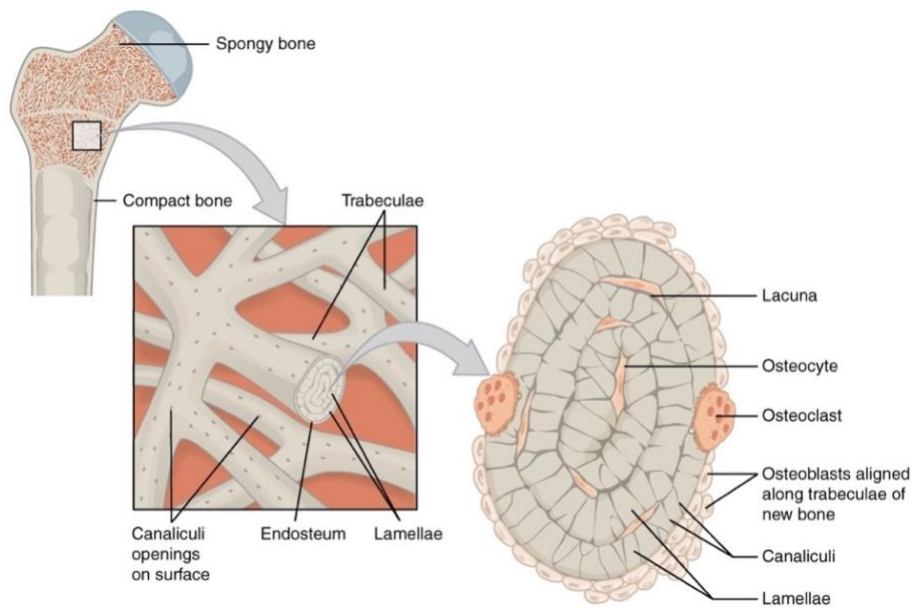


Figure 2.4 A cross-sectional view of the microstructure of trabecular bone and one of its trabeculae. Reproduced from (OpenStax, 2019) under the CC BY-SA 4.0 licence.

2.1.3 Long bones

A long bone is formed by two epiphyses, two metaphyses, and a diaphysis (Figure 2.5). The epiphyses are at both ends of the bone, filled with trabecular bone. The epiphyseal plate (growth plate) is formed by a layer of transparent cartilage in growing bone, separating the epiphysis from the diaphysis (or bone shaft). Osseous tissues replace the cartilage, and the epiphyseal plate turns into an epiphyseal line when the long bone stops growth upon reaching adulthood (around 18 to 21 years old). The outer surface of the epiphysis is a thin cartilage layer that provides friction reduction and shock absorption (Jerome et al., 2018; Maynard & Downes, 2019; OpenStax, 2013).

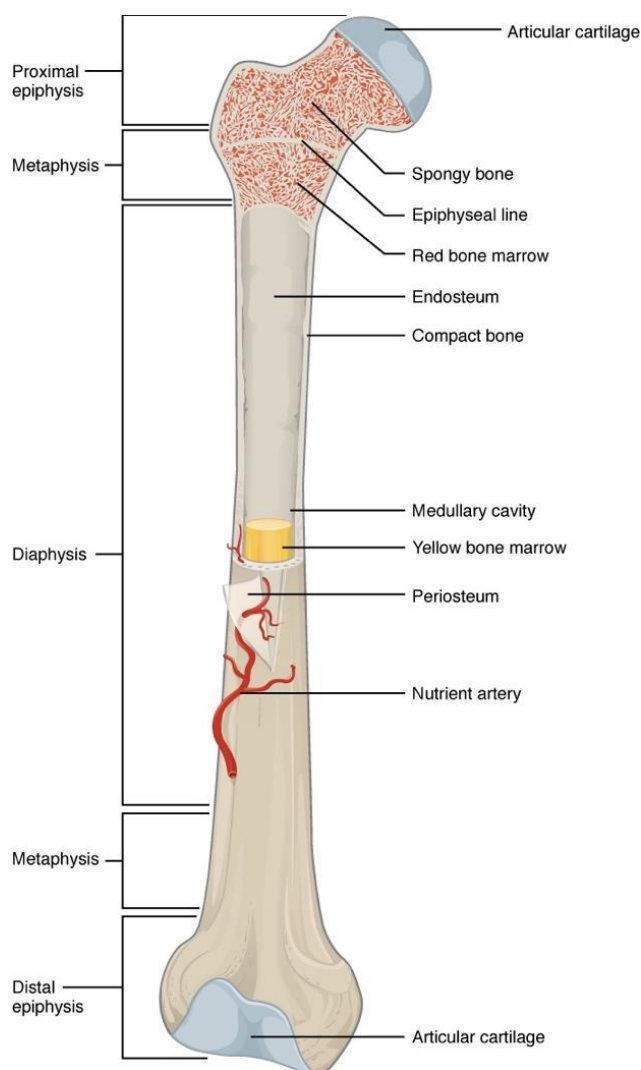


Figure 2.5 Structure of a long bone. Reproduced from (OpenStax, 2013) under the CC BY-SA 4.0 licence.

The diaphysis is in the middle of the long bone, formed by hard and dense cortical bone where bending forces predominate (Jerome et al., 2018). It is a cylindrical hollow shaft connecting the distal and the proximal ends. The medullary cavity is the empty space within the diaphysis, filled with yellow marrow in adults. The inner and outer surface of the diaphysis are the endosteum and the periosteum respectively, where bone modeling and remodeling take place. Tendons and ligaments attach on the periosteum. Lymphatics, nerves, and blood vessels in the periosteum supply nourishments to the cortical bone (OpenStax, 2013). The metaphysis is between the diaphysis and the epiphysis. It includes two regions: the primary (immature) and secondary (mature) spongiosa. The trabecular bone in the metaphysis and epiphysis mainly supports the compression from the joints (Jerome et al., 2018).

2.2 Bone growth and remodeling

2.2.1 Bone growth

Bone growth is a critical mechanism during childhood and adolescence. It increases the size, minimizes the mass, and optimizes the shape and strength of bone (Hoenig et al., 2022; Wang & Seeman, 2008), building a foundation for which the characteristics can remain later in life (Wang et al., 2009). The peak bone mass of young adulthood is dependent on the bone growth occurring in earlier ages, which can largely determine the risk of osteoporosis and fracture when one grows old (Luiza Loro et al., 2000).

There are two different growth mechanisms: intramembranous ossification and endochondral ossification (Mackie et al., 2008; OpenStax, 2013; Provot et al., 2021; Väänänen, 1993). Intramembranous ossification happens in the embryo, which is the case for most parts of the skull and some other bones such as scapula and clavicle.

- In the beginning, mesenchymal cells differentiated into osteoblasts in a cluster namely ossification center, responsible for bone formation (Figure 2.6 a).
- Osteoblasts are trapped by osteoid (unmineralized organic matrix), becoming osteocytes (Figure 2.6 b).

- The trabecular matrix is formed by osteoid around the capillaries, and the periosteum is formed by the osteoblasts on the trabecular surface (Figure 2.6 c).
- The cortical bone is further developed by periosteum. The blood vessels are crowded by trabecular bone, which turns into red marrow in the end (Figure 2.6 d).

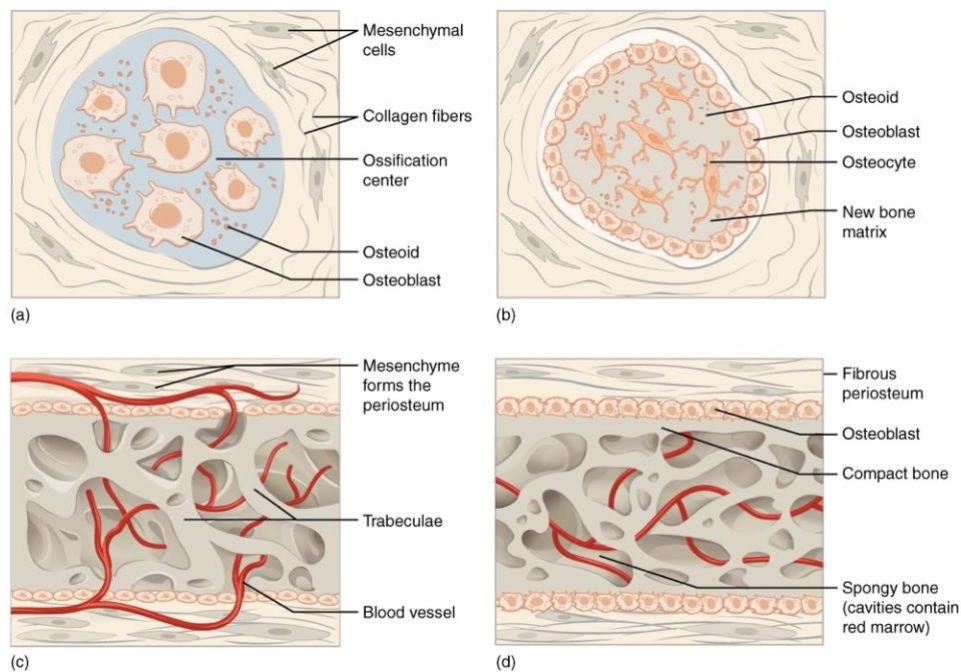


Figure 2.6 Intramembranous ossification of bone. Reproduced from (OpenStax, 2013) under the CC BY-SA 4.0 licence.

The rest of the bones (most bones) are formed through endochondral ossification (or cartilage bone formation), which takes much more time than intramembranous ossification.

- Mesenchymal cells originated from sclerotome firstly differentiate into chondrocytes, which in turn form a cartilage template (Figure 2.7 A). The proliferated and enlarged chondrocytes signal the differentiation of osteoblasts from perichondrial cells.
- Osteoclasts of hematopoietic origin erode the calcified cartilage, and osteoblasts synthesize and mineralize new bone matrix (Figure 2.7 B). The cartilage invasion by the cells and capillaries are in the middle of the bone first then at both ends to form the primary and secondary ossification centers respectively (Figure 2.7 C, D).

- Once the ossification centers are formed, the growth plate (epiphyseal plate) appears in between and performs longitudinal bone growth (Figure 2.7 D). The growth plate disappears once the bone reaches maturity, with metaphysis and epiphysis fused together (Figure 2.7 E). The articular cartilage at both ends of the bone is the only cartilage left.

Bone modeling is defined as bone formation by osteoblasts and resorption by osteoclasts. Both types of modeling help bone grow and maintain in shape under the mechanism of endochondral ossification (Allen & Burr, 2019; Jee, 2001; Maynard & Downes, 2019).

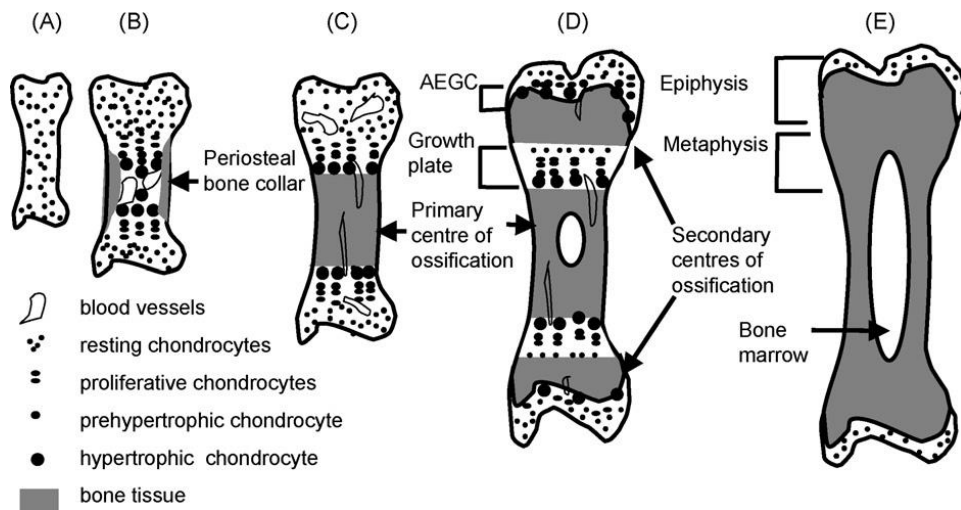


Figure 2.7 Endochondral ossification of bone. Reproduced from (Mackie et al., 2008) with permission of the copyright owner.

Bone growth can take place radially and longitudinally. Longitudinal bone growth occurs at the growth plate from each end of the bone, where cartilage is formed on the epiphyseal side and ossified on the diaphyseal side, adding new trabecular bone to the existing spongiosa and new cortical bone to the existing cortex (Jee, 2001; OpenStax, 2013, 2019; Rauch, 2005). Growth plates are monopolar, they are “pushed” further and further from the bone shaft (the primary growth center) when bone grows in length by adding more and more osseous tissue to the diaphysis (Villemure & Stokes, 2009; White et al., 2011). Inside the growth plate (Figure 2.8), chondrocytes go through cell division then enlargement in four zones: the reserve zone, the proliferative zone, the hypertrophic zone, and the calcification zone (OpenStax, 2013; Villemure & Stokes, 2009).

- Reserve zone: the chondrocytes protect the growth plate from the epiphyseal bone tissue.

- Proliferative zone: new chondrocytes are produced (divided) via mitosis.
- Hypertrophic zone: the chondrocytes are enlarged and matured.
- Calcification zone: invaded by osteoblasts and capillaries, most of the chondrocytes undergo apoptosis because of matrix calcification.

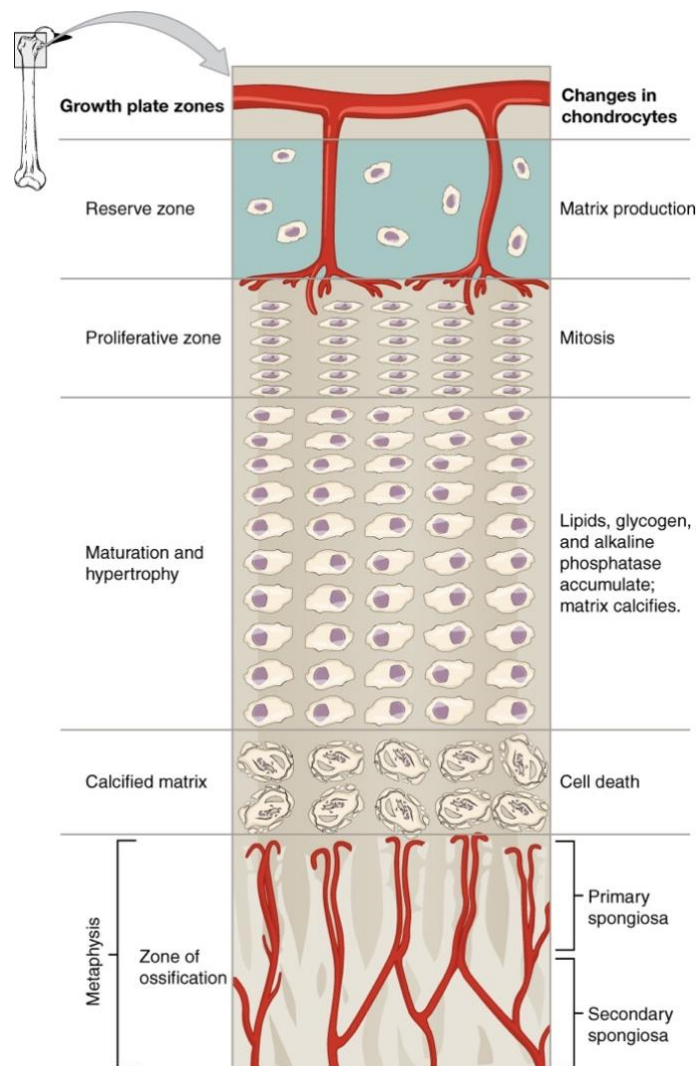


Figure 2.8 Cellular mechanisms in growth plate for the longitudinal bone growth. Reproduced from (OpenStax, 2013) under the CC BY-SA 4.0 licence.

Starting from the bone collar on the diaphyseal cartilage in the endochondral ossification (Figure 2.7 B), radial growth (appositional growth) is coupled with formation modeling on the periosteum

and resorption modeling on the endosteum as shown in Figure 2.9 (OpenStax, 2013; Rauch, 2005). Cortical thickness is preserved, and the width of the bone is increased over time. Radial growth can happen even after the cessation of longitudinal growth, and it is sexually dimorphic. Men generally have larger bone diameters upon reaching maturity, because periosteal bone formation can be stimulated by testosterone but inhibited by estrogen (Allen & Burr, 2019).

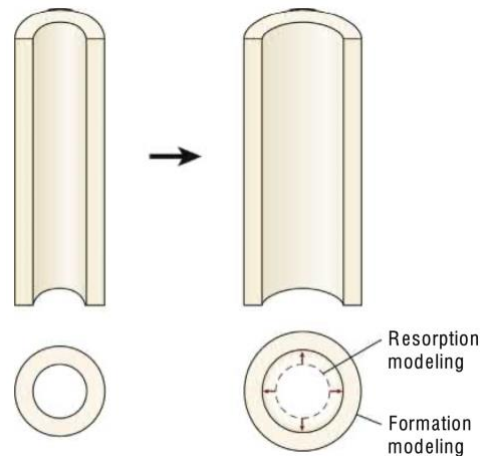


Figure 2.9 Radial bone growth over time. Reproduced from (Allen & Burr, 2019). with permission of the copyright owner.

2.2.2 Growth evaluation

Experimental studies in the past have evaluated the longitudinal bone growth using various methods as shown in Table 2.1. The conventional technique is based on histology, where one or two injections of a fluorescent labelling substance such as tetracycline and calcein are given to the animal before sacrifice (Hansson et al., 1972; Hunziker & Schenk, 1989; Turner, 1994). The injected substance can bind to the bone mineralization front, forming a fluorescent band corresponding to each injection (Álvarez et al., 2000; Hansson et al., 1972). The bone tissues around the targeted growth plate are processed and observed under microscope. Using the microscopic images, a growth distance is measured between the two fluorescent bands (in the case of two injections) or between the fluorescent band and another osseous landmark (in the case of one injection).

Table 2.1 Comparison of the bone growth evaluation methods.

Reference	Animal model	Growth evaluation method	Key contributions
(Hansson et al., 1972)	Young and adult rats, tibia	Growth plate (<i>ex vivo</i>), Histology (tetracycline)	One of the earliest studies to measure growth rate using the fluorescent labelling method
(Hunziker & Schenk, 1989)	Young rats, tibia	Growth plate (<i>ex vivo</i>), Histology (calcein)	Relationship between chondrocyte activities and longitudinal growth
(Turner, 1994)	Adult rats, tibia	Growth plate (<i>ex vivo</i>), Histology (calcein)	Detailed illustration of the calcein label measurement methodologies
(Álvarez et al., 2000)	Young rats, tibia	Growth plate (<i>ex vivo</i>), Histology (calcein)	Growth rate determines the synthesis and degradation of growth plate cartilage
(Stokes et al., 2006)	Young rats, rabbits, calves; loading on caudal vertebra and proximal tibia	Growth plate (<i>ex vivo</i>), Histology (calcein, xyleneol orange)	Relationship between growth alteration and sustained stress alteration of different magnitudes
(Stern et al., 2015)	Young mice, long bones (ulna, radius, humerus, femur, tibia, fibula)	Whole bone (<i>ex vivo</i>), Micro-CT & Image registration	The isometric scaling of long bones is preserved from childhood to adulthood
(Altman et al., 2015)	Young and adult rats treated by parathyroid hormone (PTH), tibia	Growth plate (<i>in vivo</i>), Micro-CT & Image registration	One of the earliest studies to measure growth rate using the <i>in vivo</i> micro-CT-based method
(Lu et al., 2016)	Adult mice, tibia	Whole bone (<i>in vivo</i>), Micro-CT & Image registration	A reproducible protocol to follow up local bone adaptation in a spatio-temporal manner
(Mustafy, Benoit, et al., 2018)	Young rats under radiation exposure, tibia	Growth plate (<i>ex vivo</i>), Histology (calcein)	Effects of different levels of radiation exposure on longitudinal tibial growth
(Mustafy, Londono, Moldovan, et al., 2019)	Young rats, loading on tibia	Growth plate (<i>ex vivo</i>), Histology (calcein)	Effects of different levels of mechanical loading on longitudinal tibial growth

Figure 2.10 shows an example of using one injection, where the upper arrows correspond to a chosen landmark (in this case, the vascular invasion at the growth plate) and the lower arrows correspond to the fluorescent band. Dividing the growth distance by the corresponding time lapse derives the longitudinal growth rate. The histology-based method has been widely used to quantify longitudinal bone growth in animal studies (Mustafy, Benoit, et al., 2018; Mustafy, Londono, Moldovan, et al., 2019; Stokes et al., 2006). However, it is destructive: animals must be sacrificed for tissue processing and assessment under microscope. It is not possible to follow up the growth curve of the same group of animals in an *in vivo* manner.

Some studies in the last decade have leveraged the power of microcomputed tomography (micro-CT) for bone growth evaluation. Using an image registration algorithm, micro-CT images taken over time become comparable by transforming them to the same spatial coordinates. Then, bone growth distance can be measured by tracking important landmarks (Stern et al., 2015), by looking at the change of the whole bone length (Lu et al., 2016), or by tracking the microstructure of the trabecular bone on the metaphyseal side of the growth plate (Altman et al., 2015). The micro-CT images can be taken *in vivo* or *ex vivo*, providing flexibility for the study design. In the case of a longitudinal *in vivo* study, the growth curve of the same animal can be tracked in time without the need to sacrifice them.

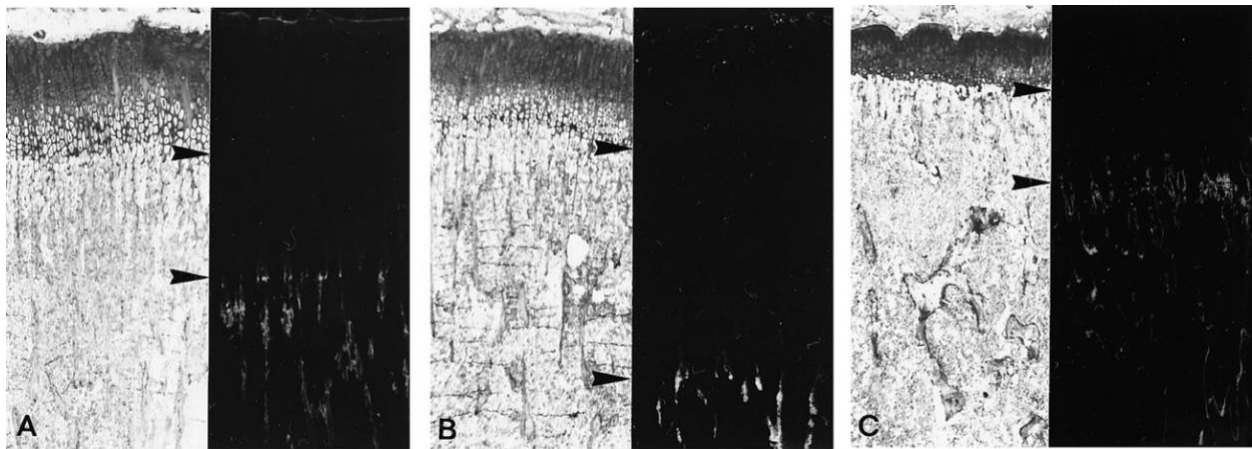


Figure 2.10 Longitudinal growth distance measured at growth plate viewed by bright-field microscopy after toluidine blue staining (left) and by incident-light fluorescence microscopy (right) for rats of (A) 21, (B) 35, and (C) 80 days old. Note that the original image was produced in monochrome. Adapted from (Álvarez et al., 2000) with permission of the copyright owner.

Figure 2.11 shows an example of how the longitudinal growth rate was calculated using a novel micro-CT-based method. Micro-CT images were taken on the same bone sample on day 0 and day 4 respectively, shown in the red and the green rectangles (Figure 2.11 A). By using an image registration algorithm, the common volume of interest (VOI) between the two scans can be located (Figure 2.11 B). Finally, overlapping the registered images in the same coordinate frame reveals the longitudinal growth distance related to this time gap (Figure 2.11 C). The micro-CT-based method is somewhat similar to the conventional histology-based method in terms of how the growth distance is measured: the distance by which the metaphyseal trabecular bone has moved towards the diaphysis is considered equivalent to the longitudinal growth distance of the corresponding growth plate. The key difference is in the way that the landmarks are tracked: by using fluorescent labelling (histology-based method) or by using image registration (micro-CT-based method).

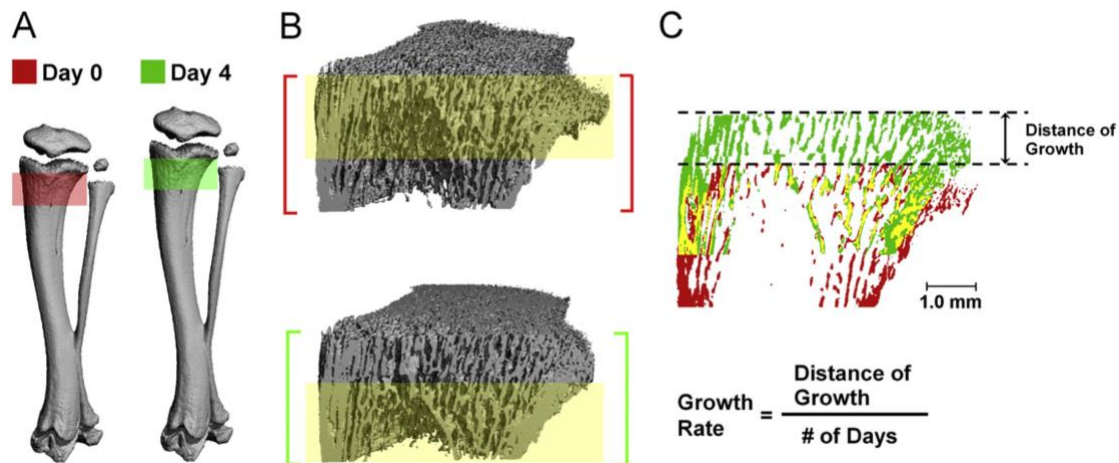


Figure 2.11 Longitudinal growth distance and rate measured by a micro-CT-based method. (A) micro-CT images of the bone sample at day 0 and day 4, (B) common bone regions identified by an image registration algorithm, (C) trabecular microstructure overlaid to demonstrate the growth distance. Reproduced from (Altman et al., 2015) with permission of the copyright owner.

To our knowledge, the study of Altman et al. was the first in the literature to utilize the *in vivo* micro-CT-based method to evaluate longitudinal bone growth (Altman et al., 2015). The authors applied this approach on rats from 1-month-old to over 3-month-old, covering the entire peak growth period. The image registration algorithm was based on an open source platform ITK

(Johnson et al., 2015), making it possible to reproduce the method in future studies. However, this relatively new method was not validated against the conventional histology-based method (fluorescent labelling). The precision errors of the micro-CT-based method and the correlation between the two methods remain to be studied.

2.2.3 Bone remodeling

Throughout life, bone undergoes remodeling, which is a process involving sequential bone resorption and formation. Old or damaged bone is resorbed by osteoclasts and new bone is laid by osteoblasts as replacement, which decreases the degree of calcification (Allen & Burr, 2019; Hadjidakis & Androulakis, 2006; Jee, 2001). Bone remodeling performs regulation of the body mineral homeostasis, and improves bone mechanical properties (Väänänen, 1993). The remodeling frequency determines the calcification distribution, which is an indirect indicator of bone quality (Nango et al., 2022). The baseline bone remodeling can cover around 5% to 10% of an adult skeleton on an annual basis. Bone remodeling mediated repairs can be triggered by physical exercises and injuries (OpenStax, 2013).

The mechanism of bone remodeling is similar in cortical and trabecular bone, based on a group of cells (osteoclasts and osteoblasts) and their associated blood vessels called bone (or basic) multicellular unit (BMU) under five sequential phases (Figure 2.12): activation, resorption, reversal, formation, and quiescence (Allen & Burr, 2019; Bellido et al., 2019; Parfitt, 2021; Väänänen, 1993):

- In the activation phase, resting cells are activated by signals from apoptotic osteocytes. Pre-osteoclasts are recruited and differentiated into mature osteoclasts.
- In the resorption phase, the mineralized bone matrix is exposed to the osteoclasts when the lining cells are removed from the surface. The activated osteoclasts are guided and attached to bone surface to perform resorption of mineralized bone and liberation of collagen fragments, which can be used as biomarkers (measurable in blood and urine). The osteoclasts go through apoptosis once the resorption is completed.

- The reversal phase provides a transition from the resorption to the formation, where mononuclear cells appear in the resorption lacunae to prepare for bone formation.
- In the bone formation phase, preosteoblasts are activated and differentiated into osteoblasts. The osteoid is laid down by osteoblasts and mineralized to fill up the resorption lacunae. About 90% of the osteoblasts undergo apoptosis, which are replaced by new ones if the bone formation is still needed. Some of them are entrapped into the osteoid matrix and turn into osteocytes.
- Lastly, in the phase of quiescence (resting), the rest of the osteocytes are flattened into bone lining cells to cover the bone quiescent surfaces. These cells can be activated again for bone formation. Most of the bone surfaces in the body are in the phase of quiescence.

In response to mechanical or hormonal stimulations, osteocytes perform the regulation of osteoclasts and osteoblasts through every step of the remodeling cycle (Bellido et al., 2019). A complete remodeling cycle can take four to six months in the human body. Over 75% of this time is spent on formation and mineralization (Allen & Burr, 2019).

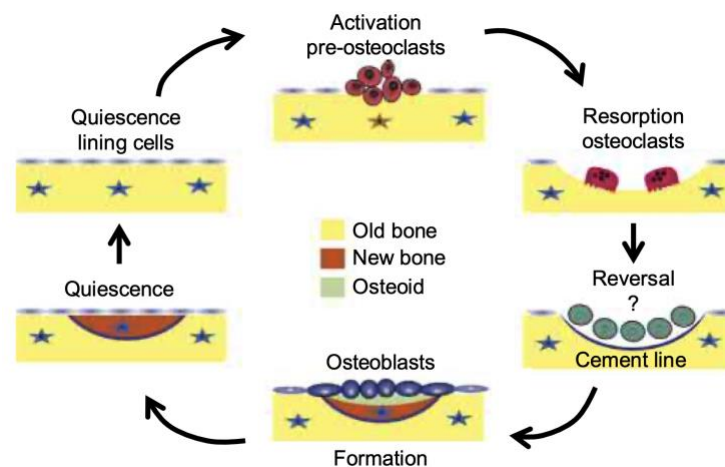


Figure 2.12 The process of bone remodeling involving different cells. Adapted from (Bellido et al., 2019) with permission of the copyright owner.

Bone modeling and remodeling are comparable as shown in Table 2.2, and the fundamental differences are in their different goals (Allen & Burr, 2019). Bone modeling shapes the bone and increases bone mass primarily in childhood, whereas bone remodeling can happen throughout life

as a renewal process. Due to different objectives, they have distinct mechanisms: bone modeling is either in activation-formation or activation-resorption, mediated by osteoblasts or osteoclasts independently; but bone remodeling happens in a sequential activation-resorption-reversal-formation-quiescence manner where both osteoblasts and osteoclasts are involved. Bone modeling can happen on three types of bone envelopes: periosteal, endosteal and trabecular. Bone remodeling can take place on an additional type of bone envelop which is intracortical. Bone modeling and remodeling work cooperatively to optimize for bone shape and mass, and ensure the skeletal stability throughout lifetime (Kostenuik, 2021). Since the micro-CT-based evaluation of bone formation and resorption are not capable of identifying their exact sources (either modeling or remodeling), the parenthesized term “(re)modeling” is appropriate in such context to refer to both mechanisms (Birkhold et al., 2015; Young et al., 2022). Therefore, “(re)modeling” is especially a well-suited term under the context of young animals, which undergo significant growth (modeling) accompanied by the life-long remodeling activities.

Table 2.2 Comparison between bone modeling and remodeling. Adapted from (Allen & Burr, 2019) with permission of the copyright owner.

	Bone modeling	Bone remodeling
Goal	Shape bone, increase bone mass	Renew bone
Cells	Osteoclasts or osteoblasts, and their precursors	Osteoclasts, osteoblasts, and their precursors
Bone Envelop	Periosteal, endosteal, trabecular	Periosteal, endosteal, trabecular, intracortical
Mechanism	Activation-formation Activation-resorption	Activation-resorption-reversal-formation-quiescence
Timing	Primarily childhood	Throughout life
Bone Mass	Increase	Maintain or slight decrease

2.2.4 Remodeling evaluation

Bone remodeling parameters are conventionally evaluated using dynamic histomorphometry (histology), shown in Table 2.3. Animals are injected with fluorescent labels days before sacrifice, after which the bone tissue undergoes a series of processing steps for microscopic observation.

Remodeling parameters such as bone formation and resorption thickness, surface area, and volume are calculated (Vignery & Baron, 1980; Willie et al., 2013). However, this experimental approach is destructive. The parameters can only be measured at terminal time points of the study due to the requirement for *ex vivo* tissue processing. The entire protocol can be laborious to perform, which complicates the study design if a large number of samples is required.

Table 2.3 Comparison of the bone remodeling evaluation methods.

Reference	Animal model	Remodeling evaluation method	Key contributions
(Vignery & Baron, 1980)	Young rats, alveolar bone, cortical bone	Histomorphometry	One of the earliest experimental studies on bone remodeling
(Waarsing et al., 2004)	Adult rats sham operated or ovariectomized (OVX), tibia, cortical and trabecular bone	<i>In vivo</i> micro-CT (prototype) & 3D image registration	One of the first studies to apply 3D image registration on micro-CT images to track local microstructural changes in bone
(Schulte et al., 2011)	Adult mice, loading on caudal vertebra, trabecular bone	<i>In vivo</i> micro-CT & 3D image registration, histomorphometry	Reproducibility of both methods, result comparison between 2D and 3D parameters
(Willie et al., 2013)	Adult mice, loading on tibia, cortical and trabecular bone	Histomorphometry	The response to mechanical loading in trabecular bone of older mice was reduced and delayed compared to young mice
(Lan et al., 2013)	Adult rats, tibia, cortical and trabecular bone	<i>In vivo</i> micro-CT & 3D image registration	Precision of the 3D image registration; difference between registering on cortical and trabecular bone
(Birkhold et al., 2014a, 2014b)	Adult mice, loading on tibia, trabecular and cortical bone	<i>In vivo</i> micro-CT & 3D image registration, histomorphometry	Reproducibility and accuracy of both methods; result comparison between 2D and 3D parameters
(de Bakker et al., 2015)	Adult rats treated by alendronate or PTH, tibia, cortical and trabecular bone	<i>In vivo</i> micro-CT & 3D image registration, histomorphometry	Correlation analysis between the results obtained from both methods
(Altman et al., 2015)	Young and adult rats treated by PTH, tibia, cortical and trabecular bone	<i>In vivo</i> micro-CT & 3D image registration	Evaluation of bone (re)modeling for young and adult rats under PTH treatments
(Harrison et al., 2022)	Adult rabbits treated by PTH, tibia, cortical bone	<i>In vivo</i> synchrotron micro-CT & 3D image registration	First study to measure <i>in vivo</i> longitudinal erosion rate (LER) using synchrotron radiation micro-CT

Using a prototype *in vivo* micro-CT, a study in 2004 showed the possibility of evaluating bone remodeling in a non-destructive manner (Waarsing et al., 2004). The authors demonstrated how 3D image registration can help to process the micro-CT images and to track microstructural changes in local bone over time. Later studies took a step further by applying customized remodeling algorithms on micro-CT images (Birkhold et al., 2014a, 2014b; Schulte et al., 2011). Figure 2.13 shows an example of applying image registration on micro-CT images of rat tibia. The registration strategy depends on the study design: maximizing the microstructural alignment of trabecular bone will lead to the result on the left (Figure 2.13 A) and applying it for the cortical shell will lead to the result on the right (Figure 2.13 B). To measure the remodeling parameters on trabecular bone, the image registration should be based on trabecular bone. To measure the remodeling parameters on cortical bone, the registration should be based on cortical bone. It is usually impossible to align both types of microstructures at the same time because metaphyseal trabecular bone undergoes coalescence to the cortical bone as part of bone growth (Altman et al., 2015; Cadet et al., 2003), especially in young animals.

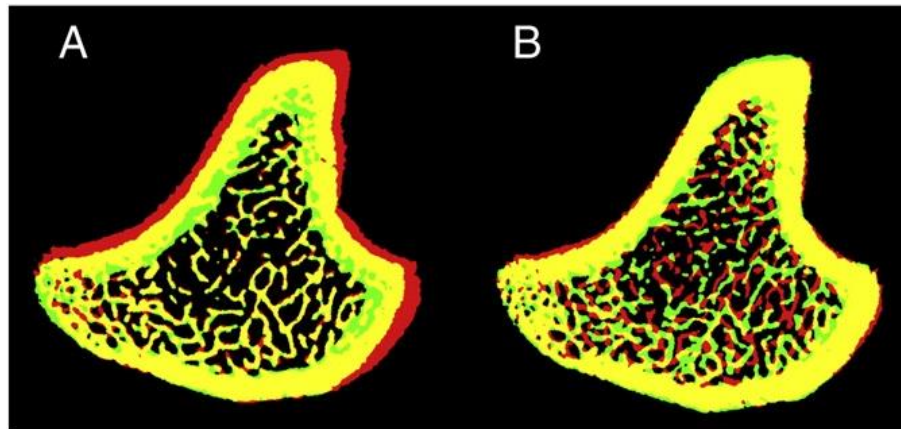


Figure 2.13 Micro-CT images (cross-sectional view) of a rat tibia overlaid by performing image registration based on (A) trabecular microstructure or (B) cortical shell. The bone volume in red is from the image of day 0, and the volume in green is from the image of day 14. The common volume between the two time points is in yellow. Reproduced from (Lan et al., 2013) with permission of the copyright owner.

The *in vivo* micro-CT-based method has undergone rigorous validation. In a 2011 study, the micro-CT-based method was compared to the conventional histomorphometry by assessing the

reproducibility of both methods (Schulte et al., 2011). Results measured in 2D were also compared to their counterparts in 3D. Two later studies performed validation of the micro-CT-based method versus histomorphometry on trabecular (Birkhold et al., 2014a) and cortical (Birkhold et al., 2014b) VOIs respectively, showing the reproducibility and accuracy of both methods. The micro-CT-based method has been applied in longitudinal studies of pharmaceutical treatments (Altman et al., 2015; de Bakker et al., 2015), confirming the power of alendronate and parathyroid hormone (PTH) on regulating the bone remodeling activities. The algorithms applied on images of laboratory micro-CT can be easily adapted to other imaging modalities. In a recent study (Harrison et al., 2022), a micro-CT platform supplied by a synchrotron source was used to quantify the longitudinal erosion rate (LER) *in vivo* for the first time. This advancement was made possible with the improved image quality of synchrotron micro-CT.

2.3 Bone microdamage

2.3.1 Microdamage accumulation and propagation

Human skeleton accumulates microscopic tissue damage (microdamage) during daily activities in response to physiological loadings (Hoenig et al., 2022). Microdamage accumulation is an important process to dissipate energy (Lambers et al., 2013; Tang & Vashishth, 2007; Vashishth et al., 1997). In healthy skeletons, the damages are constantly being repaired by bone remodeling. However, excessive amount of microdamage will lead to stress and fatigue fractures when damage accumulation outpaces repair (Martin, 1995; Muir et al., 1999). Athletes and soldiers receiving rigorous training have higher risk of developing stress fractures (Kardouni et al., 2021), for which women are more vulnerable than men due to lower energy availability (Rizzone et al., 2017; Sacher et al., 2022). Regulating the extent of microdamage is vital in maintaining bone health (Allen & Burr, 2019). As shown in Table 2.4, there are three types of microdamage: linear microcracks, diffuse damage, and trabecular microfractures, which can be distinguished based on different dimensions, stress modes and repair mechanisms (Burr, 2011; Burr et al., 1997; Diab & Vashishth, 2005; Hoenig et al., 2022; Lambers et al., 2013; Wenzel et al., 1996).

Table 2.4 Comparison of the three types of microdamage. Adapted from (Burr, 2011) under the CC BY-NC-SA 4.0 licence.

	Linear microcracks	Diffuse microdamage	Trabecular microfractures
Dimension	$\sim 1 \times 80 \times 300 \text{ } \mu\text{m}$	$\leq 10 \text{ } \mu\text{m}$ wide	Complete fracture
Stress mode	Compression	Tension	Bending, shear
Tissue properties	More brittle	More ductile	Off-axis orientation
Predominant location	Interstitial	Within osteons and trabecular packets	Trabecular
Age	Older	Younger	Older
Repair	Targeted remodeling	Remodeling	Endochondral ossification

Primarily formed in interstitial tissue, linear microcracks are shaped like ellipses with sharp border lines (Figure 2.14 A, pointed by long arrows), whose major axis is in the longitudinal direction of the bone and minor axis in the transversal plane (Donahue & Galley, 2006; O'Brien et al., 2000). Diffuse microdamages are usually found in osteons or trabecular packets near the surface (Figure 2.14 A, pointed by arrowheads), appearing as an area of pooled staining without clear view of individual cracks (Burr, 2011; Diab & Vashishth, 2005; Vashishth, Koontz, et al., 2000). They serve to dissipate energy, which helps to prevent and postpone fracture (Diab et al., 2006; Diab et al., 2005; Diab & Vashishth, 2007; Fazzalari et al., 1998; Herman et al., 2010). Linear microcracks and diffuse microdamages can be repaired by remodeling, with different signaling mechanisms.

Entirely different from the previous two types of “crack-like” damages, trabecular microfractures are complete fatigue fractures through isolated trabeculae (Fazzalari, 1993; Todd et al., 1972). Callus of woven bone are formed via endochondral ossification near the site of trabecular microfractures (Okazaki et al., 2014; Vernon-Roberts & Pirie, 1973), which is similar to woven bone formation on fractured diaphysis for healing purposes (Fazzalari, 1993). Trabecular microfractures are frequently observed in osteoporotic patients with weakened trabecular network (Figure 2.14 B), though the damage itself can be accumulated through normal daily activities (Okazaki et al., 2014). The rest of this review will focus on the discussion of the “crack-like” microdamages: linear microcracks and diffuse microdamages.

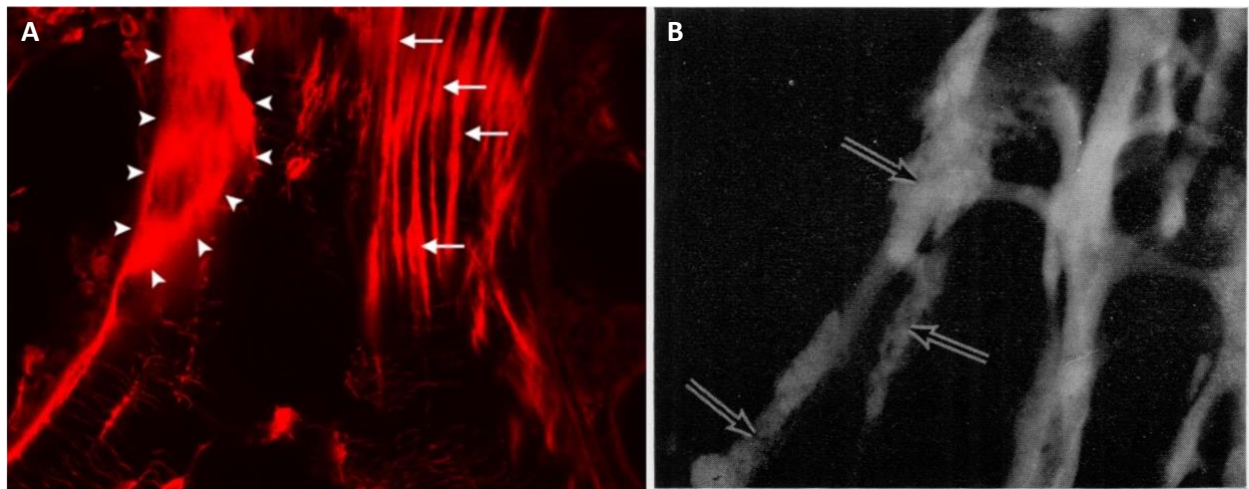


Figure 2.14 Different types of microdamages observed in human samples. (A) diffuse microdamages (pointed by arrowheads) and linear microcracks (pointed by long arrows); (B) trabecular microfractures (pointed by long arrows) and the surrounding woven bone callus. Adapted from (Li et al., 2013) and (Vernon-Roberts & Pirie, 1973) respectively, with permission of the copyright owners.

Experimental observations determined that diffuse microdamages can occur at various fatigue levels (Seref-Ferlengez et al., 2015) whereas linear microcracks appear at later stages (Burr et al., 1997; Burr et al., 1998; Herman et al., 2010; Parsamian & Norman, 2001; Seref-Ferlengez et al., 2014). Some believed that diffuse microdamages might be precursors to linear microcracks (Diab & Vashishth, 2007; Herman et al., 2010), though the two types of damage did not seem to correlate with each other (Seref-Ferlengez et al., 2015; Vashishth, Koontz, et al., 2000; Wenzel et al., 1996). Microdamages are distributed stochastically in bone (Gargac et al., 2014; Huiskes et al., 2000; Vashishth, Koontz, et al., 2000). They may initiate easily in cortical bone, but the propagation is slowed down by microstructural barriers including osteocyte lacunae, osteons, and cement lines (Akkus & Rinnac, 2001; Burr et al., 1988). The majority of cracks are either ceased outside the osteon or diverted into longitudinal propagation (Donahue & Galley, 2006; Nalla et al., 2005; O'Brien et al., 2005). Under such a protectional mechanism, microcracks are greatly limited in length (Moore & Gibson, 2001; Schaffler, 2003; Wang & Niebur, 2006), which makes timely repairs before leading to fracture (Burr, 1993; Wu et al., 2013). In trabecular bone, microdamages

are more accumulated in thinner and rod-like trabeculae (Green et al., 2011; Shi, Liu, et al., 2010; Shi, Sherry Liu, et al., 2010; Wang & Niebur, 2006; Wu et al., 2013). The density of trabecular microdamage is correlated with bone volume fraction (BV/TV) and structure model index (SMI) (Karim & Vashishth, 2011; Shi, Liu, et al., 2010; Wu et al., 2013).

Microdamage accumulation is also related to aging. Younger bones can endure higher levels of stress before generating microdamage (Green et al., 2011) where diffuse microdamage is formed more frequently than linear microcracks (Diab et al., 2005; Tang & Vashishth, 2010). In aging individuals, the increased formation of linear microcracks outpaces skeleton's ability of damage repair (Schaffler et al., 1995; Sobelman et al., 2004), which results in increased bone fragility and higher fracture risks (Burr et al., 1997; Diab et al., 2006).

2.3.2 Microdamage and remodeling

There are two types of remodeling in bone: stochastic and targeted remodeling (Burr, 2002; Donahue & Galley, 2006). Stochastic remodeling serves to maintain and adapt bone tissue to the external environments. Targeted remodeling is more associated with microdamage repair where osteoclasts remove damaged bone matrix and osteoblasts replace them with new bone in a specific manner (Verborgt et al., 2000; Zhang et al., 2018). Microdamage accumulation can activate targeted remodeling (Bentolila et al., 1998; Burr, 2002; Burr et al., 1985; Hoenig et al., 2022; Mori & Burr, 1993). The extent of bone remodeling is correlated with the number of linear microcracks present (Bentolila et al., 1998). It was hypothesized that target remodeling prioritizes the longest cracks to repair and keeps the crack length short instead of removing them completely (Martin & Sobelman, 2004; Sobelman et al., 2004). Pharmaceutical treatments that can inhibit bone remodeling impose some risks to bone mechanics (Seref-Ferlengez et al., 2015).

On the cellular level, osteocyte apoptosis is associated with bone remodeling events (Burr, 2002). Depending on the strain level, bone fatigue induces osteocyte apoptosis in areas of linear microcracks (Noble et al., 2003; Verborgt et al., 2000), which in turn activates targeted bone remodeling for damage repair (Bentolila et al., 1998; Cardoso et al., 2009; Kennedy et al., 2012; Noble & Reeve, 2000; Seref-Ferlengez et al., 2014). The signaling process between damage onset and osteocyte apoptosis is based on the intensity change of interstitial fluid transport (Duncan &

Turner, 1995; Muir et al., 2007; Tami et al., 2002). As another type of microdamage, diffuse microdamages are not able to initiate remodeling because they are not large enough to alter osteocyte viability (Gargac et al., 2014; Herman et al., 2010). The repair of diffuse microdamage is based on a process namely direct mineral filling (Boyde, 2003; Seref-Ferlengez et al., 2014; Zhang et al., 2018).

However, the increased remodeling activities for damage repair might temporarily increase porosity in both cortical and trabecular bone thus causing some loss of mechanical properties, which is especially dangerous in the context of osteoporosis and osteopenia (Donahue & Galley, 2006; Milgrom et al., 1985). The resorption cavities in trabecular bone can facilitate microdamage propagation (Goff et al., 2015; Hernandez, 2008; McNamara et al., 2006). To counter the negative effects of damage repair, woven bone formation on the periosteum enhances bone strength by increasing the cross-sectional area of bone (Silva & Touhey, 2007). The amount of woven bone formed depends on the damage level (Lynch & Silva, 2008; Uthgenannt et al., 2007), and it is not affected by treatment of risedronate, a bisphosphonate (Kidd et al., 2011).

2.3.3 Microdamage and mechanical properties

Mechanical loading on cortical bone can introduce a microcrack zone (frontal process zone) around the pre-existing macrocrack or notch (Malik et al., 2003; Parsamian & Norman, 2001; Vashishth et al., 1997; Vashishth, Tanner, et al., 2000; Vashishth et al., 2003). This frontal process zone can further turn into a wake of microcracks (process zone wake) when the macrocrack moves forward (Schaffler et al., 1994; Vashishth et al., 1997; Vashishth, Tanner, et al., 2000; Vashishth et al., 2003; Zioupos & Currey, 1998). Both the frontal process zone and process zone wake are toughening mechanisms to decelerate macrocrack propagation.

Microdamage generated *in vitro* or *ex vivo* present similar morphologies as what is observed *in vivo* (Donahue & Galley, 2006; Fazzalari et al., 1998). The propagation of microcracks initiated *in vitro* are largely affected by microstructural barriers as the *in vivo* case (O'Brien et al., 2003). Altered and mixed loading modes such as shear and multiaxial loadings can lead to increased crack propagation beyond the microstructural barriers (Lambers et al., 2013; Wang & Niebur, 2006; Wu et al., 2013). Similar to the fatigue behavior in composite materials (Carter & Hayes, 1977), bone

microdamage accumulation may gradually and progressively lead to degraded mechanical properties including elastic modulus (Burr et al., 1997; Burr et al., 1998; Goff et al., 2015; Pattin et al., 1996), stiffness (Carter & Hayes, 1977; Wu et al., 2013), and toughness (Zioupos et al., 1996a, 1996b).

The loss of stiffness can be monitored by compliance increase in ulna loading models (Bentolila et al., 1998; Seref-Ferlengez et al., 2014; Verborgt et al., 2000). However, due to the presence of soft tissues near the joints, the stiffness changes in forelimbs are not equal to the changes in ulna (Buettmann & Silva, 2016; Lambers et al., 2013; Uthgenannt & Silva, 2007). In aging bone tissues, toughness loss caused by decreased collagen quality might be more related to bone microdamage than stiffness (Donahue & Galley, 2006; Zioupos, 2001). Elastic modulus is believed to be the least sensitive parameter to predict microcrack density (Morgan et al., 2005; Tang & Vashishth, 2007).

Accompanied by progressive loss of stiffness, microcrack accumulation and propagation affect the fatigue life of bone gradually. When measured in number of cycles to failure, fatigue life is dependent on the range of total strain but not the level of mean strain (Carter et al., 1981). Most of the microdamage accumulation happens late in fatigue life, presenting a non-linear relationship (Lambers et al., 2013). The relationship between applied strain and fatigue life is non-linear as well (Donahue & Galley, 2006; Martin, 1992). Under the same loading magnitude, the fatigue life is shorter for tension than in compression (Cezayirlioglu et al., 1985; Pattin et al., 1996; Vashishth et al., 2001). This can be explained by the fact that human body is more adapted to compressive strain (walking, jumping) on a routine basis (Donahue & Galley, 2006).

The type of mechanical loading determines the type of microdamage developed (Boyce et al., 1998). Smaller cracks like diffuse microdamages are more associated with tension, and larger cracks like linear microcracks are more related to compression (Boyce et al., 1998; Burr et al., 1998; Carter et al., 1981; Diab & Vashishth, 2005; Reilly & Currey, 1999; Seref-Ferlengez et al., 2015). This difference in the location of response might be related to the electric potentials generated in bone, where tension leads to the positive and compression leads to the negative (Carter et al., 1981). Diffuse microdamages are primarily caused by the creep (time) component of the fatigue loading, and linear microcracks are more related to its cyclic nature (Caler & Carter, 1989; Carter & Caler, 1983; George & Vashishth, 2005; Seref-Ferlengez et al., 2014). As the sum of

creep and cyclic strain, maximum strain shows a better correlation with the damage volume detected (Lambers et al., 2013; Moore & Gibson, 2004; Nalla et al., 2003).

2.3.4 Microdamage evaluation

Frost was one of the first to propose the existence of *in vivo* microdamage (Frost, 1960). He observed human rib samples stained by basic fuchsin under microscope, noticed that the damages were mostly in planes of cement lines or outside osteons. These damages were believed to be linear microcracks based on subsequent progression of the field. The conventional method to quantify microdamage is based on histomorphometry (histology), where the number, length, and density (crack number per surface area) of cracks are calculated (Mori & Burr, 1993). However, histomorphometry is destructive to the samples, limited to two-dimensional damage evaluation, and quite laborious to perform (Tang & Vashishth, 2007).

Leveraging the three-dimensional capability of micro-CT, Tang et al. developed a novel approach based on a parameter “damaged volume per bone volume” (DV/BV) evaluated on human trabecular bone samples stained by lead-uranyl acetate as a contrast agent (Tang & Vashishth, 2007). The same authors proposed another parameter “damaged surface per damaged volume” (DS/DV) in a subsequent study to distinguish between diffuse damage and linear microcracks due to their different morphologies (surface to volume ratio) (Tang & Vashishth, 2010). Based on the same principle of contrast-enhanced micro-CT, Leng et al. designed a protocol using barium sulfate (BaSO_4) as the contrast agent (Leng et al., 2006; Leng et al., 2008) and validated the novel method against the conventional histomorphometry (Landrigan et al., 2011) using human femoral samples. The barium-based contrast agent is more practical than other staining agents because of wide availability of the reagents including barium chloride, sodium sulfate and acetone. Also, BaSO_4 particles were observed to penetrate into smaller damages where calcein and fuchsin are not able to enter (Choudhari et al., 2016). Figure 2.15 shows examples of cortical microdamage evaluated by the BaSO_4 staining method.

The contrast-enhanced micro-CT-based method is dependent on the image resolution. Without using a contrast agent, CT scanners are able to observe openings of about 10% of the image resolution (Breunig et al., 1993). A resolution of 10 μm is enough to detect most of the

microdamages in animal and human samples (Tang & Vashishth, 2010). However, it is not possible to distinguish between linear microcracks and diffuse microdamages at this resolution. A resolution of 1 μm will make it possible to assess cortical porosity (Palacio-Mancheno et al., 2014), which is not available in most of the *in vivo* micro-CT systems. One of the drawbacks of the micro-CT-based method is that the contrast agents are not specific to microdamages, which means that they can diffuse or precipitate into all the void spaces in bone including porosities and vessels (Landrigan et al., 2011; Leng et al., 2008; Tang & Vashishth, 2010; Turnbull et al., 2011; Zhang et al., 2018). Consequently, the damage level measured by contrast agents showed higher variation compared to that measured by conventional histomorphometry (Landrigan et al., 2011).

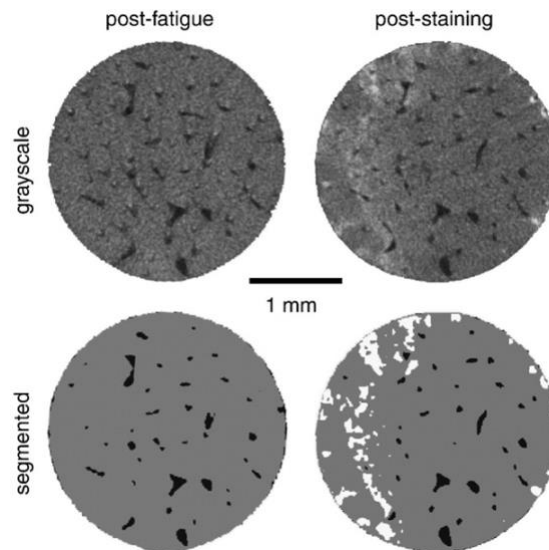


Figure 2.15 Micro-CT images of a human cortical specimen. The left column shows the post-fatigue status (no staining), and right column shows the post-staining status (stained by BaSO_4). The top row images are in grayscale and the bottom row images are segmented. The detected microdamages are shown in bright white pixels, which were not visible before staining.

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2.3.5 Animal studies

Animal models are widely used in microdamage research. It is much easier to control the extent of damage in animal models than in human specimens. The *in vivo* forelimb loading model with various loading designs is the state of the art to study microdamage and stress fracture in cortical

bone, which accounts for more than 90% of the ulna bone mass (Cowin, 2001). Stress fractures are common in ulna and radius for athletes in several domains such as tennis, softball, weightlifting and gymnastics (Cole et al., 2020; Hoenig et al., 2022). As shown in a finite element simulation of the forelimb loading model (Figure 2.16), the most vulnerable position on the ulna shaft is from the mid-diaphysis to the mid-third distal area due to their triangular cross-section and relatively thinner cortex than their adjacent area (Cole et al., 2020; Crasto et al., 2015; Zhang et al., 2018).

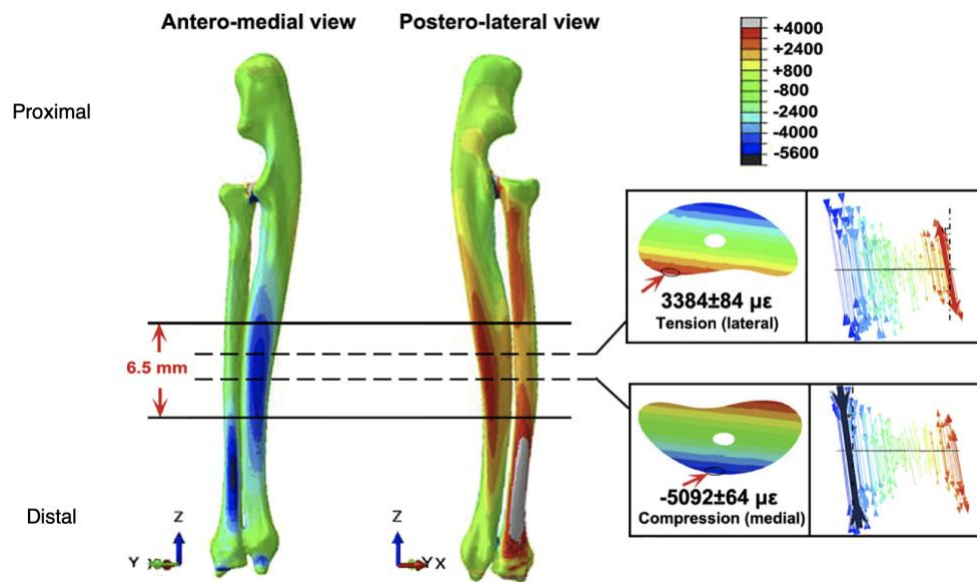


Figure 2.16 Strain distribution on ulna and radius using a micro-CT-based finite element model with a peak force of 30.5 N applied on the ulna. Reproduced from (Zhang et al., 2018) with permission of the copyright owner.

Table 2.5 summarizes the development of the forelimb model in a chronological order. Bentolila et al. were one of the first to apply *in vivo* mechanical loading on rat forelimb to generate microdamage (Bentolila et al., 1998). They observed intracortical remodeling related to the fatigue loading applied, which was an important condition for using rodents in microdamage research. A subsequent study closely evaluated the osteocyte morphology and hypothesized that osteocyte apoptosis might be related to the activation of remodeling-based repair of microdamage (Verborgt et al., 2000). This hypothesis was validated in a later study where the authors discovered that the remodeling mediated microdamage repair was only for linear microcrack but not for diffuse microdamage (Herman et al., 2010).

Table 2.5 Comparison of the studies using a forelimb loading model on small animals to study *in vivo* bone microdamage or stress fracture. The parenthesized term in the “animal model” column refers to the way that the mechanical loading was controlled. In the “evaluation” column, † denotes histomorphometry(histology)-based evaluation and * refers to micro-CT-based evaluation.

Reference	Animal model	Evaluation	Key contributions
(Bentolila et al., 1998)	Adult rats, loading on forelimb (stiffness)	Microdamage [†] , osteocyte integrity [†]	Fatigue loading can initiate intracortical remodeling in rat long bones, which might be related to osteocyte damage and death
(Verborgt et al., 2000)	Female adult rats, loading on forelimb (stiffness)	Osteocyte integrity [†]	Hypothesis: osteocyte apoptosis activates targeted bone remodeling for fatigue induced damages
(Hsieh & Silva, 2002)	Female adult rats, loading on forelimb (displacement)	Microstructure [†] , mechanical properties	Periosteal woven bone formation in response to fatigue induced damage can offset the reduced structural properties
(Uthgenannt & Silva, 2007)	Male adult rats, loading on forelimb (displacement)	Stress fracture*, mechanical properties	Establishment of an ulnar loading model with various sub-fracture damage levels
(Uthgenannt et al., 2007)	Male adult rats, loading on forelimb (displacement)	Microstructure ^{†*} , mechanical properties	Woven bone formation in response to mechanical loading is dependant of damage level
(Herman et al., 2010)	Female adult rats, loading on forelimb (cycle number)	Microdamage [†] , osteocyte integrity [†]	Osteocyte apoptosis can activate bone remodeling for linear microcracks but not for diffuse microdamages
(Kidd et al., 2011)	Female adult rats treated by risedronate, loading on forelimb (displacement)	Stress fracture [†] , microstructure [†]	Stress fracture healing was impaired by high dose of risedronate; the formation and consolidation of woven bone was not affected by the bisphosphonate treatment

Table 2.5 Continued.

Reference	Animal model	Evaluation	Key contributions
(Buettmann & Silva, 2016)	Female adult rabbits, loading on forelimb (stiffness)	Stress fracture*	Forelimb fatigue model based on rabbits; the forelimb stiffness gradually increases until the formation of macrocracks
(Zhang et al., 2018)	Male adult rats, loading on forelimb (cycle number)	Microdamage**†	Validation of the contrast-enhanced micro-CT based method for microdamage quantification of loaded ulna
(Bakr et al., 2019)	Female adult rats treated by parathyroid hormone (PTH), loading on forelimb (displacement)	Stress fracture†, microstructure†, remodeling†	A single dose of PTH treatment increased the remodeling related to stress fractures
(Bakr et al., 2020)	Female adult rats treated by PTH and alendronate (ALN), loading on forelimb (displacement)	Stress fracture†, microstructure†, remodeling†	Combined intermittent treatment of PTH and ALN increased the remodeling related to stress fractures
(Coates & Silva, 2020)	Female adult rabbits sham operated or ovariectomized (OVX), loading on forelimb (displacement)	Microdamage†, microstructure*, remodeling†, mechanical properties	Ovariectomy did not lead to osteoporosis but rather osteopenia; the sub-fracture microdamage levels were not consistent due to large peak force applied; very thin borderline between destructive and non-destructive loading modes
(Yan et al., 2021)	Male adult rats, loading on forelimb (displacement)	Microstructure*, mechanical properties	One week of post-loading recovery did not completely offset the loading-reduced stiffness upon impact testing (drop-tower compression)

In response to fatigue related damage, the diaphyseal bone has the intrinsic ability for woven bone formation which can offset (to some extent) the structural impact (Hsieh & Silva, 2002). To better understand the relationship between damage level and woven bone formation (Uthgenannt et al., 2007), researchers from the same group established a forelimb loading model with various damage levels (Uthgenannt & Silva, 2007). To our knowledge, they were the first to adapt the forelimb model in rabbits to create stress fractures (Buettmann & Silva, 2016). The use of rabbits seemed interesting since smaller animals such as rats and mice lack baseline intracortical remodeling (Allen, 2017; Lelovas et al., 2008). In this regard, the rabbit model is closer to the human condition than the rodent models. In a more recent study, Coates et al. adapted the rabbit model in the context of osteoporosis (Coates & Silva, 2020). The study showed a creative experimental design, but it presented several technical challenges. Firstly, the authors determined that ovariectomy was not sufficient to induce osteoporosis. The condition reached in the experiments was instead a mild osteopenia. Secondly, due to the large peak force applied in the mechanical loading, the damage level of the stress fracture was not consistent. Finally, the authors concluded that the boundary is very thin between destructive and non-destructive damages. They recommended using smaller peak forces with higher numbers of fatigue cycles to generate a more consistent damage level.

Pharmaceutical treatments were also tested in the forelimb loading model. Kidd et al. performed a trial of risedronate, a type of bisphosphonate, to study its impact on stress fracture healing (Kidd et al., 2011). The authors concluded that the remodeling mediated damage healing was reduced by high dose of risedronate. However, the formation and consolidation of woven bone were not affected by the use of the bisphosphonate. Researchers from the same group conducted subsequent studies on treatments of alendronate (ALN), another type of bisphosphonate, and parathyroid hormone (PTH) (Bakr et al., 2019; Bakr et al., 2020). The results showed that a single dose of PTH or combinational treatment of PTH and ALN increased the remodeling mediated stress fracture repair.

Two of the most recent studies brought our attention back to microdamage from stress fracture. Zhang et al. adapted the BaSO₄ staining protocol for use in rat ulna, which was successfully validated against histomorphometry (Zhang et al., 2018). The BaSO₄-based micro-CT imaging shows great potential for use in animal studies. Yan et al. focused on the aspect of mechanical properties related to microdamage accumulation (Yan et al., 2021). The authors applied drop-tower

testing on bone samples undergoing different resting periods, which showed that one week of post-loading resting was not enough to restore the fatigue damage related stiffness loss.

Regarding the technical aspects of the forelimb loading model, most studies used adult rats or rabbits around 5 to 8 months old. The *in vivo* mechanical loading was mostly applied at a physiological frequency of 2 to 4 Hz, with a peak force around 10 to 25 N for rats, more than 100 N for rabbits. Some studies adopted a constant peak force rate based on body weight, which might not be ideal when using ovariectomized animals since they tend to overweight easily (Chen & Heiman, 2001; McElroy & Wade, 1987). The mechanical loading was mostly controlled by a percentage of stiffness change, accumulated actuator displacement, or number of loading cycles. Changes in stiffness reflect damage only, but changes in actuator displacement can additionally reflect creep (Uthgenannt & Silva, 2007). Practically speaking, changes in displacement and number of cycles are easier to track than stiffness. Figure 2.17 shows an example setup of the *in vivo* ulna loading model.

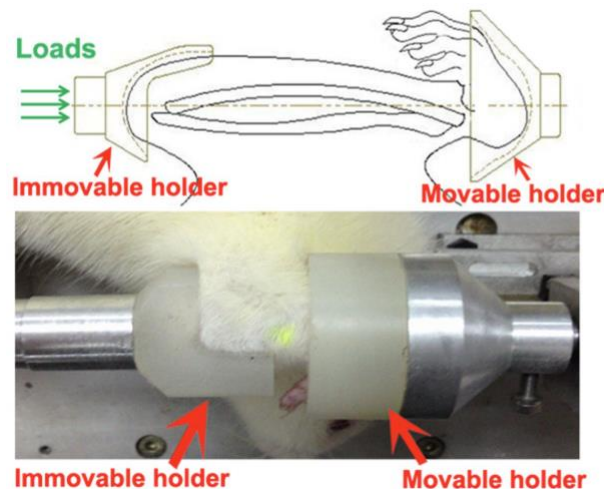


Figure 2.17 An example setup of the *in vivo* ulna loading model. Reproduced from (Zhang et al., 2018) with permission of the copyright owner.

When it comes to data collection, the mostly evaluated parameters were microdamage, stress fracture, remodeling, microstructural properties, and mechanical properties. We saw an evolution from the conventional histomorphometry-based method to the novel micro-CT-based method to evaluate microdamage, stress fracture, and microstructural properties. However, none of the studies

applied a micro-CT-based method for remodeling quantification, which has already become the state-of-the-art standard in other research directions (Altman et al., 2015; de Bakker et al., 2015). As for the evaluation of mechanical properties, three-point-bending was the most popular choice as it is easy to implement in small animal models.

Taken together, previous studies using the *in vivo* forelimb loading model showed great progress in the research of microdamage and stress fracture. Performing the mechanical loading *in vivo* enables the observation of damage accumulation, propagation, and repair under physiological conditions. The causes and mechanisms of remodeling-mediated damage repair were identified. Woven bone formation in response to fatigue damage was observed and quantified. A recent study by Coates et al. using an ovariectomized rabbit model showed great potential of the forelimb loading model in osteoporosis-related research (Coates & Silva, 2020). Future studies can leverage the most recent imaging techniques and combine the evaluation of bone remodeling, microdamage, and mechanical properties in the same *in vivo* study to investigate their interactions in the context of cortical microdamage.

2.4 Osteoporosis

2.4.1 Background

Osteoporosis is a common disease causing serious medical and financial burdens worldwide. Osteoporotic patients have inferior bone quality due to lower bone mass and deteriorated bone microstructure, leading to higher risk of fractures (Lelovas et al., 2008; Li & Xu, 2020; Wallace, 2019). In Canada, more than two million people of forty years and older are diagnosed with osteoporosis, among which women account for more than 80% (PHAC, 2021). The higher osteoporotic risk for women is generally caused by reduced level of estrogen during menopause, especially for those who are fifty years and older. When estrogen levels drop, osteoclast maturation and activation are increased, leading to accelerated bone resorption, and increased bone loss (Li & Xu, 2020). Figure 2.18 shows an example of bone loss due to aging and osteoporosis in trabecular and cortical samples.

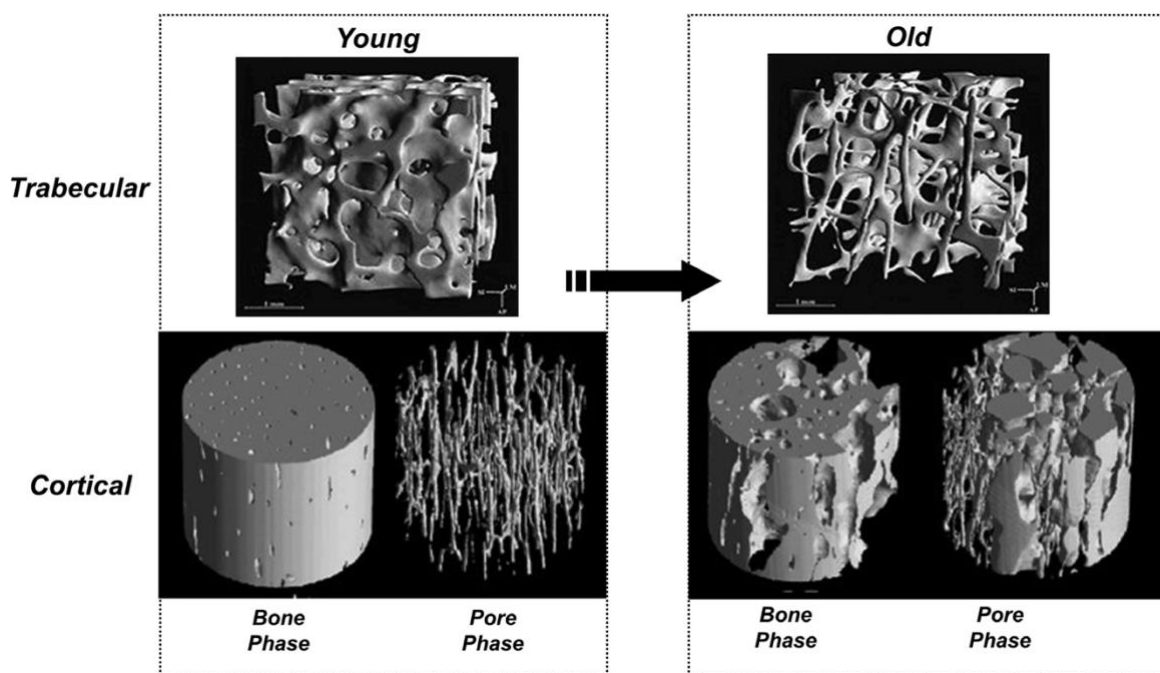


Figure 2.18 Aging and osteoporosis-related bone loss in trabecular and cortical bone. Reproduced from (Fathi Kazerooni et al., 2020) under the CC BY 4.0 licence.

According to a consensus panel of the National Institutes of Health (NIH), bone mineral density (BMD) determines about 70% of the bone strength, which makes it a common measure for osteoporosis diagnosis (Nango et al., 2022; NIH, 2001). The World Health Organization (WHO) defines osteoporosis and osteopenia (low bone mass) based on levels of bone mineral content (BMC) and bone mineral density (BMD). The thresholds are 2.5 and 1.0 standard deviation (SD) below the reference mean value of young adults for osteoporosis and osteopenia respectively (WHO, 1994). Osteoporotic patients can encounter spontaneous fracture during normal physical activities. Although osteopenic patients suffer from decreased bone and muscle strength as well, they usually do not experience fracture without injuries or fall accidents (Jee, 2001). In clinical practices, T-score and Z-score are calculated based on an individual's BMD measurement, the mean and standard deviation of normal young adults, or the patient's age-matched control (Dimai, 2017; Licata, 2006). The T-score is used for osteoporosis diagnosis and the Z-score is for predicating an individual's fracture risk compared to their age-matched peers.

2.4.2 Treatments

Treatments for osteoporosis and osteopenia include anti-catabolic and anabolic agents (Burr, 2016). The “gold-standard” anti-catabolic drugs are bisphosphonates (BPs) which have been used clinically since 1995 to prevent bone loss (Diab et al., 2021). BPs can inhibit the activities of osteoclasts, leading to increased bone stiffness (Allen, Iwata, Phipps, et al., 2006) and strength (Liu et al., 2017; Mashiba et al., 2017). However, bone toughness can be reduced with prolonged usage of BPs (Allen & Burr, 2007; Allen et al., 2008; Tang et al., 2009). BPs can reduce remodeling mediated microdamage repair (Burr, 2002). Patients treated with BPs had higher microdamage accumulation in terms of crack density, potentially increasing the risk of fragility fracture (Allen, 2018; Jin et al., 2017; Ma et al., 2017; Mashiba et al., 2000). One of the major concerns of using BP treatments is the risk of developing atypical femur fractures (AFFs), though a study has shown that the benefits outweigh the risks (Black et al., 2021). Among the different types of BPs, zoledronate (zoledronic acid, or ZOL) is believed to have the strongest potency against bone resorption (Pazianas et al., 2014), being ten times more effective than alendronate in reducing bone loss (Gasser et al., 2008).

An alternative to BPs is raloxifene (RAL), a type of selective estrogen receptor modulators (SERMs) to treat post-menopausal osteoporotic women (Jiang et al., 2021). RAL can improve the material properties of bone through estrogen receptor as a cellular mechanism and hydration as non-cellular effects (Bivi et al., 2016; Gallant et al., 2014; Meixner et al., 2017). Unlike BPs, the improvement of bone mechanical properties for RAL treated patients is not related to BMD or bone volume improvement (Allen et al., 2007; Allen, Iwata, Sato, et al., 2006). Preclinical studies have demonstrated beneficial effects on bone mechanics in mice receiving sequential treatments of ZOL and RAL (Meixner et al., 2017).

Another widely applied anticatabolic is denosumab, which inhibits the differentiation and activity of osteoclasts (McClung, 2021). Denosumab is more potent than BPs to increase strength and stiffness of bone, without the side effect of sacrificing toughness (Burr, 2016). Sequential treatment of denosumab and alendronate (a bisphosphonate) can reduce risks of humeral fracture which is a common condition among post-menopausal women (Haider et al., 2022). Due to its nature as an

antibody and its effect degradation over time, the administration is based on a semi-annual schedule (Bone et al., 2011; Eastell et al., 2011).

Teriparatide (TPTD), a form of parathyroid hormone (PTH), is approved for treating postmenopausal osteoporosis as an anabolic agent (Cosman, 2021). TPTD improves bone strength by improving BMD and bone material properties by regulating bone remodeling (Paschalis et al., 2003). The enhancement of the material properties is believed to be related to the increase of tissue heterogeneity (Katsamenis et al., 2015; Misof et al., 2003; Wang et al., 2016). There have been many discussions on combinational or sequential treatments of TPTD with anti-catabolic agents such as BPs and denosumab (Pazianas, 2015; Silva & Bilezikian, 2015; C. Zhang et al., 2020).

2.4.3 Animal models

Various animal models have been used to test treatments for osteoporosis and osteopenia (Turner et al., 2001). Rat is one of the preferred models for bone loss research thanks to its relatively low cost and high availability compared to other animal models. Although the rat skeleton is not fully representative of the human skeleton, specific traits still bear some similarities making it worth studying (Kimmel et al., 2020; Lelovas et al., 2008). One of the most significant drawbacks of using rodents is that they lack baseline intracortical remodeling (Allen, 2017; Jowsey, 1966). However, it is reported that external stimulation such as microdamage accumulation can initiate intracortical remodeling activities (Bentolila et al., 1998). Therefore, any observation of remodeling mediated damage repair is caused by experimental manipulation, which is not necessarily a disadvantage (Cardoso et al., 2009).

Among the other small animal models, rabbits are good alternatives to rodents thanks to their ability to perform intracortical remodeling on a regular basis (Coates & Silva, 2020; Harrison et al., 2020). Since rabbits generally have a bigger skeleton than rats, they require higher strain levels to produce microdamage (Baumann et al., 2015; Buettmann & Silva, 2016). Past studies also investigated bigger animal models including canines and primates, which bear more resemblance to humans. However, these models are becoming less popular due to higher expenses, longer life span (thus longer study design), and ethical issues (Turner et al., 2001).

There are several methods to induce osteoporosis or osteopenia in animal models. Ovariectomy (OVX) and immobilization on mature rats are commonly used in preclinical studies because they can resemble bone loss in human (Iwaniec & Turner, 2013; Lelovas et al., 2008; Turner, 2001). As a “gold standard” for preclinical experiments (Jee, 2001), OVX rats are primarily studied for post-menopausal osteoporosis and osteopenia (Allison et al., 2022; Danielsen et al., 1993; Feher et al., 2010; Ke et al., 1993). Estrogen level decreases in the animal body after ovariectomy, which increases bone turnover rate (bone resorption exceeds bone formation) (Kimmel et al., 2020). The consequential bone loss is rapid in the beginning, slower thereafter when bone remodeling reaches a more balanced state (Kalu, 1991; Lelovas et al., 2008). The loss of estrogen also leads to faster weight gain in OVX rats, which can increase bone mass and partially counter the bone loss caused by ovariectomy or ageing (Peng et al., 1997). Therefore, it is more appropriate to consider OVX as a model for osteopenia than for osteoporosis (Turner, 2001).

The immobilization models are primarily for studying skeletal disuse osteoporosis due to conditions such as paralysis, stroke, spinal cord injury, and long-term bedrest (Cardozo & Bauman, 2021; Kimmel et al., 2020). Surgical methods include neurectomy (Chow et al., 1996; Tuukkanen et al., 1991; Zeng et al., 1996) and tenotomy (Thompson & Rodan, 1988; Weinreb et al., 1989). Examples of conservative methods are tail suspension (Laib et al., 2000; S. Wang et al., 2022; Wronski & Morey-Holton, 1987) and limb bandaging (Li & Jee, 1991; Li et al., 1990; Maeda et al., 1993). It is possible to combine OVX with immobilization to reduce the time needed for apparent bone loss (Bagi et al., 1993; Cavolina et al., 1997; Lelovas et al., 2008; Okumura et al., 1987).

To evaluate osteoporosis and osteopenia related bone loss, histomorphometry is a conventional method which evaluates bone architecture in two dimensional views with microscopic resolutions (Lelovas et al., 2008; Nango et al., 2022). When it comes to non-invasive methods, biochemical markers are widely used in animal models to perform measurements of calcium, phosphorus, and magnesium from blood and urine samples (Lelovas et al., 2008). Another non-invasive method is densitometry based on the measurements of bone mineral density (BMD) and bone mineral content (BMC) (Cavolina et al., 1997; Lelovas et al., 2008; Nango et al., 2022). However, it can be difficult to interpret the results of BMD due to the change of bone geometry and mineral content over time (Turner et al., 2001). The most recent development of non-invasive evaluation is through micro-

CT (Lelovas et al., 2008). Thanks to its three-dimensional capability, micro-CT can provide a full picture of bone microstructure with high resolutions. Using specialized software developed by the micro-CT supplier or open-source communities, bone structural and material properties can be evaluated based on grey-scale images, providing abundant information about bone quality (Longo et al., 2016; Scheuren et al., 2020).

2.5 Bone mechanics

2.5.1 Mechanical testing modes

Bone strength is determined by three factors: bone mass, geometry, and material properties. The geometry of a bone includes its size, shape, and material distribution (Wallace, 2019). Conducting mechanical tests helps to evaluate various structural and material properties of bone samples (Leali et al., 2011). Figure 2.19 shows the common mechanical testing modes applied on a long bone including tension, compression, shear, torsion, and bending (An & Draughn, 2020; Sharir et al., 2008; Turner & Burr, 2001; Wallace, 2019).

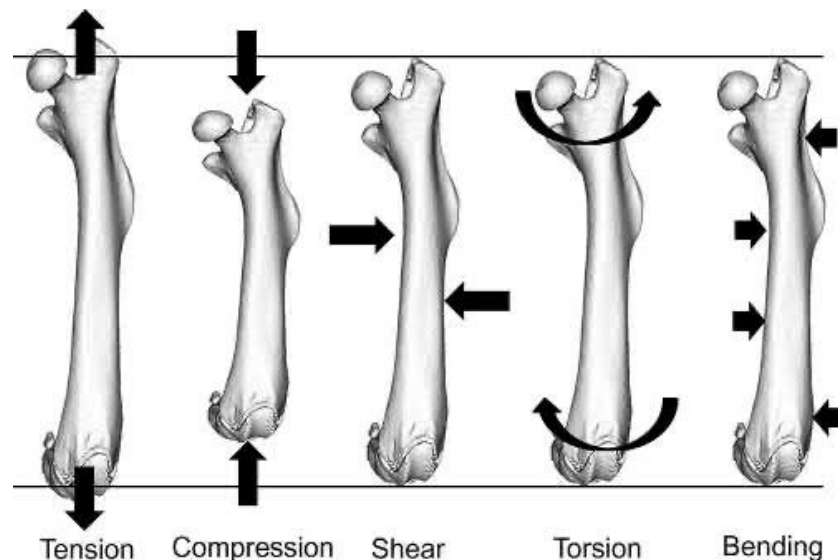


Figure 2.19 Common mechanical testing modes applied on a long bone. Reproduced from (Wallace, 2019) with permission of the copyright owner.

Tension and compression are applied when the two ends of a bone are pulled apart or pushed together. Tensile tests are more accurate than compressive tests without generating a bending moment. But compressive test might be more practical with smaller samples. Both tensile and compressive tests are unpopular for rodent models due to the difficulty with sample preparation in small sizes. Shear is induced by loading across the longitudinal axis. Torsion is created by twisting the two ends of the bone in opposite radial direction, which is usually applied for whole bone testing. However, it can be difficult to install the ends of the specimen into the testing holders.

Bending a long bone about its longitudinal axis creates a curve where part of the sample undergoes compression, and the other part receives tension. Bending is one of the most commonly applied mechanical tests on long bone due to its simplicity to perform and to interpret the results. Apart from the aforementioned classical testing modes, indentation tests are also useful for examining the hardness and Young's modulus of the sample based on the loading and unloading force-displacement curves (Turner, 2009; Zysset, 2009). In this case, a hard tip is applied on a flat bone surface of interest till a pre-defined load. Reference point indentation (RPI), which is a more recent variant of the indentation testing, shows theoretical potential to measure mechanical properties *in vivo* (Allen et al., 2015).

Testing and storage conditions can influence the biomechanical results (Turner & Burr, 2001). It is important to keep bone hydrated before and during testing, because properties such as Young's modulus, bone strength and toughness can change depending on the hydration of the sample. A common method is to wrap the bone in PBS (phosphate buffered saline) soaked gauze which simulates a physiological hydration environment. Once the bone samples are harvested and wrapped, they can be kept at -20 °C for short term storage without significant effects on bone mechanical properties (An & Draughn, 2020). However, the degradation of enzymes is not stopped at this temperature (Tomford et al., 1983). Lower temperatures around -70 °C to -80 °C are more ideal for long term storage (Tiefenboeck et al., 2020). Some experimental designs require freezing and thawing samples multiple times due to practical reasons. Past studies showed that doing so might change some of the mechanical properties of the sample (Boutros et al., 2000; Kang et al., 1997; Linde & Sørensen, 1993).

Testing temperature is critical as well. The difference between common room temperature (around 23 °C) and the body temperature (around 37 °C for humans and rodents) is significant enough that

the fatigue life can be twice as long at room temperature (Carter & Hayes, 1976; Uthgenannt & Silva, 2007). Since maintaining body temperature is difficult for *ex vivo* testing, using a consistent room temperature can be helpful. Besides the environmental conditions, the strain rate applied during loading can affect the strength and stiffness tested. Physiological strain rates are between 0.01 and 0.08/s (Rubin & Lanyon, 1982), and higher strain rates might bring more damage to the sample (Schaffler et al., 1989).

2.5.2 Three-point bending

There are two commonly applied bending tests: three-point bending and four-point bending (An & Draughn, 2020; Wallace, 2019). Bone samples are placed evenly on sample supports, then one or two external loading points apply vertical loading to the middle of the sample. Three-point bending uses one external loading point and two sample supports (Figure 2.20 left) and four-point bending uses two external loading points and two sample supports (Figure 2.20 right). Three-point bending is a more popular choice in bone research because of its simplicity. However, the results generated from this testing mode might not be representative of the whole bone, due to the local shear stress around the loading point. This drawback can be overcome by four-point bending using two loading points instead of one. Four-point bending ensures pure bending without transverse shear stress, so that the fracture occurs at the weakest location. The disadvantage of four-point bending is the requirement of equal force delivered by both loading points, which is hard to achieve for irregularly shaped bone samples.

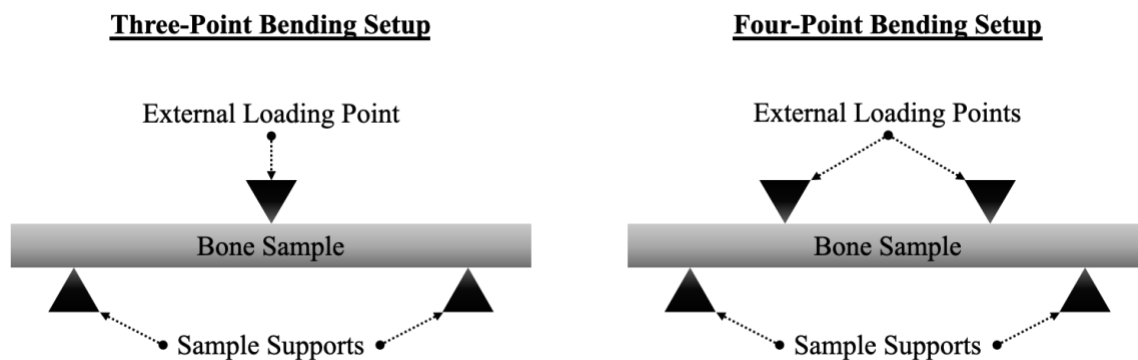


Figure 2.20 The setups of three-point bending (left) and four-point bending (right).

A force-displacement curve is generated by the loading apparatus after the bending test, as shown in Figure 2.21. The vertical axis shows force (F), and the horizontal axis shows displacement (δ). Based on the force-displacement curve, some structural parameters can be derived (Schriefer et al., 2005). The yield point is where the material starts to behave plastically and receives permanent damage (Ross et al., 2021; Turner & Burr, 2001). The slope before the yield point is the structural stiffness (S , N/mm). The yield force is noted as (F_y , N). The ultimate force is the maximal force on the curve (F_u , N). The failure force (F_f , N) is the force at the moment of failure (end point). The area under the curve is the energy absorbed throughout the loading, where the part acceding the yield point is the post-yield energy to failure (U_{post} , mJ) relative to the post-yield displacement (D_{post} , mm). Similarly, the pre-yield energy (U_{pre} , mJ) corresponds to the pre-yield displacement (D_{pre} , mm).

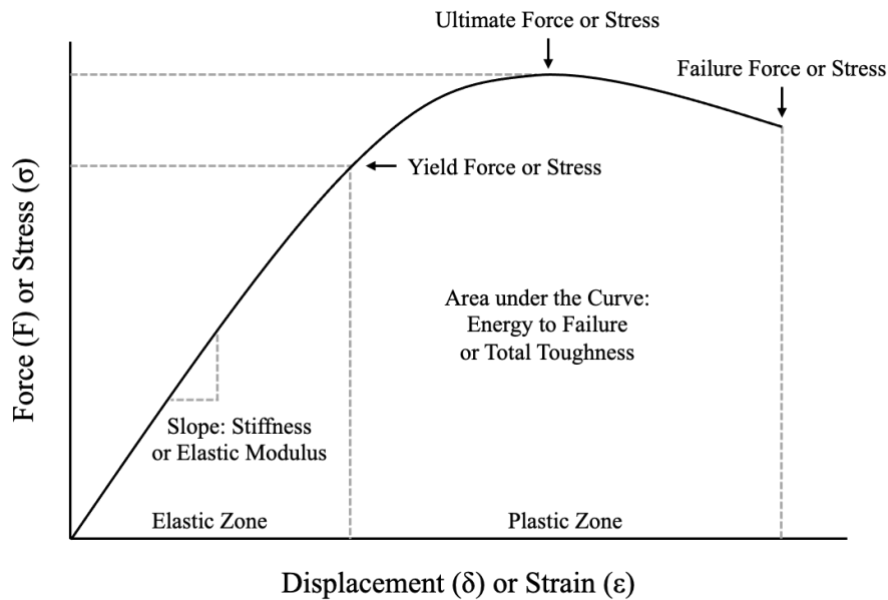


Figure 2.21 Force-displacement or stress-strain curve. Adapted from (Burr, 2020) with permission of the copyright owner.

To calculate material (instead of structural) properties, a stress-strain curve can be plotted by taking into consideration of the geometry of the samples (Wallace, 2019). The stress-strain curve was the same shape as the corresponding force-displacement curve as shown in Figure 2.21. The following equations derive the material properties in the case of three-point bending (Schriefer et al., 2005;

Turner & Burr, 2001): ultimate stress $\sigma_u = F_u \frac{Lc}{4I_x}$ (MPa), strain $\epsilon = \frac{6Dd}{L^2}$, Young's modulus $E = S \frac{L^3}{48I_x}$ (GPa), and modulus of toughness $u = U \frac{3d^2}{4I_x L}$ (MPa). The calculation is based on the following geometrical properties of the sample and setup: span between the two sample supports (L ; mm), radius of the bone perpendicular to the neutral axis (c ; mm), maximal diameter of the bending section (d ; mm), and second moment of area about the long axis (I_x ; mm⁴).

There are three major assumptions to ensure meaningful three-point bending tests (An & Draughn, 2020; Wallace, 2019). First of all, the sample is straight and uniform in cross section. Most of the animal long bone samples do not fulfill this criterion well. However, it can usually be satisfied within a local region of the sample. Secondly, the sample material should be isotropic, elastic, and homogeneous. This criterion is violated too in the context of bone as a somewhat viscoelastic, isotropic, and inhomogeneous material. Therefore, the equation mentioned above to derive strain based on displacement is only valid in the elastic region. The post-yield strain obtained with this equation should be interpreted carefully. Third, it is recommended to have at least a ratio of 20 between the length and the width of the sample to reduce the effect of shear stresses. However, this is also difficult to meet in many animal samples. In practice, it is suggested to increase the span between the two sample supports and keep it consistent among all the samples tested.

2.6 Graphical summary

The following two diagrams illustrate how the subjects presented in this literature review are interconnected. In Figure 2.22, the flowchart starts with bone growth, which takes place in childhood and adolescence. Bone growth improves bone size and mass and optimizes for shape and strength. Optimized bone growth can increase the peak bone mass achievable in young adult bone. In adulthood, factors such as aging and menopause can lead to osteoporotic or osteopenic bone, with increased bone loss, turnover, porosity, and microdamage, and decreased bone strength, collagen quality, and bone mineral density. The eventual consequence of osteoporosis or osteopenia is bone fracture. At the bottom of Figure 2.22, bone remodeling is shown to play critical roles in young and aging bones. Bone remodeling takes place throughout the whole life, and it is responsible for bone renewal, repair, and maintenance of the mineral homeostasis.

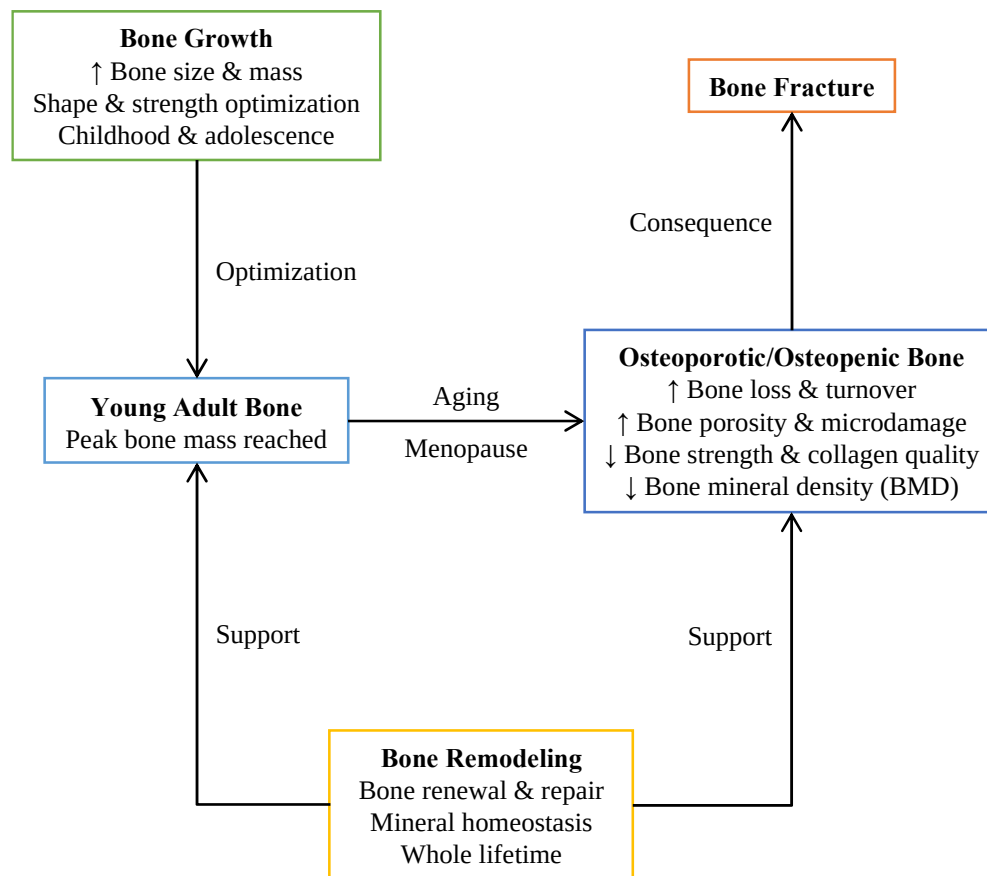


Figure 2.22 Literature summary on bone growth, remodeling, and osteoporosis/osteopenia.

In Figure 2.23, several mechanisms are centered around bone microdamage, which can be evaluated by histology and micro-CT-based methods. Bone microdamage can be divided into three categories: linear microcracks, diffuse microdamage, and trabecular microfractures. They have different morphologies and repair/propagation mechanisms, but with similar mechanical effects in general: microdamage accumulation leads to reduced fatigue life, stiffness, toughness, and Young's modulus. There are several causes of microdamage accumulation, where aging and estrogen depletion make a connection to osteoporosis/osteopenia, manifested through increased bone loss and porosity, and reduced bone mineral density and strength. As a treatment to osteoporosis and osteopenia, the BPs can affect damage repair and bone mechanical properties: the positive effects include improvement on bone stiffness and strength, while the negative effects are reduced toughness and increased microdamage density caused by remodeling inhibition. The eventual consequence of microdamage over accumulation is bone fracture.

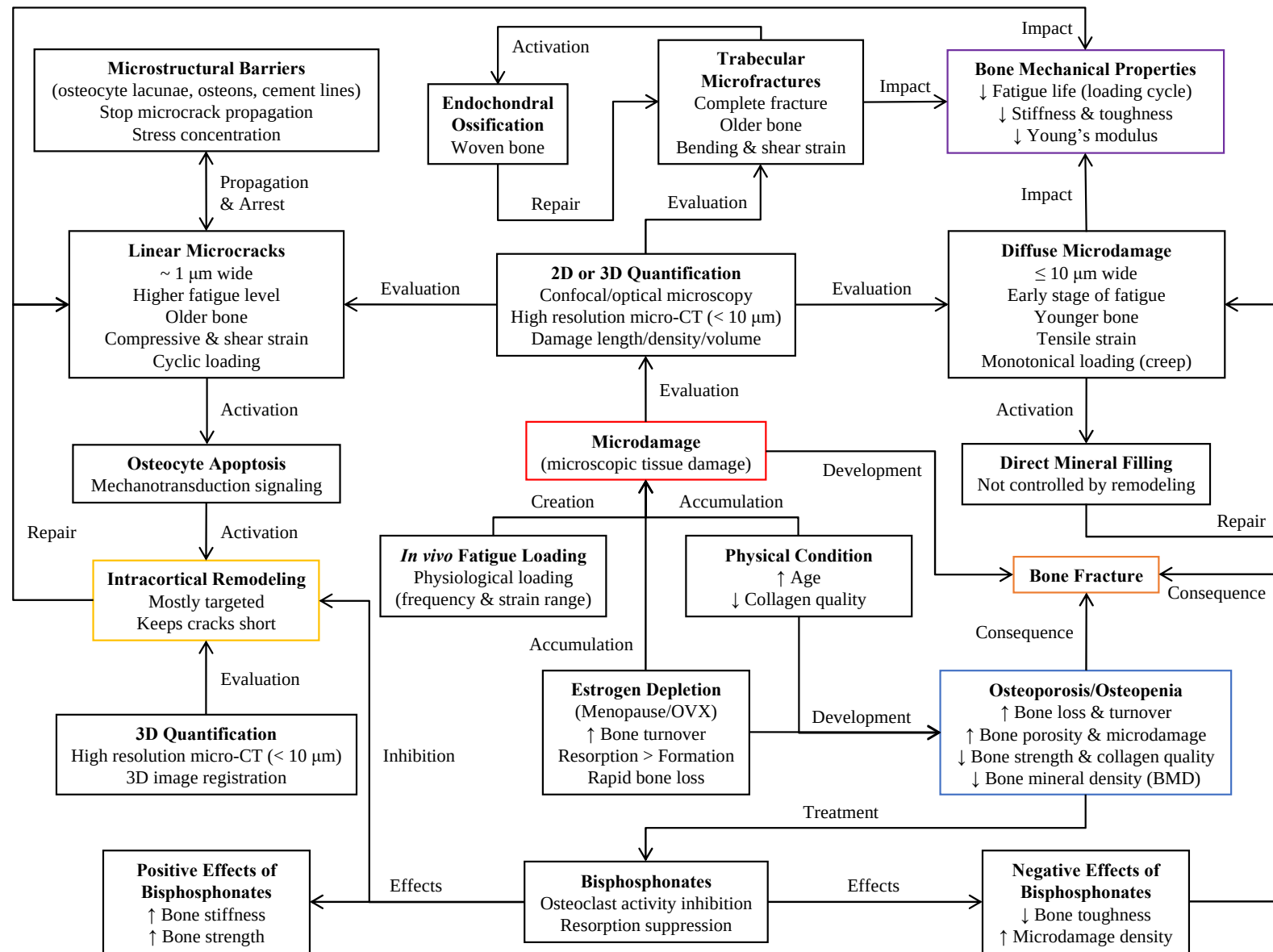


Figure 2.23 Literature summary centered on microdamage, involving various mechanisms and role players.

CHAPTER 3 PROJECT RATIONALE, RESEARCH QUESTIONS AND OBJECTIVES

3.1 Rationale

Bone is a composite material with self-repairing capabilities. The external dense shell formed by cortical bone and the internal trabecular network provide structural support and protection to the human body. Bone growth takes place during childhood and adolescence. Optimization of bone growth determines the peak bone mass achievable upon reaching adulthood, which in turn influences the bone quality later in life. The evaluation of bone growth using the conventional histology-based method is destructive to the sample and laborious to perform. The novel *in vivo* micro-CT-based method enables non-destructive evaluation in a three-dimensional manner, making it possible to track bone growth of individual animals over time. However, the micro-CT-based method has not been validated against conventional histology, with unknown accuracy and reproducibility.

Bone remodeling happens during the whole lifetime in the human body to perform bone renewal and repair. It is a cyclic process including activation, resorption, reversal, formation, and quiescence. A complete remodeling cycle can take over five months. The evaluation of bone remodeling based on conventional dynamic histomorphometry is a destructive method which requires sacrificing animals. The *in vivo* micro-CT-based method can track the (re)modeling parameters non-destructively, including bone formation and resorption thickness, surface area, and volume. The micro-CT-based method has been validated against dynamic histomorphometry in literature studies.

Bone microdamage accumulation is a physiological process that occurs in our daily life. The amount of microdamage can be efficiently managed by the repair mechanisms in healthy individuals. On the contrary, microdamage accumulation in osteoporotic and osteopenic patients can outpace their repair capability, leading to increased skeletal fragility and higher fracture risks. Linear microcracks can initiate targeted remodeling for damage repair. Diffuse damage is repaired by direct mineral filling. Microdamage accumulation can lead to reduced mechanical properties including elastic modulus, stiffness, and toughness. The evaluation of microdamage by conventional histology is limited to two-dimensional observation under microscope. The novel

contrast-enhanced micro-CT-based method can perform damage evaluation of three-dimensional bone volumes, providing more comprehensive results. The micro-CT-based method has been validated against the conventional histology-based method. The rat forelimb loading model has been extensively used in experimental studies to investigate microdamage and stress fracture in cortical bone. Under well controlled mechanical loading conditions, the level of microdamage generated is consistent within the same group of animals. It shows great potential for pre-clinical research.

Osteoporosis and osteopenia are pathologies leading to excessive bone loss, defined by low bone mineral density. Common pharmaceutical treatments include bisphosphonates, raloxifene, denosumab and teriparatide. Patients treated by bisphosphonates showed higher accumulation of microdamage, raising concerns about using this type of anti-catabolic drugs. Animal models are widely used in osteoporotic and osteopenic research, among which ovariectomized rats are the “gold standard” to assimilate the conditions of human bone loss. Additionally, mechanical testing on animal bone samples help to evaluate their structural and material properties. Among the various testing methods, three-point-bending is the most popular choice in animal studies due to its simplicity for testing and result interpretation.

In summary, bone (re)modeling, bone microdamage and their mechanical responses are tightly linked under the complex bone architecture. They are critical mechanisms in the development and treatment of bone loss. Osteoporosis and osteopenia reflect the reduced bone quality in aging individuals, whose peak bone mass at younger ages was optimized by bone growth. Leveraging the power of *in vivo* micro-CT, bone growth and (re)modeling can be evaluated non-destructively in a spatio-temporal manner. Three-dimensional microdamage can be quantified using contrast-enhanced micro-CT. Bone structural and material properties can be assessed using three-point bending tests.

A concurrent three-dimensional assessment of the complex interplay between bone microdamage accumulation, (re)modeling and mechanical properties is lacking to provide a comprehensive scientific basis for evaluating age-related microdamage and fracture risks. It is critical to develop a platform with such evaluations on laboratory animal models, which can provide biomechanical evidence transferable to future studies on bone loss and fracture.

3.2 Research questions

1. Bone growth evaluation

Is the *in vivo* micro-CT-based method a reproducible alternative to the conventional histology-based method to evaluate the longitudinal bone growth?

2. Bone growth and (re)modeling of young rats

Does physiological cyclic compression applied during puberty influence the longitudinal bone growth and bone (re)modeling of growing rats?

3. Bone microdamage accumulation in rat cortical bone

How much cyclic compressive loading can lead to microdamage accumulation and eventually fractures in adult rat cortical bone?

4. Bone microdamage accumulation in ovariectomized rats

What is the extent of microdamage accumulation in normal, ovariectomized and BP-treated adult rats?

3.3 Objectives

3.3.1 General objective

The general objective was to develop an experimental platform which enables quantification of bone growth, (re)modeling, microdamage and mechanical properties in laboratory rats. Assuming that the results are transferable to the human skeleton, this research project will provide insights to future studies in the context of pubertal bone growth and estrogen-related bone loss.

3.3.2 Specific objectives

1. Quantification of bone growth

Develop and validate an *in vivo* micro-CT-based method to evaluate longitudinal growth rates in rat long bone.

2. Quantification of bone (re)modeling

Adapt and apply an *in vivo* micro-CT-based method to evaluate 3D bone (re)modeling parameters in rat long bone.

3. Quantification of bone microdamage

Adapt and apply a contrast-enhanced micro-CT-based method to evaluate 3D bone microdamage accumulation in rat cortical bone.

4. Relationships between bone microdamage and (re)modeling

Conduct a concurrent evaluation of bone microdamage, (re)modeling, and mechanical properties in healthy, ovariectomized, and BP-treated adult rats. Establish relationships between the three key factors in order to provide insights to further testing of bone loss treatments.

The following Table 3.1 shows how each of the chapters in the main body of the dissertation answers their corresponding research questions and specific objectives.

Table 3.1 Chapters, their corresponding research questions, and specific objectives.

Chapters	Research Questions	Specific Objectives
Chapter 4 (Article 1)	Research Question 1	Specific Objective 1
Chapter 5 (Article 2)	Research Question 2	Specific Objective 2
Chapter 6 (Preliminary Experiments)	Research Question 3	Specific Objective 3
Chapter 7 (Article 3)	Research Question 4	Specific Objective 4

CHAPTER 4 ARTICLE 1: VALIDATION OF AN IN VIVO MICRO-CT-BASED METHOD TO QUANTIFY LONGITUDINAL BONE GROWTH OF PUBERTAL RATS

4.1 Article information

- This chapter presents the first article written in the context of this dissertation, which answers the research question 1 and the specific question 1 as shown in Table 3.1
- The article was first published in *Bone*, volume 154, article 116207, on 2021-09-20 by Elsevier, reproduced with permission from Elsevier
- Permanent link: <https://doi.org/10.1016/j.bone.2021.116207>
- The contribution of the first author, Bohao Ning, was about 85%
- Co-authors: Irène Londono, Catherine Laporte, Isabelle Villemure

4.2 Author contribution

- Bohao Ning: conceptualization, methodology, software, data curation, validation, formal analysis, writing - original draft, writing - review & editing, visualization
- Irène Londono: conceptualization, methodology, data curation, resources, writing - review & editing
- Catherine Laporte: conceptualization, methodology, writing - review & editing, supervision
- Isabelle Villemure: conceptualization, methodology, writing - review & editing, supervision, funding acquisition

4.3 Abstract

Bone growth is an essential part of skeletal development during childhood and puberty. Accurately characterizing longitudinal bone growth helps to better understand the determining factors of peak bone mass, which has impacts on bone quality later in life. Animal models were largely used to study longitudinal bone growth. However, the commonly used histology-based method is destructive and unable to follow up the growth curve of live animals in longitudinal experiments. In this study, we validated an *in vivo* micro-CT-based method against the histology-based method to quantify longitudinal bone growth rates of young rats non-destructively. CD (Sprague Dawley) IGS rats aged 35, 49 and 63 days received the same treatments: two series of repeated *in vivo* micro-CT scans on their proximal hindlimb at a five-day interval, and two calcein injections separated by three days. The longitudinal bone growth rate was quantified by registering time-lapse micro-CT images in 3D, calculating the growth distance on registered images, and dividing the distance by the five-day gap. The growth rate was also evaluated by measuring the 2D distance between consecutive calcein fluorescent bands on microscopic images, divided by the three-day gap. The two methods were both validated independently with reproducible repeated measurements, where the micro-CT-based method showed higher precision. They were also validated against each other with low relative errors and a strong Pearson sample correlation coefficient (0.998), showing a significant ($p < 0.0001$) linear correlation between paired results. We conclude that the micro-CT-based method can serve as an alternative to the histology-based method for the quantification of longitudinal growth. Thanks to its non-invasive nature and true 3D capability, the micro-CT-based method helps to accommodate *in vivo* longitudinal animal studies with highly reproducible measurements.

4.4 Keywords

Bone growth, longitudinal growth, *in vivo* micro-CT, image registration, histology, calcein labeling

4.5 Introduction

Bone growth is an important mechanism allowing bone mass accrual and bone shape maintenance. Human bone growth takes place during childhood and puberty, and stops in the twenties with a complete ossification of the epiphyseal plate (OpenStax, 2013). Peak bone mass is normally achieved right after the growing period when skeletal differences are established, building foundations for bone quality later in life (Luiza Loro et al., 2000; Wang et al., 2009; Wang & Seeman, 2008). Quantifying longitudinal bone growth helps to evaluate the impacts of various factors on pubescent musculoskeletal development, including key aspects such as nutrient intake (Bass et al., 2013; Weaver et al., 2016; Welch & Weaver, 2005; Yadav et al., 2020) and physical exercise (Mosley & Lanyon, 2002; Stokes, 2002; Welch et al., 2008; Zimmermann et al., 2017).

The widely adopted histology-based method uses fluorochrome labelling to quantify longitudinal bone growth in animal studies. Upon injection, oxytetracycline (Hansson, 1967; Hansson et al., 1972) or calcein (Álvarez et al., 2000; Cancel et al., 2009; Hunziker & Schenk, 1989; Ménard et al., 2014; Mustafy, Benoit, et al., 2018; Mustafy, Londono, Moldovan, et al., 2019; Stokes et al., 2006; Valteau et al., 2011) can bind to the mineralizing tissue, forming a fluorescent band observable under microscope. Measuring the distance between two separate fluorescent bands and dividing the corresponded injection interval give the longitudinal growth rate. However, the histology-based method is destructive and time-consuming. Animals must be sacrificed for tissue preparation to quantify their bone growth, which makes it impossible to follow the growth profile of the same animals in a long-term survival context. Also, various human factors involved in the procedure can potentially introduce biased results.

The emergence of the *in vivo* micro-CT has made it possible to non-destructively quantify morphological parameters in animal studies (Bouxsein et al., 2010). Using a 3D image registration technique, Waarsing et al. firstly tracked the local microstructural changes of rat tibia over time (Waarsing et al., 2004). Subsequent studies have extensively investigated the reproducibility of the registration approach (Boyd et al., 2006; Lan et al., 2013; Nishiyama et al., 2010; Schulte et al., 2014). Validated against the histology-based method (Birkhold, 2016; Birkhold et al., 2014a, 2014b; Schulte, 2011; Schulte et al., 2011), the *in vivo* micro-CT-based method can extract time-lapse bone remodeling parameters in small animal models (Altman et al., 2015; Birkhold et al.,

2016; de Bakker et al., 2015). Several studies also evaluated longitudinal bone growth using the micro-CT-based method (Altman et al., 2015; Lu et al., 2016; Lu et al., 2018; Stern et al., 2015), making it possible to track growth over any time in any given animal. However, the micro-CT-based evaluation of longitudinal bone growth has not yet been validated against the widely adopted histology-based method. The precision and the accuracy of the registration-based approach for bone growth measurements hence remains unclear.

In this study, we set to complete the lacking validation on longitudinal bone growth quantification. Using the same cohort of rats, we applied both the micro-CT-based method and the histology-based method to quantify the bone growth rate at the proximal tibial growth plate. Our objectives were: (1) to evaluate each of the two methods regarding their precision errors with repeated measurements; (2) to quantify the accuracy and precision of the 3D image registration algorithm applied by the micro-CT-based method; (3) to assess the repeatability of the 2D distance measurement tool used within the histology-based method; (4) to validate the two approaches against each other using relative error and Pearson's correlation analysis.

4.6 Material and methods

4.6.1 General experimental design

Eighteen male CD (Sprague Dawley) IGS rats aged 21, 35 and 49 days were acquired from Charles River Laboratories (Montreal, QC, Canada). At the time of growth rate evaluation, they were aged 35 (n=6), 49 (n=6), and 63 (n=6) days, respectively. All rats were given one week of acclimation in the animal facility of Sainte-Justine University Hospital Research Center before the beginning of the experiment. Rats had free access to food and water in cages of two and lived a light/dark circle of 12 hours at about 23 °C and 21% humidity. The present study was approved by the Institutional Committee for Good Animal Practices in Research of the Sainte-Justine University Hospital Center and the animal use and care followed the guidelines of the Canadian Council on Animal Care (CCAC). All rats received the same treatments to quantify longitudinal bone growth rates on the proximal side of their right tibia using two different approaches concurrently: the micro-CT-based method and the histology-based method (Figure 4.1).

For the micro-CT-based method (Figure 4.1, top), each rat received two series of repeated *in vivo* micro-CT scans at their proximal tibia on day 1 (scans 1 and 2) and day 6 (scans 3 and 4). The rat was repositioned between the repeated scans to assess the reproducibility of the method. Using the four *in vivo* scans acquired at two different time points, a 3D image registration algorithm was applied to the four combinations of scan pairs. Based on the registration results, the 3D growth distance was first evaluated, and the longitudinal bone growth rate was further calculated using the 5-day interval. The repeated *in vivo* scans (at 18 μm) of the same day were registered to evaluate the accuracy and precision of the 3D registration algorithm. Additional *ex vivo* repeated scans (at 9 and 18 μm) of the left tibia were also acquired to study the effect of image resolution on registration.

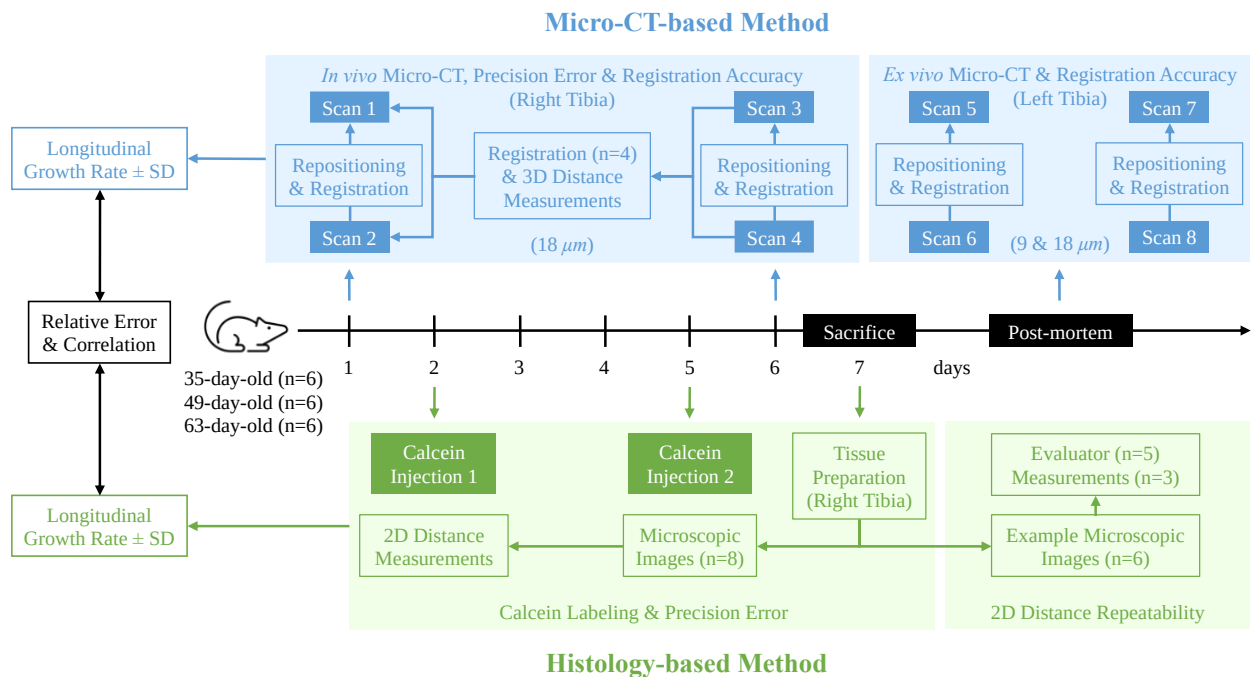


Figure 4.1 General experimental design. All rats received the same treatments in both the micro-CT-based method (top) and the histology-based method (bottom).

For the histology-based method, the rats received two doses of calcein injection, on days 2 and 5. On day 7, the rats were euthanized by CO₂ asphyxiation under isoflurane anesthesia followed by decapitation. The right tibiae were prepared for histology. Microscopic images were taken on bone

tissue slices at the proximal growth plate. The growth distance was measured between the two calcein fluorescent bands to derive the longitudinal growth rate within the 3-day interval.

One rat was excluded from the analyses due to a failed calcein administration on day 2, and two others were excluded for the same reason on day 5. A total of 15 rats remained available for the complete comparative analysis, including five rats from the 35-day-old group, six rats from the 49-day-old group, and four rats from the 63-day-old group.

4.6.2 Micro-CT-based bone growth measurement

4.6.2.1 *In vivo* micro-CT scans

A SkyScan 1176 microtomograph system (Bruker-microCT N.V., Kontich, Belgium) was used to perform the *in vivo* scans on each rat's right hindlimb. During scan sessions, the rat was under anesthesia of 2.5% isoflurane (Baxter, Deerfield, IL, USA) supplied by 1.25 L/min oxygen. The rat was positioned along the longitudinal direction of the animal bed, fixed with custom-made stabilizers made of styrofoam. The physiological conditions of the rat were closely monitored using the built-in camera of the microtomograph system and the window on the micro-CT door. The scan was set to a "medium" spatial resolution of 18 μm and to 65 kVp, 385 μA , 350 ms exposure time, 0.5°/180° rotation step, 2 average frames, using an aluminum filter of 1 mm. The total scan duration was about 11 minutes, resulting in a radiation dose of about 1.63 Gy per scan, calculated by the CTion software (Bruker-microCT N.V., Kontich, Belgium). At the end of each scanning day, two calibration phantoms of calcium hydroxyapatite (0.25 and 0.75 $\text{g}\cdot\text{cm}^{-3}$ CaHA) were scanned using the same settings for BMD (bone mineral density) calibration purposes.

Micro-CT image reconstruction and processing were performed as shown in Figure 4.2. Raw images were reconstructed using the NRecon software (Bruker-microCT N.V., Kontich, Belgium). A circle shape tool was used to include both tibia and fibula in selection. Fine-tuning was performed to compensate for possible movement artifacts based on the initial automatic alignment value proposed by the software. A beam hardening correction of 30% and ring artifact correction of 5 were applied for all the reconstructions. No smoothing was applied. The reconstruction parameters were established from trial scans of a pilot study.

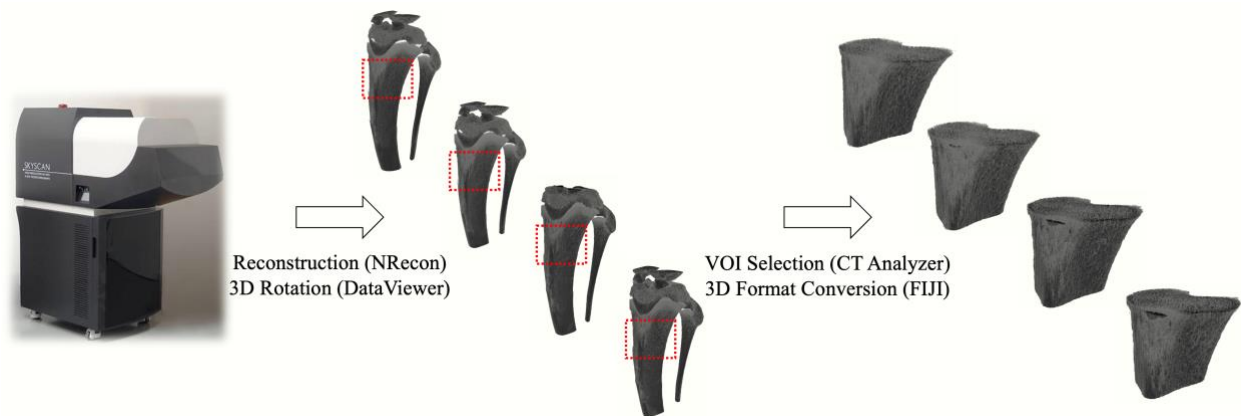


Figure 4.2 Micro-CT image reconstruction and processing pipeline shown for each rat (4 scans in total). From left to right: image acquisition (SkyScan 1176), reconstruction (NRecon) and 3D rotation (DataViewer), VOI selection (CT Analyzer) and 3D format conversion (FIJI). The red dotted rectangle shows the position of tibia VOI in their respective original micro-CT reconstructions.

Following reconstruction, image slices were imported in DataViewer (Bruker-microCT N.V., Kontich, Belgium) to perform a 3D rotation to compensate for variations in scanning positions. The images were aligned along the global longitudinal z axis, and the proximal growth plate was parallel to the transverse x-y plane. The images were then imported in CT Analyser (Bruker-microCT N.V., Kontich, Belgium) for volume of interest (VOI) analysis. The chosen VOI started from 100 slices distal to the proximal growth plate of the tibia, and included 300 slices in total, which approximately corresponded to a thickness of 5.2 mm. A circle shape tool with adaptive interpolation performed a semi-automatic selection of the tibia. The VOI was then saved as a new image sequence, imported in FIJI (Schindelin et al., 2012) for format conversion, saved for the subsequent 3D image registration.

4.6.2.2 3D image registration

A customized 3D image registration algorithm was implemented using the open-source platform ITK (Kitware, Clifton Park, NY, USA) (Johnson et al., 2015). A two-stage registration scheme aligned the micro-CT scans from day 1 with the ones from day 6, where the first stage performed general bone orientation alignment and the second stage aimed at the trabecular microstructure

alignment (Figure 4.3). The registration was rigid, because bone volumes are either deposited or absorbed over time, without any deformation (Stern et al., 2015). This registration approach was overall based on previous publications using a similar strategy (Altman et al., 2015; de Bakker et al., 2015; Lan et al., 2013), with the addition of a multi-resolution design in each calculation stage as well as the simplification of landmark matching used in previous studies.

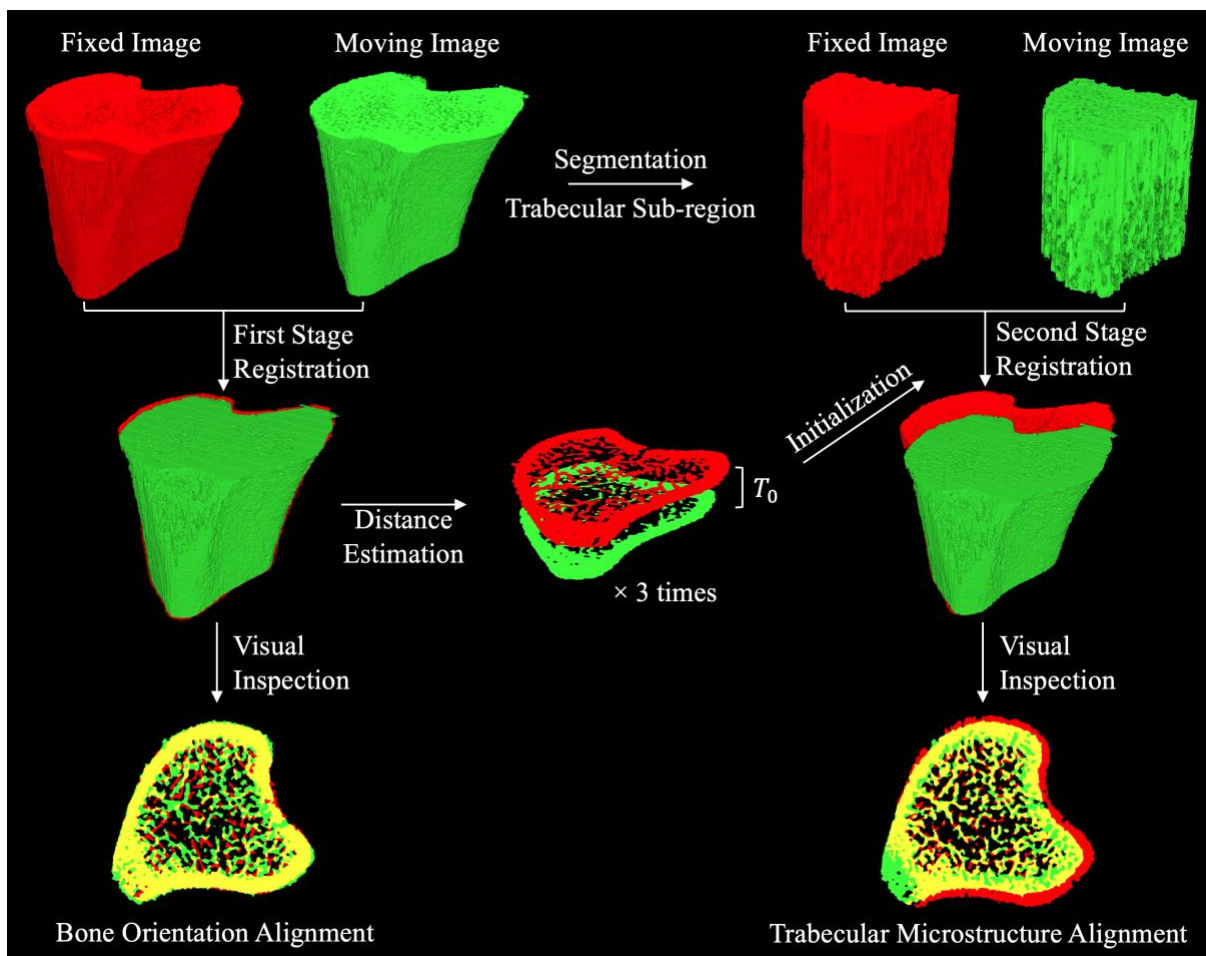


Figure 4.3 Image registration pipeline for the first stage (left) and the second stage (right). The top row shows input images for registration, the middle row shows example results viewed in 3D.

The bottom row shows example results viewed as 2D overlaid image slices. The day 1 bone volume is in red, and the day 6 bone volume is in green. The overlapping area is thus in yellow. The middle center shows the distance estimation which initializes the second stage registration.

At the first stage, the image from day 1 was set as the moving image, and the one from day 6 was set as the fixed image (Figure 4.3, top left). Mutual information (Viola & Wells III, 1997) was chosen as the optimization metric, and the regular step gradient descent based on unit quaternion gradients (Hamilton, 1866) was applied as the optimizer. A rigid 3D transform with six degrees of freedom and a linear interpolation were used. To avoid being trapped in local extrema: the calculation started at a coarse resolution with images subsampled at one eighth of the original image dimension, then moved on using subsampling factors of one sixth and one quarter subsequently. The finest resolution was not used in this stage of registration as this was not necessary for general cortical shape alignment, according to our preliminary tests where nearly identical results were obtained using either the original images or their one-quarter subsampled version. The registration program output was a transformation vector, where T_{z1} in the sixth position corresponded to the linear translation along the longitudinal z axis.

After the first registration stage, the 3D image from day 1 was spatially aligned with the image of day 6 based on the overall shape of the proximal tibia, by including both cortical and trabecular bone. Although the overall cortical shape was well aligned, the trabecular patterns were still misaligned after the first registration stage (Figure 4.3, bottom left). This phenomenon was reported in the literature by comparing registration results of maximizing cortical bone alignment to those based on trabecular bone (Lan et al., 2013). In fact, since bone apposition modeling on the endosteum and resorption modeling on the periosteum take place alongside the longitudinal bone growth to maintain the bone shape (Burr & Allen, 2019), it is basically impossible to simultaneously align the cortical shell and the trabecular microstructure, especially for rapidly growing rats. Also, trabecular bone in the metaphysis coalesces to cortical bone (Altman et al., 2015). This results in a longitudinal offset between trabecular structures of the two images when the cortical shell is well aligned (de Bakker et al., 2015). Thus, a second registration stage was implemented for a finer alignment of the trabecular bone microstructure, which is directly related to the measurement of longitudinal bone growth at the growth plate.

Before performing the second registration stage, the outputs of the first stage were visually inspected in ParaView (Kitware, Clifton Park, NY, USA) to avoid any spatial misalignment. The registration results were also quantitatively verified against the minimally required mutual information as shown in the second row in Table 4.1. The underlying assumption is that higher

absolute values of mutual information reflect a better overlapping between the two images. The minimum values were defined with some preliminary tests based on visual inspection.

Table 4.1 Absolute value of mutual information for both stages of 3D image registration: the minimum requirement for a correct registration (determined by some preliminary tests based on visual inspection), and average results $\pm$ standard deviation for each group of rats.

	Requirement	35-day-old	49-day-old	63-day-old
First stage	≥ 0.60	0.76 ± 0.07	0.90 ± 0.05	1.04 ± 0.06
Second stage	≥ 0.08	0.13 ± 0.04	0.15 ± 0.02	0.23 ± 0.08

Since the capture radius of the optimizer is usually limited (Brooks, 2008), it is difficult to perform accurate registration without a reasonable initial guess for the transformation parameters. Alongside the visual and quantitative inspection, an initial translation along the longitudinal direction was manually estimated by comparing the morphology of transversal slices from the two images (Figure 4.3, middle center). This estimation was performed three times for each image pair (requiring about 2 min) and an average value T_0 was obtained. Then, trabecular bone was automatically segmented using a custom-developed algorithm (Buie et al., 2007). Segmented images were visually verified to ensure the proper separation between cortical and trabecular bone. A cuboid-shaped sub-region containing trabecular bone only from each segmented image served as input image for the second registration step (Figure 4.3, top right).

At the second stage, the registration was initialized using the previously determined longitudinal translation of T_0 to facilitate the optimization process. The same transform, interpolator, metric, and optimizer were applied as in the first stage. The calculation started with a subsampled image of one third of the original dimension, then used the full resolution image to determine the final transformation parameter vector where T_{z2} in the sixth position corresponded to the linear translation along the longitudinal z axis. The output transformation parameter vectors of the first and second stages were applied together to resample the moving image onto the fixed image of the first stage. In the end, the program output was the registered image of day 1, which was resampled in the coordinates of the day 6 image. Output files were visually inspected in ParaView for

trabecular microstructural alignment (Figure 4.3, bottom right). The absolute values of mutual information were also verified against the minimum requirement as shown in the third row of Table 4.1. Three registrations of one rat from the 49-day-old group did not show satisfying results upon visual inspection. In these cases, the calculations were repeated using a single-level registration where the images were not subsampled, yielding well aligned results. The calculation took about 5 minutes at the first stage and 10 minutes at the second stage for each image pair, using a desktop computer equipped with a 3.2 GHz 6-core Intel Core i7 processor.

4.6.2.3 3D growth rate measurement

After the 3D image registration of each image pair, the growth distance was calculated as the sum of T_{z1} and T_{z2} since all the images were taken from the exact same position relative to the proximal tibial growth plate. The same value could be obtained by directly measuring the distance on lateral overlaid views of registered 3D images (Figure 4.4). The growth rate was calculated by dividing the growth distance by the five-day interval between the two scans.

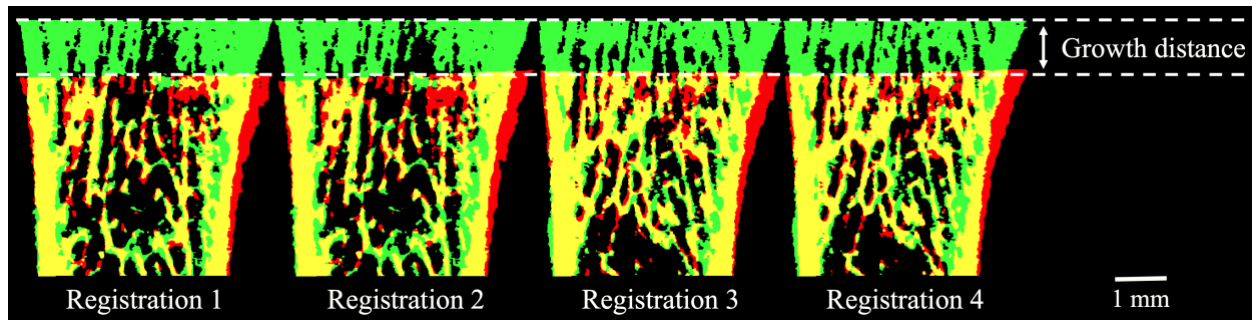


Figure 4.4 Measurement of longitudinal growth distance directly on the overlaid images. From left to right: four different combinations of registration for each rat, deriving an average growth distance. The day 1 bone volume is in red, and the day 6 bone volume is in green. The overlapping area is thus in yellow.

As each rat received two series of *in vivo* micro-CT scans on day 1 as well as day 6, either of the two scans from day 1 (scans 1 and 2) could serve as the moving image and either one from day 6 (scans 3 and 4) could be the fixed image. There were hence four possible image combinations available for 3D image registration as shown in Figure 4.1 (top) and Figure 4.4. Each registration

result led to a longitudinal growth rate ($\mu\text{m}/\text{day}$), giving a total of four repeated measures. An average value was calculated for the four repeated measures, denoted as $\bar{\tau}_i$ for rat i .

4.6.3 Histology-based bone growth measurement

4.6.3.1 Calcein labeling

Calcein (Sigma-Aldrich, St. Louis, MO, USA) was injected twice intraperitoneally (1.5 mg/kg) to each rat, on days 2 and 5. The rats were closely monitored after injection for the appearance of fluorescent urine as a verification of successful calcein administration. For each injection, the calcein bound to the mineralization front of the rat tibia and formed a fluorescent band, which was observable under the microscope.

After the sacrifice on day 7, proximal tibia segments about 10 mm thick were fixed in formalin solution (Anachemia, Montreal, QC, Canada) for 48 hours, dehydrated in graded ethanol solutions, clarified in xylene, and embedded using methylmethacrylate (MMA) (Fisher Scientific Canada, Nepean, ON, Canada). A microtome (Leica SM2500) cut the tissue blocks in four series of four slides, containing two 6 μm -thick sections each. Sectioning was in the sagittal plane, starting from the medial side of the tibial plate, reaching about one third of the entire tibial thickness. A microscope (Leica DMR) was used to evaluate the sections from each rat under $2.5\times$ magnification. All sections were visually inspected and selected to make sure that the four different locations within the growth plate were included and that the two fluorescent bands were clearly defined, giving eight images available per rat for further 2D distance measurement.

4.6.3.2 2D growth rate measurement

A single experienced evaluator (blind to the registration results) performed the distance analysis for all the microscopic images. For each image, the evaluator defined the longitudinal direction, and marked points on each of the two fluorescent lines (Figure 4.5). A custom-built application interpolated the continued points into splines and calculated the average distance between the two splines along the longitudinal direction using MATLAB R2018b (MathWorks, Natick, MA, USA). Dividing the measured distance by an interval of three days (between day 2 and day 5) provided

the longitudinal growth rate ($\mu\text{m}/\text{day}$) for each section. An average growth rate was calculated over the eight repeated measures, denoted as $\bar{\tau}_i^*$ for rat i .

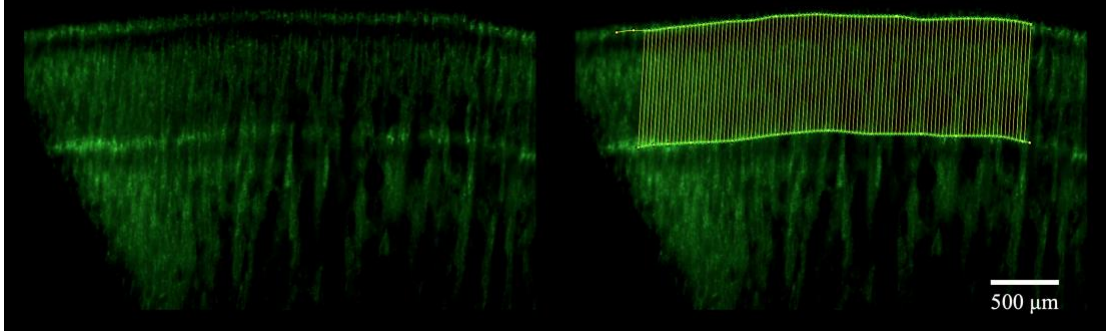


Figure 4.5 Distance measurement between calcein fluorescent bands. Left: original microscopic image. Right: fluorescent lines defined as splines, and growth distances ($n=100$) between the two splines shown. The average growth distance for this photo was the mean value of the 100 distances measured.

4.6.4 Validation

4.6.4.1 Precision error of the two methods

To investigate the reproducibility of the micro-CT-based and histology-based methods, precision errors were evaluated for the longitudinal growth rate of each rat (Birkhold et al., 2014b; Glüer et al., 1995; Nishiyama et al., 2010). The standard deviation SD_i ($\mu\text{m}/\text{day}$) of growth rate for rat i was calculated for n_i repeated measurements ($n_i = 4$ for the micro-CT-based method, and $n_i = 8$ for the histology-based method). The individual relative precision error was expressed as the coefficients of variation of the n_i repeated measurements for rat i . The overall precision error SD_{method} was then calculated for both methods as the root-mean-square average of the individual precision error SD_i for $i \in [1, 15]$. The 95% confidence interval (CI) of either method was also calculated.

To evaluate the effect of age on longitudinal bone growth rates, ANOVA test (General Linear Model with Tukey HSD Post Hoc Test) was performed using SPSS Statistics v26 (IBM Corp, Armonk, NY, USA) among the three groups of rats for each method.

4.6.4.2 Accuracy and precision of 3D image registration

To assess the accuracy of 3D image registration, repeated *in vivo* scans of the same day were registered to evaluate the percentage of non-matching voxels (ratio between the non-matching voxel count and the total number of bone voxels), followed by 3D Gaussian filtering ($\sigma = 1, support = 5$) and binarization. Global thresholds of $0.28269 \text{ g}\cdot\text{cm}^{-3}$ CaHA for trabecular VOI and of $0.37649 \text{ g}\cdot\text{cm}^{-3}$ CaHA for cortical VOI were used. Precision errors of standard histomorphometry parameters were also calculated for each registered scan pair, including bone volume fraction (BV/TV), average cortical thickness (Ct.Th), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) (Bouxsein et al., 2010; Nishiyama et al., 2010).

The same analysis was also performed for repeated *ex vivo* micro-CT scans on the left tibia harvested from each sacrificed rat. The left hindlimb of each rat was cut at the diaphyseal femur, and the whole tibia with soft tissue attached was placed in small transparent plastic bags to simulate the *in vivo* scanning condition. The only difference was the absence of any potential movement artifact during the *ex vivo* scanning. The *ex vivo* scans at $18 \mu\text{m}$ were performed using the exact same parameters as for the *in vivo* scans, whereas another set of higher resolution scans were acquired at $9 \mu\text{m}$, using a setting of 65 kVp, 300 μA , 1200 ms exposure time, $0.5^\circ/180^\circ$ rotation step, no average frames, with a 1 mm aluminum filter. Each tibia was scanned twice at proximal metaphysis with a repositioning in between (Figure 4.1, top). The same reconstruction and image processing were applied as described in section 4.6.2.1. As for the 3D image registration, only the first stage of calculation was performed using the same parameters presented in section 4.6.2.2, because the *ex vivo* bone samples did not undergo any modeling or remodeling and, therefore, did not differ in terms of bone microstructure. The same principle applied to the repeated *in vivo* scans of the same day.

To evaluate the effect of scanning condition on image registration, ANOVA test (General Linear Model with Tukey HSD Post Hoc Test) was performed using SPSS Statistics v26 (IBM Corp, Armonk, NY, USA) for the percentage of non-matching voxels among the three scanning conditions.

4.6.4.3 Repeatability of 2D distance measurement

To evaluate the repeatability of the 2D microscopic image analysis, five evaluators were invited to perform independent measurements on six randomly chosen images. The evaluators performed three independent trials for each image, including the repeated definition of the longitudinal direction and point selection along the two calcein fluorescent bands. The intra-evaluator standard deviation of each evaluator and each image was calculated, expressed as the coefficients of variation. The inter-evaluator standard deviation for each image was calculated for repeated measurements from all the evaluators, also expressed as the coefficients of variation.

4.6.4.4 Agreement between the two methods

The average longitudinal growth rates measured by the micro-CT-based method ($\bar{\tau}_i$) and the histology-based method ($\bar{\tau}_i^*$) were compared for each rat by calculating relative error (Golub & Van Loan, 1996) where $\bar{\tau}_i^*$ was served as reference value. A Pearson correlation analysis was performed using Excel (Microsoft, Redmond, WA, USA) by comparing results measured by the two methods $\bar{\tau}_i$ and $\bar{\tau}_i^*$ for each rat i . The sample correlation coefficient r was calculated. Since the population coefficient ρ is unknown, the t-statistic was computed to evaluate the significance of the linear correlation. The null hypothesis H_0 was defined as $\rho = 0$ (no significant linear correlation between the two measurement methods in the population), and the alternative hypothesis H_a was set to $\rho \neq 0$ (a significant linear correlation between the two methods in the population).

4.7 Results

4.7.1 Precision error of the two methods

The longitudinal bone growth rate of the proximal rat tibiae measured by the two methods are shown in Figure 4.6. As expected, the younger rats had significantly higher growth rates than the older ones ($p < 0.001$ between any of the three groups, for both methods). Comparing the average growth rate of each rat measured by the two methods, they generally overlapped well within the interval of ± 1 standard deviation.

Table 4.2 shows the precision errors evaluated for the two methods, expressed as standard deviations and as coefficients of variation. The histology-based method shows higher variability and larger confidence intervals than the micro-CT-based method.

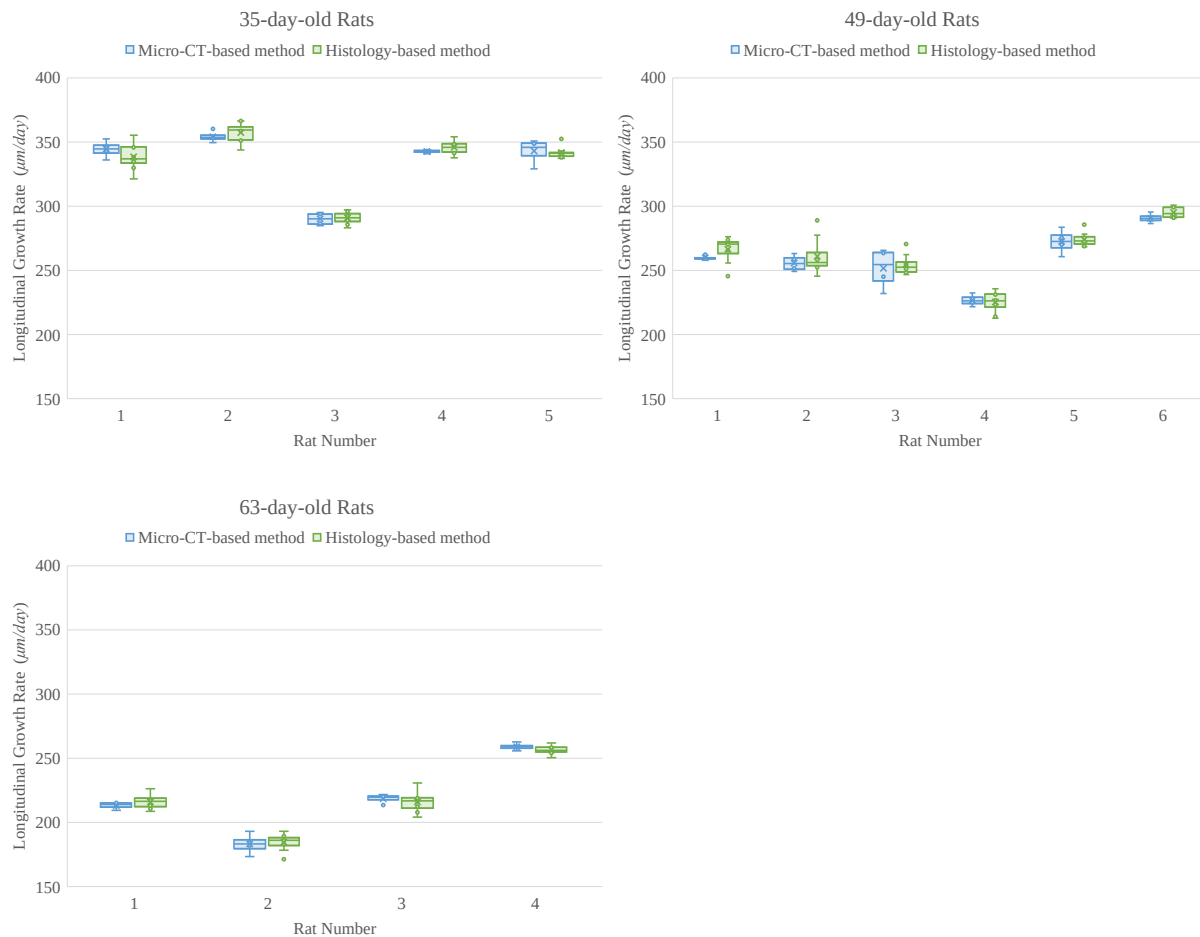


Figure 4.6 Comparison of the longitudinal growth rates measured by the two methods. Top left: the 35-day-old group (n=5). Top right: the 49-day-old group (n=6). Bottom: the 63-day-old group (n=4).

Table 4.2 Precision error of the two methods.

	Micro-CT-based method	Histology-based method
Standard deviation ($\mu\text{m/day}$)	6.85	7.81
Coefficients of variation	2.50%	2.84%
95% confidence interval ($\mu\text{m/day}$)	[5.68, 8.63]	[6.95, 8.93]

4.7.2 Accuracy and precision of 3D image registration

Table 4.3 shows the percentage of non-matching voxels calculated based on repeated scans of proximal tibia. The non-matching percentage is higher for trabecular ($< 6\%$) than cortical ($< 2\%$) VOI. This is mainly caused by the accuracy of the micro-CT at the applied spatial resolution (9 and 18 μm) for young rat bone imaging: cortical thickness ($\sim 500 \mu\text{m}$) is much larger than that of the trabeculae ($\sim 80 \mu\text{m}$) (Longo et al., 2016), thus higher accuracy was found with cortical VOI ($p < 0.001$). For the trabecular VOI, 9 μm scans showed a significantly lower non-matching percentage than for the two 18 μm conditions. For the cortical VOI, no effect of scanning conditions was observed.

Table 4.3 Percentage of non-matching voxels for trabecular and cortical VOI from the repeated scans at three different scanning conditions, expressed in average value $\pm$ standard deviation. ^a Significant difference from the *in vivo* 18 μm group ($p < 0.001$). ^b Significant difference from the *ex vivo* 18 μm group ($p < 0.001$). ^c Significant difference from the *ex vivo* 9 μm group ($p < 0.001$).

	Percentage of non-matching voxel	
	Trabecular VOI	Cortical VOI
<i>In vivo</i> repeated scan pair at 18 μm (n=36)	5.19% $\pm$ 1.59% ^c	1.81% $\pm$ 0.59%
<i>Ex vivo</i> repeated scan pair at 18 μm (n=18)	5.40% $\pm$ 0.91% ^c	1.59% $\pm$ 0.48%
<i>Ex vivo</i> repeated scan pair at 9 μm (n=18)	3.40% $\pm$ 1.29% ^{ab}	1.89% $\pm$ 0.56%

Table 4.4 presents precision error of standard histomorphometry parameters evaluated on registered repeated scan pair under three different scanning conditions. The 9 μm scans showed better precision for all the parameters besides BV/TV. The 18 μm scans showed similar results for both *in vivo* and *ex vivo* conditions, except for Tb.Sp where the *ex vivo* scans had higher precision.

Table 4.4 Precision error of standard histomorphometry parameters evaluated on registered repeated scan pair at three different scanning conditions.

	BV/TV	Ct.Th	Tb.Th	Tb.N	Tb.Sp
<i>In vivo</i> repeated scan pair at 18 μm (n=36)					
Precision error	0.85%	1.80%	1.63%	1.73%	1.17%
95% confidence interval low	0.69%	1.46%	1.32%	1.41%	0.95%
95% confidence interval high	1.11%	2.34%	2.12%	2.25%	1.52%
<i>Ex vivo</i> repeated scan pair at 18 μm (n=18)					
Precision error	0.92%	1.96%	2.43%	1.95%	0.30%
95% confidence interval low	0.70%	1.48%	1.84%	1.47%	0.23%
95% confidence interval high	1.37%	2.89%	3.60%	2.89%	0.45%
<i>Ex vivo</i> repeated scan pair at 9 μm (n=18)					
Precision error	1.44%	0.74%	1.14%	1.12%	0.11%
95% confidence interval low	1.09%	0.56%	0.86%	0.85%	0.08%
95% confidence interval high	2.14%	1.10%	1.68%	1.66%	0.16%

4.7.3 Repeatability of 2D distance measurement

Figure 4.7 (left) shows the results of the intra-evaluator error regarding the 2D growth distance measurement. The average coefficient of variation of all the evaluators is $0.93\% \pm 0.27\%$ (Table 4.5). The measurement is very consistent within each evaluator and most of the images evaluated. Evaluators 1, 3 and 4, who were more experienced with the 2D distance measuring tool, showed better repeatability with coefficients of variation less than 1%, in contrast with evaluators 2 (1.14%) and 5 (1.29%), who were first-time users.

As for the inter-evaluator error (Figure 4.7, right), the average coefficient of variation was 1.32% (Table 4.5), suggesting that the 2D measurement tool is highly reproducible and consistent between users for all the images tested. Images 5 and 6 show smaller variation values, probably due to the

learning process of the evaluators as the images were analyzed in ascending numerical order from 1 to 6.

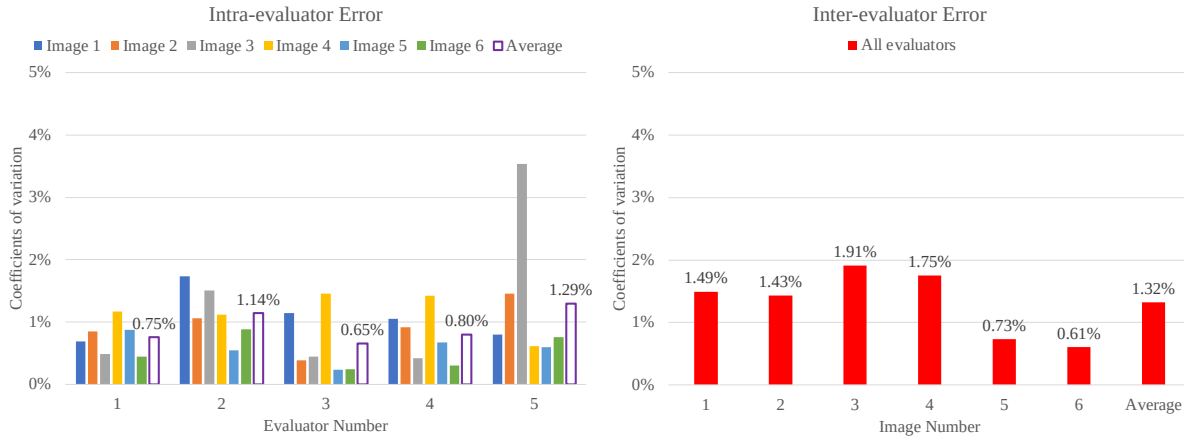


Figure 4.7 Repeatability results for the 2D growth distance measurement. Left: intra-evaluator errors. Right: inter-evaluator errors.

Table 4.5 Average intra- and inter-evaluator errors, expressed in standard deviation and coefficients of variation.

	Average intra-evaluator error	Average inter-evaluator error
Standard deviation (μm)	7.88 ± 2.30	11.09 ± 4.38
Coefficients of variation	$0.93\% \pm 0.27\%$	$1.32\% \pm 0.53\%$

4.7.4 Agreement between the two methods

Relative errors of longitudinal growth rate measured by the two methods are shown in (Figure 4.8, left). The highest relative error (2.45%) was found on rat 1 of the 49-day-old group, while the others generally lied between 1% and 2%. The overall agreement between the two methods is very high given an average relative error of 1.12%, which translates in approximately 2.5 to 3.5 μm per day, or 12.5 to 17.5 μm in total for the five-day interval. This discrepancy is well under the resolution limits of the micro-CT (18 μm). Also, comparing to the average intra-evaluator error (7.88 μm) and inter-evaluator error (11.09 μm) of the histology-based method (Table 4.5), over half of the disparities might have been originated from the microscopic photo evaluation step of the histology-based method.

The Pearson correlation analysis resulted in a sample correlation coefficient of 0.998 ($p < 0.0001$), showing a significantly positive linear correlation between growth rates evaluated by the two methods (Figure 4.8, right).

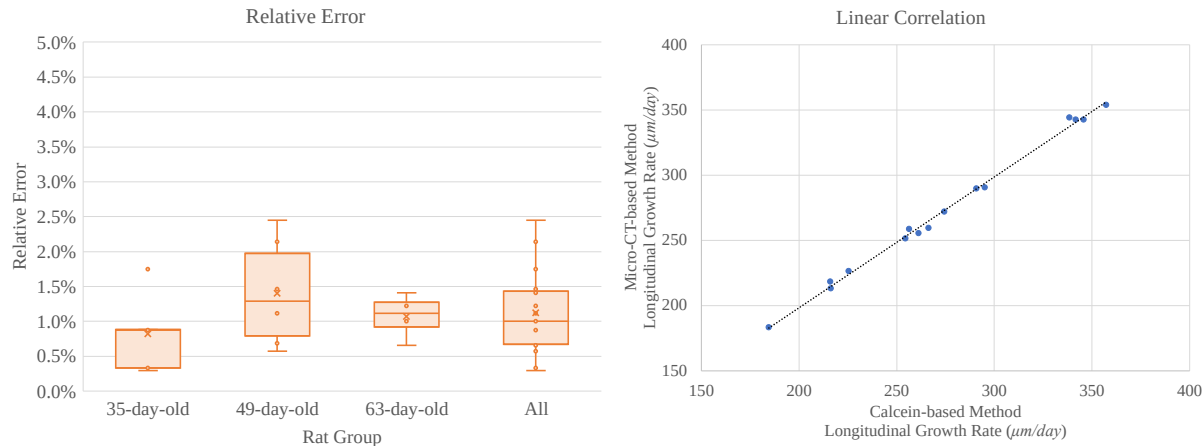


Figure 4.8 Agreement between results calculated by the two methods. Left: relative error. Right: linear correlation.

4.8 Discussion

The present study aimed at validating the micro-CT-based method against the histology-based method to quantify longitudinal bone growth rates. First, both methods showed great reproducibility with low precision errors. For the micro-CT-based method, 3D registration on repeated scans was highly accurate for both trabecular and cortical bone. The 2D distance measurement within the histology-based method showed low intra- and inter-evaluator variability. Finally, longitudinal growth rates obtained from both methods agreed well with low relative errors and significantly high linear correlation.

The commonly used histology-based method is less reproducible than the micro-CT-based method, with the former showing about 12% higher precision errors (Table 4.2). This difference is not only due to the 2D vs 3D distance measurement, but also because more human operation factors are involved in the histology-based method. The tissue sample preparation is quite laborious and involves manual variations, especially during the block cutting steps. Three rats were unfortunately

rejected because of failed calcein injections. In the present study, it took about three weeks just to prepare the samples using the histology-based method, whereas one week was sufficient to analyze all the data using the micro-CT-based method. Another source of human variability in the histology-based method can be found within the 2D distance measurement using the custom-built MATLAB measurement tool. Comparing the general precision error of 7.81 $\mu\text{m}/\text{day}$ of the histology-based method with the average inter-evaluator error of 11.09 μm (about 3.70 $\mu\text{m}/\text{day}$), about half of the variations in the histology-based method might have been caused by microscopic photo evaluation. Although experienced users can yield higher repeatability, intra- and inter-evaluator variations still exist (Figure 4.7). Finally, the destructive nature of the histology-based method makes it difficult to accommodate time-lapse longitudinal studies, where the growth data are usually only available at terminal time points (Cancel et al., 2009; Ménard et al., 2014; Mustafy, Benoit, et al., 2018; Mustafy, Londono, Moldovan, et al., 2019; Valteau et al., 2011).

On the other hand, the micro-CT-based method is highly reproducible (Table 4.2). An average standard deviation of 6.85 $\mu\text{m}/\text{day}$ is a satisfactory result given that the spatial resolution of our micro-CT images is 18 μm . Considering the scan interval of five days, the total standard deviation would be $6.85 \times 5 = 34.25$ μm , approximatively double of the image resolution, which is influenced by partial volume effects (Palacio-Mancheno et al., 2014). The method will be even more reproducible when using a finer image resolution, as shown in the accuracy results of the image registration algorithm (section 4.7.2). The overall non-matching voxel percentage is under 2% for cortical bone and under 6% for trabecular bone (Table 4.3), comparable to similar analyses in the literature (Birkhold et al., 2014a, 2014b). The precision error of standard histomorphometry parameters on registered scans (Table 4.4) is also comparable to past results (Nishiyama et al., 2010). The repeated scans at 9 μm showed similar registration accuracy on cortical bone to their 18 μm counterparts (Table 4.3), probably due to the limitation of the linear interpolator employed in the current algorithm (Schulte et al., 2014). Nonetheless, the finer image resolution contributed to better registration accuracy on trabecular bone as well as higher precision towards histomorphometry analysis (Table 4.4). However, higher resolution images usually require longer scanning time (longer anesthesia time) and impose higher radiation doses, which consequently lead to higher risks to the animal's health (Brouwers et al., 2007; Longo et al., 2016; Sacco et al., 2017). Overexposure to X-rays was shown to have negative effects on bone microstructure (Klinck et al.,

2008; Mendes et al., 2020; Mustafy, Benoit, et al., 2018), which might alter the validity of some experimental designs. In the present study, our choice of *in vivo* micro-CT setting was based on a previous dosimetric study within our laboratory using the same animal model (Mustafy, Benoit, et al., 2018). Based on our accuracy results with 9 μm scans, the methodology presented in the current study will be entirely applicable to micro-CT scans under 18 μm of resolution if required.

Regarding the experimental design (Figure 4.1), we did not apply the same time interval for the micro-CT-based method and the histology-based method primarily due to practical reasons (limited procedure time during a day). The three-day interval employed in the histology-based method was used in previous studies of our laboratory, yielding repeatable results (Cancel et al., 2009; Ménard et al., 2014; Mustafy, Benoit, et al., 2018; Mustafy, Londono, Moldovan, et al., 2019; Valteau et al., 2011). To make sure that the growth rate measured by both methods are comparable, the two series of *in vivo* micro-CT scans were acquired one day before and one day after the two calcein injections respectively, giving an interval of five days. However, image registration becomes more difficult when the time interval increases, due to the rapid growth of young rats. To overcome this obstacle, we followed the recommendation of de Bakker et al. to separate the registration into two stages (de Bakker et al., 2015), where the first stage was focused on aligning the overall structure of the two images and the second stage took a step further to maximize alignment of trabecular microstructure. Although we minimized the risk of human factors involved in the registration process by inspecting the results both qualitatively (3D spatial alignment) and quantitatively (absolute value of mutual information), the micro-CT-based method is not completely free of human factors. The 3D rotation in the image processing steps, the segmentation of trabecular bone and the two-stage image registration are all subjective to some degree. Further improvements can be made towards a higher degree of automation, e.g., using learning-based algorithms (Eppenhof et al., 2018; Eppenhof & Pluim, 2019; Sergeev et al., 2012).

Our successful evaluation of the longitudinal growth rate of the young rats under 2 months old implies that the current methodology remains valid for older rats, since the image registration is the most challenging at this age, especially paired with a moderate image resolution of 18 μm . However, one need to pay close attention to the image resolution and the scan interval when using older rats, as the precision might not be the same. The longitudinal growth of rats substantially reduces upon reaching maturity (Burr & Allen, 2019). For example, the growth rate of a 3-month-

old rat is around 80 $\mu\text{m}/\text{day}$ (Álvarez et al., 2000), in contrast to about 220 $\mu\text{m}/\text{day}$ for a 2-month-old rat (Figure 4.6). Giving the average standard deviation of 6.85 $\mu\text{m}/\text{day}$ obtained in the current study, it is recommended to extend the scanning interval to at least 3-fold to obtain a similar precision level, if using an image resolution of 18 μm . At a more advanced age, e.g., 6-month-old, the growth rate will be even lower (Altman et al., 2015), requiring finer image resolutions (such as 9 μm) and longer scanning period. As for the calcein-based method, the precision might stay on a similar level regardless of the rat age, since microscopes can usually provide much better resolution than *in vivo* micro-CT. However, most studies on longitudinal bone growth focused on younger rats, as the external factors related to bone growth primarily took effect during puberty (Mustafy, Londono, Moldovan, et al., 2019; Wang et al., 2009; Wang & Seeman, 2008).

Finally, the micro-CT-based method is less time-consuming for data analysis, compared to histology. Users can usually take advantage of application packages provided by micro-CT manufacturers and open-source software to ensure highly reproducible image analysis (Bouxsein et al., 2010). Its 3D nature also contributes to a true spatial understanding of the bone microstructure, eliminating the possibility of biased evaluation due to the use of 2D microscopic images. The imaging modality itself is non-destructive, making it a better candidate for longitudinal studies where follow-up parameters are continually assessed (Waarsing et al., 2004). Paired with 3D image registration, the *in vivo* micro-CT measurement method is capable of tracking bone modeling and remodeling, which fully takes advantage of the spatiotemporal information contained in the tomographs (Altman et al., 2015; Birkhold et al., 2014a, 2014b; Boyd et al., 2006; Campbell & Sophocleous, 2014; de Bakker et al., 2015; Lan et al., 2013; Schulte et al., 2011; Waarsing et al., 2004). With the wide adoption of *in vivo* micro-CT in translational studies, the histology-based method might not be the “gold-standard” anymore. The image analysis tools developed for animal studies using micro-CT can be easily adapted to clinical studies using HR-pQCT, since the ratio between the resolution of the two imaging modalities is close to that between the trabecular thickness of rat and human bones (Altman et al., 2015).

To summarize, the micro-CT-based method was successfully validated against the histology-based method on longitudinal bone growth quantification with low relative error and significantly high linear correlation. Since we used very young rats aged 1 to 2 months old and a moderate micro-CT resolution of 18 μm , the method remains valid for older animals with slower bone growth, and

micro-CT scans using finer resolutions. Combined with other 3D registration-based tools for in vivo bone modeling and remodeling quantification, the micro-CT-based methods can serve as good alternatives to dynamic histomorphometry, especially for longitudinal animal studies. Some research groups are also investigating deep learning-based approaches to further exploit the rich information contained in the tomographs (Asgharzadeh et al., 2020; B. Zhang et al., 2020). We believe that imaging systems with higher-resolution and safer radiation doses, paired with highly automated and reproducible software packages will be a future trend of active research.

4.9 Acknowledgements

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4.10Funding

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4.11References

Please refer to the general list of references provided at the end of the dissertation (page 152).

CHAPTER 5 ARTICLE 2: IMPACT LOADING INTENSIFIES CORTICAL BONE (RE)MODELING AND ALTERS LONGITUDINAL BONE GROWTH OF PUBERTAL RATS

5.1 Article information

- This chapter presents the second article written in the context of this dissertation, which answers the research question 2 and the specific question 2 as shown in Table 3.1
- The article was first published in *Biomechanics and Modeling in Mechanobiology*, on 2023-03-31 by Springer Nature, reproduced with permission from Springer Nature
- Permanent link: <https://doi.org/10.1007/s10237-023-01706-5>
- The contribution of the first author, Bohao Ning, was about 80%
- Co-authors: Tanvir Mustafy, Irène Londono, Catherine Laporte, Isabelle Villemure

5.2 Author contribution

- Bohao Ning: conceptualization, methodology, software, data curation, formal analysis, writing - original draft, writing - review & editing, visualization
- Tanvir Mustafy: conceptualization, methodology, data curation, writing - review & editing, visualization
- Irène Londono: methodology, data curation, resources, writing - review & editing
- Catherine Laporte: conceptualization, methodology, writing - review & editing, supervision
- Isabelle Villemure: conceptualization, methodology, writing - review & editing, supervision, funding acquisition

5.3 Abstract

Physical exercise is important for musculoskeletal development during puberty, which builds bone mass foundation for later in life. However, strenuous levels of training might bring adverse effects to bone health, reducing longitudinal bone growth. Animal models with various levels of physical exercise were largely used to provide knowledge to clinical settings. Experiments from our previous studies applied different levels of mechanical loading on rat tibia during puberty accompanied by weekly *in vivo* micro-CT scans. In the present article, we apply 3D image registration-based methods to retrospectively analyze part of the previously acquired micro-CT data. Longitudinal bone growth, growth plate thickness, and cortical bone (re)modeling were evaluated from rats' age of 28 to 77 days. Our results show that impact loading inhibited proximal bone growth throughout puberty. We hypothesize that impact loading might bring different growth alterations to the distal and proximal growth plates. High impact loading might lead to pathological consequence of osteochondrosis and catch-up growth due to growth inhibition. Impact loading also increased cortical bone (re)modeling before and after the peak proximal bone growth period of young rats, of which the latter case might be caused by the shift from modeling to remodeling as the dominant activity towards the end of rat puberty. We confirm that the tibial endosteum is more mechano-sensitive than the periosteum in response to mechanical loading. To our knowledge, this is the first study to follow up bone growth and bone (re)modeling of young rats throughout the entire puberty with a weekly time interval.

5.4 Key words

Bone growth, bone modeling, bone remodeling, micro-CT, image registration

5.5 Introduction

Adolescence is a critical period to build foundations for reaching an optimal peak bone mass at adulthood, which helps to reduce the chances of developing osteoporosis later in life (Weaver, 2002). Regular physical exercise during childhood and puberty are beneficial to musculoskeletal

development (Rosengren et al., 2022; Weeks et al., 2008), with effects remaining in adulthood (Mustafy et al., 2020; Rosengren et al., 2022). However, high intensity exercise at a young age might bring adverse effects to bone health such as insufficient bone growth, as shown in animal (Bourrin et al., 1994; Forwood & Parker, 1987) and clinical (Caine et al., 2001; Theintz et al., 1993) studies, due to abnormal mechanical loadings (Stokes, 2002; Villemure & Stokes, 2009).

In pre-clinical animal models, different loading routines were tested including bending (Turner et al., 1991), axial compression (Gohin et al., 2020; Lynch et al., 2010; Main et al., 2014), running (Huang et al., 2003; Niehoff et al., 2004; Wallace et al., 2007), tower climbing (Notomi et al., 2001), swimming (Forwood & Parker, 1987; Huang et al., 2003; Snyder et al., 1992), and free fall (Cho et al., 2015), which bring different effects to young rodent bones. Both static and dynamic loading can suppress longitudinal and radial growth (Robling et al., 2001), the degree of which is proportional to the load magnitude (Ohashi et al., 2002; Robling et al., 2001). By controlling the frequency, stress and strain of dynamic loading, animal models help to find the “boundary” between beneficial and detrimental exercise routines (Bourrin et al., 1994; Villemure & Stokes, 2009).

Mechanical parameters were usually assessed to evaluate the structural benefits in animal studies (Main et al., 2014; Niehoff et al., 2004; Wallace et al., 2007). Bone geometrical parameters were measured by histology (Huang et al., 2003; Niehoff et al., 2004; Ohashi et al., 2002), pQCT (Wallace et al., 2007), and micro-CT (Cho et al., 2015; Gohin et al., 2020; Main et al., 2014). Bone (re)modeling was traditionally assessed using dynamic histomorphometry (Cheong et al., 2020; Lynch et al., 2010; Mosley & Lanyon, 2002), and longitudinal bone growth was determined by histology. However, most of the measurements acquired in the past studies were either temporal or end-point data. Considering the wide adoption of *in vivo* micro-CT-based methods to evaluate bone (re)modeling (Altman et al., 2015; Birkhold et al., 2016; de Bakker et al., 2015) and longitudinal bone growth (Altman et al., 2015; Lu et al., 2016; Ning et al., 2022; Stern et al., 2015), it seems interesting to revisit the established animal loading models with the novel non-destructive evaluation methods.

Previous studies by our research group presented a tibia loading model on pubertal rats undergoing different strain levels (Mustafy, Londono, Moldovan, et al., 2019; Mustafy et al., 2020). We performed *in vivo* micro-CT scans, post-mortem three-point-bending tests, and growth plate

histomorphometry to evaluate the effect of loading on tibial mechano-adaptation during the rapid bone growth period. Our results showed that impact loading improved bone microstructural qualities and mechanical properties towards the end of puberty, despite leading to reduced longitudinal bone growth (Mustafy, Londono, Moldovan, et al., 2019). The beneficial effect of high impact loading on rats at puberty persisted into adulthood (Mustafy et al., 2020). Nonetheless, we did not evaluate bone (re)modeling parameters around the loading site, which could have elucidated the origins of the skeletal improvements in response to the impact loading. Neither did we perform a complete follow-up of the rat growth curve due to the destructive nature of histology. The bone growth rate and growth plate thickness evaluated at the terminal point of the experiment hardly provide a full picture of the growth inhibition.

The present article complements the data analysis for our previous studies. Image registration-based methods analyzed the existing *in vivo* micro-CT scans in a spatio-temporal manner. The objectives were: (1) to quantify longitudinal bone growth and growth plate thickness of the growing rats; (2) to quantify bone (re)modeling throughout rats' puberty. To our knowledge, this is the first study to follow up bone growth and bone (re)modeling of young rats throughout the entire puberty with a weekly time interval.

5.6 Material and methods

5.6.1 Experimental design

The present study analyzes the *in vivo* micro-CT data of 48 male Sprague-Dawley rats from two previous studies by our research group. Data with severe motion artifacts and incomplete datasets were excluded from analysis as single time points (about 6.6%) (Mustafy, Londono, Moldovan, et al., 2019; Mustafy et al., 2020; Mustafy, Londono, et al., 2018).

In brief, rats arrived from Charles River Laboratories (Montreal, Canada) at the age of 21 days, and received axial cyclic loading on their right tibia (Mustafy, Londono, et al., 2018) starting from the age of 28 days until the age of 77 days (Figure 5.1a). The loading was based on a 2 Hz haversine waveform of 1200 cycles per day, 5 days per week, generating different strains on the tibial medio-proximal surface (Figure 5.1b): 450 $\mu\epsilon$ (the low impact group, $n=12$), 850 $\mu\epsilon$ (the medium impact

group, $n=12$), and $1250 \mu\epsilon$ (the high impact group, $n=12$). The sham group rats ($n=12$) received the same experimental setups (anesthesia, *in vivo* micro-CT scans, cage activities) without any cyclic loading to serve as baseline controls. The loading strains were determined based on the peak strain level of the human tibia while performing walking, running, and jumping exercises (Burr et al., 1996; De Souza et al., 2005; Lanyon et al., 1975; Milgrom et al., 2002; Yang et al., 2011).

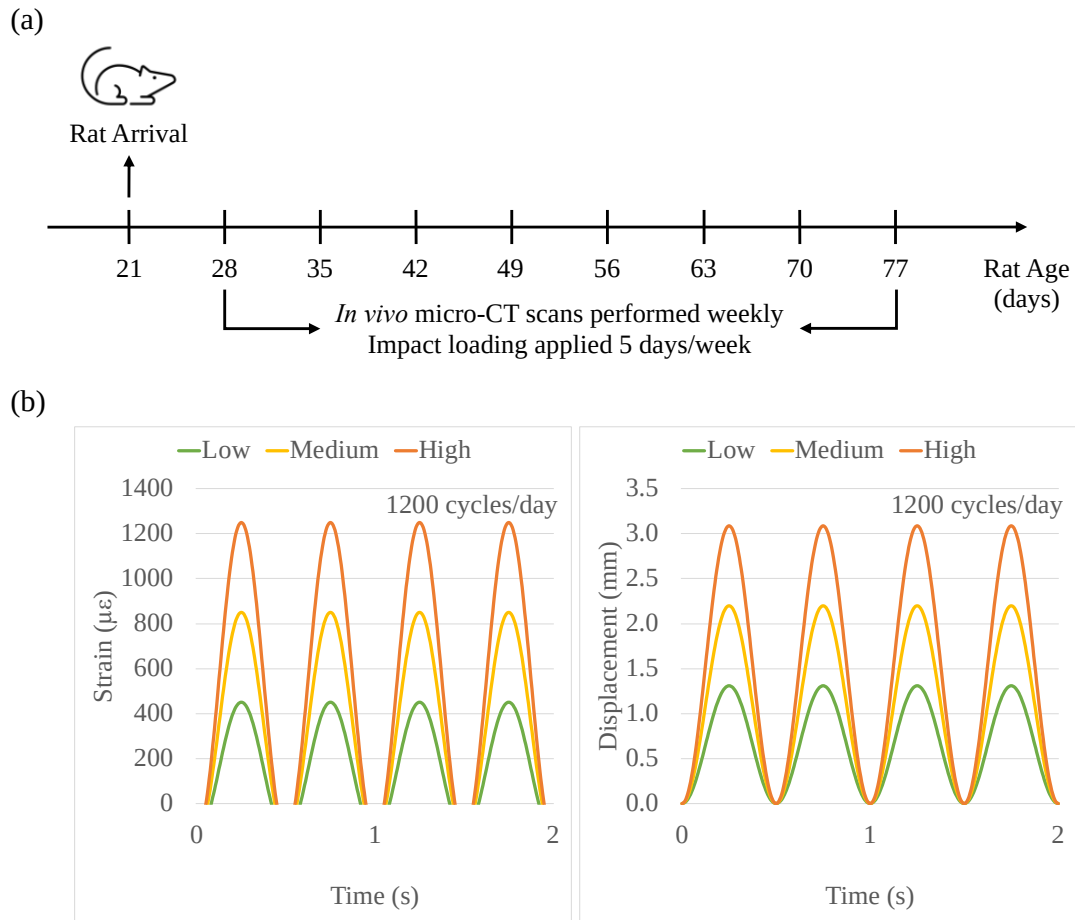


Figure 5.1 Experimental design: (a) treatment timeline and (b) the strain and the displacement curves of the impact loading shown in example of 4 cycles (2 seconds).

The rats also received weekly *in vivo* micro-CT scans using a SkyScan 1176 microtomograph (Bruker-microCT N.V., Kontich, Belgium) on their entire right tibia to capture the time-lapse microstructural changes (Figure 5.1a). The scans were acquired at 65 kVp, 384 μA , 350 ms exposure time, $0.65^\circ/180^\circ$ rotation step, using a 1 mm aluminum filter, with an image resolution

of 18 μm (Mustafy, Benoit, et al., 2018). Post alignment, beam hardening correction and ring artifacts reduction were applied during the reconstruction of the scans using NRecon software (v.1.6.10, Bruker-microCT N.V., Kontich, Belgium). The subsequent analysis of proximal and diaphyseal VOIs were based on the distance information provided by scout views.

The mechanical loading and the *in vivo* micro-CT scans were performed while rats were under anesthesia supplied by isoflurane. The Institutional Committee for Good Animal Practices in Research of the Sainte-Justine University Hospital Center approved the animal experiments related to the data analyzed in the present study. Animal usage followed the Canadian Council on Animal Care (CCAC) guidelines.

5.6.2 Preprocessing and registration of micro-CT scans

DataViewer (Bruker-microCT N.V., Kontich, Belgium) was used to perform 3D rotation to align the tibia along the longitudinal z-axis. CT Analyser (Bruker-microCT N.V., Kontich, Belgium) was employed to extract VOIs (volume of interests) at tibial metaphysis and diaphysis. The proximal metaphysis was chosen to measure the longitudinal growth rate, which can account for about 55% to 80% of total tibial longitudinal growth (Altman et al., 2015; Stern et al., 2015). The VOIs started from the proximal growth plate of each scan, expanding about 8 mm on the proximal metaphysis. To calculate the bone (re)modeling parameters of cortical tibia, the mid-diaphysis, where peak strain level developed from axial compression, was analyzed (Birkhold, 2016; Bouxsein et al., 2010; Patel et al., 2014). The VOIs were centered around the tibial mid-diaphysis, spanning for about 30% of the tibial total length. Once the 2D image sequence was exported from CT Analyser, another image processing software “FIJI” (Schindelin et al., 2012) was used to convert it into a 3D format to perform the subsequent 3D image registration.

A customized intensity-based 3D registration algorithm registered VOIs from consecutive time points of the same rats. Inspired by previous studies (Altman et al., 2015; de Bakker et al., 2015), the registration methodology was implemented using an open platform ITK (Kitware, Clifton Park, NY, USA) (Johnson et al., 2015), with mutual information (Viola & Wells III, 1997) as metric, regular step gradient descent as optimizer, linear interpolation and rigid 3D transform with six degrees of freedom. For the metaphyseal VOIs, the registration was performed in two stages of

different resolutions to calculate proximal bone growth rate. The first stage aligned the cortical bone overall and the second stage maximized trabecular alignment automatically with a manual input between the two stages (Ning et al., 2022). As for the diaphyseal VOIs, only the first stage was applied since they were primarily cortical bone.

5.6.3 Longitudinal bone growth

Using a validated method previously reported by our group (Ning et al., 2022), the longitudinal bone growth rate of proximal tibia was measured by overlapping registered images from consecutive time points of the same rats. The 3D image registration was based on the alignment of trabecular bone (Figure 5.2a). Each registration result gave a growth distance (Figure 5.2b), from which we derived the proximal growth rate (PGR, $\mu\text{m}/\text{day}$) based on a 7-day scanning interval. The proximal growth distance (PGD, mm) was calculated as the sum of the growth distances of all the past time points as a cumulative measure.

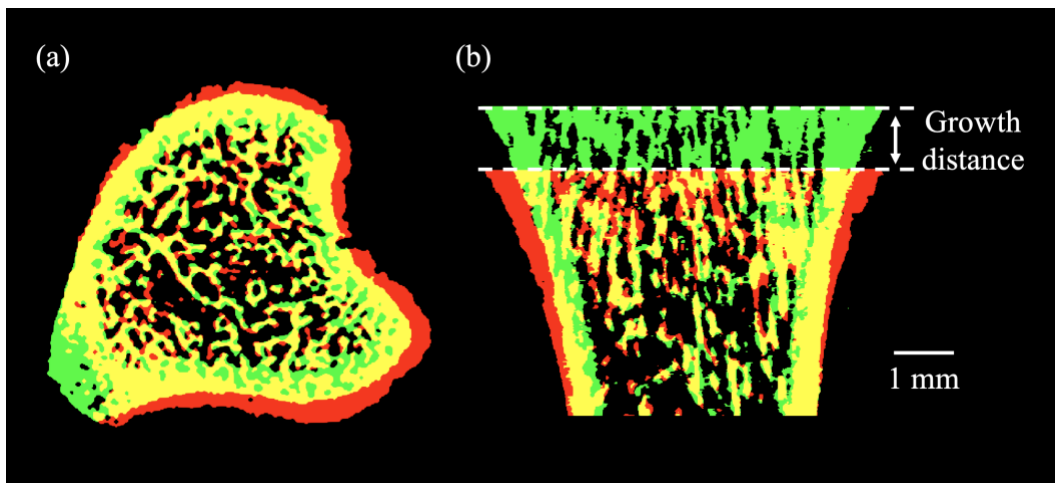


Figure 5.2 A pair of example sample registered on trabecular bone to calculate proximal growth distance: (a) a transversal overlaid view of trabecular alignment, (b) the growth distance is calculated in a longitudinal overlaid view. Bone volumes of the previous time point are in red, volumes of the subsequent time point are in green, overlapping areas are thus in yellow.

As for the distal side, it was more difficult to perform 3D image registration based on some of our preliminary tests. The distal metaphysis has generally smaller cross-sectional area and less dense

trabecular pattern than its proximal counterpart, providing less information for the registration algorithm to work on. In the end, we were not able to measure the distal bone growth rate accurately and consistently for the current dataset. However, the whole bone length was measured to account for the bone growth contributed by the distal growth plate. Total tibial length (TTL, mm) was measured using the 2D scout view provided in the CT Analyser software for each dataset. The total growth distance (TGD, mm) was derived as the difference of TTL between each time point (35 to 77 days old) and the starting point (28 days old), which can be roughly considered as the whole bone counterpart of PGD. The whole bone counterpart of PGR was not available due to the lack of 3D image registration on the distal side. Furthermore, PGD was divided by TGD at 77 days old for each rat to estimate the proportion of proximal growth over total growth (PGRatio).

5.6.4 Growth plate thickness

Since the longitudinal bone growth is contributed by both the distal and proximal growth plates, their thicknesses were measured from the micro-CT images to assess the effect of impact loading on their morphologies. VOIs of about 3 mm (proximal side) and 2 mm (distal side) thick (in the longitudinal direction) were saved using CT Analyser, covering the entire growth plate area. The transversal image sections were then imported into MATLAB R2018b (MathWorks, Natick, MA, USA) for thickness measurement by a customized script based on the methodology developed in a previous study (Shazeeb et al., 2018). The proximal growth plate thickness (PGPTh, μm) was successfully measured for all the data from 28 to 77 days old. As for the distal growth plate thickness (DGPTh, μm), it was more difficult to measure due to its smaller size than the proximal side. Under the current image resolution, we were not able to accurately measure the distal growth plate thicknesses beyond 49 days old, where the standard deviation could surpass the group average thickness itself.

5.6.5 Cortical bone (re)modeling

Figure 5.3 shows an example of the cortical registration in overlaid views. A previously validated MATLAB-based program calculated the bone modeling and remodeling parameters on both

endosteum and periosteum (Birkhold, 2016; Birkhold et al., 2014b, 2016), where a global bone threshold was set to 65/255 (0.413 g/cm³ CaHA). The output parameters were evaluated between all the consecutive time points, including volume ratios: newly mineralized bone volume per bone volume (MV/BV) and newly eroded bone volume per bone volume (EV/BV); surface ratios: newly mineralized bone surface per bone surface (MS/BS) and newly eroded surface per bone surface (ES/BS); and 3D rates: mineral apposition rate (MAR, $\mu\text{m}/\text{day}$), mineral resorption rate (MRR, $\mu\text{m}/\text{day}$), bone formation rate per bone surface (BFR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{day}$), and bone resorption rate per bone surface (BRR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{day}$). For each parameter, two variants were calculated on the periosteum and the endosteum respectively.

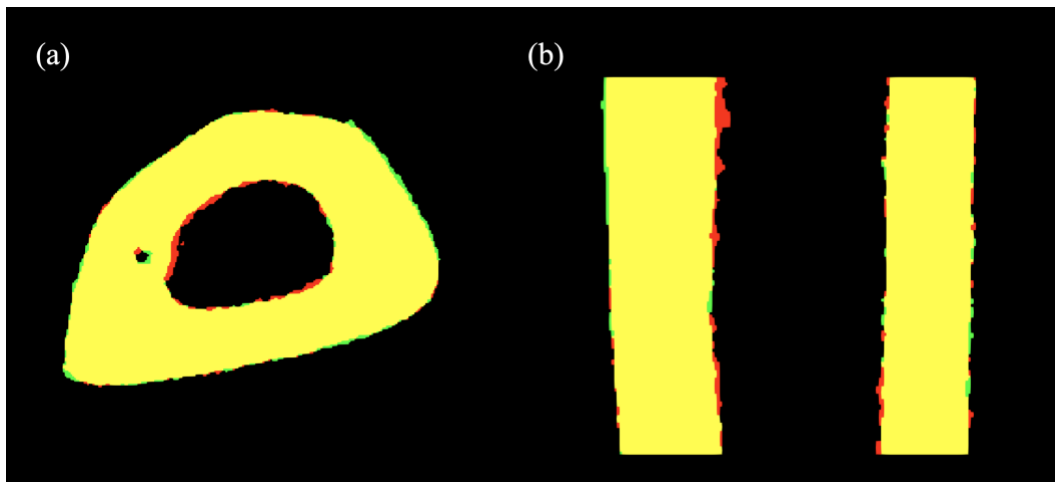


Figure 5.3 A pair of example sample registered on cortical bone to calculate bone (re)modeling parameters: (a) a transversal overlaid view of cortical alignment, (b) a longitudinal overlaid view of cortical alignment. Bone volumes of the previous time point are in red, volumes of the subsequent time point are in green, overlapping areas are thus in yellow.

5.6.6 Statistical tests

SPSS Statistics v26 (IBM Corp, Armonk, NY, USA) was used to perform the statistical tests. For the growth rate, distance, and growth plate thickness, two-way mixed ANOVA was applied to account for the between-subject factor (loading groups: sham, low, medium, and high), and the within-subject factor (time points/periods: from 28 days to 77 days old). For the (re)modeling parameters, three-way mixed ANOVA was applied to account for the two between-subject factors

(loading groups: sham, low, medium, and high; location: endosteum and periosteum), and the within-subject factor (time periods: from 28 days to 77 days old).

Outliers were identified by verifying boxplots, of which none was excluded given the low percentage (less than 2% for the growth-related parameters, and less than 2.5% for the (re)modeling parameters). All the parameters were normally distributed in general as assessed by normal Q-Q plots. Levene's test of homogeneity of variance was applied, showing that data from a few time points violated the homogeneity. The mixed ANOVA tests were carried out anyway because they are robust to heterogeneity of variance if group samples sizes are approximately equal (Altman, 1991; Keppel, 2004; Laerd Statistics, 2015). Since the assumption of sphericity was not always met using Mauchly's test of sphericity, the Greenhouse–Geisser correction was applied for the p-value calculation of the within-subjects' effects. Bonferroni correction was applied for the p-values in the post hoc tests and the multiple comparisons. The significance level (alpha) of all the statistical tests was set to 0.05.

5.7 Results

5.7.1 Single factor effects, two-way interactions, and three-way interactions

As shown in Table 5.1, all the growth-related parameters had significant effects of time and loading groups, as well as the two-way interaction between time and loading groups. As for the (re)modeling parameters (Table 5.2), almost all of them had significant effects of time, loading groups and location. Many presented significant two-way interaction of time * loading and time * location as well. Fewer parameters showed significant two-way interaction of loading * location and three-way interaction of time * loading * location. The follow-up *post hoc* tests and multiple comparisons are presented in the next sections.

Table 5.1 *p*-values of the single factor effects, two-way interactions, and three-way interactions for the growth-related parameters. Only values of statistical significance were shown. “-” refers to no statistical significance ($p \geq 0.05$). Greenhouse–Geisser correction applied for testing the within-subjects’ effects due to violation of sphericity.

Factors	PGR	PGD	TTL	TGD	PGPTh	DGPTh
Time	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Loading	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Time * Loading	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001

Table 5.2 *p*-values of the single factor effects, two-way interactions, and three-way interactions for the remodeling-related parameters. Only values of statistical significance were shown. “-” refers to no statistical significance ($p \geq 0.05$). Greenhouse–Geisser correction applied for testing the within-subjects’ effects due to violation of sphericity.

Factors	MV/BV	EV/BV	MS/BS	ES/BS	MAR	MRR	BFR/BS	BRR/BS
Time	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Loading	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Location	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001	< 0.001	< 0.001
Time * Loading	0.012	0.004	0.045	0.018	< 0.001	< 0.001	-	0.004
Time * Location	0.001	0.021	< 0.001	-	0.005	-	0.001	0.031
Loading * Location	< 0.001	0.005	-	-	-	-	< 0.001	0.006
Time * Loading * Location	-	0.024	-	-	-	-	-	0.019

5.7.2 Longitudinal bone growth

The proximal growth parameters PGR and PGD are shown in Figure 5.4 (a, b). When looking at the PGR of each group, the sham group maintained a peak level around 28 to 49 days old, which was from 28 to 42 days old for the low impact rats, from 35 to 49 days old for the medium impact rats, and from 35 to 63 days old for the high impact rats. The PGR of all the rats declined over time, especially after the age of 35 to 42 days old, which is a common period of all the rats' peak proximal growth. The sham group had significantly higher PGR for most of the time periods, and higher PGD for all the time points. The three impact loading groups did not consistently show different PGR and PGD except for the beginning of the experiment (28 to 42 days old).

The total tibial length and growth parameters TTL and TGD are presented in Figure 5.4 (c, d). The TTL increased over time for all the rats as expected, where the sham rats showed higher length at some of the time points compared to the impact rats. The high impact rats had significantly shorter tibia than the sham rats at all time points except for the 77 days old. When looking at the TGD, the total growth of the high impact rats was the highest among all the rats starting from 49 days old. This explains how the high impact rats were able to reduce their length difference with the sham rats over time as seen in Figure 5.4c. As for the proximal growth ratio PGRatio, the sham rats showed $81\% \pm 8\%$, the low impact rats were $59\% \pm 6\%$, the medium impact rats measured $68\% \pm 6\%$, and the high impact rats were only $51\% \pm 5\%$. The sham and the medium impact rats both showed significant differences compared to all the other groups ($p < 0.05$ after Bonferroni correction).

5.7.3 Growth plate thickness

The growth plate thicknesses of both growth plates are shown in Figure 5.5. The DGpTh (Figure 5.5a) declined through time, with the high impact rats having higher values at 3 time points. The PGpTh (Figure 5.5b) declined through time as well, showing that the high impact group rats were at the highest levels at most of the time points. At 77 days old, there were no between-group difference at all. The proximal growth plates were about two times thick of the distal growth plates at all the comparable time points.

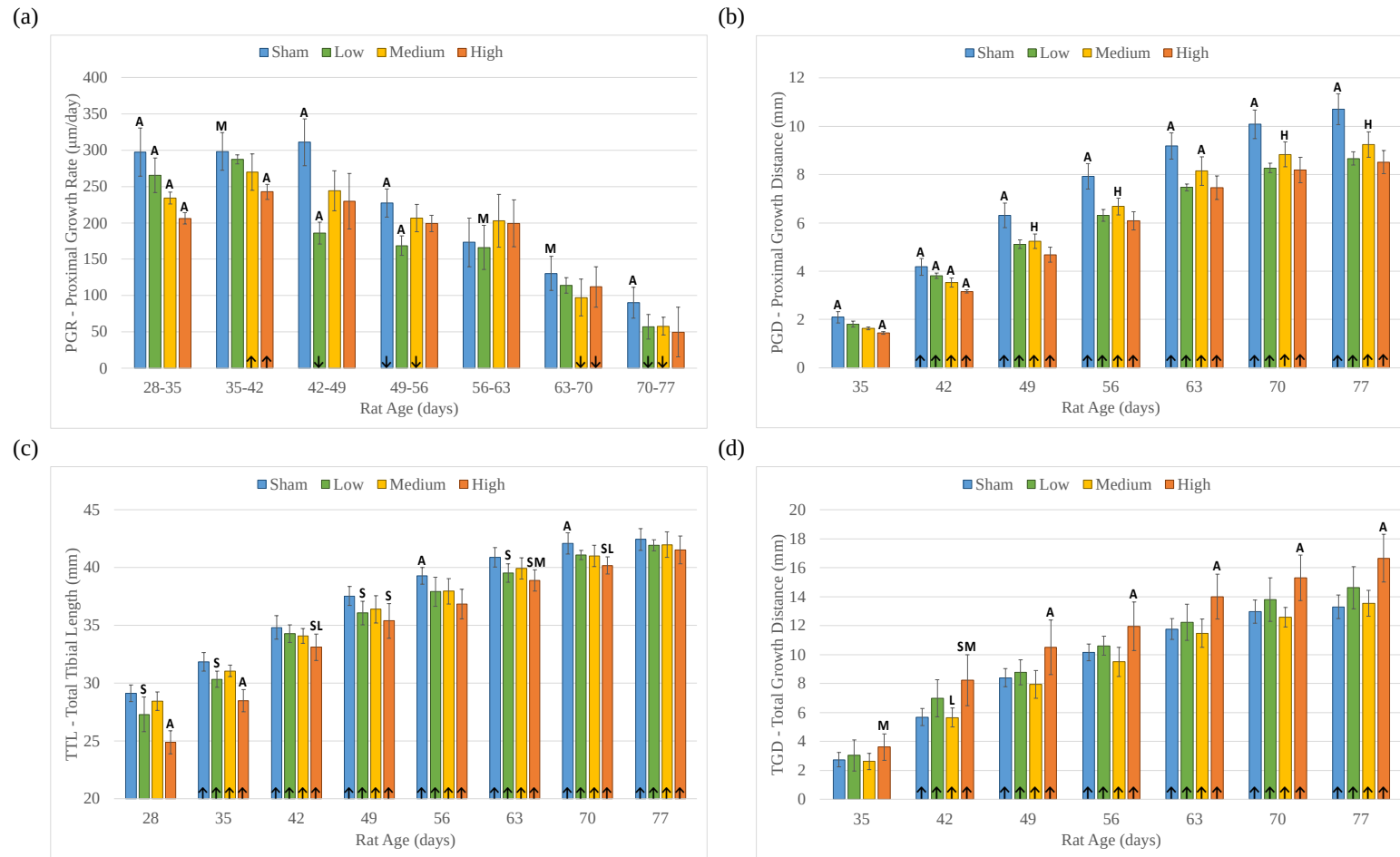


Figure 5.4 Longitudinal growth parameters: (a) PGR, (b) PGD, (c) TTL and (d) TGD. Statistical significance symbols were marked on top of each bar for the effects of loading groups, and at the bottom for the effects of time. “A” / “S” / “L” / “M” / “H”: significant compared to all the other groups / the sham group / the low impact group / the medium impact group / the high impact group, respectively. “↑” / “↓”: significantly increased / decreased value compared to the previous time, respectively. Bonferroni correction was applied for the *p*-values in the *post hoc* tests and the multiple comparisons. The significance level (alpha) was set to 0.05.

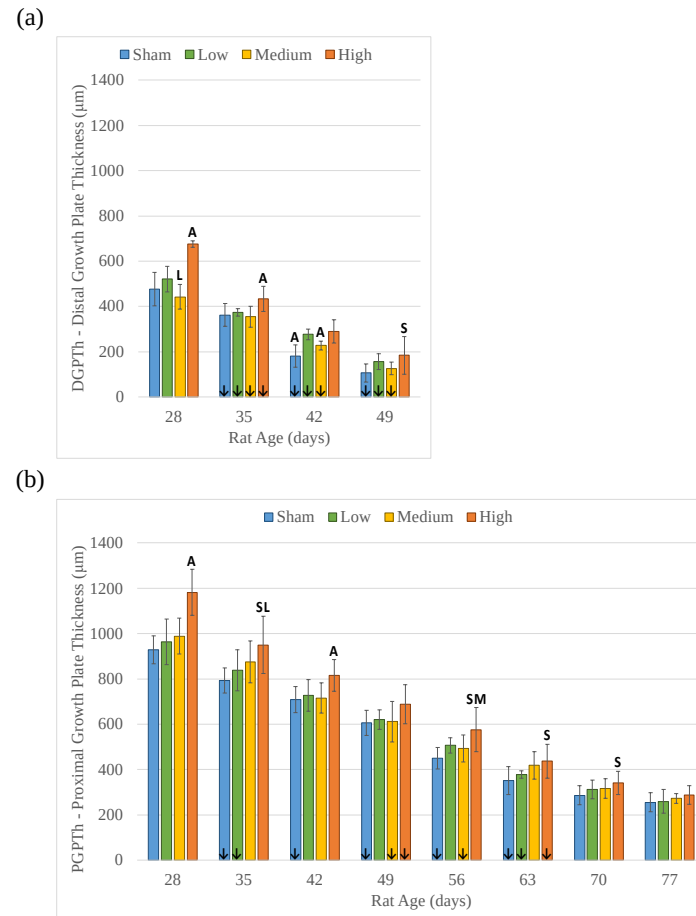


Figure 5.5 Growth plate thickness: (a) DGPTH and (b) PGPTH. Statistical significance symbols were marked on top of each bar for the effects of loading groups, and at the bottom for the effects of time. “A” / “S” / “L” / “M” / “H”: significant compared to all the other groups / the sham group / the low impact group / the medium impact group / the high impact group, respectively. “↑” / “↓”: significantly increased / decreased value compared to the previous time, respectively. Bonferroni correction was applied for the p -values in the *post hoc* tests and the multiple comparisons. The significance level (alpha) was set to 0.05.

5.7.4 Bone (re)modeling on the periosteum

Table 5.3 shows the (re)modeling parameters on the periosteum of mid-diaphysis. The effect of loading groups was analyzed for each time period. The impact loading groups, especially the medium and high impact groups, showed significantly higher bone formation parameters than the sham group at the beginning of the loading protocol (28 to 42 days old). Similar statistical differences were shown towards the end of puberty as well (70 to 77 days old).

For the effect of time, the formation parameters declined in general, and the resorption parameters stayed at a relatively lower level, though not always statistically significant when comparing the consecutive time points (Table 5.3). Thus, our analysis focused on comparing the effect of every 14 days: from 28-35 to 42-49 days, from 42-49 to 56-63 days, and from 56-63 to 70-77 days, where the statistical significance symbols were marked in the lines of 42-49, 56-63 and 70-77 respectively (Table 5.3). The high impact group showed reduced MV/BV and MS/BS at 42-49 days comparing to 28-35 days. Apart from these, very few significant effects of time were shown on the periosteum.

5.7.5 Bone (re)modeling on the endosteum

Table 5.4 shows the (re)modeling parameters on the endosteum of mid-diaphysis. The high impact group showed higher values of most resorptive parameters than the other groups at the beginning of the experiment (28 to 35 days old). The medium and high impact groups had higher values of most formation and resorption parameters compared to the sham group towards the end of puberty (63 to 77 days old).

The effect of time was analyzed using the same methodology as presented in the previous section for the periosteal parameters. From the beginning till 49 days old, the high impact group showed significant decrease in MV/BV, EV/BV, ES/BS and BRR/BS. Towards the end of the experiment, both the medium and the high impact groups showed significantly increased values for most parameters.

Table 5.3 Bone (re)modeling parameters on the periosteum of mid-diaphysis of rat tibia. The data was presented as “average $\pm$ standard deviation”. For the effect of loading groups, the statistically significant data points were in bold. “S” / “L” / “M”: significant compared to the sham group / the low impact group / the medium impact group, respectively. For the effect of time periods, up and down arrows were marked in the lines of 42-49, 56-63, and 70-77 respectively. “ $\uparrow$ ” / “ $\downarrow$ ”: significantly increased / decreased value compared to the previous 14 days, respectively. Bonferroni correction was applied for the p -values in the *post hoc* tests and the multiple comparisons. The significance level (alpha) was set to 0.05.

VOI: Periosteum									
Age (days)	Group	MV/BV ($\mu\text{m}^3/\mu\text{m}^3$)	EV/BV ($\mu\text{m}^3/\mu\text{m}^3$)	MS/BS ($\mu\text{m}^2/\mu\text{m}^2$)	ES/BS ($\mu\text{m}^2/\mu\text{m}^2$)	MAR ($\mu\text{m}/\text{day}$)	MRR ($\mu\text{m}/\text{day}$)	BFR/BS ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	BRR/BS ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)
28-35	Sham	7.08% $\pm$ 0.74%	0.06% $\pm$ 0.11%	88.96% $\pm$ 7.09%	0.71% $\pm$ 1.38%	7.07 $\pm$ 0.51	1.41 $\pm$ 1.52	2.78 $\pm$ 0.32	0.02 $\pm$ 0.04
	Low	7.21% $\pm$ 1.43%	0.08% $\pm$ 0.12%	84.00% $\pm$ 13.77%	0.88% $\pm$ 1.36%	6.37 $\pm$ 1.05	2.75 $\pm$ 1.93	2.72 $\pm$ 0.49	0.03 $\pm$ 0.05
	Medium	6.00% $\pm$ 1.56%	0.21% $\pm$ 0.30%	74.55% $\pm$ 16.41% S	2.74% $\pm$ 3.89%	5.69 $\pm$ 1.66 S	2.29 $\pm$ 1.70	2.33 $\pm$ 0.57	0.08 $\pm$ 0.12
	High	8.96% $\pm$ 0.86% SLM	0.09% $\pm$ 0.09%	92.41% $\pm$ 5.76% M	0.90% $\pm$ 0.94%	7.32 $\pm$ 0.83 M	2.89 $\pm$ 2.28	2.93 $\pm$ 0.29 M	0.03 $\pm$ 0.03
35-42	Sham	4.67% $\pm$ 1.09%	0.32% $\pm$ 0.47%	70.69% $\pm$ 15.17%	4.86% $\pm$ 7.07%	5.74 $\pm$ 0.87	3.16 $\pm$ 1.64	2.23 $\pm$ 0.51	0.15 $\pm$ 0.23
	Low	6.06% $\pm$ 0.77% S	0.25% $\pm$ 0.47%	80.56% $\pm$ 11.26%	3.41% $\pm$ 6.68%	7.12 $\pm$ 0.52 S	2.87 $\pm$ 1.65	2.65 $\pm$ 0.40	0.11 $\pm$ 0.20
	Medium	6.41% $\pm$ 1.00% S	0.11% $\pm$ 0.26%	86.48% $\pm$ 12.14%	1.46% $\pm$ 3.29%	7.29 $\pm$ 0.75 S	2.23 $\pm$ 1.19	2.94 $\pm$ 0.47 S	0.05 $\pm$ 0.11
	High	7.07% $\pm$ 0.98% S	0.09% $\pm$ 0.14%	90.51% $\pm$ 8.03% S	1.21% $\pm$ 1.84%	7.65 $\pm$ 0.53 S	2.79 $\pm$ 1.64	2.90 $\pm$ 0.30 S	0.04 $\pm$ 0.06
42-49	Sham	3.11% $\pm$ 0.94%	0.41% $\pm$ 0.48%	53.88% $\pm$ 15.11% $\downarrow$	7.14% $\pm$ 8.36%	5.42 $\pm$ 1.19	3.25 $\pm$ 1.26	1.72 $\pm$ 0.54	0.22 $\pm$ 0.25
	Low	3.20% $\pm$ 1.12%	0.61% $\pm$ 0.53%	53.84% $\pm$ 17.12% $\downarrow$	10.73% $\pm$ 10.00%	5.70 $\pm$ 1.60	3.46 $\pm$ 1.63	1.65 $\pm$ 0.54	0.31 $\pm$ 0.27
	Medium	4.23% $\pm$ 0.93%	0.24% $\pm$ 0.45%	71.40% $\pm$ 15.03% L	4.17% $\pm$ 8.39%	5.92 $\pm$ 0.69	2.82 $\pm$ 0.80	2.33 $\pm$ 0.54 SL	0.13 $\pm$ 0.24
	High	3.94% $\pm$ 1.04% $\downarrow$	0.24% $\pm$ 0.32%	64.74% $\pm$ 15.48% $\downarrow$	3.94% $\pm$ 5.34%	5.30 $\pm$ 1.51	2.66 $\pm$ 0.27	2.06 $\pm$ 0.52	0.13 $\pm$ 0.17
49-56	Sham	3.24% $\pm$ 0.79%	0.07% $\pm$ 0.08%	59.40% $\pm$ 13.76%	1.24% $\pm$ 1.41%	5.26 $\pm$ 1.11	2.35 $\pm$ 0.75	1.92 $\pm$ 0.46	0.04 $\pm$ 0.05
	Low	4.38% $\pm$ 1.61%	0.43% $\pm$ 0.47%	70.25% $\pm$ 20.37%	7.62% $\pm$ 9.01%	6.92 $\pm$ 0.81 S	3.22 $\pm$ 0.88	2.46 $\pm$ 0.80	0.26 $\pm$ 0.30
	Medium	3.16% $\pm$ 0.94%	0.29% $\pm$ 0.33%	59.28% $\pm$ 16.36%	5.29% $\pm$ 5.84%	5.09 $\pm$ 1.44 L	3.26 $\pm$ 1.24	1.93 $\pm$ 0.60	0.17 $\pm$ 0.19
	High	4.56% $\pm$ 1.17%	0.32% $\pm$ 0.75%	77.70% $\pm$ 18.35%	5.57% $\pm$ 12.75%	6.85 $\pm$ 0.88 SM	2.73 $\pm$ 1.56	2.60 $\pm$ 0.66	0.18 $\pm$ 0.41
56-63	Sham	2.32% $\pm$ 0.53%	0.34% $\pm$ 0.37%	47.15% $\pm$ 10.81%	6.93% $\pm$ 7.54%	4.06 $\pm$ 0.77	3.11 $\pm$ 0.86	1.49 $\pm$ 0.36	0.21 $\pm$ 0.23
	Low	2.17% $\pm$ 0.74%	0.61% $\pm$ 0.62%	43.31% $\pm$ 14.22%	12.38% $\pm$ 12.66%	4.82 $\pm$ 1.16	3.30 $\pm$ 1.14	1.38 $\pm$ 0.45	0.39 $\pm$ 0.40
	Medium	3.02% $\pm$ 0.66%	0.27% $\pm$ 0.46%	62.54% $\pm$ 11.96% L	6.15% $\pm$ 10.97%	4.96 $\pm$ 1.01	3.22 $\pm$ 1.42	1.99 $\pm$ 0.43 L	0.19 $\pm$ 0.33
	High	2.71% $\pm$ 1.48%	0.76% $\pm$ 0.79%	52.31% $\pm$ 22.50%	16.24% $\pm$ 17.11%	5.28 $\pm$ 1.70	3.88 $\pm$ 1.67	1.66 $\pm$ 0.80	0.49 $\pm$ 0.51
63-70	Sham	1.99% $\pm$ 0.87%	0.38% $\pm$ 0.25%	41.57% $\pm$ 16.84%	7.97% $\pm$ 5.36%	4.32 $\pm$ 1.58	3.06 $\pm$ 0.71	1.31 $\pm$ 0.58	0.25 $\pm$ 0.17
	Low	2.83% $\pm$ 1.18%	1.22% $\pm$ 0.67%	54.96% $\pm$ 20.90%	24.38% $\pm$ 13.69%	6.82 $\pm$ 1.21 S	6.73 $\pm$ 1.02 S	1.82 $\pm$ 0.83	0.77 $\pm$ 0.42
	Medium	1.45% $\pm$ 0.83% L	2.21% $\pm$ 1.02% SL	30.92% $\pm$ 16.75%	49.07% $\pm$ 24.22% SL	6.89 $\pm$ 0.66 S	6.72 $\pm$ 1.33 S	0.99 $\pm$ 0.56 L	1.52 $\pm$ 0.71 SL
	High	1.96% $\pm$ 1.20%	1.92% $\pm$ 1.09% S	41.01% $\pm$ 24.07%	40.87% $\pm$ 23.67% S	6.54 $\pm$ 1.24 S	6.52 $\pm$ 1.12 S	1.31 $\pm$ 0.79	1.27 $\pm$ 0.72 S
70-77	Sham	1.22% $\pm$ 0.49%	0.51% $\pm$ 0.37%	26.73% $\pm$ 10.95%	11.35% $\pm$ 8.34%	3.44 $\pm$ 1.05	3.33 $\pm$ 0.95	0.83 $\pm$ 0.35	0.35 $\pm$ 0.25
	Low	1.96% $\pm$ 0.69%	1.65% $\pm$ 0.64% S	42.14% $\pm$ 14.49%	36.38% $\pm$ 15.87% S	6.96 $\pm$ 0.68 S	6.16 $\pm$ 1.78 S	1.31 $\pm$ 0.46	1.12 $\pm$ 0.45 S
	Medium	3.28% $\pm$ 1.38% SL	0.94% $\pm$ 0.70%	66.30% $\pm$ 22.97% SL	20.58% $\pm$ 15.94%	7.79 $\pm$ 0.71 S	6.31 $\pm$ 1.33 S	2.19 $\pm$ 0.97 SL	0.61 $\pm$ 0.43 L
	High	3.03% $\pm$ 1.49% S	1.07% $\pm$ 0.80%	61.78% $\pm$ 26.11% S	23.26% $\pm$ 17.59%	7.43 $\pm$ 1.06 S	6.72 $\pm$ 1.49 S	1.99 $\pm$ 0.97 S	0.71 $\pm$ 0.53 L

Table 5.4 Bone (re)modeling parameters on the endosteum of mid-diaphysis of rat tibia. The data was presented as “average $\pm$ standard deviation”. For the effect of loading groups, the statistically significant data points were in bold. “S” / “L” / “M”: significant compared to the sham group / the low impact group / the medium impact group, respectively. For the effect of time periods, up and down arrows were marked in the lines of 42-49, 56-63, and 70-77 respectively. “ $\uparrow$ ” / “ $\downarrow$ ”: significantly increased / decreased value compared to the previous 14 days, respectively. Bonferroni correction was applied for the *p*-values in the *post hoc* tests and the multiple comparisons. The significance level (alpha) was set to 0.05.

VOI: Endosteum									
Age (days)	Group	MV/BV ($\mu\text{m}^3/\mu\text{m}^3$)	EV/BV ($\mu\text{m}^3/\mu\text{m}^3$)	MS/BS ($\mu\text{m}^2/\mu\text{m}^2$)	ES/BS ($\mu\text{m}^2/\mu\text{m}^2$)	MAR ($\mu\text{m}/\text{day}$)	MRR ($\mu\text{m}/\text{day}$)	BFR/BS ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	BRR/BS ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)
28-35	Sham	7.92% $\pm$ 1.52%	0.68% $\pm$ 0.31%	49.40% $\pm$ 7.61%	8.42% $\pm$ 2.95%	4.31 $\pm$ 0.72	3.04 $\pm$ 0.24	3.09 $\pm$ 0.58	0.26 $\pm$ 0.11
	Low	8.60% $\pm$ 5.57%	1.14% $\pm$ 1.54%	44.17% $\pm$ 16.80%	10.14% $\pm$ 7.39%	4.49 $\pm$ 1.81	3.27 $\pm$ 1.63	3.30 $\pm$ 2.34	0.46 $\pm$ 0.67
	Medium	7.84% $\pm$ 4.00%	1.66% $\pm$ 1.28%	45.09% $\pm$ 21.30%	17.98% $\pm$ 14.23%	5.32 $\pm$ 2.10	3.73 $\pm$ 0.96	3.09 $\pm$ 1.60	0.66 $\pm$ 0.52
	High	13.75% $\pm$ 7.92%	5.23% $\pm$ 5.47% SLM	31.91% $\pm$ 21.69%	32.07% $\pm$ 28.87% SL	5.39 $\pm$ 1.47	4.80 $\pm$ 2.17 S	4.45 $\pm$ 2.38	1.80 $\pm$ 1.92 SL
35-42	Sham	7.09% $\pm$ 2.92%	0.47% $\pm$ 0.48%	67.81% $\pm$ 16.71%	7.04% $\pm$ 6.51%	5.20 $\pm$ 1.21	3.55 $\pm$ 1.59	3.39 $\pm$ 1.36	0.22 $\pm$ 0.21
	Low	13.50% $\pm$ 6.68% S	0.80% $\pm$ 1.04%	65.00% $\pm$ 17.27%	8.45% $\pm$ 7.84%	6.57 $\pm$ 1.85	3.54 $\pm$ 0.84	5.77 $\pm$ 2.69	0.33 $\pm$ 0.40
	Medium	10.63% $\pm$ 4.72%	1.60% $\pm$ 1.72%	56.28% $\pm$ 27.23%	17.74% $\pm$ 17.66%	6.31 $\pm$ 2.15	4.44 $\pm$ 1.34	4.86 $\pm$ 2.13	0.76 $\pm$ 0.82
	High	15.17% $\pm$ 6.17% S	0.75% $\pm$ 0.84%	65.22% $\pm$ 10.52%	8.11% $\pm$ 5.51%	6.29 $\pm$ 2.23	3.53 $\pm$ 1.31	6.13 $\pm$ 2.43	0.30 $\pm$ 0.33
42-49	Sham	3.13% $\pm$ 1.05%	1.08% $\pm$ 0.95%	45.72% $\pm$ 13.42%	14.70% $\pm$ 8.62%	3.74 $\pm$ 0.58	4.21 $\pm$ 1.75	1.76 $\pm$ 0.63	0.60 $\pm$ 0.50
	Low	5.12% $\pm$ 4.51%	1.18% $\pm$ 1.77%	46.75% $\pm$ 22.69%	16.48% $\pm$ 17.00%	5.01 $\pm$ 2.29	3.64 $\pm$ 1.30	2.71 $\pm$ 2.34	0.66 $\pm$ 1.03
	Medium	5.65% $\pm$ 2.91%	0.94% $\pm$ 2.17%	55.54% $\pm$ 20.15%	9.71% $\pm$ 12.94%	4.93 $\pm$ 1.04	3.59 $\pm$ 1.84	3.07 $\pm$ 1.52	0.51 $\pm$ 1.18
	High	3.95% $\pm$ 2.66% $\downarrow$	0.81% $\pm$ 1.04% $\downarrow$	40.02% $\pm$ 16.66%	12.60% $\pm$ 12.77% $\downarrow$	4.18 $\pm$ 1.67	3.13 $\pm$ 0.58	2.10 $\pm$ 1.45	0.42 $\pm$ 0.54 $\downarrow$
49-56	Sham	4.10% $\pm$ 2.05%	0.32% $\pm$ 0.20%	56.28% $\pm$ 14.77%	6.42% $\pm$ 2.90%	4.25 $\pm$ 1.00	3.24 $\pm$ 0.63	2.42 $\pm$ 1.16	0.19 $\pm$ 0.11
	Low	9.39% $\pm$ 2.48% S	1.21% $\pm$ 0.77%	61.79% $\pm$ 9.00%	14.69% $\pm$ 8.67%	7.23 $\pm$ 1.41 S	5.21 $\pm$ 1.65 S	5.31 $\pm$ 1.27	0.70 $\pm$ 0.47
	Medium	4.10% $\pm$ 5.45% L	0.52% $\pm$ 0.58%	44.94% $\pm$ 19.62%	8.59% $\pm$ 8.46%	4.19 $\pm$ 2.50 L	3.87 $\pm$ 1.47	2.51 $\pm$ 3.40	0.31 $\pm$ 0.32
	High	7.13% $\pm$ 5.43%	1.13% $\pm$ 1.32%	57.23% $\pm$ 16.01%	12.65% $\pm$ 12.13%	5.89 $\pm$ 2.74	4.18 $\pm$ 1.23	4.10 $\pm$ 3.15	0.64 $\pm$ 0.75
56-63	Sham	2.08% $\pm$ 1.11%	0.41% $\pm$ 0.45%	43.78% $\pm$ 11.47%	8.02% $\pm$ 5.24%	3.32 $\pm$ 0.53	3.16 $\pm$ 0.91	1.32 $\pm$ 0.67	0.26 $\pm$ 0.28
	Low	2.48% $\pm$ 2.22%	2.43% $\pm$ 2.01%	36.99% $\pm$ 23.40%	25.34% $\pm$ 18.30%	4.58 $\pm$ 2.19	5.24 $\pm$ 1.35 S $\uparrow$	1.60 $\pm$ 1.47	1.50 $\pm$ 1.21
	Medium	3.18% $\pm$ 1.69% $\downarrow$	1.76% $\pm$ 3.00%	44.75% $\pm$ 24.37%	20.38% $\pm$ 26.35%	4.20 $\pm$ 0.92	4.71 $\pm$ 2.47	1.99 $\pm$ 0.96	1.09 $\pm$ 1.75
	High	2.68% $\pm$ 2.26%	1.80% $\pm$ 2.48%	41.36% $\pm$ 22.20%	19.33% $\pm$ 17.74%	4.28 $\pm$ 1.68	4.71 $\pm$ 2.84	1.69 $\pm$ 1.38	1.20 $\pm$ 1.71
63-70	Sham	1.71% $\pm$ 1.50%	0.94% $\pm$ 0.67%	30.53% $\pm$ 14.81%	17.55% $\pm$ 10.39%	3.44 $\pm$ 0.90	3.75 $\pm$ 0.93	1.07 $\pm$ 0.86	0.62 $\pm$ 0.45
	Low	8.60% $\pm$ 6.52%	4.61% $\pm$ 4.62%	36.66% $\pm$ 32.54%	42.77% $\pm$ 35.42%	9.36 $\pm$ 4.79	7.41 $\pm$ 5.03	5.71 $\pm$ 4.37	3.07 $\pm$ 3.14
	Medium	7.28% $\pm$ 7.17%	4.83% $\pm$ 3.01%	50.74% $\pm$ 35.26%	26.73% $\pm$ 22.01%	9.75 $\pm$ 6.93	7.25 $\pm$ 1.98	4.85 $\pm$ 4.69	3.32 $\pm$ 2.04
	High	8.20% $\pm$ 6.07%	6.44% $\pm$ 5.39% S	49.46% $\pm$ 39.17%	39.73% $\pm$ 37.92%	9.84 $\pm$ 6.23	8.98 $\pm$ 4.50 S	5.33 $\pm$ 3.91	4.31 $\pm$ 3.52 S
70-77	Sham	1.38% $\pm$ 1.11%	0.77% $\pm$ 0.74%	27.02% $\pm$ 13.24%	16.03% $\pm$ 13.18%	3.43 $\pm$ 0.88	3.27 $\pm$ 0.76	0.94 $\pm$ 0.76	0.52 $\pm$ 0.51
	Low	5.34% $\pm$ 3.55%	5.78% $\pm$ 5.71%	42.44% $\pm$ 29.90%	36.86% $\pm$ 32.39%	7.81 $\pm$ 3.36 S	7.86 $\pm$ 4.20	3.55 $\pm$ 2.33	3.89 $\pm$ 3.88
	Medium	10.60% $\pm$ 7.19% S $\uparrow$	7.22% $\pm$ 6.02% S $\uparrow$	27.41% $\pm$ 18.59%	54.11% $\pm$ 26.19% S $\uparrow$	9.98 $\pm$ 3.10 S $\uparrow$	10.19 $\pm$ 5.37 S $\uparrow$	7.07 $\pm$ 4.86 S $\uparrow$	4.84 $\pm$ 4.03 S $\uparrow$
	High	10.53% $\pm$ 9.17% S $\uparrow$	7.03% $\pm$ 5.25% S $\uparrow$	36.50% $\pm$ 32.33%	49.48% $\pm$ 36.35% S	9.69 $\pm$ 5.08 S	10.95 $\pm$ 5.60 S $\uparrow$	6.82 $\pm$ 6.02 S $\uparrow$	4.69 $\pm$ 3.62 S $\uparrow$

5.7.6 Bone (re)modeling comparison between the periosteum and the endosteum

Table 5.5 shows the p -values of the multiple comparisons performed between the results of each (re)modeling parameter on the periosteum and the endosteum of each loading group respectively. MS/BS and MAR had significantly higher values on the periosteum for all the time points except for 63 to 77 days old, while endosteum had higher values for the significant comparisons of all the other parameters (except for ES/BS at 63 to 70 days old). The impact groups, especially the medium and high impact groups showed more differences between endosteum and periosteum, which is most obvious towards the beginning (28 to 42 days old) and the end (63 to 77 days old) of the experiment.

Table 5.5 p -value of multiple comparisons between the results of each (re)modeling parameter on periosteum and endosteum with Bonferroni correction applied. The significance level (alpha) was set to 0.05. Only the cases of statistical significance were shown. The **bold underlined** numbers indicate higher values on the endosteum, otherwise higher values on the periosteum. “-” refers to no statistical significance.

Age (days)	Group	MV/BV	EV/BV	MS/BS	ES/BS	MAR	MRR	BFR/BS	BRR/BS
28-35	Sham	-	-	<0.001	-	<0.001	<u>0.017</u>	-	-
	Low	-	-	<0.001	-	0.002	-	-	-
	Medium	-	-	<0.001	<u>0.002</u>	-	-	-	-
	High	<u>0.008</u>	<u><0.001</u>	<0.001	<u><0.001</u>	0.005	<u>0.015</u>	<u>0.019</u>	<u><0.001</u>
35-42	Sham	-	-	-	-	-	-	-	-
	Low	<u><0.001</u>	-	0.045	-	-	-	<u><0.001</u>	-
	Medium	<u>0.013</u>	<u><0.001</u>	<0.001	<u>0.001</u>	-	<u><0.001</u>	<u>0.004</u>	<u>0.018</u>
	High	<u><0.001</u>	-	<0.001	<u>0.023</u>	0.047	-	<u><0.001</u>	<u>0.031</u>
42-49	Sham	-	-	-	<u>0.034</u>	0.006	-	-	-
	Low	<u>0.010</u>	-	-	<u>0.021</u>	-	-	<u>0.023</u>	<u>0.037</u>
	Medium	-	-	0.029	-	-	-	-	-
	High	-	-	0.001	-	-	-	-	-
49-56	Sham	-	-	-	-	-	-	-	-
	Low	<u><0.001</u>	<u>0.018</u>	-	-	-	<u>0.005</u>	<u>0.010</u>	<u>0.001</u>
	Medium	-	-	-	-	-	-	-	-
	High	<u>0.031</u>	-	-	-	-	<u>0.045</u>	<u>0.024</u>	-
56-63	Sham	-	-	-	-	-	-	-	-
	Low	-	<u>0.039</u>	-	-	-	<u>0.014</u>	-	-
	Medium	-	<u>0.021</u>	0.016	<u>0.023</u>	-	<u>0.037</u>	-	<u>0.024</u>
	High	-	-	-	-	-	-	-	-
63-70	Sham	-	-	-	-	-	-	-	-
	Low	<u>0.006</u>	<u>0.011</u>	-	-	-	-	<u>0.005</u>	<u>0.010</u>
	Medium	<u>0.001</u>	<u>0.022</u>	0.038	0.027	-	-	<u>0.001</u>	<u>0.018</u>
	High	<u><0.001</u>	<u><0.001</u>	-	-	<u>0.048</u>	<u>0.019</u>	<u>0.001</u>	<u><0.001</u>
70-77	Sham	-	-	-	-	-	-	-	-
	Low	-	<u>0.031</u>	-	-	-	-	-	<u>0.035</u>
	Medium	<u><0.001</u>	<u><0.001</u>	<0.001	<u><0.001</u>	<u>0.047</u>	<u>0.006</u>	<u><0.001</u>	<u><0.001</u>
	High	<u><0.001</u>	<u><0.001</u>	0.014	<u>0.006</u>	<u>0.029</u>	<u>0.004</u>	<u><0.001</u>	<u><0.001</u>

5.8 Discussion

5.8.1 Impact loading altered longitudinal bone growth throughout rat puberty

Our results showed that the proximal growth rate PGR slightly increased around 28 to 42 days old, then decreased afterwards for most rats (Figure 5.4a). This was expected as rats usually reach peak proximal growth around the age of 30 days (Álvarez et al., 2000; Hansson et al., 1972). Despite the same general tendency for all the rats, the impact loading groups showed significantly lower PGR than the sham group for most of the time periods. The PGR at the first week of the experiment was the most different among the rat groups, showing the highest average for the sham group, then the low, medium, and high impact groups as the loading intensity increases. This distinguishable pattern diminished over time, with no significant difference on PGR among the three impact loading groups towards the end of the experiment. The results confirm the cause-and-effect relationship between impact loading and growth reduction (Bourrin et al., 1994; Robling et al., 2001) for growing rats. The inhibition of proximal bone growth seems more obvious for PGD than PGR, with the sham group having the highest level of PGD and the low and the high impact groups having the lowest levels, showing that even low impact loading can cause growth inhibition.

However, when looking at the duration of the peak proximal growth period, the high impact rats was the longest (section 5.7.2). The inhibitive effects of impact loading were also less pronounced when looking at total tibial length TTL and total growth distance TGD (Figure 5.4c, d). The sham rats had the longest TTL at several time points, but their difference with the high impact rats decreased over time. At 77 days old, when the high impact rats might have achieved some “catch-up growth”, there was no significant difference on TTL between groups. The TGD shows that the high impact rats had the longest total growth at most of the time points, whereas the other rats showed very few between-group differences. How did the high impact rats grow longer when their proximal growth was restrained? We might find an answer in the results of the proximal growth ratio: the total proximal growth of the high impact rats only accounted for about half of their total growth, and this proportion ranged from about 60% to 80% for the other groups. These distinct ratios of proximal tibial growth are in line with rodent data documented in the literature (Altman et al., 2015; Stern et al., 2015), with values ranging from about 55% to over 80%. In humans, the ratio can also vary from 50% to more than 90% depending on the age and the type of long bone

(Pritchett, 1991, 1992). In our study, this discrepancy was instead caused by different levels of impact loading. All the loading levels led to reduced proximal growth, and the high impact loading resulted in increased total growth.

We hypothesize that the impact loading had different effects on proximal and distal growth plates due to their different cross-sectional areas and volumes (Breur et al., 1991) and more curved structure at the proximal metaphysis than at the distal metaphysis (Lanyon, 1980). As such, the two growth plates might have undergone different strain levels despite being loaded with the same compressive forces, resulting in different alterations on longitudinal growth. Future studies can verify this hypothesis by using histological analysis of the growth plates and finite element simulation of compressive loadings on rat tibia (Mustafy, Londono, & Villemure, 2019).

5.8.2 High impact loading led to thicker tibial growth plates

The results in Figure 5.5 showed that the thickness of the growth plates declined over time due to growth plate senescence, as reported in the literature (Nilsson & Baron, 2004; Villemure & Stokes, 2009). Besides, the high impact loading increased the thickness of both distal and proximal growth plates. At the age of 77 days old, there was no significant difference between groups. However, our previous histological analysis on the same rat cohort showed different statistical results with similar average values (Mustafy, Londono, Moldovan, et al., 2019). This difference might be caused by the larger standard deviations associated with the micro-CT-based measurements compared with the histology-based measurements. Nonetheless, both analyses showed thicker growth plates of the high impact rats compared to the sham rats at most time points. Some past studies showed thinner growth plates in response to mechanical loading (Cancel et al., 2009; Ménard et al., 2014; Valteau et al., 2011), and some others showed thicker measurements (Ohashi et al., 2002; Robling et al., 2001; Swissa-Sivan et al., 1989). The latter case was sometimes interpreted as a pathological consequence of osteochondrosis or dyschondroplasia (Duff, 1986a, 1986b; Robling et al., 2001). Osteochondrosis includes several types of growth-related disorders, and it is especially common among athletes due to excessive physical exercise (Orava & Virtanen, 1982). It seems only the high impact rats in our study might have developed osteochondrosis (Mustafy, Londono, Moldovan, et al., 2019).

Another explanation for the thicker growth plates of the high impact rats is “catch-up growth”, which was mentioned in section 5.8.1 for the results of TTL and TGD. Catch-up growth is a mechanism of faster-than-normal growth rates in terms of bone length and organ mass after a period of growth inhibition (Finkelstein et al., 2013). It has been associated with various inhibitive factors such as diet control (Farnum et al., 2003), hypothyroidism (Marino et al., 2008) and external loading (Reich et al., 2008). Evidence from the literature suggests that growth inhibition can delay the growth plate senescence (thus thicker than normal), which leads to faster growth than the normal age-matched controls (Lui et al., 2011; Marino et al., 2008). Considering the following points of the high impact rats compared to the sham rats: lower PGR before 56 days old (Figure 5.4a), indifferent TTL at 77 days old (Figure 5.4c), higher TGD since 42 days old (Figure 5.4d), and higher DGPT_h and PGPT_h at most time points (Figure 5.5), the high impact rats might have undergone some catch-up growth. The only missing point here is that the impact loading was applied continuously in our study design, in contrast to the unloading periods given in many of the catch-up growth studies. However, we can consider that the rats might have adapted to the loading intensity after a certain period (Niehoff et al., 2004), which might be somewhere between 42 and 56 days old based on the results in Figure 5.4 (a, d). In one of our previous studies, the rats underwent an unloading period after the 77 days of age to study the remaining effects of growing-period loading in adulthood (Mustafy et al., 2020). The medium and high impact rats showed higher muscle weights, improved structural and material properties, and better microstructural properties than their aged-matched sham rats. These benefits could have partially been caused by some catch-up growth. However, the growth-related changes might not be obvious since the long bone growth declines steadily upon rat reaching maturity around 3 months old (Hunziker & Schenk, 1989; Roach et al., 2003; Walker & Kember, 1972).

To conclude for the sections 5.8.1 and 5.8.2: impact loadings can alter the longitudinal bone growth of rats and might bring different effects on the proximal and the distal growth plates; high impact loading led to thicker growth plates, which might be related with osteochondrosis and catch-up growth. In general, mechanical stimulation helps to achieve higher peak bone mass (Fritton et al., 2005), which is beneficial to bone quality later in life. However, the intensity of adolescent training should be well controlled to avoid bone growth alteration (Caine et al., 2001; Weaver, 2002).

5.8.3 Impact loading increased bone formation before and after the peak proximal bone growth period, especially on the periosteum

The loading experiment began at the rat age of 28 days, before the peak proximal bone growth period (35 to 42 days old) as explained in section 5.7.2. Based on the tendencies of MV/BV, MS/BS, MAR, and BFR/BS (Table 5.3) from 28 to 42 days old, the high impact loading resulted in an immediate response of bone formation at the beginning of the experiment. This initial response was not always statistically significant compared to the sham group, which is probably due to the initial individual differences (Prodinger et al., 2018; Torcasio et al., 2012). While the loading experiment continued from 42 days old onward, few significant differences between groups were found for any of the (re)modeling parameters until 63 days old (towards the end of puberty). This might be caused by a gradual mechanical adaptation of the rat tibiae from the beginning of the loading experiment (Chermside-Scabbo et al., 2020; Main et al., 2014; Razi et al., 2015).

For the last two weeks of the loading protocol (63 to 77 days old), the impact loading groups, especially the medium and high impact groups, presented higher bone formation than the sham group, however not always distinguishable among each other. Evidence from the literature shows that, towards the end of the rapid growth period, mechanical loading on rat tibiae enhances blood perfusion, which might be related to bone formation (Gohin et al., 2020). We can hypothesize that this increased response to mechanical loading is caused by the shift from modeling to remodeling as the predominant activity in rat skeletons when they approach sexual maturity (Erben, 1996; Jee & Yao, 2001; Lelovas et al., 2008): the effect of impact loading might have been blunted by the predominant modeling activity during the peak proximal bone growth stage, then reversed afterwards when remodeling began to dominate over modeling. As reported in the literature, load-induced changes in the cortical tibia can increase from mid to end-puberty (Main et al., 2014). The increased bone formation before and after the peak proximal bone growth period might have contributed to the enhanced bone quality of the high impact group rats as reported in one of our previous studies (Mustafy, Londono, Moldovan, et al., 2019).

5.8.4 Impact loading resulted in higher bone resorption before and after the peak proximal bone growth period, especially on the endosteum

The high impact group showed higher EV/BV, ES/BS, MRR and BRR/BS (Table 5.4) than the other groups towards the beginning of the experiment (28 to 35 days old), which is probably due to the initial response to the intense loading level as well. All the resorptive parameters showed higher values for the medium and the high impact groups towards the end of puberty (63 to 77 days old) compared to the sham group. This can be explained with a similar hypothesis as in section 5.8.3: the increase in bone resorption might be caused by the shift of predominant activity from bone modeling to remodeling. When the rats are rapidly growing, formation modeling is the main activity on the periosteum and resorption modeling predominates on the endosteum (Allen & Burr, 2019) as part of the radial growth of cortical bone (Notomi et al., 2001). The impact loadings have enhanced the modeling on both surfaces in the first half of the experiment (Table 5.3, Table 5.4). This distinctive formation-resorption pattern became less evident when rats grew towards maturity, where formation and resorption remodeling happened on both surfaces.

5.8.5 The endosteum is more mechano-sensitive than the periosteum

When looking at the effect of time periods in Table 5.3, Table 5.4, the tendencies on the endosteum stood out more. The high impact loading group showed reduced endosteal resorption parameters 14 to 21 days after the start of the experiment under the effect of rats' rapid growth, and both the medium and high impact groups had increased endosteal (re)modeling activities towards the end of the experiment. However, the effect of time was not clear on the periosteum. When analyzing the results presented in Table 5.5, the endosteum stood out again, with higher values of most (re)modeling parameters (all except for MS/BS and MAR) throughout the experiment compared to their periosteal counterparts. However, this disparity between periosteum and endosteum in response to mechanical loading does not seem to be related to the intensity of loading, as shown in Table 5.1, where very few parameters presented significant two-way interaction for loading * location.

Taken together, the tibial endosteum responds more strongly than the periosteum under impact loading, with larger bone (re)modeling volume, surface, and thickness in most time periods. A previous study using a mouse model observed a similar finding with increased bone formation on both cortical surfaces and reduced endosteal bone resorption (Birkhold et al., 2016). One of the key differences between the two studies is that the animals in our study were loaded at very young ages (adolescence, rapid bone growth), which is right before the starting point of Birkhold et al.'s study (adulthood, slower bone growth). That is probably why we did not observe any reduction of endocortical resorption as our rats were still very young.

5.8.6 Limitations

Although the non-invasive micro-CT-based measurements made it possible to follow-up the bone growth and remodeling parameters over time, the repeated anesthesia sessions might have caused adverse effects to rats' well-being. Isoflurane anesthesia has been widely used in animal studies where repeated micro-CT imaging sessions were necessary (Altman-Singles et al., 2017; Wehrle et al., 2019). The potential toxicity of isoflurane has been reported in the literature (Wehrle et al., 2019), including immunosuppression (Stollings et al., 2016), liver damage (Stevens et al., 1975), DNA damage (Kim et al., 2006), and overexpression of oncogenes (Kádár et al., 2007). The use of isoflurane can also change calcium metabolism (Hotchkiss et al., 1998) which might in turn affect how bone grows and remodels (Allen & Burr, 2019; Burr, 2019). In addition to the effects directly related to isoflurane, repeated immobilization of rats can increase their stress and corticosterone level, decrease their osteocalcin and vertebral bone growth (Patterson-Buckendahl et al., 2001). Higher level of corticosterone might also lead to increased osteoblastic apoptosis and thus, to reduced bone formation (Silvestrini et al., 2000).

Unlike the widely reported growth suppression effect of the non-steroidal anti-inflammatory drugs (NSAIDs) (Burch et al., 2021; Chang et al., 2005; Cottrell & O'Connor, 2010; Pountos et al., 2012), the direct effects of isoflurane on bone growth and remodeling are not well documented in the literature. The general risk of performing repeated isoflurane anesthesia in mice was assessed as "mild" in a recent severity classification study (Hohlbaum et al., 2017). As for our study design, the rats were under isoflurane anesthesia in two scenarios: the *in vivo* mechanical loading and the

in vivo micro-CT imaging. The former one was applied 10 minutes per day, 5 days per week, in total of 8 weeks. The later one was performed 8 times which lasts about 5 to 12 minutes each. A group of control rats without any mechanical loading (and therefore without loading-related anesthesia) was used to control for the effects of isoflurane exposure and showed no significant difference with sham rats in terms of growth, microstructural, structural and material parameters (Mustafy, Londono, Moldovan, et al., 2019). Based on these results, we did not include the control rats in the present study and assumed that the sham rats can already serve as unloaded controls. However, the control rats in the aforementioned article were still exposed to isoflurane during the *in vivo* micro-CT imaging sessions. To control for this, future studies can include a group of rats without any anesthesia-related manipulation, which can be sacrificed at a specific time point for histology analysis in order to compare with isoflurane-exposed rats.

A spatial resolution of 18 μm was applied for the micro-CT scans to ensure a safe radiation dose as determined by a preliminary study (Mustafy, Benoit, et al., 2018), which is especially important in the context of repeated weekly scans throughout rat puberty. However, this level of resolution was limiting for the subsequent analysis of remodeling parameters. For example, the MRR of the sham rats was as low as 1.4 $\mu\text{m}/\text{day}$, which is equivalent to a resorption distance of 9.8 μm weekly, just a bit higher than half of the image resolution (18 μm). Any apposition or resorption depth lower than 18 μm would have been measured as 18 μm , and any change smaller than half of the resolution (9 μm) could not be detected due to the partial volume effect (Palacio-Mancheno et al., 2014). The resolution of 18 μm is also limiting to measure the growth plate thickness, especially for the distal growth plate as explained in section 5.6.4. Compared to histology-based measurements, the micro-CT-based results showed higher standard variations as explained in section 5.8.2. This aspect can be possibly improved with newer generations of micro-CT and nano-CT scanners (Bouchard et al., 2021; Bouxsein et al., 2010; Kampschulte et al., 2016), which can provide better image quality with optimized radiation dose.

The (re)modeling parameters were measured on cortical bone of mid-diaphyseal tibia. In fact, the metaphyseal tibia is usually analyzed as well in the literature to quantify (re)modeling activities in trabecular bone (Bouxsein et al., 2010). In one of our preliminary tests, the (re)modeling parameters were calculated on a few registered trabecular samples using a similar strategy as for the cortical bone. However, the trabecular microstructural changes seemed overwhelmingly large to ensure a

meaningful analysis of the (re)modeling parameters under an image resolution of 18 μm . For almost all the datasets tested, there were difficulties for properly aligning trabecular surfaces, which might be a similar problem for having relatively larger variations of surface parameters (MS/BS, ES/BS) than the other parameters in Table 5.3 and Table 5.4. It was determined that the trabecular (re)modeling analysis was not suitable for the current dataset, especially due to the rapid growth of rats and the micro-CT scanning interval of 7 days. On the other hand, the proximal bone growth was successfully evaluated even though it was based on a similar strategy of registering trabecular bone. The difference here is that the bone growth calculation is less demanding in terms of the image quality and registration accuracy: the consistent alignment of a couple of trabeculae across different longitudinal sections can provide a growth distance estimate with a satisfying precision (Ning et al., 2022).

Thus, the weekly micro-CT scanning schedule might not be the most optimal interval to evaluate bone (re)modeling parameters. Since the remodeling cycle in rodents can vary from 2 weeks to over a month (Baron et al., 1984; Jilka, 2013; Van et al., 1982; Weinstein et al., 1998), the weekly scans might have taken place in different “phases” within a regular remodeling cycle, causing the large standard deviations at several time points of the results. Nonetheless, our current study was able to recognize the general changes of the cortical (re)modeling parameters throughout rats’ puberty. Subsequent studies can focus on a shorter time span with shorter scanning intervals to be able to discern the different remodeling phases. Using a synchrotron-sourced micro-CT scanner, it will be possible to track specific remodeling events in small animal bones (Harrison, 2022; Harrison et al., 2022). However, it is much less convenient to do so considering the very limited access to the synchrotron sources, as well as the generally higher radiation doses generated thus fewer longitudinal scans possible.

5.9 Conclusion

Our results show that impact loading inhibited proximal bone growth throughout puberty. We hypothesize that impact loading might bring different growth alterations to the distal and proximal growth plates. High impact loading might lead to pathological consequence of osteochondrosis and catch-up growth due to growth inhibition. Impact loading also increased cortical bone (re)modeling

before and after the peak proximal bone growth period of young rats, of which the latter case might be caused by the shift from modeling to remodeling as the dominant activity towards the end of rat puberty. We confirm that the tibial endosteum is more mechano-sensitive than the periosteum in response to mechanical loading. The present study provides insights to future clinical studies on optimization of physical exercise for children and adolescents. Similar image processing techniques can be applied to clinical imaging modalities. Newer generations of imaging technologies with better resolution and lower radiation doses may be more suitable for testing the hypothesis advanced in this study.

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5.12 References

Please refer to the general list of references provided at the end of the dissertation (page 152).

CHAPTER 6 PRELIMINARY EXPERIMENTS: QUANTIFICATION OF BONE MICRODAMAGE

6.1 Introduction

This chapter summarizes the preliminary experiments performed for the third specific objective of the present dissertation: adapt and apply a contrast-enhanced micro-CT-based method which evaluates 3D bone microdamage accumulation in rat cortical bone. First of all, an axial compressive loading protocol was developed to create microdamage in rat forelimb (section 6.2). Then, the loading protocol was applied to determine the forelimb fracture point in rats (section 6.3). Afterwards, the BaSO₄ staining procedure was adapted and applied for the contrast-enhanced micro-CT imaging (section 6.4). Moreover, two sub-fracture loading levels were tested in preparation of the subsequent *in vivo* experiments (section 6.5). Lastly, the effects of BaSO₄ staining on bone mechanical properties were investigated (section 6.6).

6.2 Development of an axial compressive loading protocol

6.2.1 Objectives

This preliminary experiment was required to develop an experimental setup for the compressive loading on forelimbs of mature female rats (around 5 months old). A loading profile was designed to deliver compressive loadings that are comparable to past studies in the literature (Herman et al., 2010; Zhang et al., 2018).

6.2.2 Methods

The experimental setup is shown in Figure 6.1. The compressive loading is applied by a Mach-1 micromechanical system (Biomomentum Inc., Laval, QC, Canada). Since the loading is supposed to take place while the rat is under anesthesia, a fume adsorber is placed near the loading bed for the evacuation of excessive isoflurane vapour (anesthetic). A warming pad with automatic temperature control is placed on the loading bed to keep the rat's body warm. The forelimb sizes

of two sacrificed mature female rats (reused from other research groups) have been measured, and two forelimb fixators made of stainless steel were designed, which are installed on each side of the compressive loading design. The loading setup was subsequently tested on the intact body of two sacrificed 5-month-old female rats for the fit of their forelimbs in proportion to the fixators.

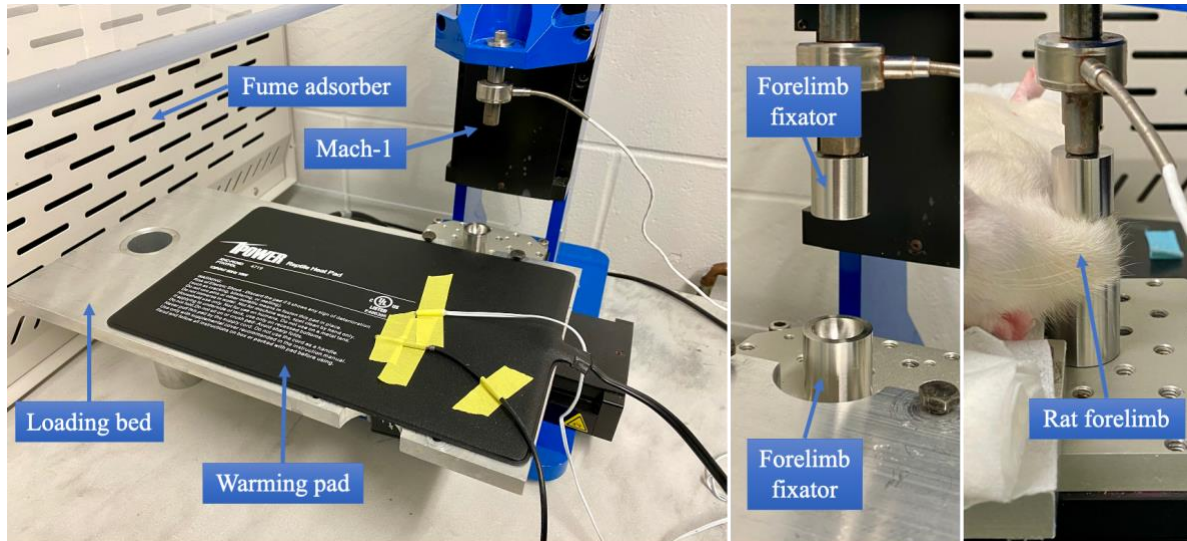


Figure 6.1 Experimental setup for forelimb compressive loading on rats.

Furthermore, a loading profile was designed for the Mach-1 micromechanical system, which can deliver loading curves comparable to past studies in the literature (Herman et al., 2010; Uthgenannt et al., 2007; Uthgenannt & Silva, 2007; Zhang et al., 2018). The complete loading session consists of three consecutive steps: preparation, preconditioning, and main loading.

During the preparation step: the load cell is calibrated, the forelimb fixators are installed, the data acquisition rate is set to 20 Hz, and the function “Zero Load” is executed.

During the preconditioning step, the following sub-steps are performed to condition the soft tissues at the two ends of the rat forelimb using 50 cycles of sinusoidal compression:

1. “Find Contact” function (speed = 0.1 mm/s, criteria = 1 N).
2. “Sinusoid” function (amplitude = 1 mm, frequency = 2 Hz, cycles = 50).
3. “Move Relative” function (amplitude = -1 mm, speed = 0.2 mm/s).
4. “Zero Position” function.

This is followed by the main loading step, which involves force adjustment to a determined peak value at each repetition. The sinusoidal loading is performed for 360 cycles, with each repetition lasting about 3.5 minutes. The user can configure the total number of repetitions for the main loading. The following sub-steps describe the sequence of a single repetition:

1. “Find Contact” function (speed = 0.1 mm/s, criteria = desired peak force).
2. “Move Relative” function (amplitude = -1 mm, speed = 0.2 mm/s).
3. “Sinusoid” function (amplitude = 1 mm, frequency = 2 Hz, cycles = 360).
4. “Move Relative” function (amplitude = -1 mm, speed = 0.2 mm/s).

6.2.3 Results and discussion

Figure 6.2 shows the contact force and the actuator displacement over time for one of the tested forelimbs. The loading protocol allowed readjusting to the peak force at each repetition of the main loading step, and the loading lasted about 90 minutes before reaching the fracture point. The actuator displacement accumulated gradually, displaying a “z-shaped” curve similar to observations in past studies with similar loading designs (Buettmann & Silva, 2016; Uthgenannt & Silva, 2007). Based on *ex vivo* tests with two female rats of 5-month-old (4 forelimbs in total), it was concluded that the loading protocol could deliver consistent loading results measured by the contact force and actuator displacement. The present compressive loading protocol was applied in subsequent experiments, including the main *in vivo* study documented in Chapter 7.

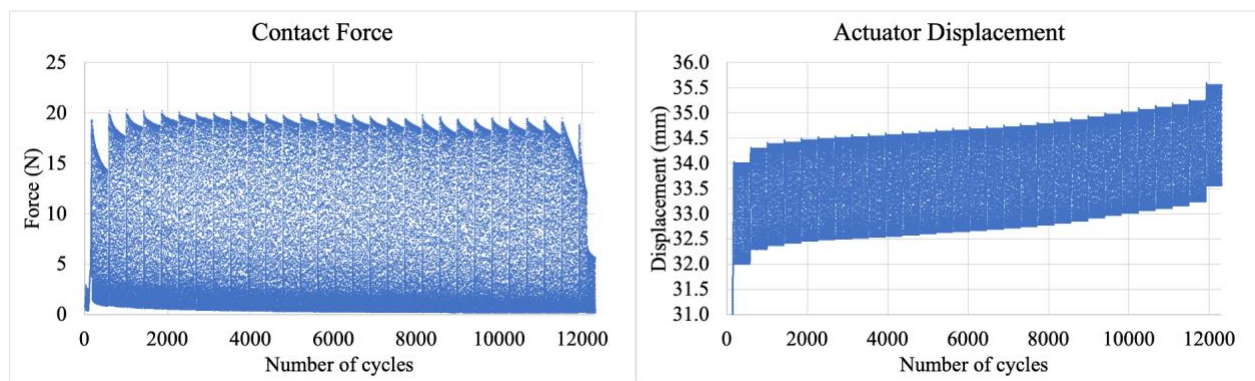


Figure 6.2 Contact force and actuator displacement measured for one of the tested forelimbs.

6.3 Determination of the rat forelimb fracture point

6.3.1 Objectives

This preliminary experiment was conducted to prepare for the *in vivo* sub-fracture loading which was used to create microdamage in normal and ovariectomized rats. The fracture point expressed in actuator displacement and number of loading cycles was used for controlling the extent of the sub-fracture loading and for preventing unexpected bone fracture.

6.3.2 Methods

Twenty female rats around 5 months old were used in this experiment, of which 6 were normal rats and 14 rats received bilateral ovariectomy at the age of 3 months. All the rats received a single session of *in vivo* mechanical loading on both of their forelimbs while under isoflurane anesthesia (3.0%) supplied by oxygen (1.8 L/min). The loading was based on sinusoidal waveforms of 2 Hz with peak forces ranging from 14 to 24 N and lasting for a maximum of 3 hours.

After the loading, the rats were immediately euthanized, and the output files were analyzed using the Mach-1 Analysis software. The fracture point was characterized by a significant drop in contact force and concurrently increased displacement (Hsieh & Silva, 2002; Uthgenannt & Silva, 2007). The accumulated actuator displacement was defined as the difference of the actuator's absolute position between the first and the last loading cycle of the main loading step (section 6.2.2). A two-tailed t-test with unequal variance was performed to compare the average of accumulated actuator displacement between the ovariectomized rats and the normal rats ($p < 0.05$).

6.3.3 Results and discussion

The accumulated actuator displacement of the fracture point was not different between ovariectomized and normal rats ($p = 0.34$). Since a single level of sub-fracture loading was desired in the subsequent *in vivo* experiments, the results of the fracture point were combined for all the rats. The average accumulated actuator displacement for a complete forelimb fracture was 1.0391 ± 0.3091 mm, which was in line with values obtained from normal rats in the literature (Uthgenannt

et al., 2007; Uthgenannt & Silva, 2007). It was noticed that the majority of the rats did not encounter a fracture within 0.60 mm of accumulated actuator displacement. Therefore, a sub-fracture loading with a stopping point around this value was considered to prevent bone fracture most likely.

A loading of 4320 or 5040 cycles corresponded to an accumulated actuator displacement of 0.56 mm or 0.60 mm, respectively. In a past study performed on rabbit forelimbs, the authors recommended using small constant peak forces for more consistent loading results (Coates & Silva, 2020). Other studies used a constant peak force rate of 0.055 N/g based on rat body weight (Herman et al., 2010; Uthgenannt et al., 2007; Uthgenannt & Silva, 2007; Zhang et al., 2018), which was not applicable in our study design since the ovariectomized rats could be overweight compared to the normal rats. In the end, it was decided to apply a constant peak force of 16 N, which was about 0.040 N/g for the ovariectomized rats and 0.050 N/g for the normal rats.

Having the fracture point of the rat forelimb based on accumulated actuator displacement and on the number of loading cycles, the following two levels of sub-fracture loading were proposed for subsequent experiments:

1. Peak force of 16 N, stopping at 4320 cycles or 0.56 mm (about 54% of the fracture point).
2. Peak force of 16 N, stopping at 5040 cycles or 0.60 mm (about 58% of the fracture point).

Before testing these sub-fracture loading levels, the methodologies of the BaSO₄ staining and the contrast-enhanced micro-CT imaging need to be adapted and applied for microdamage quantification (section 6.4).

6.4 Adaptation and optimization of the contrast-enhanced micro-CT

6.4.1 Objectives

This preliminary experiment was designed to adapt the BaSO₄-staining-based contrast-enhanced micro-CT imaging, and to compare the effects of micro-CT resolution on the capacity of microdamage quantification.

6.4.2 Methods

The BaSO₄-based contrast-enhanced micro-CT imaging for bone microdamage quantification has been validated against the histology-based method in the literature (Landrigan et al., 2011; Zhang et al., 2018). In this study, forelimb samples from 15 sacrificed rats were used to adapt the BaSO₄ staining procedure. The right forelimbs were loaded to sub-fracture levels ranging from 4000 to 13000 cycles, and the left forelimbs served as unloaded controls. After the loading, the samples were collected and dissected for microdamage evaluation. The samples were stained under vacuum for 3 days in a mixture solution of BaCl₂, acetone and PBS in equal volume, then another 3 days in a mixture solution of Na₂SO₄, acetone and PBS in equal volume. Samples of 11 rats were used for testing the following parameters based on the SOPs shared by the research groups of Dr. Ryan K. Roeder (Leng et al., 2006; Turnbull et al., 2011) and Dr. Cari M. Whyne (Atkins et al., 2019; Choudhari et al., 2016): sample positioning strategy (whether or not the radius and ulna are stained in separate vials), vacuum pressure (-100 mmHg or -680 mmHg relative to the atmosphere pressure), and the concentration of the BaCl₂ and Na₂SO₄ solutions (0.10, 0.25 or 0.50 M). The rest of the sample pairs were subsequently stained using the optimized version of these parameters.

After the staining procedure, the samples were wrapped in PBS-soaked gauze, stored in transparent tubes of 5 mL, and scanned by micro-CT. The scans were performed using a SkyScan 1176 system at an image resolution of 8.74 μ m, 65 kVp voltage, 385 μ A current, 1500 ms exposure time, 1 mm Al filter, 0.25° / 180° rotation steps. To account for the effects of image resolution on the SV/BV results, one forelimb sample was scanned by two different micro-CT systems: SkyScan 1176 at 8.74 μ m, and ZEISS Xradia 520 Versa at 4.16 μ m. The ZEISS scan setting was: 60 kVp voltage, 84 μ A current, 2000 ms exposure time, LE3 filter, 0.225° / 360° rotation steps. Based on the contrast-enhanced micro-CT scans, the microdamage volume ratio SV/BV (stained volume per bone volume) was quantified using a custom-built program in MATLAB R2018b (MathWorks, Natick, MA, USA).

6.4.3 Results and discussion

The thresholds were set to 0.67 g/cm³ for the bone volume and 1.77 g/cm³ for the stained volume based on visual observation and histogram analysis in the CT Analyzer software (Bruker microCT

N.V., Kontich, Belgium). The threshold for the stained volume was verified to be well above the range of the tissue mineral density (TMD) of the forelimb cortical bone. For each pair of samples tested, the SV/BV was plotted as a function of the longitudinal slice position, showing that the peak staining position was around the mid-third diaphysis of radius and ulna (Figure 6.3), which confirms the findings from literature (Uthgenannt & Silva, 2007; Zhang et al., 2018). The subsequent microdamage analysis was concentrated on the VOI of mid-third diaphysis only. Note that the SV/BV was not always close to zero at the non-peak area (Figure 6.3 right), which was mostly caused by stains remained on the surface of the samples based on visual evaluation of the micro-CT images. This effect could be well reduced by considering a new parameter “ Δ SV/BV” which is the absolute difference of SV/BV between the contralateral bones, assuming that the surface staining and porosities of the contralateral limbs are about the same. Therefore, the derived parameter Δ SV/BV is reflective of the microdamages caused by the mechanical loading only. This damage calculation method was applied in the subsequent experiments in Section 6.5 and in Chapter 7.

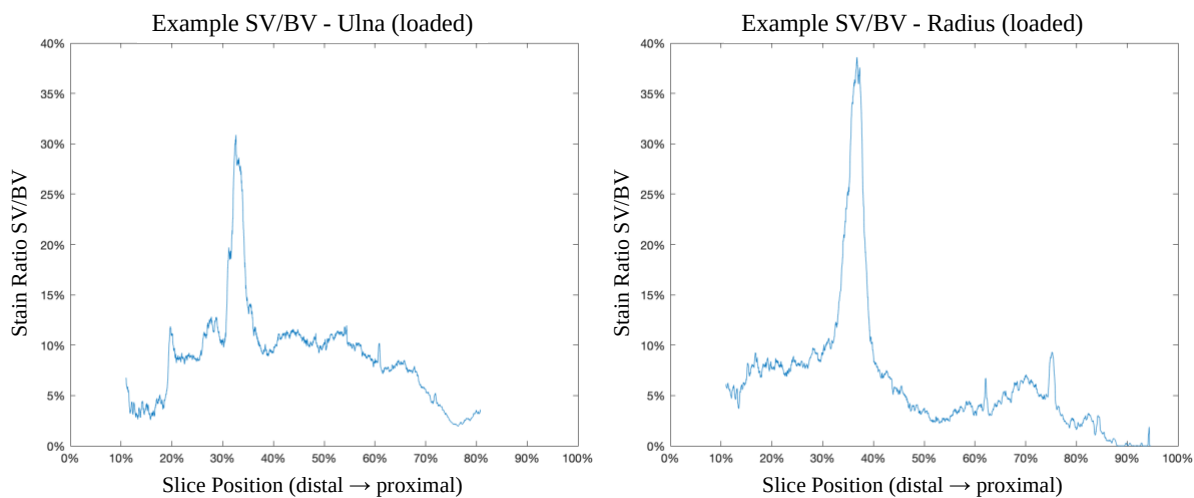


Figure 6.3 Example SV/BV levels of loaded samples as a function of the slice position.

Based on the evaluation of the SV/BV plots, placing the radius and ulna samples separately helped to increase the stains around the mid-third diaphysis. Since changes in vacuum pressure did not seem to have an impact on the staining results, it was decided to use the less demanding pressure level (-100 mmHg). The solution mixtures of 0.10 M and 0.25 M did not show enough staining on

the tested samples even if they were loaded, and the concentration of 0.50 M adequately provided damage-related staining as proposed by previous studies (Leng et al., 2006; Turnbull et al., 2011; Zhang et al., 2018). For the 4 rats which were subsequently tested with the optimal staining parameters, the loaded samples showed consistently higher SV/BV levels than their control counterparts as shown in Figure 6.4. These results confirmed the successful creation of microdamage by the compressive loading protocol.

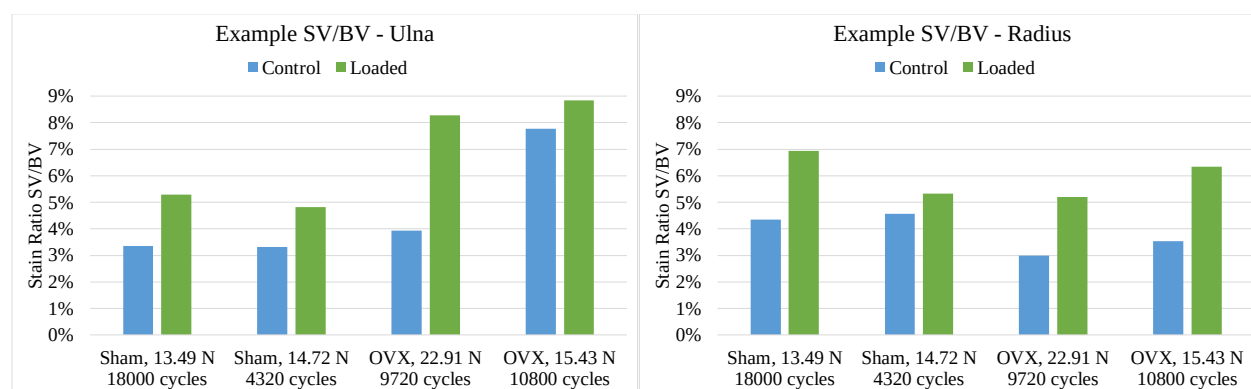


Figure 6.4 Example SV/BV of ulna and radius sample pairs.

A qualitative comparison of the same stained sample evaluated by both SkyScan and ZEISS systems is presented in Figure 6.5. On the left side, the scans performed using the ZEISS system showed cortical porosities with sharper edges, while the scans performed by both systems identified similar porosities in general. On the right side, both scans showed the same area of microdamage, though the ZEISS scans revealed more detailed stained regions. These observations are in line with results in the literature that the microdamages produced in rat ulna were mostly located at the medial area due to compressive strains, and at the lateral area due to tensile strains (Uthgenannt & Silva, 2007; Zhang et al., 2018).

A quantitative comparison between the scans of the same stained sample by the two different micro-CT systems are shown in Figure 6.6. The SV/BV calculated from the scans taken by both scanners were not directly comparable because they had different imaging settings. To account for these differences, the thresholds for the stained volume were adjusted for each system with three different options around the fixed threshold of 1.77 g/cm³. Both micro-CT systems showed a

similar tendency of the SV/BV versus the longitudinal slice position. Their relative difference was mostly between 1% and 3% inside the VOI, with a maximum value around 5%.

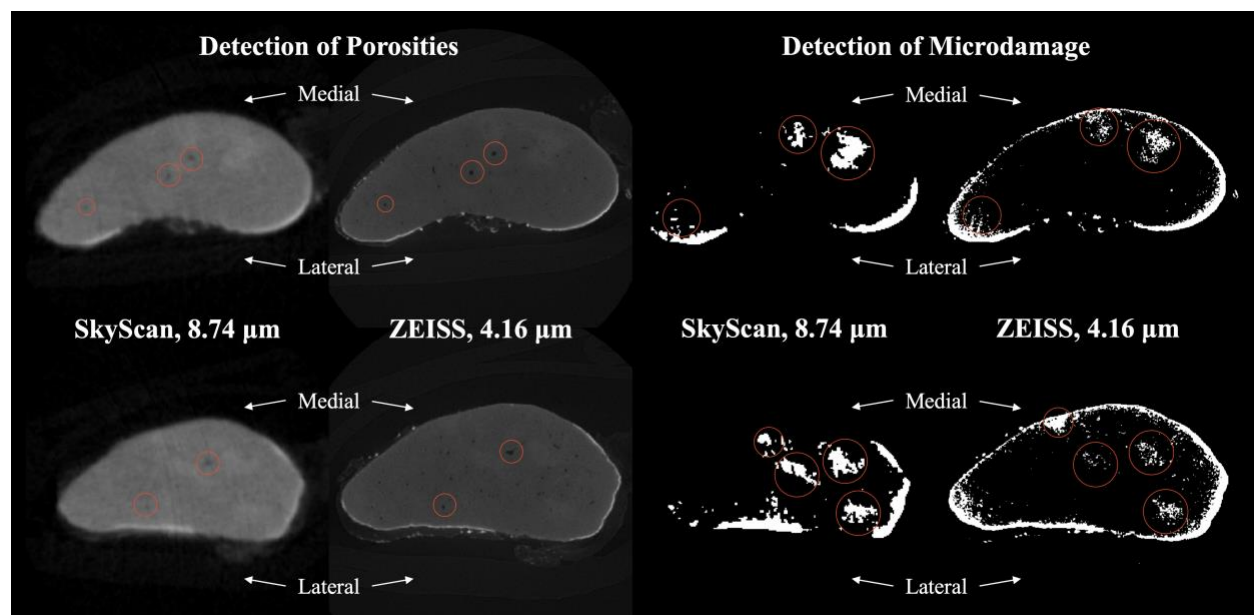


Figure 6.5 Example cross-sectional views of the same stained sample evaluated by the SkyScan and the ZEISS systems: detection of porosities shown in grey scale (left), and detection of microdamage shown in binary (right).

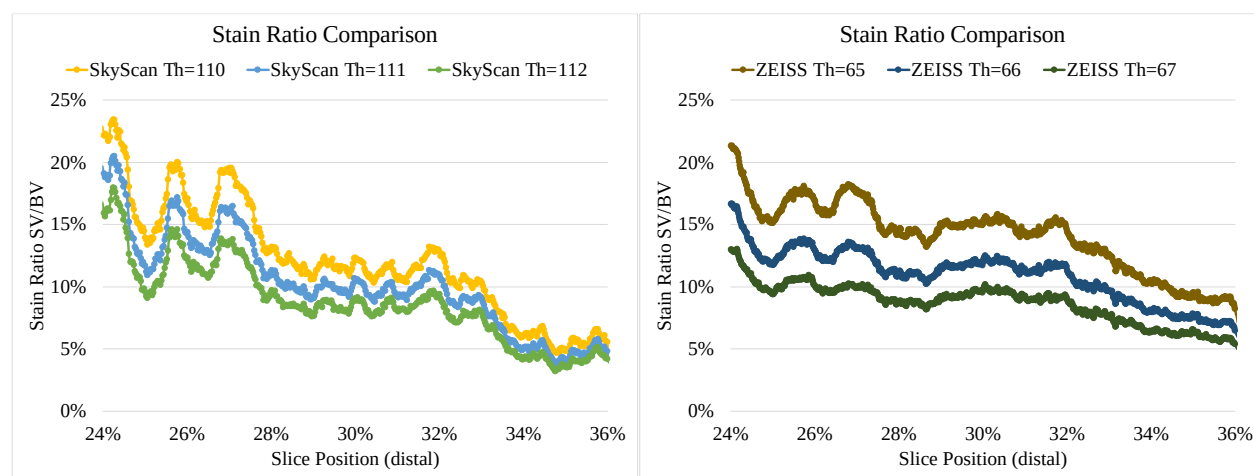


Figure 6.6 Comparison of SV/BV measured by two different micro-CT systems (SkyScan and ZEISS) using comparable thresholds. The SV/BV was plotted as a function of the longitudinal slice position (percentage relative to the distal end of the bone).

However, the ZEISS system took about 3 to 4 hours to perform a single scan, which increased the risk of sample dehydration since the mounting mechanism in the ZEISS scanner required the samples to be exposed to air. On the contrary, a scan taken by the SkyScan system only took about 25 minutes, during which the samples could be placed in a closed tube with hydration provided by PBS-soaked gauze. Besides, the 4.16 μm scan performed by the ZEISS system could only cover a portion of the samples (about 4 mm), whereas the 8.74 μm scan performed by the SkyScan system could cover a much longer area (about 20 mm) which is very close to the total length of the forelimb (around 25 mm). As reported in the literature, an image resolution of 10 μm is capable of detecting BaSO₄-stained microdamage in rodent samples (Choudhari et al., 2016; Gargac et al., 2014; Turnbull et al., 2011). Therefore, the improvement of image resolution by the ZEISS system did not compensate for its disadvantages of longer scanning time (and higher cost), sample dehydration, and smaller VOI.

To conclude, the BaSO₄-staining-based microdamage quantification was successful using the SkyScan 1176 system at an image resolution of 8.74 μm . It was decided to use the SkyScan system because it could provide consistent SV/BV results without dehydrating the samples. This method of microdamage quantification was applied in subsequent experiments including the main *in vivo* study in Chapter 7.

6.5 Testing of the sub-fracture loading levels

6.5.1 Objectives

This experiment was conducted to test the two sub-fracture loading levels which were proposed at the end of the section 6.3.3. The loading parameters and the microdamage levels were evaluated and compared for both loading levels.

6.5.2 Methods

Six female rats around 5 months old were acquired for this test. Once sacrificed, the rats were immediately loaded on their left forelimbs to one of the following sub-fracture levels while the right forelimbs served as unloaded controls:

1. Peak force of 16 N, stopping at 4320 cycles / 0.56 mm (about 54% of the fracture point).
2. Peak force of 16 N, stopping at 5040 cycles / 0.60 mm (about 58% of the fracture point).

The accumulated actuator displacement is considered a good predictor for bone fracture (Coates & Silva, 2020; Uthgenannt et al., 2007; Uthgenannt & Silva, 2007), but the number of cycles is easier to track in real time with the Mach-1 micromechanical loading device. Therefore, it was decided to define the stopping point based on both parameters to prevent bone fracture and to ensure consistent microdamage levels. The number of cycles and the accumulated actuator displacement were continually monitored, and the loading was stopped when any of the two parameters reached their predefined levels.

After the loading, the forelimb samples were collected and dissected for the BaSO₄ staining procedure. The stained samples were then scanned by micro-CT (SkyScan 1176) for microdamage evaluation using the $\Delta SV/BV$ parameter. The following parameters were compared using two-tailed T-test with unequal variance ($p < 0.05$) between the two loading levels: total loading cycle, accumulated actuator displacement, $\Delta SV/BV$ (radius or ulna).

6.5.3 Results and discussion

The results of the forelimb loading are shown in Figure 6.7. None of the measured parameters showed significant differences between the two loading levels, which might be caused by the limited sample sizes (3 rats per group) constrained by the preliminary nature of this test. However, the higher sub-fracture loading level (in terms of number of cycles) showed a higher average accumulated displacement with a smaller standard deviation, which might contribute to more consistent *in vivo* loading results. Both loading levels were able to produce microdamage that was detectable by contrast-enhanced micro-CT imaging. The results of both loading levels were similar in general.

In conclusion, the two sub-fracture loading levels were quite similar in terms of the loading performance and microdamage quantities. To achieve more consistent results on the accumulated actuator displacement (which is a good predictor of bone fracture), option 2 (peak force of 16 N, stopping at 5040 cycles / 0.60 mm) was chosen for the subsequent main *in vivo* experiment with normal and ovariectomized mature rats in Chapter 7.

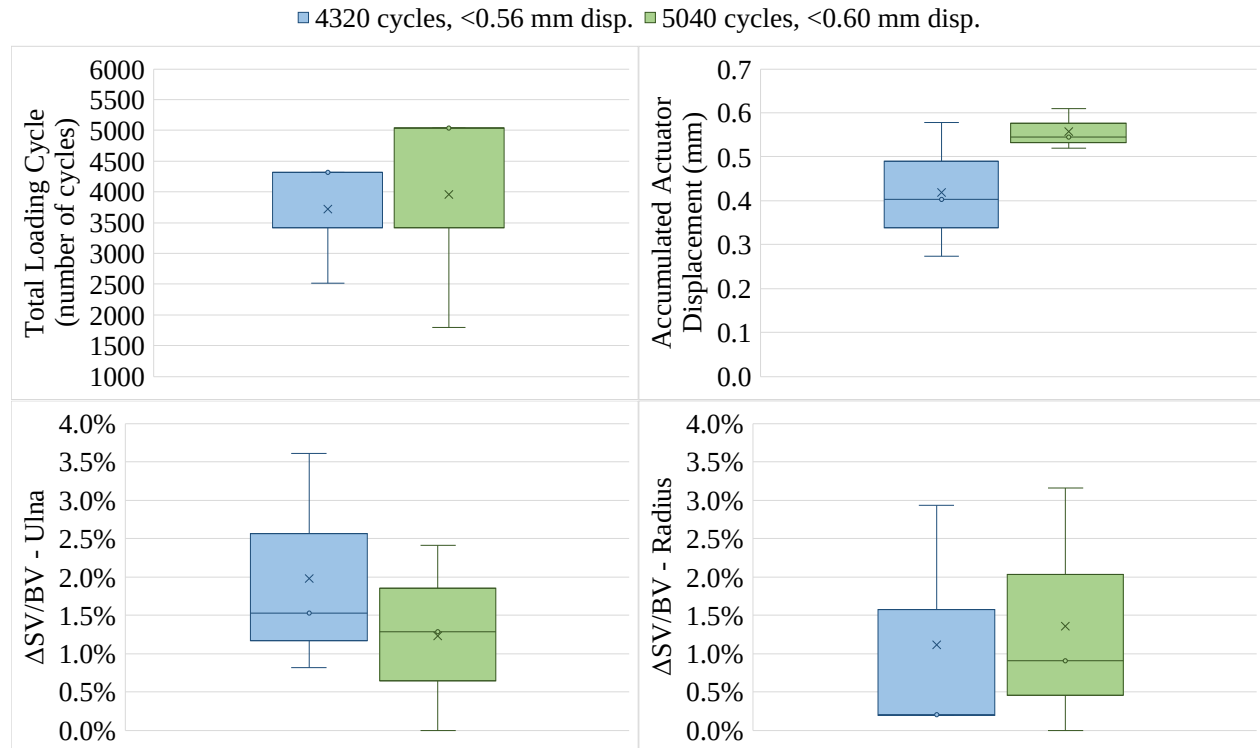


Figure 6.7 Results of the compressive loading and microdamage on the rat forelimb for both of the sub-fracture loading levels.

6.6 Effects of the staining on rat forelimb mechanical properties

6.6.1 Objectives

For the main *in vivo* study in Chapter 7, it was proposed to reuse the same rats for mechanical testing after the microdamage quantification of their forelimbs. However, it was uncertain if the BaSO₄ staining procedure could have impacted the mechanical properties measured by the three-

point-bending test. The following preliminary experiment was conducted to investigate this factor with comparative tests.

6.6.2 Methods

Six 5-month-old female rats were used in this experiment. Right after the sacrifice of the rats, their forelimbs were collected and dissected. The right radii and ulnae underwent a three-point-bending test on the day of sacrifice, and their left counterparts went through the six-day BaSO₄ staining procedure before the three-point-bending test. The mechanical properties were evaluated based on the methodology presented in section 2.5.2. Structural properties were measured from the force-displacement curves including ultimate force (F_u , N), failure force (F_f , N), yield force (F_y , N), stiffness (S , N/mm), pre-yield displacement (D_{pre} , mm), post-yield displacement (D_{post} , mm), pre-yield energy (U_{pre} , mJ), and post-yield energy to failure (U_{post} , mJ). Their corresponding material properties were calculated by considering the cross-sectional geometries measured from the micro-CT scans, deriving the ultimate stress (σ_u , MPa), failure stress (σ_f , MPa), yield stress (σ_y , MPa), Young's modulus (E , GPa), pre-yield strain (ϵ_{pre} , $\mu\epsilon$), post-yield strain (ϵ_{post} , $\mu\epsilon$), pre-yield toughness (u_{pre} , MPa), and post-yield toughness (u_{post} , MPa). The contralateral sample pairs (radius or ulna) were compared for each of the mechanical properties using two-tailed t-tests with unequal variance ($p < 0.05$).

6.6.3 Results and discussion

As shown in Table 6.1, none of the mechanical properties showed significant difference between the contralateral samples that went through different sample staining steps, except for the ultimate stress (σ_u) of radius which was about 11% lower for the stained samples.

It was concluded that the BaSO₄ staining procedure does not cause significant impacts on most of the mechanical properties of the forelimb samples. Interpreting the result of the radius ultimate stress requires extra attention if the current mechanical testing method is applied. In the present case, the advantages of reusing the same animals (which decreases the overall number of animals

used) for evaluating bone microdamage and mechanical parameters are greater than the drawback of altering a single parameter (Sneddon et al., 2017).

Table 6.1 Comparison of the mechanical properties of the samples (control or stained). The data with statistical significance ($p < 0.05$) are marked bold.

	Ulna (control)	Ulna (stained)	Radius (control)	Radius (stained)
F_u (N)	12.74 $\pm$ 2.14	13.01 $\pm$ 2.13	18.14 $\pm$ 1.72	18.58 $\pm$ 1.97
F_f (N)	10.00 $\pm$ 1.45	10.31 $\pm$ 2.09	14.78 $\pm$ 3.79	13.97 $\pm$ 1.84
F_y (N)	8.85 $\pm$ 1.69	8.73 $\pm$ 1.81	12.58 $\pm$ 1.65	13.26 $\pm$ 1.59
S (N/mm)	24.87 $\pm$ 5.22	26.77 $\pm$ 7.82	40.55 $\pm$ 4.20	47.60 $\pm$ 8.35
D_{pre} (mm)	0.47 $\pm$ 0.04	0.50 $\pm$ 0.11	0.45 $\pm$ 0.07	0.45 $\pm$ 0.03
D_{post} (mm)	1.39 $\pm$ 0.30	1.11 $\pm$ 0.29	0.88 $\pm$ 0.42	1.16 $\pm$ 0.29
U_{pre} (mJ)	2.02 $\pm$ 0.45	1.92 $\pm$ 0.39	2.81 $\pm$ 0.63	2.86 $\pm$ 0.38
U_{post} (mJ)	15.22 $\pm$ 4.19	12.21 $\pm$ 3.22	13.77 $\pm$ 6.97	18.23 $\pm$ 2.98
σ_u (MPa)	271.07 $\pm$ 21.55	247.84 $\pm$ 13.23	281.74 $\pm$ 7.12	250.16 $\pm$ 9.97
σ_f (MPa)	213.77 $\pm$ 24.07	196.88 $\pm$ 32.68	227.44 $\pm$ 45.35	188.67 $\pm$ 23.65
σ_y (MPa)	188.10 $\pm$ 21.76	165.46 $\pm$ 10.73	195.10 $\pm$ 13.79	179.07 $\pm$ 18.90
E (GPa)	27.29 $\pm$ 3.26	24.46 $\pm$ 2.72	22.17 $\pm$ 1.93	21.05 $\pm$ 2.33
u_{pre} (MPa)	0.83 $\pm$ 0.16	0.75 $\pm$ 0.11	1.24 $\pm$ 0.21	1.18 $\pm$ 0.16
u_{post} (MPa)	6.24 $\pm$ 1.44	4.80 $\pm$ 1.23	6.13 $\pm$ 3.06	7.51 $\pm$ 1.45
ϵ_{pre}	0.0091 $\pm$ 0.0013	0.0102 $\pm$ 0.0020	0.0128 $\pm$ 0.0022	0.0137 $\pm$ 0.0012
ϵ_{post}	0.0270 $\pm$ 0.0065	0.0227 $\pm$ 0.0059	0.0250 $\pm$ 0.0115	0.0351 $\pm$ 0.0078

6.7 Conclusion

In this chapter, the contrast-enhanced micro-CT-based method for quantification of bone microdamage has been successfully adapted. The compressive loading protocol was able to create consistent microdamage in rat forelimbs at a sub-fracture level, which was detectable by contrast-enhanced micro-CT imaging. The comparative tests using three-point-bending provided the experimental evidence required to reuse the same samples for the evaluation of bone microdamage and mechanical properties. The methods developed and adapted in the present chapter were applied to the main *in vivo* study in Chapter 7.

CHAPTER 7 ARTICLE 3: ZOLEDRONATE REDUCES LOADING- INDUCED MICRODAMAGE IN CORTICAL ULNA OF OVARIECTOMIZED RATS

7.1 Article information

- This chapter presents the third article written in the context of this dissertation, which answers the research question 4 and the specific question 4 as shown in Table 3.1
- The article was submitted to *Journal of the Mechanical Behavior of Biomedical Materials* (Elsevier) on 2023-06-20, and a bioRxiv preprint was made available on 2023-05-14
- Preprint link (bioRxiv): <https://doi.org/10.1101/2023.05.12.540579>
- The contribution of the first author, Bohao Ning, was about 85%
- Co-authors: Irène Londono, Catherine Laporte, Isabelle Villemure

7.2 Author contribution

- Bohao Ning: conceptualization, methodology, software, data curation, formal analysis, writing - original draft, writing - review & editing, visualization
- Irène Londono: conceptualization, methodology, data curation, resources, writing - review & editing
- Catherine Laporte: conceptualization, methodology, writing - review & editing, supervision
- Isabelle Villemure: conceptualization, methodology, writing - review & editing, supervision, funding acquisition

7.3 Abstract

As a daily physiological mechanism in bone, microdamage accumulation dissipates energy and helps to prevent fractures. However, excessive damage accumulation might bring adverse effects to bone mechanical properties, which is especially problematic among the osteoporotic and osteopenic patients treated by bisphosphonates. Some pre-clinical studies in the literature applied forelimb loading models to produce well-controlled microdamage in cortical bone. Ovariectomized animals were also extensively studied to assimilate human conditions of estrogen-related bone loss. In the present study, we combined both experimental models to investigate microdamage accumulation in the context of osteopenia and zoledronate treatment. Three-month-old normal and ovariectomized rats treated by saline or zoledronate underwent controlled compressive loading on their right forelimb to create *in vivo* microdamage, which was then quantified by barium sulfate contrast-enhanced micro-CT imaging. Weekly *in vivo* micro-CT scans were taken to evaluate bone (re)modeling and to capture microstructural changes over time. After sacrifice, three-point-bending tests were performed to assess bone mechanical properties. Results show that the zoledronate treatment can reduce cortical microdamage accumulation in ovariectomized rats, which might be explained by the enhancement of several bone structural properties such as ultimate force, yield force, cortical bone area and volume. The rats showed increased bone formation volume and surface after the generation of microdamage, especially for the normal and the ovariectomized groups. Woven bone formation was also observed in loaded ulnae, which was most significant in ovariectomized rats. Although all the rats showed strong correlations between periosteal bone formation and microdamage accumulation, the correlation levels were lower for the zoledronate-treated groups, potentially because of their lower levels of microdamage. The present study provides insights to further investigations of pharmaceutical treatments for osteoporosis and osteopenia. The same experimental concept can be applied in future studies on microdamage and drug testing.

7.4 Keywords

Microdamage, remodeling, osteoporosis, osteopenia, zoledronate, micro-CT

7.5 Introduction

Bone microscopic damage (microdamage) accumulation is a physiological process that takes place during our daily activities (Burr et al., 1985; Frost, 1960). A balanced state of microdamage accumulation and repair can help to dissipate energy and to prevent fragility fractures (Diab et al., 2006; Diab & Vashishth, 2005). However, excessive levels of microdamage could lead to impaired bone mechanical properties (Burr et al., 1998; Pattin et al., 1996; Zioupos, 2001). Past studies in the literature have investigated how microdamage is initiated (Carter et al., 1981; Fazzalari et al., 1998; Nagaraja et al., 2005; Wenzel et al., 1996), accumulated (Diab & Vashishth, 2007; Thurner et al., 2006; Vashishth, Koontz, et al., 2000), propagated (O'Brien et al., 2005; Vashishth et al., 2003; Wang & Niebur, 2006), and repaired (Bentolila et al., 1998; Burr, 2002; Mori & Burr, 1993; Verborgt et al., 2000). Pre-clinical studies applying mechanical loading on long bones of small laboratory animals showed the advantage of creating consistent damage levels that can be quantified by histology (Bentolila et al., 1998; Herman et al., 2010; Seref-Ferlengez et al., 2014) or micro-CT-based methods (Gargac et al., 2014; Turnbull et al., 2011; Zhang et al., 2018).

Some experimental studies on microdamage made links with osteoporosis and osteopenia (Coates & Silva, 2020; Fazzalari et al., 1998; Lambers et al., 2013) because they can influence damage accumulation. These pathologies are characterized by low bone mineral density (BMD) (Dimai, 2017; NIH, 2001), which is especially common among the female post-menopausal population due to estrogen deficiency (Li & Xu, 2020; WHO, 1994). Ovariectomized rodent models were widely used in pre-clinical studies due to their strong resemblance to the human conditions of estrogen-related bone loss (Iwaniec & Turner, 2013; Jee & Yao, 2001; Kalu, 1991; Lelovas et al., 2008). As one of the common treatments for bone loss, various types of bisphosphonates (BPs) were tested regarding their effects on bone quality improvement (Burr, 2016; Feher et al., 2010; Hao et al., 2015; Kettenberger et al., 2014; Liu et al., 2017). Zoledronate (zoledronic acid) is considered among the most potent BPs by inhibiting the bone resorption thus increasing the BMD (Åstrand et al., 2006; Gasser et al., 2008; Kettenberger et al., 2014).

However, the BP-reduced bone resorption can decrease the efficiency of microdamage repair (Allen & Burr, 2007; Chapurlat & Delmas, 2009; Kidd et al., 2011; Li et al., 2001), leading to increased damage density (Allen, 2018; Ma et al., 2017; Mashiba et al., 2000) and therefore

impaired bone quality (Allen & Burr, 2011; Burr, 2016, 2020). Clinical studies revealed that long term treatment with BPs can be associated with increased risk of atypical femoral fractures (Black et al., 2018; Black et al., 2021; Gedmintas et al., 2013; Shane et al., 2014), atypical forearm fractures (Moon et al., 2013; Oh et al., 2018; Ohta et al., 2022; Tan et al., 2015), and osteonecrosis of the jaw (Khosla et al., 2007; Marx, 2003; Shane et al., 2013). Although numerous translational studies were carried out to better understand these side effects (Kettenberger et al., 2014; Kidd et al., 2011; Mulcahy et al., 2015; J. Y. Wang et al., 2019), the data are still lacking in terms of the direct relationships between microdamage accumulation, remodeling, and mechanical properties in the context of BP treatment. To fill in this knowledge gap, animal models are especially appropriate as they provide well-controlled experimental conditions.

In the present study, we combined the widely used ulna microdamage model with the well-established ovariectomized rat model to study microdamage accumulation in cortical ulna. Our objectives were: (1) to create and quantify *in vivo* ulnar microdamage in ovariectomized rats further treated by saline or zoledronate; (2) to quantify ulnar (re)modeling and mechanical properties; (3) to evaluate the correlations between ulnar microdamage and (re)modeling or mechanical parameters. We hypothesized that the zoledronate treatment can reduce loading-induced microdamage by improving structural properties in cortical ulna of ovariectomized rats.

7.6 Material and methods

7.6.1 Experimental design

Three-month-old female virgin CD IGS rats (n=48) were received from the Charles River Laboratories on week 1 (Figure 7.1). The animals were housed in cages of two with free access to food and water at the animal facility of the Sainte-Justine University Hospital Center. They lived a light/dark cycle of 12 hours at about 23°C and 21% humidity. One week of acclimation was allocated before the start of the experiment on week 2. The rats were assigned to one of the following treatment groups: “NOM” (normal, n=16), “OVX” (ovariectomized, n=16) and “ZOL” (zoledronate-treated, n=16). The NOM rats did not receive any surgery, serving as baseline controls, while the OVX and ZOL rats received bilateral ovariectomy at the animal supplier on

week 0 (Figure 7.1). Within each treatment group (n=16/group), the rats were randomly assigned to two sub-groups of different sacrifice time points (n=8 at week 11 and n=8 at week 12). The effects of ovariectomy have been confirmed, with significant bone loss occurring in trabecular tibia from week 2 to week 12 (Supplementary Material). The Institutional Committee for Good Animal Practices in Research of the Sainte-Justine University Hospital Center approved the animal experiments in this study.

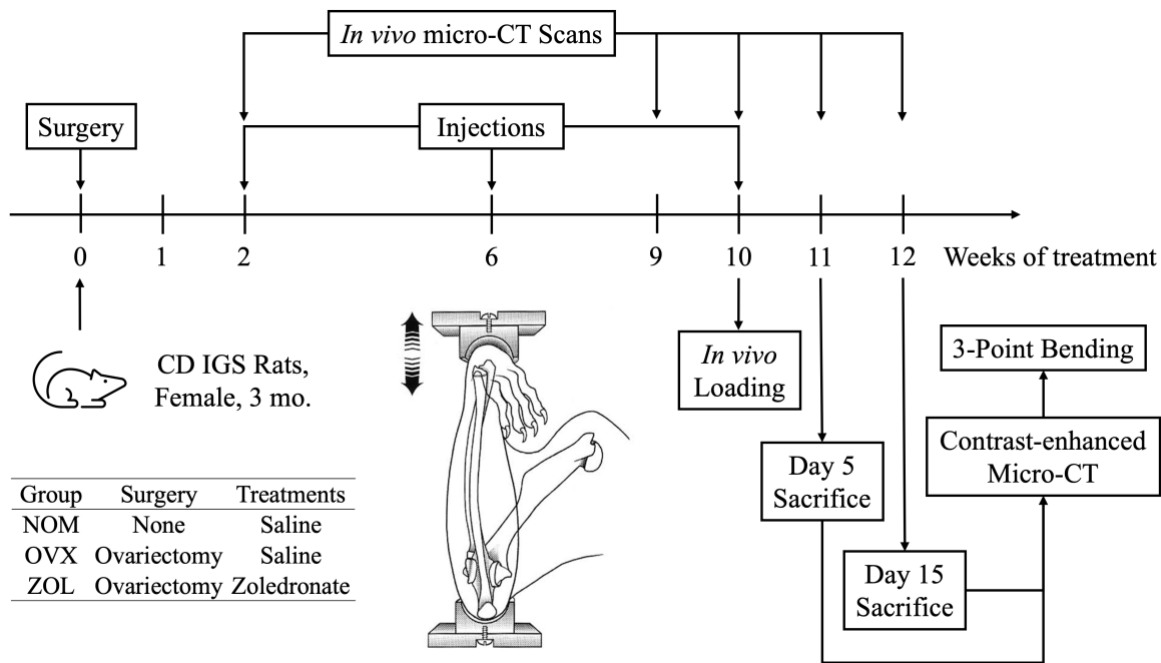


Figure 7.1 Experimental design. The forelimb loading diagram was adapted from (Robling et al., 2002) with permission obtained from the publisher.

As shown in Figure 7.1, the rats received *in vivo* micro-CT scans on weeks 2, 9, 10, 11 and 12 to follow up on the microstructural changes in their forelimbs and hindlimbs (section 7.6.2). They also received subcutaneous injections on weeks 2, 6 and 10, where the NOM and OVX rats were given sterile saline (1 mL/kg) and the ZOL rats were given zoledronate (100 µg/kg) for a total treatment period of 9 weeks. The dose of the zoledronate was based on past rodent models to reflect the clinical doses used for bone loss treatment in humans (Aref et al., 2016; Feher et al., 2010; Gasser et al., 2008). On week 10, all the rats received a single session of mechanical loading (section 7.6.3) on their right forelimb to generate microdamage *in vivo*. Half of the rats (n=8 from

each treatment group) were sacrificed 5 days post-loading (week 11), and the other half 15 days post-loading (week 12) to quantify the amount of microdamage using contrast-enhanced micro-CT imaging (section 7.6.4). After sacrifice, all the ulnar samples received a three-point-bending test to evaluate their mechanical properties (section 7.6.5).

7.6.2 *In vivo* micro-CT imaging

On week 2, *in vivo* micro-CT scans were acquired using a SkyScan 1176 microtomograph (Bruker microCT N.V., Kontich, Belgium) on rats' right forelimb (mid-diaphysis) to capture the initial bone microstructure (Figure 7.1). On weeks 9, 10, 11 and 12, additional weekly scans were performed on both sides of forelimb to calculate the (re)modeling rates. Rod-shaped phantoms of 0.25 and 0.75 g/cm³ were scanned as well for calibration purposes. All the scans were performed at an image resolution of 8.74 μ m, using a filter of 1 mm Al, exposure time of 1200 ms, voltage of 65 kVp, current of 300 μ A, rotation angle of 0.5°/180°. The rats were under isoflurane anesthesia (2.5%) supplied by oxygen (1.5 L/min) during the scans, which lasted about 11 minutes each. The radiation exposure was estimated to be about 1.08 Gy for each scan by the CTion software (Bruker microCT N.V., Kontich, Belgium) (Mustafy, Benoit, et al., 2018).

After the scans, the images were reconstructed using the NRecon software (Bruker microCT N.V., Kontich, Belgium). The dynamic range was set to [0, 0.06] (attenuation coefficient) and the post-alignment was adjusted visually starting from the default value proposed by the software. Beam-hardening correction was applied at 20% and ring-artifact correction was set to 5. For the subsequent image analysis, volumes of interest (VOIs) were extracted using CT Analyzer (Bruker microCT N.V., Kontich, Belgium). The ulnar VOIs were centered at the mid-third diaphysis, expanding for about 20% of total bone length. This location was shown to receive the most flexion in response to axial forelimb compression (Herman et al., 2010; Zhang et al., 2018). A global threshold of 75/255 (0.45 g/cm³) was set for the following image analysis, which was determined by an automatic thresholding function in CT Analyzer and visually verified in the images and histograms.

To calculate the weekly (re)modeling parameters between weeks 9 and 12, the ulnar scans from weeks 10, 11 and 12 were rigidly registered to the baseline scans of week 9 using custom software

developed using ITK (Kitware Inc., Clifton Park, NY, USA) (Johnson et al., 2015). Automatic registration was based on a regular step gradient optimizer with mutual information as the optimization metric. Once the images were registered, a previously validated MATLAB-based program was used to calculate the (re)modeling parameters on the periosteum (Birkhold et al., 2014b). The following (re)modeling parameters were analyzed in terms of volume, surface, and thickness: newly mineralized bone volume per bone volume (MV/BV), newly eroded bone volume per bone volume (EV/BV), newly mineralized bone surface per bone surface (MS/BS), newly eroded surface per bone surface (ES/BS), mineral apposition rate (MAR, $\mu\text{m}/\text{day}$), and mineral resorption rate (MRR, $\mu\text{m}/\text{day}$). The static histomorphometry was performed using CT Analyzer (Birkhold et al., 2016; Bouxsein et al., 2010). The following cortical ulnar parameters were evaluated: tissue mineral density (TMD, g/cm^3), cortical bone volume (Ct.BV, mm^3), average cross-sectional cortical area (Ct.Ar, mm^2), and average cortical thickness (Ct.Th, μm).

7.6.3 *In vivo* mechanical loading

The *in vivo* loading session was applied on week 10 (Figure 7.1). One hour before loading, the rats were given a dose of buprenorphine (0.05 mg/kg) subcutaneously as a sedative and an analgesic. Ten minutes before loading, the rats were put into anesthesia via isoflurane (3.0%) supplied by oxygen (1.8 L/min). They were laid on their back, supported by a surface warming pad (36 to 37°C). The *in vivo* loading, performed using a Mach-1 micromechanical system (Biomomentum Inc., Laval, QC, Canada), started with a preconditioning session of 50 sinusoidal cycles of cyclic compression with a frequency of 2 Hz and a displacement amplitude of 1 mm.

The subsequent main loading step was based on sinusoidal cyclic compression as well with a physiological frequency of 2 Hz and a peak force of 16 N, leading to a cumulative actuator displacement of 0.4591 mm and 4665 loading cycles on average. The loading was stopped when one of the following equivalent conditions was satisfied: the cumulative actuator displacement reached 0.60 mm, or 5040 cycles was completed. The dual stopping criteria served to prevent the forelimb from fracture, which corresponded to 58% of the fracture point (1.0391 mm of the cumulative actuator displacement) based on a preliminary test where 90% of the rats ($n=20$ in total) did not encounter a fracture within 0.60 mm of the cumulative actuator displacement (equivalent

to 5040 cycles). Right after the *in vivo* loading, the rats were given a dose of metacam (1 mg/kg) subcutaneously to relieve potential pain caused by loading. The rats were followed up 1h, 4h, 24h, 48h and 72h after the loading session for assessment of their well-being, based on which no additional analgesic was provided.

7.6.4 BaSO₄ Staining and contrast-enhanced micro-CT imaging

Once the rats were sacrificed by CO₂ asphyxiation and decapitation, the forelimbs were collected, removed of soft tissues, and dissected into individual ulnar samples. The samples were wrapped in phosphate-buffered-saline (PBS)-soaked gauze and stored in tubes of 5 mL in a freezer (-20°C). Five days after the dissection, the samples were thawed for barium sulfate (BaSO₄) contrast staining according to a previously validated method (Choudhari et al., 2016; Gargac et al., 2014; Landrigan et al., 2011; Leng et al., 2008; Turnbull et al., 2011; Zhang et al., 2018): the ulnar samples were immersed in an equally mixed solution of 0.5 M barium chloride (BaCl₂), acetone, and PBS solution for 3 days under vacuum; followed by another 3 days of staining in an equally mixed solution of 0.5 M sodium sulfate (Na₂SO₄), acetone, and PBS solution under vacuum.

After the staining, the samples were gently washed and wrapped in PBS-soaked gauze for contrast-enhanced micro-CT imaging. The scans were performed using the SkyScan 1176 at an image resolution of 8.74 µm, using a filter of 1 mm Al, exposure time of 1500 ms, voltage of 65 kVp, current of 385 µA, rotation angle of 0.25°/180°. After the scans, the samples were wrapped again in PBS-soaked gauze and put back into tubes for storage (-20°C). The micro-CT images were reconstructed using the NRecon software. The dynamic range was set to [0, 0.12] (attenuation coefficient) and the post-alignment was adjusted visually starting from a default value proposed by the software. Beam-hardening correction was applied at 20% and ring-artifact correction was set to 5.

To evaluate the microdamage accumulated in ulna, the same VOIs were used as explained in section 7.6.2. The microdamage was intended to be evaluated inside the original pre-loading bone volume. Therefore, the newly formed woven bone on the periosteum was excluded by registering the ulnar VOIs to the images of week 10 (pre-loading images). The image registration method was the same as explained in section 7.6.2. Subsequently, the VOIs were imported into MATLAB

R2018b (The MathWorks, Inc., Natick, MA, USA) for microdamage quantification. A customized script evaluated the stained volume over the total bone volume (SV/BV), which reflects the overall extent of microdamage based on methods developed in previous studies, which were validated by the histology-based methods (Turnbull et al., 2011; Zhang et al., 2018). The thresholds for the bone volume and the stained volume were 0.67 g/cm^3 and 1.77 g/cm^3 respectively, which were determined based on histogram analysis for each of the 3D datasets. The threshold for the stained volume has also been verified to be well above the dynamic range of the bone mineral density. For each pair of contralateral ulnae, the difference in SV/BV between the loaded and the control samples was calculated to derive a new parameter $\Delta\text{SV/BV}$, which reflects the overall amount of microdamage resulting from the applied *in vivo* loading, excluding the effects of daily damage as well as staining in the voids and on the bone surface. Only one pair of samples (2%) showed slightly higher SV/BV on the control sample than on the loaded sample. In this case, we took the value of $\Delta\text{SV/BV}$ to be 0 and interpreted the result as insignificant damage produced by the *in vivo* loading.

7.6.5 Three-point-bending

One week after the contrast-enhanced micro-CT, the ulnar samples were thawed to room temperature (about 23°C) for the three-point-bending procedure. The bending was performed using the Mach-1 micromechanical system and a customized bending platform. The span between the two sample supports was set to 18 mm. The samples were loaded in the medio-lateral direction (Figure 7.2) to ensure stable positioning of the samples. The actuator displacement speed was set to 0.01 mm/s and the data acquisition was performed at a frequency of 100 Hz.

After the bending tests, the force-displacement curves were analyzed using the Mach-1 Analysis software (Biomomentum Inc., Laval, QC, Canada) to measure the following structural properties: ultimate force (F_u , N), yield force (F_y , N), stiffness (S , N/mm), pre-yield displacement (D_{pre} , mm), and pre-yield energy (U_{pre} , mJ). To estimate the material (tissue) properties using beam theory equations (Burr, 2020; Burr et al., 2015; Mashiba et al., 2000; Schriefer et al., 2005; Wallace, 2019), the *ex vivo* micro-CT images of the samples were used to calculate the second moment of area, maximum diameter of the loaded section, and the distance from the neutral axis to the bone surface with the BoneJ plugin (Domander et al., 2021) of the FIJI software (Schindelin et al., 2012).

The following material properties were calculated: ultimate stress (σ_u , MPa), yield stress (σ_y , MPa), Young's modulus (E , GPa), pre-yield strain (ϵ_{pre} , $\mu\epsilon$) and pre-yield toughness (u_{pre} , MPa). The post-yield properties were not analyzed as the conversion equations are only applicable in the elastic region (Wallace, 2019).

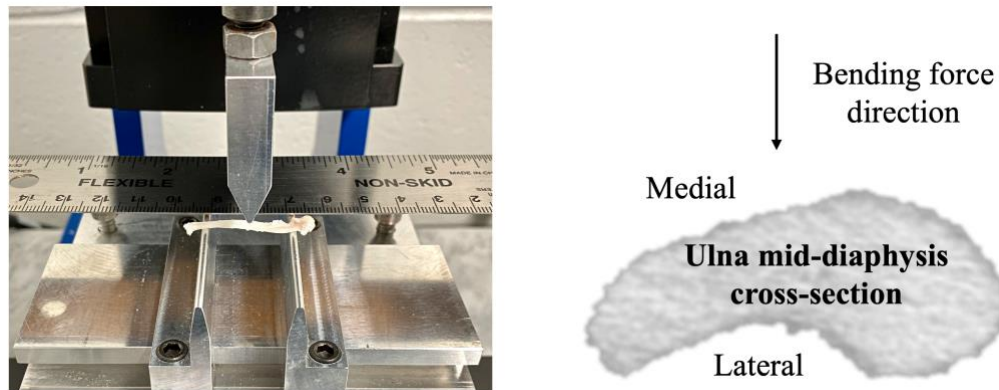


Figure 7.2 Three-point-bending setup for the ulna samples. The experimental setup is shown on the left and the cross-sectional view is shown on the right.

7.6.6 Statistics

The statistical tests were performed using SPSS Statistics v26 (IBM Corp, Armonk, NY, USA). Different ANOVA tests were performed for each parameter as shown in Table 7.1. The assumptions of the ANOVA tests were verified when applicable. The boxplots revealed very few outliers (less than 3%) which were not removed since they did not consist of data entry or measurement errors to our knowledge. The Shapiro-Wilk's test and Normal Q-Q Plots determined that the data were normally distributed in general. The Levene's test for equality of variances was performed, showing that variances were homogenous for the vast majority of the data groups. The ANOVA test was performed because it is somewhat robust to heterogeneity when the group sample sizes are equal, data are normal, and the ratio of the largest group variance to the smallest group variance is less than 3 (Altman, 1991; Jaccard, 1998; Keppel, 2004; Laerd Statistics, 2015). The Box's test determined that the homogeneity of covariances was also met for most of the data groups. As determined by the Mauchly's test of sphericity, the assumption of sphericity was violated for some data groups. Thus, the Greenhouse–Geisser correction was applied when

interpreting the within-subject effects. Bonferroni correction was applied for the p -value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05. Additional data of interest are reported for the results of bone mechanical properties ($p < 0.10$).

Table 7.1 Summary of ANOVA tests performed. The treatment factor refers to the rat group NOM, OVX and ZOL. The recovery factor refers to the sacrifice time point of day 5 or day 15 post-loading. The bone side factor refers to left (control) or right (loaded) ulna. The time points vary from week 2 to week 12 of the experiment.

Parameter	ANOVA Type	Between-subjects Factors	Within-subjects Factor
Bone microdamage	Two-way	Treatment, recovery	n/a
Bone (re)modeling	Three-way mixed	Recovery, bone side	Time Points
Bone mechanical properties	Three-way	Treatment, recovery, bone side	n/a
Static histomorphometry	Two-way mixed	Treatment	Time Points

Besides the ANOVA analyses, two-tailed t-tests were performed for the static histomorphometry parameters on week 12 to compare between left (control) and right (loaded) ulna of the same rat. The statistical significance level (alpha) was set to 0.05 for all the t-tests. The two-tailed Spearman's rank-order correlation was used to evaluate the correlations of the bone microdamage parameter SV/BV with bone (re)modeling or mechanical properties. The analysis between SV/BV and the (re)modeling parameters was only performed for the rats that were sacrificed 15 days post-loading, since the (re)modeling parameters of the day-5 rats were not available. The assumption of a monotonic relationship between all the data pairs was verified using scatterplots. The Spearman's correlation coefficient was considered significant for $p < 0.05$, and additional data of interest ($p < 0.15$) are reported as well.

7.7 Results

7.7.1 Bone microdamage

An example visualization of ulnar microdamage is shown in Figure 7.3, representative of each treatment group and the side of bone. The volume of formed, resorbed and constant bone were identified based on the registration results between the stained VOIs (week 12) and the pre-loading VOIs (week 10). The damage (stained) volume was identified based on the threshold analysis of the stained VOIs. The results of $\Delta\text{SV}/\text{BV}$ are shown in Figure 7.4. Both NOM and OVX rats had increased $\Delta\text{SV}/\text{BV}$ over time; the ZOL ulnae were at a lower level on day 15 compared to the OVX. The inter-group differences of $\Delta\text{SV}/\text{BV}$ were most significant at week 12. Therefore, the example visualization in Figure 7.3 focuses on the data from week 12.

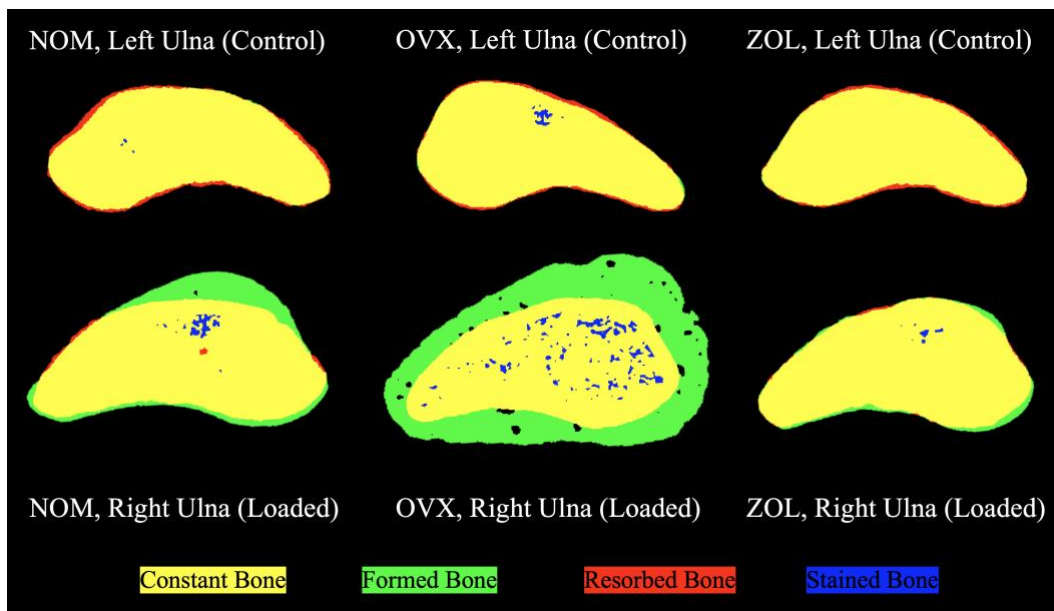


Figure 7.3 Example visualization of the ulna microdamage results shown in binarized cross-sectional area. The top row shows the control ulnae of each group, and the bottom row shows the loaded ulnae. Based on the image registration results and threshold analysis, the constant, formed, resorbed, and stained bone volume are shown in yellow, green, red, and blue, respectively.

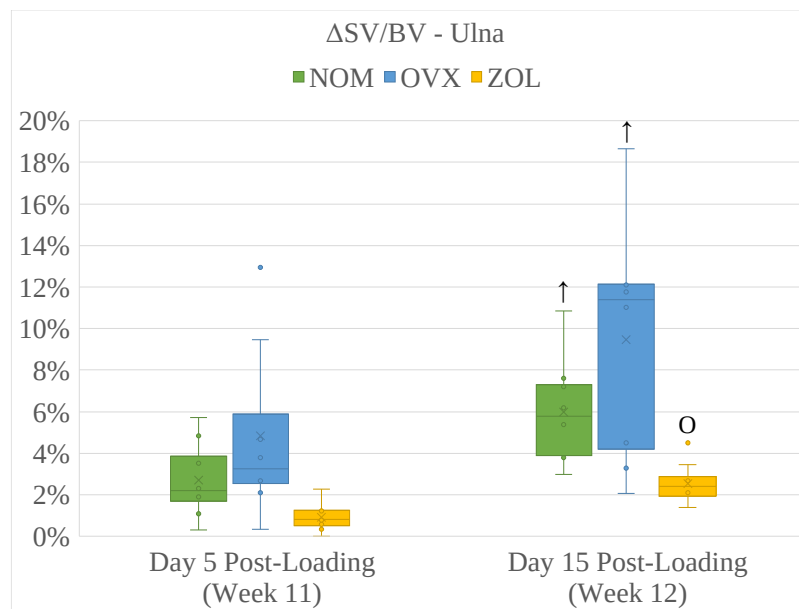


Figure 7.4 Δ SV/BV for ulna, reflecting the overall microdamage accumulated by *in vivo* loading (n=8/group). “↑” refers to a significant increase compared to the previous time point. “O” refers to a significant difference compared to the OVX group. Bonferroni correction was used for *post hoc* tests and multiple comparison adjustments, after which the statistical significance level (alpha) was set to 0.05.

7.7.2 Bone (re)modeling

The results of (re)modeling are shown in Figure 7.5. Effects of recovery time were observed for almost all the parameters. Effects of treatment groups were apparent as well. For example, the MV/BV and MS/BS showed approximatively three levels on week 12: the NOM (loaded) and OVX (loaded) on a higher level, the NOM (control) and ZOL (loaded) on the middle level, then the OVX (control) and ZOL (control) on a relatively lower level. In general, the resorption parameters showed less between-group differences than their formation counterparts. Contralateral differences of each parameter were not always clear, and statistically significant results were mostly in week 12.

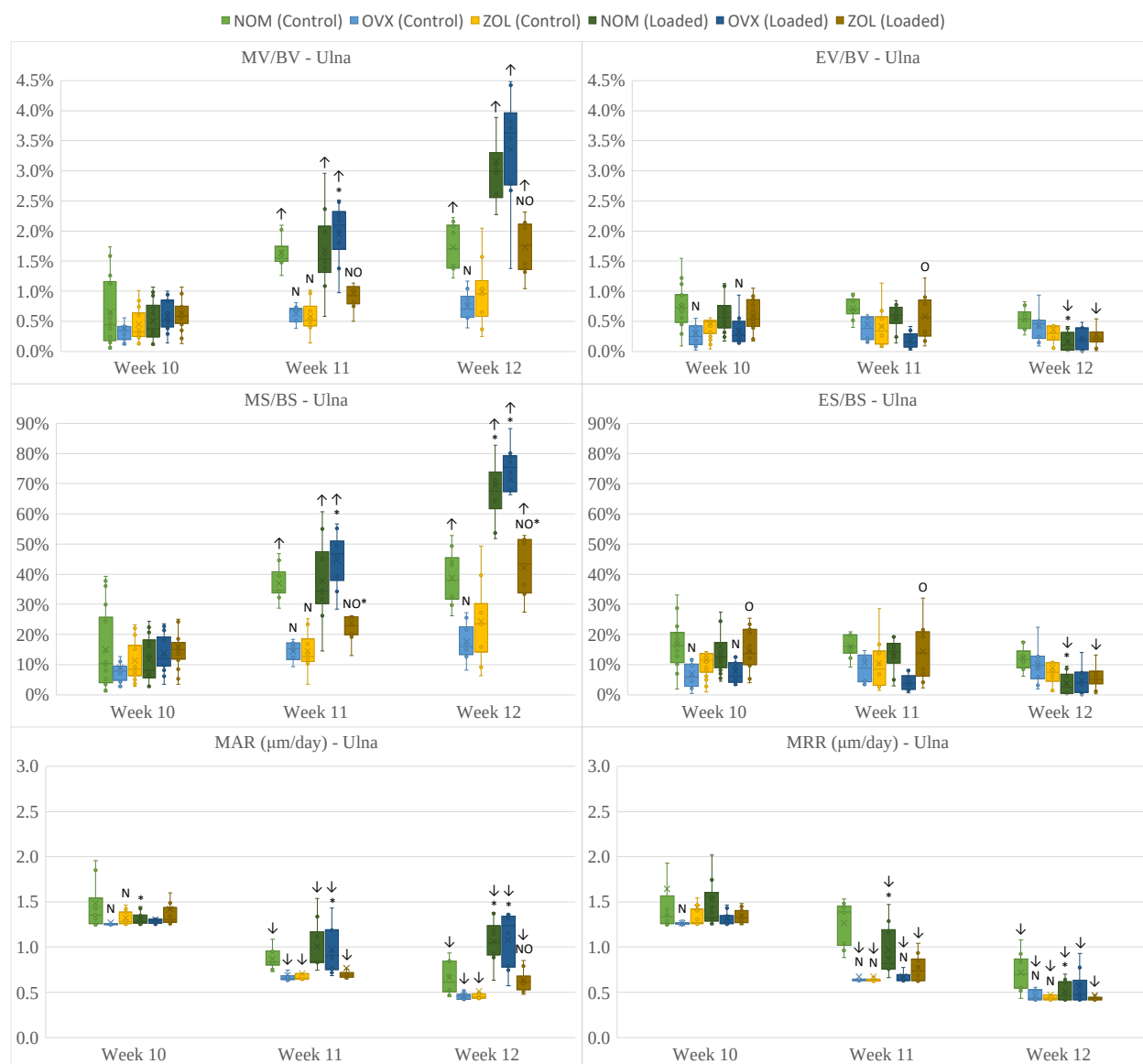


Figure 7.5 Ulnar (re)modeling parameters shown in boxplots (n=16/group at week 10, n=8/group at weeks 11 and 12). “↑” or “↓” refers to a significant increase or decrease compared to week 10. “N” or “O” refers to a significant difference compared to the NOM or OVX group. “*” refers to a significant difference between the left and right ulna. Bonferroni correction was applied for the *p*-value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05.

7.7.3 Bone mechanical properties

The results of the mechanical properties (structure and material) are shown in Table 7.2. Effects of recovery time were observed for the NOM and OVX ulnae. The ZOL ulnae showed higher F_u , F_y and S compared to the NOM or OVX ulnae at most of the relevant data points. Their material counterparts σ_u , σ_y and E showed less consistent results. There were a few data points showing contralateral differences (control vs loaded), which were mostly found in results of loaded samples on day 15 post-loading.

7.7.4 Static histomorphometry

The results of static histomorphometry are shown in Table 7.3. The effects of treatment time were significant for the OVX and ZOL rats. The ZOL ulnae (control) had higher Ct.BV than the NOM, and higher Ct.Ar than both NOM and OVX rats on week 12. Only the NOM and OVX groups showed some significant differences between their control and loaded ulnae on week 12.

7.7.5 Correlation analysis

The correlation results between SV/BV and the (re)modeling parameters are shown in Table 7.4. All the formation parameters showed significant positive correlations with the microdamage parameter SV/BV. The correlations were mostly negative for the resorption parameters, and the NOM rats had statistically significant results for all the parameters. The correlations between SV/BV and the mechanical properties are shown in Table 7.5. All the data points with statistical significance showed negative correlations.

Table 7.2 Ulnar mechanical properties tested by three-point-bending (n=8/group). The structural properties are shown on the top half of the chart, and their material counterparts are at the bottom half. “↑” or “↓” refers to a significant increase or decrease compared to day 5. “N” or “O” refers to a significant difference compared to the NOM or OVX group. “*” refers to a significant difference between the left (control) and right (loaded) ulna. Bonferroni correction was applied for the *p*-value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05. The result of a *p*-value between 0.05 and 0.10 was marked in blue. The cells of statistical significance are in bold.

Time	VOI	Group	F _u (N)	F _y (N)	S (N/mm)	D _{pre} (mm)	U _{pre} (mJ)
Day 5	Control	NOM	11.68 ± 1.37	9.06 ± 0.87	17.54 ± 2.09	0.5957 ± 0.0636	2.67 ± 0.33
		OVX	13.41 ± 1.86	10.34 ± 1.94	26.23 ± 5.66 N	0.5366 ± 0.0686	2.70 ± 0.72
		ZOL	16.42 ± 3.58 NO	11.47 ± 2.12 N	30.05 ± 13.55 N	0.5542 ± 0.1359	3.10 ± 1.02
	Loaded	NOM	11.94 ± 1.47	9.43 ± 1.23	17.70 ± 1.71	0.6159 ± 0.0559	2.87 ± 0.57
		OVX	12.69 ± 2.32	10.26 ± 1.98	25.09 ± 8.33	0.5774 ± 0.1261	2.83 ± 0.68
		ZOL	16.63 ± 3.50 NO	11.84 ± 2.66 N	26.59 ± 7.16 N	0.6056 ± 0.1025	3.43 ± 1.16
Day 15	Control	NOM	12.93 ± 2.34	10.08 ± 1.62	22.33 ± 5.84	0.6437 ± 0.0886	2.87 ± 0.48
		OVX	12.47 ± 1.38	9.37 ± 0.87	20.20 ± 3.94 ↓	0.5852 ± 0.0894	2.63 ± 0.30
		ZOL	16.65 ± 2.22 NO	11.72 ± 1.68 O	31.39 ± 5.58 NO	0.5467 ± 0.0813 N	2.99 ± 0.66
	Loaded	NOM	14.22 ± 1.61 ↑	10.39 ± 1.19	24.38 ± 3.69 ↑	0.5578 ± 0.0816 *	2.88 ± 0.62
		OVX	14.23 ± 2.22	9.87 ± 1.58	26.47 ± 7.04 *	0.4741 ± 0.0448 ↓*	2.33 ± 0.44
		ZOL	16.47 ± 2.11	11.84 ± 1.72 O	28.96 ± 4.80	0.5381 ± 0.0310	3.14 ± 0.57 O
Time	VOI	Group	σ _u (MPa)	σ _y (MPa)	E (GPa)	ε _{pre} (μϵ)	U _{pre} (MPa)
Day 5	Control	NOM	275.63 ± 14.58	214.29 ± 12.83	22.53 ± 2.10	10,998 ± 1,477	1.17 ± 0.15
		OVX	262.79 ± 20.45	202.53 ± 28.18	25.42 ± 3.07	10,718 ± 1,034	1.06 ± 0.26
		ZOL	290.13 ± 13.96	205.55 ± 26.36	24.67 ± 5.84	11,326 ± 2,368	1.16 ± 0.36
	Loaded	NOM	262.81 ± 22.70	207.34 ± 15.50	20.23 ± 1.83	11,910 ± 1,291	1.21 ± 0.18
		OVX	243.61 ± 28.97	196.84 ± 25.94	23.80 ± 6.39	11,620 ± 2,732	1.10 ± 0.27
		ZOL	288.54 ± 43.35 O	205.64 ± 34.34	21.88 ± 3.86	12,648 ± 2,102	1.25 ± 0.37
Day 15	Control	NOM	289.05 ± 22.83	214.10 ± 15.01	22.41 ± 2.32	13,507 ± 928 ↑	1.42 ± 0.18 ↑
		OVX	250.86 ± 21.28 N	188.50 ± 12.61 N	21.12 ± 3.98 ↓	11,287 ± 1,805 N	1.02 ± 0.14 N
		ZOL	283.42 ± 28.60 O	199.72 ± 23.32	25.16 ± 3.72	11,608 ± 1,876 N	1.08 ± 0.24 N
	Loaded	NOM	274.10 ± 18.20	203.02 ± 15.16	21.31 ± 1.51	13,618 ± 1,379 ↑	1.36 ± 0.20
		OVX	253.65 ± 34.43	176.50 ± 29.40 N↓	22.63 ± 3.97	9,820 ± 1,043 N↓*	0.87 ± 0.20 N↓
		ZOL	268.66 ± 23.23	192.86 ± 16.92	21.97 ± 2.52 *	11,551 ± 984 N	1.10 ± 0.17 N

Table 7.3 Ulnar static histomorphometry (n=8/group). “↑” refers to a significant increase compared to week 2. “N” or “O” refers to a significant difference compared to the NOM or OVX group. “*” refers to a significant difference between the left (control) and right (loaded) ulna. Bonferroni correction was applied for the *p*-value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05. The cells of statistical significance are in bold.

Time	VOI	Group	TMD (g/cm ³)	Ct.BV (mm ³)	Ct.Ar (mm ²)	Ct.Th (μm)
Week 2	Baseline	NOM	1.03 ± 0.03	10.04 ± 0.51	1.26 ± 0.07	157.85 ± 16.87
		OVX	0.97 ± 0.05 N	10.44 ± 0.69	1.33 ± 0.08	142.08 ± 22.36 N
		ZOL	0.99 ± 0.02	10.23 ± 1.01	1.31 ± 0.11	152.02 ± 10.48 N
Week 12	Control	NOM	1.07 ± 0.05	11.00 ± 0.80 ↑	1.35 ± 0.09 ↑	241.60 ± 9.81 ↑
		OVX	1.08 ± 0.03 ↑	12.03 ± 0.93 ↑	1.43 ± 0.11 ↑	177.92 ± 8.97 N↑
		ZOL	1.06 ± 0.06 ↑	13.01 ± 1.00 N↑	1.58 ± 0.10 NO↑	170.34 ± 26.30 N
	Loaded	NOM	1.04 ± 0.05	12.22 ± 1.16 ↑*	1.50 ± 0.16 ↑*	255.91 ± 9.02 ↑*
		OVX	1.02 ± 0.06 *	14.35 ± 1.52 N↑*	1.68 ± 0.17 ↑*	135.92 ± 42.59 N*
		ZOL	1.02 ± 0.08	12.87 ± 0.96 ↑	1.56 ± 0.10 ↑	170.35 ± 43.41 N

Table 7.4 Spearman's correlation coefficients between SV/BV and the (re)modeling parameters (n=8/group). The data are marked bold for statistical significance (“***” for *p* < 0.01, “**” for *p* < 0.05, and “†” for *p* < 0.15).

	SV/BV - Ulna					
	NOM		OVX		ZOL	
MV/BV	0.86	**	0.87	**	0.77	**
MS/BS	0.82	**	0.85	**	0.75	**
MAR	0.87	**	0.89	**	0.54	*
EV/BV	-0.72	**	-0.29		-0.48	†
ES/BS	-0.71	**	-0.27		-0.48	†
MRR	-0.51	*	0.44	†	-0.28	

Table 7.5 Spearman's correlation coefficients between SV/BV and the mechanical properties (n=16/group). The data are marked bold for statistical significance (“***” for $p < 0.01$, “*” for $p < 0.05$, and “†” for $p < 0.15$).

	SV/BV - Ulna			
	NOM	OVX	ZOL	
F_u	0.18	-0.12	-0.26	†
F_y	0.03	-0.21	-0.21	
S	0.16	-0.13	-0.19	
D_{pre}	-0.34	†	-0.13	-0.07
U_{pre}	-0.13	-0.22	-0.17	
σ_u	-0.30	†	-0.23	-0.31 †
σ_y	-0.32	†	-0.23	-0.27 †
E	-0.39	*	-0.19	-0.24
ϵ_{pre}	0.16	-0.11	-0.13	
u_{pre}	0.02	-0.14	-0.23	

7.8 Discussion

7.8.1 The zoledronate treatment improves osteopenic conditions

Ovariectomy-induced changes on cortical bone (ulna, Table 7.3) were less obvious than on trabecular bone (tibia, Supplementary Material). Evidence from the literature suggests that bone loss in ovariectomized rats was quicker in trabecular bone than in cortical bone (Jee & Yao, 2001). It is also easier to detect porosity in trabecular bone than in cortical bone using laboratory micro-CT scanners (Cooper et al., 2007). Nonetheless, the ZOL rats had lower Δ SV/BV (Figure 7.4) and higher F_u , F_y , S, σ_u , Ct.Ar (at some data points in Table 7.2 and Table 7.3) compared to the OVX rats. The beneficial effects of zoledronate treatment were found significant for improving the bone strength (F_u , σ_u) without sacrificing the bone brittleness (D_{pre} , ϵ_{pre}) (Bilston et al., 2002). Past experimental studies showed that zoledronate treatment can improve the bending strength of long bones (Aref et al., 2016; Bilston et al., 2002), which was positively correlated with BMD (Hornby et al., 2003). Trabecular (Hornby et al., 2003) and cortical (Stadelmann et al., 2011) microstructure could also be improved by the zoledronate treatment. We confirm that zoledronate can improve the bone quality of ovariectomized rats in terms of bone microstructure and bending strength (Aref et al., 2016; Gasser et al., 2008; Meixner et al., 2017).

7.8.2 The zoledronate treatment reduces cortical microdamage accumulation

The ZOL rats accumulated less microdamage than the other rats as shown by the lower $\Delta\text{SV}/\text{BV}$ compared to the OVX ulnae on day 15 post-loading. In addition, levels of $\Delta\text{SV}/\text{BV}$ remained unchanged from day 5 to day 15 in ZOL rats, while the NOM and OVX rats showed microdamage progression in ulnae (Figure 7.4). In comparison, the OVX rats had more microdamage than the NOM rats despite lacking significant differences, which might be due to the higher standard deviation of $\Delta\text{SV}/\text{BV}$ on day 15 post-loading for the OVX. In fact, the maintained damage level in ZOL rats might be associated with efficient remodeling-based damage repair (Burr, 2014; Seref-Ferlengez et al., 2014; Seref-Ferlengez et al., 2015), while the increased damage level in NOM and OVX rats might be related to an excess amount of damage propagated relative to repair in the same time period (Wang & Niebur, 2006; Wu et al., 2013). Another possible explanation is that the increase of TMD, Ct.BV and Ct.Ar in the ZOL rats could have led to a reduced strain (rate) in the cortex while receiving the external mechanical loading, so that the critical stress intensity for crack initiation or propagation was not reached (Wright & Hayes, 1976; Zioupos, 2001). Therefore, the initial amount of microdamage might be determinant for their state of repair and propagation.

Past studies with similar experimental designs on rat forelimb showed that the microdamage volume ratio (SV/BV) was the highest 5 days post-loading (Zhang et al., 2018), and the damage density (Cr.Dn) reduced after 2 weeks of recovery post-loading (Herman et al., 2010). These findings are not exactly in line with the results in the present study, where the NOM and OVX rats had increased SV/BV 15 days post-loading. These discrepancies might be caused by several differences in experimental design such as rats' gender, surgical treatments, and the methods used for microdamage quantification. A previous study using an ovariectomized ovine model revealed that the zoledronate treatment increased the average crack density but reduced average crack length based on histology analysis (Brennan et al., 2011). A study using an osteolytic rat model showed reduced SV/BV level due to zoledronate treatment, evaluated by contrast-enhanced micro-CT (Tolgyesi et al., 2022). As such, the reduced SV/BV level observed in the ZOL rats of the present study might have been caused by the reduced average length but not density, since the contrast enhanced micro-CT-based method is less sensitive than the histology-based method for damage detection. In general, the BPs treatment-related microdamage accumulation is more prominent in trabecular than in cortical bone (Allen & Burr, 2011).

Taken together, we conclude that the zoledronate treatment can reduce bone microdamage accumulation in cortical bone of osteopenic rats subjected to the same level of compressive loading. The lower accumulation of cortical microdamage might be caused by the improved structural properties in zoledronate treated rats, which might also determine the initial amount of loading-induced microdamage.

7.8.3 Microdamage accumulation correlates with periosteal bone formation

The correlation results in Table 7.4 show that more microdamage accumulation is associated with increased bone formation and decreased bone resorption on the periosteum of ulna. Based on visual observation in Figure 7.3, the bone formed on the ulnar periosteum was mostly woven bone, which can help to restore the mechanical properties after tissue damage (Uthgenannt et al., 2007; Uthgenannt & Silva, 2007; Wohl et al., 2009). Moreover, the results show that the correlation between the microdamage level and the woven bone formation is stronger for the OVX and NOM rats than the ZOL rats, which might be caused by the lower microdamage levels in ZOL rats.

A past study with similar experimental design showed that zoledronate treatment led to higher periosteal bone formation compared to vehicle treatment (Feher et al., 2010). Although ZOL rats in our study showed periosteal bone formation, this response was weaker and less correlated with microdamage accumulation compared to the NOM and OVX rats. The contrasting results might be caused by several experimental differences, where in the Feher study, the *in vivo* mechanical loading was on a milder level (15 N, 360 cycles per day) for a longer period (1 week); the zoledronate was given as a single dose of 100 µg/kg; the ovariectomized animals were six months old, which might reveal some ageing-related characteristics of bone loss; and the dynamic histomorphometry for the evaluation of bone remodeling was based on histology. Nevertheless, we can confirm the cause and effect relationship between bone microdamage accumulation and periosteal bone formation (Uthgenannt et al., 2007), the extent of which was different between the normal, ovariectomized, and zoledronate-treated animals.

7.8.4 Limitations

In this study, an ovariectomized rat model was used to represent estrogen-deficiency related bone loss in human. One of the disadvantages of rat models for microdamage studies is that rats do not have baseline intracortical remodeling due to the lack of Haversian systems (Iwaniec & Turner, 2013; Jee & Yao, 2001; Lelovas et al., 2008). This is different in the human body, which makes it harder to translate rodent-based research to clinical settings (Allen, 2017). Rabbits are advantageous in this regard because they are considered to be the smallest laboratory animals with the capability of intracortical remodeling (Buettmann & Silva, 2016; Coates & Silva, 2020; Lelovas et al., 2008; Turner, 2001). Nonetheless, it is possible to initiate intracortical remodeling in rats with external stimulations such as mechanical loading (Bentolila et al., 1998). The age of the rats is also an important factor for bone loss. The ovariectomy-induced bone loss is often considered osteopenic rather than osteoporotic at 3 months old (Iwaniec & Turner, 2013; Jee & Yao, 2001; Kimmel et al., 2020; Lelovas et al., 2008), which is considered as a “mature rat model”, in contrast to the “aged rat model” of 6 to 12 months old (Kalu, 1991). Besides the advantages of shorter experimental duration and greater availability from animal suppliers, the 3 month old rat model is also meaningful to investigate the microdamage accumulation in young adult populations who undergo rigorous physical training (Bigosinski et al., 2010; Cole et al., 2020; Hod et al., 2006).

The analgesics used for the *in vivo* mechanical loading posed potential effects on bone metabolism. Although buprenorphine is usually considered a safe analgesic for laboratory animals (Guarnieri et al., 2012), clinical findings suggest that it can reduce bone density by altering the osteoblastic activity (Coluzzi et al., 2015). Meloxicam (metacam), a type of nonsteroidal anti-inflammatory drug (NSAIDs), can potentially inhibit osteoblastic activities thus reduce bone formation (Burch et al., 2021; Chang et al., 2005; Cottrell & O'Connor, 2010; Pountos et al., 2012). Though we still observed significant periosteal bone formation post-loading, these responses might have been stronger without the use of meloxicam. Nonetheless, all the rats received the same analgesic dosage in the present study to ensure meaningful between-group comparisons. The adverse effects of the analgesics were assumed to be negligible.

The present study leveraged the non-destructive nature of the *in vivo* micro-CT imaging to evaluate bone (re)modeling. However, rats' remodeling cycle is generally longer than the weekly scan

interval (Baron et al., 1984; Jilka, 2013; Van et al., 1982; Weinstein et al., 1998). Therefore, the (re)modeling parameters obtained were not representative of a complete formation or resorption cycle, but rather local averaged rates including both modeling and remodeling events. Furthermore, the repeated anesthesia and immobilization associated with the *in vivo* imaging might have brought adverse effects to the animals' well-being (Hohlbaum et al., 2017; Hotchkiss et al., 1998; Kádár et al., 2007; Kim et al., 2006; Patterson-Buckendahl et al., 2001; Stevens et al., 1975; Stollings et al., 2016). There is no direct evidence that the repeated irradiation performed caused significant bone loss to the scanned sites. Newer generations of micro-CT scanners might improve the image quality while reducing the scan duration, thus reducing the radiation doses. Deep learning algorithms also showed great potential for improving the interpretation of micro-CT scans (Wang et al., 2020).

In regard to the contrast-enhanced micro-CT imaging for microdamage evaluation, we used the same image resolution as for the *in vivo* scans but with higher voltage and current, longer exposure time, and more projections. This level of resolution was shown to be sufficient for detecting BaSO₄ stained microdamage in rodent long bones (Turnbull et al., 2011) and human samples (Landrigan et al., 2011). Despite not being able to detect individual microdamages at this level of detail, the micro-CT-based SV/BV was proved to be well correlated with the histology-based measurement of microcrack density (Cr.Dn) (Landrigan et al., 2011; Zhang et al., 2018). Future studies can leverage the power of deep learning algorithms for microdamage detection in micro-CT images (Buccino et al., 2023; Caron et al., 2023).

Lastly, the process of BaSO₄ staining might cause some alteration of the mechanical results. To account for this possibility, we conducted a preliminary test with 6 pairs of contralateral forelimb samples, where the results showed that none of the mechanical properties were significantly different between the contralateral ulnar samples. Therefore, we assumed that the between-group differences were not significantly changed by staining since all the samples went through the same processing steps. We considered that the advantages of reusing the same animals for mechanical testing outweighed their disadvantages based on the 3R principles (Sneddon et al., 2017).

7.9 Conclusion

In the present study, we developed and exploited an *in vivo* experimental platform combining the evaluation of bone microdamage, (re)modeling, mechanical properties as well as their correlations in the same cohort of animals receiving different treatments. Based on our results and evidence from the literature, we conclude that zoledronate treatment can reduce cortical microdamage accumulation and improve osteopenic conditions of ovariectomized rats, which might be explained by the enhancement of several bone structural properties. Microdamage accumulation showed positive correlations with periosteal bone formation, while exhibiting negative correlations with bone resorption and mechanical properties. The extent of these correlations is different in normal, ovariectomized and zoledronate-treated animals. Our results provide insights towards further investigation of pharmaceutical treatments for osteoporosis and osteopenia. The same experimental concept can be applied in future studies on microdamage and drug testing.

7.10 Acknowledgements

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7.12 References

Please refer to the general list of references provided at the end of the dissertation (page 152).

7.13 Supplementary Material: Analysis of trabecular tibia

The right hindlimb (proximal metaphysis) of all the rats were scanned by micro-CT on week 2 and week 12 to confirm the effects of ovariectomy and zoledronate treatment. The scan settings were the same as explained in section 7.6.2 of the article. The proximal tibial VOIs of 2 mm long were taken starting from 2 mm distal to the proximal growth plate. The trabecular VOIs were then segmented using a semi-automatic selection tool in CT Analyzer. A global threshold of 80/255 (0.49 g/cm³) was set for the following trabecular analysis, which was determined by an automatic thresholding function in CT Analyzer and visually verified in the images and histograms. The following trabecular parameters were analyzed: bone mineral density (BMD, g/cm³), trabecular bone volume (Tb.BV, mm³), trabecular bone surface area (Tb.BS, mm²), trabecular bone volume fraction (Tb.BV/TV), trabecular thickness (Tb.Th, μ m), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, μ m), and connectivity density (Conn.Dn, 1/mm³).

Table 7.6 Tibial dynamic histomorphometry (n=16/group). “↑” or “↓” refers to a significant increase or decrease compared to week 2. “N”, “O”, or “A” refers to a significant difference compared to the NOM group, the OVX group, or all the other groups. Bonferroni correction was applied for the *p*-value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05. The cells of statistical significance are in bold.

Time	Group	BMD (g/cm ³)			Tb.BV (mm ³)			Tb.BS (mm ²)			Tb.BV/TV (1)		
Week 2	NOM	0.44 ± 0.05	A		9.26 ± 1.13			555.12 ± 75.28			45.15% ± 8.83%	A	
	OVX	0.28 ± 0.04	A		5.16 ± 1.44	N		460.73 ± 122.69			21.95% ± 5.59%	A	
	ZOL	0.38 ± 0.03	A		8.24 ± 1.23	O		630.64 ± 124.22	O		35.11% ± 4.88%	A	
Week 12	NOM	0.47 ± 0.05	A		10.15 ± 1.21	A		548.31 ± 76.30	A		49.83% ± 6.65%	A	
	OVX	0.22 ± 0.03	A↓		3.26 ± 0.68	A↓		233.60 ± 57.50	A↓		17.71% ± 2.48%	A	
	ZOL	0.66 ± 0.06	A↑		22.57 ± 5.77	A↑		704.38 ± 109.81	A		73.09% ± 7.74%	A↑	
Time	Group	Tb.Th (μ m)			Tb.N (1/mm)			Tb.Sp (μ m)			Conn.Dn (1/mm ³)		
Week 2	NOM	67.73 ± 7.42	A		6.63 ± 0.79			88.52 ± 9.68			2579.78 ± 350.05		
	OVX	49.37 ± 6.04	A		4.46 ± 1.08	N		131.10 ± 30.91	N		1700.91 ± 579.30		
	ZOL	55.82 ± 5.56	A		6.29 ± 0.88	O		94.82 ± 11.31	O		2661.36 ± 648.74	O	
Week 12	NOM	73.27 ± 7.14			6.80 ± 0.58	A		84.80 ± 6.56	A		2607.99 ± 421.45		
	OVX	72.14 ± 6.68	↑		2.48 ± 0.49	A↓		153.97 ± 14.28	A		949.94 ± 297.08	A↓	
	ZOL	89.96 ± 10.59	NO↑		8.17 ± 0.75	A↑		67.95 ± 8.46	A↓		2482.04 ± 738.30		

CHAPTER 8 GENERAL DISCUSSION

This chapter summarizes the key experimental concepts explored in the present dissertation. Each sub-section presents how the method was developed or adapted, followed by a brief discussion on its advantages and limitations. This discussion chapter is complementary to the discussions in each article (Chapter 4, Chapter 5, Chapter 7) and the ones in Chapter 6.

8.1 Animal models

Two animal models were used in the present dissertation: a pubertal rat model (Chapter 4 and Chapter 5) to study longitudinal bone growth, and a mature rat model (Chapter 7) to study microdamage accumulation in the context of estrogen-related bone loss. Rats are among the most popular laboratory animal models thanks to extensive evidence in the literature, wide availability, and low costs (Lelovas et al., 2008). Although the rat's skeleton is not the same as in the humans body, it still bears resemblance in response to mechanical stimulations and drugs (Frost & Jee, 1992). The pubertal rat model (1 to 2.5 months old) has been studied in the literature regarding the various types of external stimulation on bone growth (Huang et al., 2003; Kutsenko et al., 2021; Mustafy, Londono, Moldovan, et al., 2019). Rats reach sexual maturity with reduced longitudinal bone growth around 2.5 to 3 months old (Álvarez et al., 2000; Hunziker & Schenk, 1989), and the ovariectomized rats have been used to represent post-menopausal bone loss in humans (Feher et al., 2010; Kalu, 1991; Xu et al., 2022). Associated with estrogen deficiency, weight gain can be significant 2 weeks after surgery (Peng et al., 1997; Witte et al., 2010), and bone loss can be stable 8 weeks later (Jee & Yao, 2001; Lelovas et al., 2008).

Although rat models are important in pre-clinical research, there are several notable differences between rat and human long bones. Firstly, rodents do not encounter growth plate closure at adulthood, which is different than in humans (Allen, 2017). Therefore, the bone formation and resorption rates measured in rodent bones could be originated from both bone modeling and remodeling activities. Besides, rats lack a well-developed Haversian system, which is responsible for the remodeling-mediated microdamage repair (Iwaniec & Turner, 2013). However, the mechanical stimulation was shown to stimulate intracortical remodeling (Bentolila et al., 1998). Both of these physiological differences have been well accounted for in our *in vivo* studies in

Chapter 4, Chapter 5, and Chapter 7, where we used the “(re)modeling” term in the case of young rats, and applied *in vivo* mechanical loading in the microdamage study. Lastly, the present dissertation only investigated bone growth in rat tibiae and cortical microdamage in rat forelimbs (ulnae and radii). Further studies could explore more skeletal locations such as the femur, humerus, and vertebrae as the bone growth and microdamage mechanisms might show different traits depending on the bone type.

8.2 *In vivo* micro-CT imaging

In vivo micro-CT imaging was the most important experimental method used in the present dissertation. Using a SkyScan 1176 microtomograph system, it was possible to follow up the microstructural changes in rat long bones over time:

- *In vivo* micro-CT imaging was applied to quantify longitudinal bone growth rate, which was validated against the conventional histology-based method in Chapter 4.
- Bone growth, growth plate thickness, and bone (re)modeling rates were evaluated in a pubertal rat model undergoing different levels of mechanical loading in Chapter 5.
- Bone (re)modeling related to microdamage accumulation and bone microstructural changes through time were quantified in Chapter 7.

The primary advantage of the *in vivo* micro-CT imaging method lies in its non-destructive nature, as the same animals can be imaged repeatedly to quantify time lapse microstructural changes.

However, there are limitations associated with *in vivo* imaging. Firstly, the X-ray-based CT scans irradiate bone tissues and might result in adverse effects, especially with repeated scans. Several studies in the literature report effects of different radiation dosages on rodent bones (Klinck et al., 2008; Mustafy, Benoit, et al., 2018; Pendleton et al., 2021), and a weekly dosage of 0.83 Gy for 7 scans in total was considered safe for rats (Mustafy, Benoit, et al., 2018). Secondly, the image resolution of the laboratory micro-CT scanners might be limiting for certain study designs. The majority of *in vivo* micro-CT scanners can provide an image resolution of 9 to 18 μm (Brouwers et al., 2007; Scheuren et al., 2020; Wehrle et al., 2019). While this level of detail is usually enough

to detect microstructural changes in small animal models, it does not allow to visualize cortical porosity or microdamage (Cooper et al., 2007; Palacio-Mancheno et al., 2014) without the use of contrast agents. Consequently, there is always a compromise between image resolution and radiation dose: finer images can provide more details about the bone microstructure, but they also come with higher radiation doses. In Chapter 4, a resolution of 18 μm was used, assuming that the validation based on a coarse image resolution is transferable to finer resolutions. In Chapter 5, a resolution of 18 μm was used based on a preliminary dosimetry study in the context of repeated weekly scanning (Mustafy, Benoit, et al., 2018). In Chapter 7, a finer resolution of 9 μm (8.74 μm) was used to ensure a more precise weekly (re)modeling evaluation. All in all, the choice of image resolution was based on the objectives of the studies. The last limitation is related to the repeated anesthesia and immobilization of animals. Studies in the literature have extensively investigated these concerns that can lead to pathological consequences (Kádár et al., 2007; Kim et al., 2006; Patterson-Buckendahl et al., 2001; Pereira et al., 2021; Stevens et al., 1975; Stollings et al., 2016). The overall risk was considered mild in a European severity classification study (Hohlbaum et al., 2017). Still, some care is required when interpreting the results of the repeatedly scanned animals.

8.3 3D image registration

3D image registration made it possible to compare micro-CT scans taken from different time points by transforming them to the same spatial coordinates (Johnson et al., 2015; Waarsing et al., 2004). In this dissertation, we used an image registration method for the evaluation of longitudinal bone growth (Chapter 4 and Chapter 5), of bone (re)modeling (Chapter 5 and Chapter 7), and of woven bone identification (Chapter 7). The transform applied in all these scenarios was rigid with 6 degrees of freedom (3 rotations and 3 translations) based on a hypothesis that bone does not deform significantly (Stern et al., 2015). The optimizer, metric and interpolation methods were tested in the preliminary experiments for an optimal choice. In the end, we applied the regular gradient optimizer, mutual information similarity metric, and tri-linear interpolation to ensure consistent registration results with acceptable computation time. A multi-resolution strategy was applied in all the registrations, where the calculation started with a coarse resolution, followed by finer and eventually the original resolutions to converge faster, thus potentially saving computation time.

Although 3D image registration was successful in time-lapse analysis of micro-CT images, there were a few limitations. First of all, the algorithm still needs a robust initialisation to be able to find the global optimum. This initialisation can be automatic when the morphological changes in micro-CT scans are limited. However, manual initialisation was given when registering the images of young rat bones undergoing peak growth. Thus, the overall methodology was not completely free of human interventions. Furthermore, the registration process can be time consuming depending on the hardware and the image file sizes. In our case, the registration of one image pair can take from 10 minutes to over 2 hours depending on the dataset. It can take several days to perform the registration task for all the images in a single study. Nonetheless, the calculation itself does not require manual inputs and can be completed as background tasks. There is increasing interests in learning-based algorithms to improve the degree of automation, which can potentially eliminate the influence of human operations (Asgharzadeh et al., 2020; Eppenhof & Pluim, 2019; Sergeev et al., 2012).

8.4 Bone growth quantification

Bone growth is conventionally evaluated by histology-based methods, which are laborious to perform and require animal sacrifice (Álvarez et al., 2000; Villemure & Stokes, 2009). In Chapter 4, we validated a novel micro-CT-based method versus the histology-based method for growth quantification. Empowered by image registration, the micro-CT-based method was able to follow the growth curve of each animal in a longitudinal manner. The image registration was based on a double-stage strategy, where the first stage was focused on the alignment of cortical bone and the second stage on the alignment of trabecular bone (de Bakker et al., 2015). In the end, the growth distance could be obtained from the registration outputs. Our study showed reproducible results and significantly high correlation between the measurements of the micro-CT-based and histology-based methods.

The micro-CT-based evaluation of bone growth inherits a limitation from the *in vivo* micro-CT imaging, which is the image resolution. In comparison, the histology-based method can provide growth evaluation at a cellular level (Cancel et al., 2009; Ménard et al., 2014; Valteau et al., 2011), which is not possible with the current generations of *in vivo* micro-CT scanners. To take advantage

of the non-invasive measurement with the micro-CT-based method, it is important to properly select the image resolution and its associated scanning interval depending on the animal model. Older rats with slower growth would require finer resolutions and longer scanning intervals. Younger rats with faster growth increase the difficulty of image registration, since it is more difficult to track the morphological changes. Nevertheless, the micro-CT-based method serves as a good alternative to the histology-based method and is the only documented method providing *in vivo* longitudinal measurements of bone microstructure.

8.5 Bone (re)modeling quantification

The conventional method for quantifying remodeling is also based on histology, which requires animal sacrifice (Dempster et al., 2013). The micro-CT-based evaluation of bone (re)modeling bears the advantage of non-destructive *in vivo* analysis. First of all, 3D image registration was performed on time-lapse scans of the same bone. Since we evaluated bone (re)modeling on cortical bone, a single stage of registration on cortical bone was necessary, compared to the two-step process for trabecular bone registration. Afterwards, a MATLAB-based algorithm was applied on the registered images to perform (re)modeling evaluation. For this, we adapted an algorithm developed by Dr. Bettina M. Willie's group, which has been previously validated against the histology-based method (Birkhold et al., 2014b).

In the subsequent studies (Chapter 5 and Chapter 7), the algorithm was applied on the images of rat tibia, radius, and ulna by adjusting the segmentation parameters. The micro-CT-based evaluation of bone (re)modeling has the same limitation regarding image resolution as discussed in Sections 8.1 and 8.4. Finer resolutions would enable more accurate evaluation of the (re)modeling events, which can be as low as several microns per week. However, this would also impose a higher radiation dose on the animals, which would reduce the number of longitudinal scans that can be acquired safely. There has been active research in improving micro-CT images using deep learning algorithms (Buccino et al., 2023; Wang et al., 2020), which might make it possible to acquire low-radiation scans and output high-quality images.

8.6 Contrast-enhanced micro-CT imaging

The gold standard for quantifying microdamage was based on histology evaluation of fuchsin-stained samples (Burr et al., 1985; Fazzalari et al., 1998). In the past decade, microdamage evaluation based on contrast-enhanced micro-CT imaging has been developed and validated in the literature using contrast agents such as lead-uranyl acetate (Tang & Vashishth, 2007, 2010) and barium sulfate (Gargac et al., 2014; Landrigan et al., 2011; Turnbull et al., 2011; Zhang et al., 2018). Due to the same limit on image resolution as discussed in the previous sub-sections (8.1, 8.4 and 8.5), contrast-enhanced micro-CT imaging cannot provide detailed damage morphologies as it is possible with histology.

However, the key advantage of the micro-CT-based method here is that the samples can be kept intact for subsequent analysis such as mechanical testing. In our study in Chapter 7, we were able to reuse the same animals for the evaluation of bone (re)modeling, microdamage and mechanical properties, which would require at least double amount of the animals if the histology-based method was applied. Thanks to this study design, we were able to demonstrate correlations between microdamage accumulation, bone (re)modeling and mechanical properties. Further improvements can be made to achieve more detailed damage evaluation using synchrotron radiation micro-CT (SR micro-CT) (Harrison, 2022) and deep learning algorithms (Caron et al., 2023).

8.7 *In vivo* mechanical loading

In vivo mechanical loading was applied in two different scenarios in the present dissertation: loading on rat hindlimb as an external stimulation during rat puberty (Chapter 5) and loading on adult rat forelimb to create microdamage in cortical bone (Chapter 6, Chapter 7). Both types of *in vivo* mechanical loading were adapted from commonly used models in the literature (Mustafy, Londono, Moldovan, et al., 2019; Stadelmann et al., 2015; Uthgenannt & Silva, 2007; Wang & Niebur, 2006). In both cases, the loading sessions were performed while rats were under isoflurane anesthesia, in form of cyclic compression with a physiological frequency of 2 Hz.

Applied weekly (5 days/week) during the entire rat puberty, hindlimb loading was used to represent three different levels of exercises based on strain gauging: walking, running, and jumping. The

loading was applied through haversine curves of different amplitudes. The results showed that the high impact loading led to increased bone (re)modeling and altered bone growth of young rats (Chapter 5). As for the forelimb loading, it was only applied once to generate *in vivo* microdamage. A peak force of 16 N was fixed and the loading was given based on sinusoidal curves till about 58% of the fracture point. Based on evaluation by contrast-enhanced micro-CT imaging, the forelimb loading model was able to produce consistent levels of microdamage (Chapter 6, Chapter 7).

8.8 Three-point-bending

In Chapter 6 and Chapter 7, three-point-bending tests were performed on radius and ulna samples to assess their mechanical properties. The bending procedure generated force-displacement curves, which were used to measure structural properties such as force, stiffness, displacement, and energies. To evaluate the material properties, cross-sectional geometries were considered using micro-CT scans, deriving parameters such as stress, elastic modulus, strain, and toughness (Burr et al., 2015; Schriefer et al., 2005; Wallace, 2019). Our results showed different mechanical properties between rat groups, highlighting the benefits of the zoledronate treatment (Chapter 7).

However, the results were not always statistically significant, which might be caused by several factors including individual animal differences (Prodinger et al., 2018), sample processing and storage (Tiefenboeck et al., 2020), and the accuracy of the three-point-bending itself (Wallace, 2019). Four-point bending is considered a better alternative thanks to its ability to reduce the impacts of shear stresses around the loading tips (Aref et al., 2016; Wallace et al., 2007). However, both of the loading tips in the four-point-bending design need to receive the same force, which is difficult to achieve with bones that have irregular shapes along their longitudinal axis (Wallace, 2019). Another alternative mechanical testing method is the indentation test, which shows great advantages for *in vivo* testing of bone material properties (Allen et al., 2015; Hao et al., 2015; Liu et al., 2017; Seref-Ferlengez et al., 2014), for which the testing standards of the reference point indentation (RPI) have been developed in recent studies (Setters & Jasiuk, 2014; Thiagarajan et al., 2018). The downsides of the RPI techniques include the localized nature of the measurements

(Carriero et al., 2014; J. Wang et al., 2019) and their inability to separate the responses of elasticity and plasticity unlike the conventional tensile and compressive tests (Chang et al., 2018).

8.9 3D experimental platform

Overall, the 3D experimental platform developed in the present thesis provided synchronous evaluation of bone growth, (re)modeling, microdamage and mechanical properties in animal models. Thanks to the micro-CT-based methods empowered by advanced image processing algorithms, the direct relationships between the critical bone mechanisms and properties have been analyzed. The same methodologies can be applicable to future preclinical studies on pubertal skeletal development as well as bone loss management. Clinical studies on drug testing and pharmaceutical treatment optimization can also benefit from similar experimental techniques by performing customized adaptation and implementation in accordance with the clinical imaging modalities. The developed experimental approach shows great potential in both academic and industrial research settings.

Moreover, the present experimental platform has been developed under an ISO-13485 Quality Management System (QMS) within the TransMedTech Institute (Montréal, Canada), where all the experimental procedures have been strictly carried out according to the relevant documentations. The key equipments, such as the SkyScan 1176 micro-CT and the Mach-1 micromechanical system, have been calibrated and validated periodically according to the manufactures' guidelines. Management protocols, such as project planning, data management and risk mitigation, have also been well carried out with the help of the quality assurance personnel. The implementation of the QMS within this PhD project contributed to ensure the standardized experimental processes and data integrity, enhancing research quality and ethical standards.

CHAPTER 9 CONCLUSION AND RECOMMENDATIONS

9.1 Conclusion

The general objective of the present dissertation was to develop an experimental platform, which enables quantification of bone growth, (re)modeling, microdamage, and mechanical properties in laboratory rats. First of all, an *in vivo* micro-CT-based method for bone growth quantification was developed and validated against the conventional histology-based method. Then, an algorithm for bone (re)modeling quantification based on *in vivo* micro-CT was adapted and applied in a pubertal rat model. Furthermore, a method for microdamage quantification was adapted using contrast-enhanced micro-CT, and it was tested in a forelimb microdamage model using normal and ovariectomized rats. Lastly, the micro-CT-based toolsets were applied in an ovariectomized rat study with zoledronate treatment, in which bone microdamage, (re)modeling, and mechanical properties were quantified, and their correlations were demonstrated.

The first specific objective was to develop and validate an *in vivo* micro-CT-based method, which evaluates longitudinal growth rates in rat long bones (Section 3.3.2, point 1). In Chapter 4, a validation study was performed to compare the bone growth measurements performed by the micro-CT-based and the histology-based methods. The results showed that both methods were able to produce consistent measurements, where the micro-CT-based method showed higher precision. There were low relative errors and a significant correlation between the paired results from the two methods.

The second specific objective was to adapt and apply an *in vivo* micro-CT-based method, which evaluates 3D bone (re)modeling rates in rat long bones (Section 3.3.2, point 2). In Chapter 5, an *in vivo* micro-CT-based algorithm for bone (re)modeling evaluation was adapted and applied in a retrospective data analysis study. Longitudinal bone growth and growth plate thickness were evaluated as well to investigate the effects of different levels of mechanical loading on growing rats. The results showed that impact loading intensified bone (re)modeling and altered bone growth of pubertal rats. The impact loading might bring different effects on the distal and proximal growth plates due to their anatomical differences. The high impact loading, stimulating bone growth and (re)modeling, might have caused catch-up growth and osteochondrosis in response to the loading-induced growth inhibition.

The third objective was to adapt and apply a contrast-enhanced micro-CT-based method, which evaluates 3D bone microdamage accumulation in rat cortical bone (Section 3.3.2, point 3). In Chapter 6, preliminary experiments were performed to adapt a contrast-enhanced micro-CT method for the quantification of cortical bone microdamage. Firstly, a forelimb loading protocol has been developed to create microdamage in rat cortical bone. Once the loading setup was determined, normal and ovariectomized rats were loaded till forelimb fracture to determine the fracture point in terms of number of loading cycles and actuator displacement. The loaded samples were then collected and tested for the contrast-enhanced micro-CT method, including a BaSO₄ staining procedure. The results showed that the forelimb loading model was able to produce consistent microdamage levels, detectable by the contrast-enhanced micro-CT imaging. Moreover, the three-point-bending test was applied on the forelimb samples, including radius and ulna, to assess their mechanical properties. A preliminary study compared the mechanical properties of freshly collected bone samples and those that undergo the BaSO₄ staining procedure. The results showed that the staining procedure did not significantly change the mechanical properties of the samples, except for one parameter (σ_u on radius). These preliminary experiments were deemed successful towards the main microdamage experiment of the next objective.

The fourth and final objective was to conduct a concurrent evaluation of bone microdamage, (re)modeling, and mechanical properties in healthy, ovariectomized, and BP-treated adult rats (Section 3.3.2, point 4). In Chapter 7, an *in vivo* experimental platform was showcased by applying the methodology acquired through previous objectives of the present dissertation. The results showed reduced microdamage accumulation and improved structural qualities for the zoledronate-treated rats compared to the ovariectomized rats. The microdamage accumulation showed strong correlation with the periosteal bone formation, and this response was different between the different treatment groups.

To sum up, the present dissertation developed and validated an *in vivo* micro-CT based method for bone growth evaluation. Thanks to its non-destructive nature, the micro-CT-based method can serve as a good alternative to histology for bone growth quantification and allows *in vivo* longitudinal studies. Combining the non-invasive evaluation of bone growth and (re)modeling, this was one of the first studies to demonstrate the effects of mechanical loading on bone growth and (re)modeling through the entire rat puberty based on evidence from weekly *in vivo* micro-CT scans.

Furthermore, an *in vivo* experimental platform has been developed for concurrent evaluation of bone microdamage, (re)modeling, and mechanical properties. The results provided guidance to further optimization of pharmaceutical treatments for bone loss. Similar experimental methodologies can be applied in future experimental studies on bone microdamage and drug testing.

9.2 Responses to the research questions

This dissertation included four research questions. They are recalled below together with their corresponding responses, based on completed experiments and associated results:

1. Is the *in vivo* micro-CT-based method a reproducible alternative to the conventional histology-based method to evaluate the longitudinal bone growth?

As shown in Chapter 4, the *in vivo* micro-CT-based method could replace the histology-based method for the quantification of longitudinal bone growth. The micro-CT-based evaluation is especially useful for *in vivo* studies requiring repeated measurements.

2. Does physiological cyclic compression applied during puberty influence the longitudinal bone growth and bone (re)modeling of growing rats?

As shown in Chapter 5, the impact loading inhibits proximal growth, and the high impact loading promotes total growth in tibia of young rats. (Re)modeling activities can be increased by loading as well.

3. How much cyclic compressive loading can lead to microdamage accumulation and eventually fractures in adult rat cortical bone?

As shown in Chapter 6, cumulative actuator displacements of 0.60 mm and 1.04 mm can lead to microdamage accumulation and eventually fractures in rat forelimb, respectively.

4. What is the extent of microdamage accumulation in normal, ovariectomized and BP-treated adult rats?

As shown in Chapter 7, the zoledronate-treated rats had lower amount of microdamage compared to the normal and ovariectomized rats. The ovariectomized rats seemed to have

accumulated the highest level of microdamage, though not statistically different from the normal rats.

9.3 Recommendations for future studies

The following aspects could be explored in future experimental studies:

- Testing different animal models, bone types and structures.

A pubertal rat model was studied for bone growth and (re)modeling, and an osteopenic rat model was investigated for the effects of zoledronate treatment on microdamage accumulation. Testing different animal models (e.g., different age, gender, species, bone types and structures) might bring more insights regarding the bone metabolism and biomechanics studied in this dissertation. For example, using a senile rat model around 18 to 24 months old could reveal more ageing-related changes (N. Wang et al., 2022; Zhou et al., 2022).

- Testing different *in vivo* loading regimes.

Three different levels of weekly loading were applied in the pubertal rat model for the simulation of sportive exercises, and one single level of loading was conducted in the osteopenic rat model to create *in vivo* microdamage. Testing different loading regimes (e.g., different duration, frequency, amplitude, number of cycles, actuator displacement, types of loading devices) might lead to different experimental observations, therefore improve our knowledge of the mechanobiology of external stimuli in bone. For example, applying daily *in vivo* loading instead of a single session could more closely simulate how microdamages are accumulated in daily lives (Burr et al., 1997; Liu et al., 2019).

- Testing different imaging modalities and algorithms.

Nearly all of the micro-CT-based measurements acquired in this dissertation were based on a single micro-CT scanner (SkyScan 1176) at two different image resolutions (9 and 18 μm). Performing similar experiments using scanners of newer generations (e.g., different micro-CT, SR micro-CT, nano-CT) and learning-based image processing algorithms might improve the overall image quality, which can potentially deepen our understanding of the bone mechanisms investigated in this dissertation. For example, an SR micro-CT scanner could provide more

detailed bone morphologies compared to laboratory micro-CT scanners (Harrison, 2022; Larrue et al., 2011), though the VOI sizes of the formers are more restrictive (Turner et al., 2006).

- Testing different treatments for the osteoporotic/osteopenic animal model.

Zoledronate, a type of bisphosphonate, is only one of the numerous pharmaceutical treatments available for bone loss as explained in Section 2.4.2. Each type of treatment has its own advantages and limitations, and these aspects can be very well studied in animal models. Testing these treatments in various study designs (e.g., different drugs, combined treatments, sequential treatments, drug holidays) can help to provide more translational evidence. For example, sequential treatments of an anti-resorptive agent and an anabolic drug could potentially improve the treatment effects (Compston, 2020; Langdahl, 2020).

In summary, various types of animal models, *in vivo* testing methods and treatments can be explored beyond the *in vivo* experimental platform developed in the present dissertation. Further animal trials could help to improve our fundamental understandings of bone tissue metabolism and mechanobiology, providing guidance for optimization of pubertal growth and clinical management of bone loss.

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APPENDIX A PRELIMINARY EXPERIMENT: QUANTIFICATION OF BONE GROWTH USING THE IN VIVO MICRO-CT

Introduction

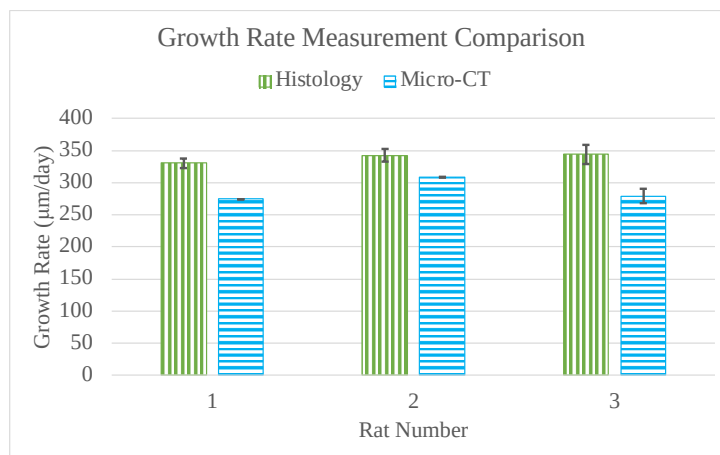
This preliminary experiment was designed to test the scan conditions of the *in vivo* micro-CT imaging for the quantification of bone growth. Three conditions were tested to optimize the parameters allowing adequate image resolution for subsequent analyses, while considering a minimum irradiation time for the animals.

Methods

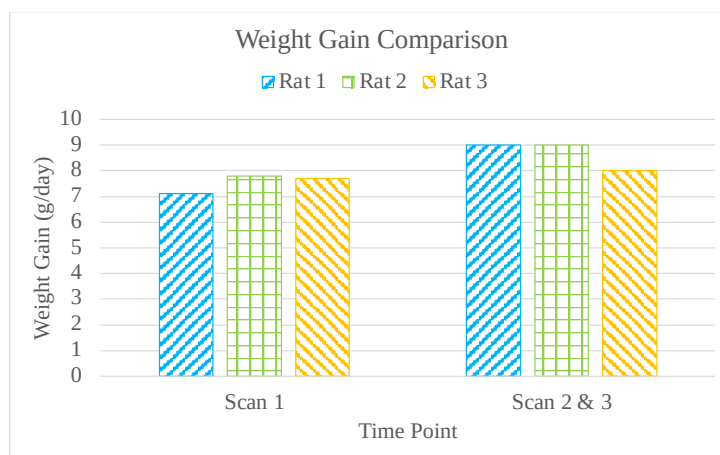
Three one-month-old rats were scanned using an *in vivo* micro-CT (SkyScan 1176) with three different scanning conditions, which imposed radiation doses of 0.83 Gy, 1.65 Gy and 2.47 Gy respectively. The right hindlimb of each rat was scanned three times within a 6-day interval. Calcein injections were given to each rat between scans to quantify bone growth rate using the conventional histology-based method. Image registration was performed on the time-lapse scans to quantify bone growth using the micro-CT-based method. The results measured by both methods were compared for each rat.

Results and discussion

The comparison of the bone growth rate measurement was shown in Appendix Figure 1, and the weight gain for each rat at each scan point was shown in Appendix Figure 2. For each rat, the growth rates measured by the two methods were comparable though with a relative difference of 15% on average, which might be caused by an inconsistency of the longitudinal axis between the two methods. This aspect could be improved further by carrying out the manual data analysis more carefully. Among the three rats, their bone growth rate was on a similar level (coefficient of variation less than 7%), which showed that the irradiation effect did not seem to affect the measurement of growth rate in this experimental design. The rats did not experience unusual behaviors related to the X-ray exposure since their mobility, ability to feed, and weight gain progression were similar for all three animals at each time point.



Appendix Figure 1 Comparison of growth rate measurements by the histology-based and the *in vivo* micro-CT-based methods for the three rats.



Appendix Figure 2 Comparison of weight gain for the three rats receiving repeated *in vivo* micro-CT scans at different times points.

Conclusion

As the image registration was successful for the three scanning conditions, and the irradiation impact did not seem to affect the animals, we proposed to use the scan condition in the middle (1.65 Gy) for the continuation of this project. This scan condition was considered a reasonable compromise between the image quality and the radiation doses.

APPENDIX B PRELIMINARY EXPERIMENT: OPTIMIZATION OF THE IN VIVO MICRO-CT SCAN CONDITIONS

Introduction

The results of the study in Chapter 5 indicated that the image resolution (18 μm) was one of the limits, especially for the evaluation of *in vivo* bone (re)modeling parameters. We decided to test a better image resolution (9 μm) for the study in Chapter 7. This preliminary experiment consisted of testing various scanning conditions by visually evaluating their performance while considering the associated radiation doses.

Methods

A sacrificed female rat of 320 g (reused from another research team) was tested in this preliminary study. To reproduce the rat body positioning of the *in vivo* scanning scenario, the intact rat body was used for the test scans. The mid-diaphysis of the left forelimb was scanned using several scan conditions starting from a baseline condition: 9 μm resolution, 1 mm Al filter, 65 kVp voltage, 385 μA courant, 1200 ms exposure time, 0.25°/180° rotation step. A summary of the last batch of tested conditions is shown in Appendix Table 1.

Appendix Table 1 Test conditions summary for the *in vivo* test scans on a mature rat.

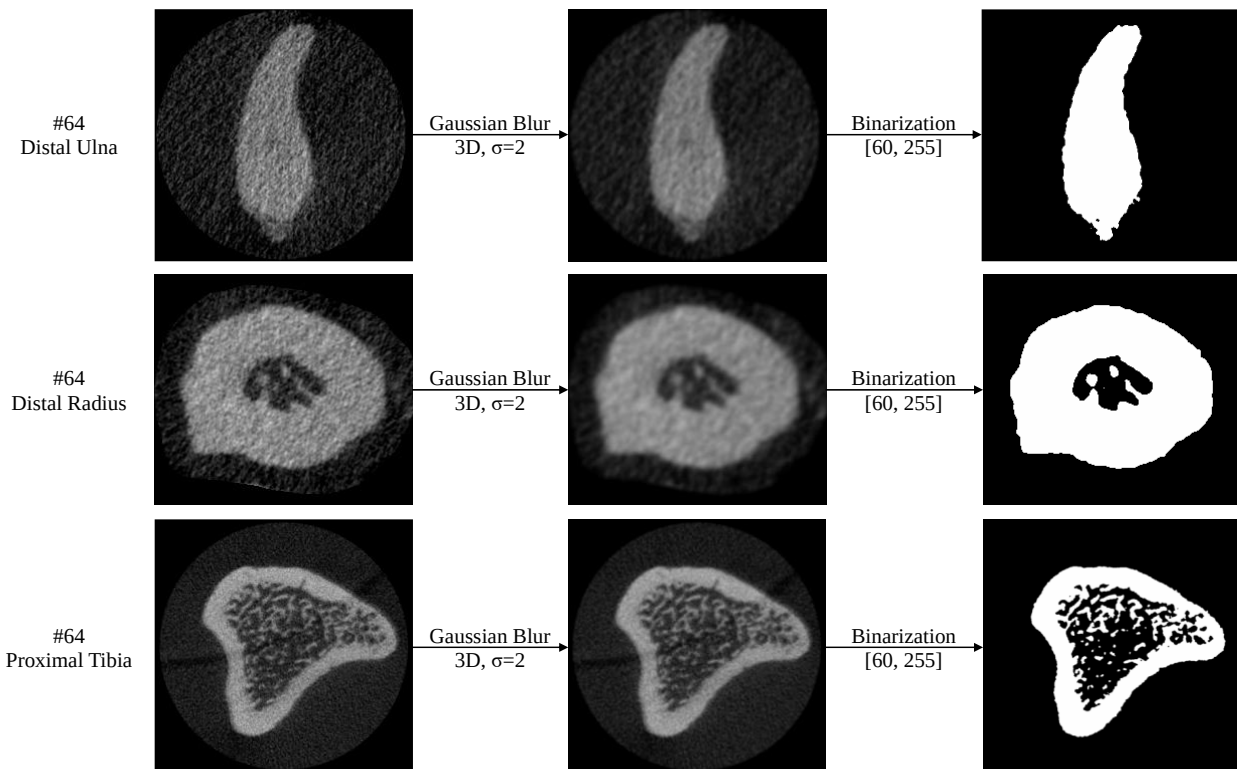
#	Image Resolution	Filter	Voltage	Courant	Exposure Time	Rotation Step	Duration (min)	Ulna Dose (mGy/min)	Ulna Dose (mGy)	Tibia Dose (mGy/min)	Tibia Dose (mGy)	Scan Schedule
60	9 μm	1mm Al	65 kV	385 μA	1500 ms	0.25° / 180°	24.9	62	1544	132	3287	<i>Ex vivo</i> scan
61	9 μm	1mm Al	65 kV	350 μA	1500 ms	0.60° / 180°	10.4	57	593	120	1248	3 - 5 scans weekly
62	9 μm	1mm Al	65 kV	350 μA	1200 ms	0.50° / 180°	10.5	57	599	120	1260	3 - 5 scans weekly
63	9 μm	1mm Al	65 kV	300 μA	1500 ms	0.60° / 180°	10.4	49	510	103	1071	3 - 5 scans weekly
64	9 μm	1mm Al	65 kV	300 μA	1200 ms	0.50° / 180°	10.5	49	515	103	1082	3 - 5 scans weekly
65	9 μm	1mm Al	65 kV	250 μA	1500 ms	0.60° / 180°	10.4	41	426	86	894	3 - 5 scans weekly
66	9 μm	1mm Al	65 kV	250 μA	1200 ms	0.50° / 180°	10.5	41	431	86	903	3 - 5 scans weekly

Results and discussion

The test scan of all the conditions were visually verified: the conditions #61 and #62 provided better microstructural results but the radiation doses were high for *in vivo* scans; the conditions #65 and #66 had the lowest doses but the background noises were significant; the conditions #63 and #64 reached a middle ground for the image quality and the radiation dose. Between the conditions #63 and #64, the later one could provide a higher number of projections thus providing more spatial information along the rotation axis. Some cross-sectional examples of the original, denoised, and binarized scans of the condition #64 is shown in Appendix Figure 3.

Conclusion

The condition #64 was selected for the *in vivo* imaging of the hindlimb and forelimb in the subsequent mature rat model (Chapter 7).



Appendix Figure 3 Result visualization in cross-sectional view for the scan condition #64.

APPENDIX C SUPPLEMENTARY DATA OF RAT RADIUS

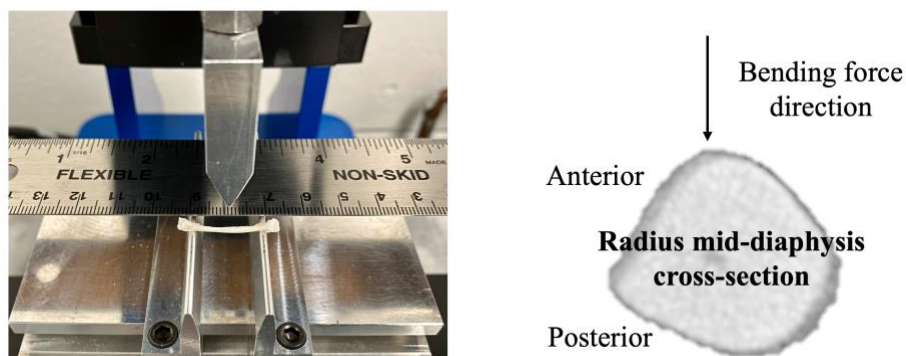
Introduction

While the article in Chapter 7 was concentrated on ulna, the same data analyses were performed for radius as well since it is also part of the rat forelimb. This appendix provides some supplementary data of radius and some comparisons with ulna.

Methods

The radius samples are collected from the same forelimbs as with the ulnae. The sample processing and analysis were performed in the exact same way as introduced in Chapter 7 except for a few differences outlined in the next paragraph.

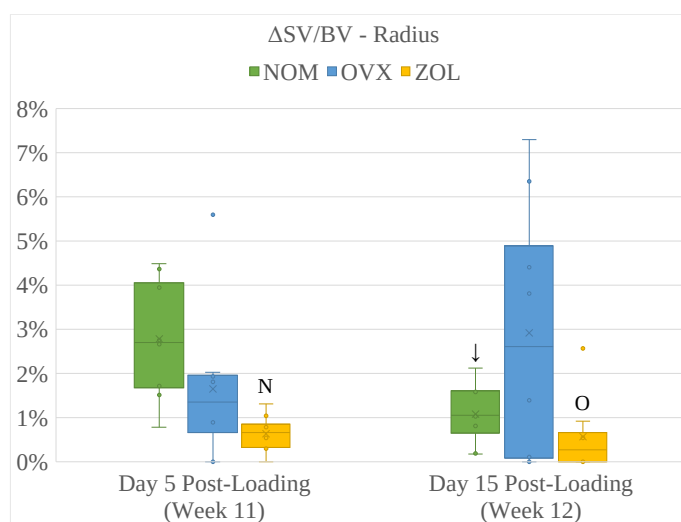
For the evaluation of bone microdamage (SV/BV), the registration step was not performed for the radial VOIs as their newly formed bone was not voluminous enough to affect the staining procedure based on visual verifications. As for the three-bending testing, the radii were loaded in the anterior-posterior direction (Appendix Figure 4). The other loading parameters are the same as with ulna, including a sample support span of 18 mm, an actuator displacement speed of 0.01 mm/s, and a data acquisition frequency of 100 Hz. Besides the ANOVA analyses performed in Chapter 7, two-tailed t-tests were also performed for the bone microdamage parameter $\Delta\text{SV/BV}$ and the bone mechanical properties to compare between the ulna and radius for each rat group.



Appendix Figure 4 Three-point-bending setup for the radius samples. The experimental setup is shown on the left and the cross-sectional view is shown on the right.

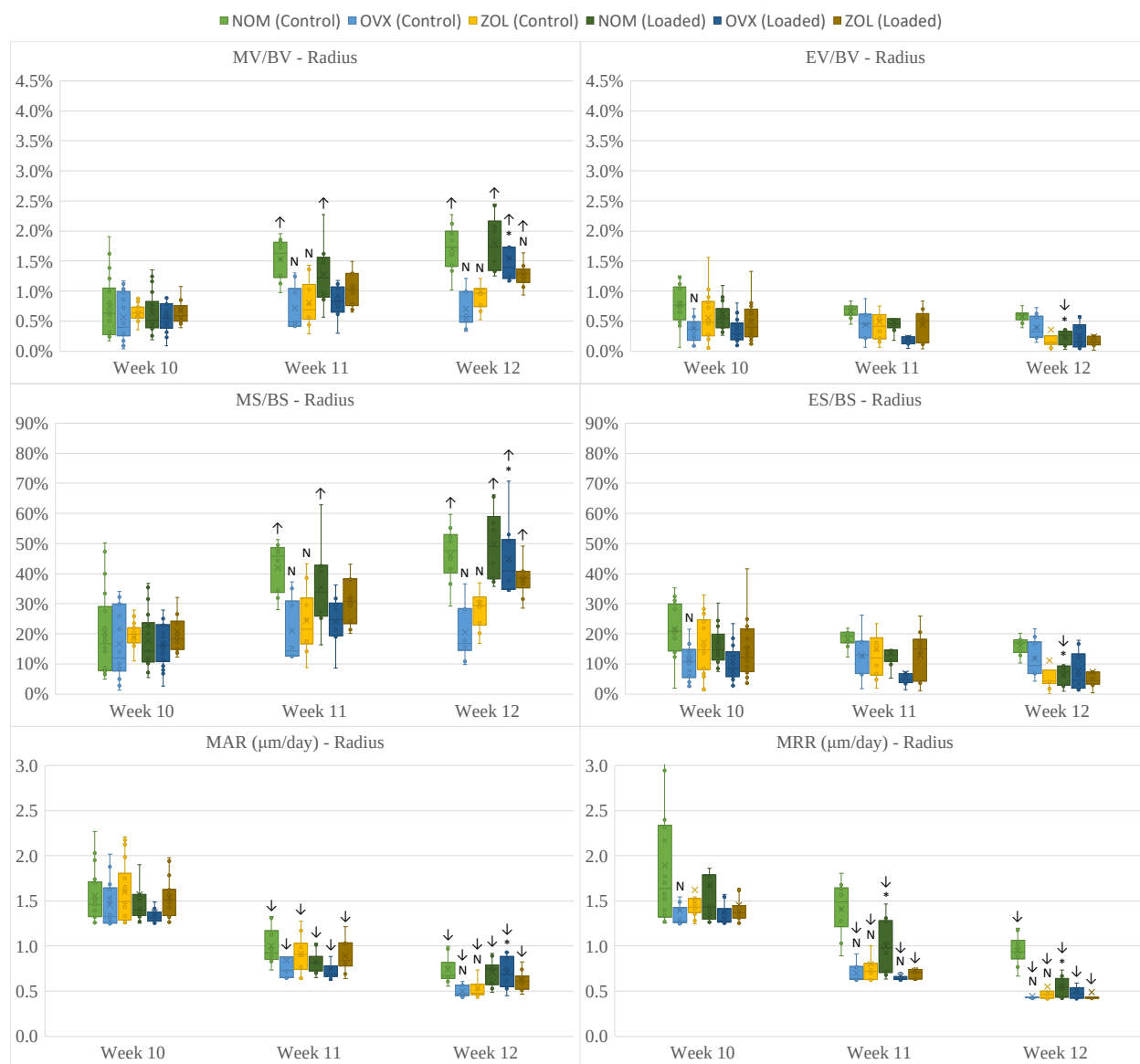
Results and discussion

The results of radial microdamage are shown in Appendix Figure 5. The NOM rats had decreased values from day 5 to day 15; the ZOL rats showed relatively lower levels compared to the NOM rats on day 5 post-loading, and to the OVX rats on day 15. Based on the t-test performed between $\Delta\text{SV}/\text{BV}$ of radius and ulna, the radius showed less microdamage than the ulna for all the three rat groups ($p < 0.01$), which might be due to the shielding effects of ulna on radius while undergoing the *in vivo* mechanical loading.



Appendix Figure 5 $\Delta\text{SV}/\text{BV}$ for radius, reflecting the overall microdamage accumulated by *in vivo* loading ($n=8/\text{group}$). “↓” refers to a significant decrease compared to the previous time point. “N” or “O” refers to a significant difference compared to the NOM or OVX group. Bonferroni correction was used for *post hoc* tests and multiple comparison adjustments, after which the statistical significance level (alpha) was set to 0.05.

The results of bone (re)modeling are shown in Appendix Figure 6. Radius and ulna showed similar general tendencies including effects of recovery time, effects of treatment groups and the effects of loading. The results of bone mechanical properties are shown in Appendix Table 2. Compared to ulna, radius had significantly ($p < 0.01$) stronger structural parameters except for U_{pre} . The differences between ulna and radius are less consistent for the material properties. In general, there were more data points of statistical significance in radius than in ulna for the mechanical properties, showing that the effects of ovariectomy are site-specific.



Appendix Figure 6 Radial (re)modeling parameters shown in boxplots ($n=16/\text{group}$ at week 10, $n=8/\text{group}$ at weeks 11 and 12). “↑” or “↓” refers to a significant increase or decrease compared to week 10. “N” refers to a significant difference compared to the NOM group. “*” refers to a significant difference between the left and right radius. Bonferroni correction was applied for the p -value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05.

Appendix Table 2 Radial mechanical properties tested by three-point-bending (n=8/group). The structural properties are shown on the top half of the chart, and their material counterparts are at the bottom half. “↑” or “↓” refers to a significant increase or decrease compared to day 5. “N”, “O” or “A” refers to a significant difference compared to the NOM group, the OVX group, or all the other groups, respectively. “*” refers to a significant difference between the left (control) and right (loaded) radius. Bonferroni correction was applied for the *p*-value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05. The result of a *p*-value between 0.05 and 0.10 was marked in blue. The cells of statistical significance are in bold.

Time	VOI	Group	F _u (N)	F _y (N)	S (N/mm)	D _{pre} (mm)	U _{pre} (mJ)
Day 5	Control	NOM	16.60 ± 2.41 A	13.09 ± 1.53	34.50 ± 5.01	0.4765 ± 0.0155	3.06 ± 0.34
		OVX	20.47 ± 2.63 A	15.29 ± 1.70 N	62.97 ± 14.73 N	0.4242 ± 0.0276 N	3.18 ± 0.31
		ZOL	24.37 ± 3.91 A	17.90 ± 2.60 NO	64.91 ± 17.09 N	0.4429 ± 0.0256 N	3.88 ± 0.49 NO
	Loaded	NOM	16.52 ± 1.75	12.63 ± 1.29	33.94 ± 3.92	0.4668 ± 0.0404	2.99 ± 0.33
		OVX	19.63 ± 2.67 N	14.51 ± 2.19	59.65 ± 16.25 N	0.4182 ± 0.0211 N	2.90 ± 0.44
		ZOL	23.42 ± 3.34 NO	16.66 ± 1.94 NO	61.18 ± 16.85 N	0.4379 ± 0.0458	3.63 ± 0.32 NO
Day 15	Control	NOM	20.46 ± 3.16 ↑	15.13 ± 2.04 ↑	46.41 ± 9.39 ↑	0.4638 ± 0.0255	3.42 ± 0.45 ↑
		OVX	20.84 ± 2.68	15.43 ± 1.86	52.15 ± 8.50 ↓	0.4464 ± 0.0208	3.34 ± 0.47
		ZOL	23.92 ± 1.81 NO	17.85 ± 2.28 NO	65.54 ± 10.75 NO	0.4448 ± 0.0293	3.93 ± 0.65 NO
	Loaded	NOM	20.83 ± 2.49 ↑	15.43 ± 1.94 ↑	49.50 ± 9.61 ↑	0.4490 ± 0.0414	3.39 ± 0.47 ↑
		OVX	20.89 ± 2.04	14.75 ± 1.21	51.45 ± 6.88	0.4326 ± 0.0298	3.08 ± 0.29
		ZOL	23.37 ± 1.83	17.10 ± 1.77 O	63.63 ± 9.40 N	0.4439 ± 0.0120	3.60 ± 0.47 O
Time	VOI	Group	σ _u (MPa)	σ _y (MPa)	E (GPa)	ε _{pre} (μ€)	u _{pre} (MPa)
Day 5	Control	NOM	271.71 ± 13.75	215.06 ± 11.65	20.15 ± 1.92	13,413 ± 990	1.41 ± 0.05
		OVX	272.87 ± 8.60	204.40 ± 14.92	27.26 ± 2.08 N	12,863 ± 555	1.30 ± 0.12
		ZOL	281.62 ± 7.91	207.51 ± 11.35	23.16 ± 2.71 NO	14,182 ± 1,404	1.44 ± 0.10
	Loaded	NOM	258.40 ± 13.91 *	197.67 ± 10.91 *	19.10 ± 2.04	13,026 ± 1,320	1.31 ± 0.11
		OVX	263.48 ± 16.89	194.44 ± 12.39	26.43 ± 3.91 N	12,482 ± 903	1.16 ± 0.11 *
		ZOL	273.95 ± 17.99	195.88 ± 19.97	22.34 ± 1.86 NO	13,690 ± 1,162	1.34 ± 0.17 O
Day 15	Control	NOM	275.55 ± 25.75	215.42 ± 20.95	24.49 ± 4.39 ↑	12,477 ± 1,915	1.19 ± 0.20 ↓
		OVX	270.77 ± 14.02	200.71 ± 11.07	21.35 ± 1.50 N↓	14,117 ± 551 N↑	1.37 ± 0.11 N
		ZOL	274.44 ± 11.71	203.84 ± 11.13	22.56 ± 2.53	14,764 ± 1,318 N	1.49 ± 0.17 N
	Loaded	NOM	261.98 ± 10.72 *	191.79 ± 14.88 *	21.81 ± 3.14 ↑*	11,566 ± 1,978 ↓	1.10 ± 0.19 ↓
		OVX	266.14 ± 17.03	188.04 ± 10.30 *	20.79 ± 1.82 ↓	13,619 ± 1,034 N↑	1.23 ± 0.11 *
		ZOL	267.69 ± 11.93	195.90 ± 15.36	22.24 ± 2.53	14,531 ± 628 N	1.35 ± 0.12 N*

The results of static histomorphometry are shown in Appendix Table 3. Similar to ulna, the ZOL radius samples showed higher Ct.BV and Ct.Ar than the NOM and OVX rats at some of the data points. The results of correlations with SV/BV are shown in Appendix Table 4 for the (re)modeling parameters and in Appendix Table 5 for the mechanical properties. The bone formation parameters were less correlated to SV/BV for radius when compared to ulna, which might be explained by the relatively lower levels of Δ SV/BV in radii. Otherwise, radius showed similar results as in ulna: positive correlations between SV/BV and most of the bone formation parameters; negative correlations between SV/BV and most of the bone resorption or mechanical properties.

Appendix Table 3 Radial static histomorphometry (n=8/group). “↑” refers to a significant increase compared to week 2. “N” or “O” refers to a significant difference compared to the NOM or OVX group. Bonferroni correction was applied for the *p*-value calculation in the *post hoc* tests and multiple comparisons, after which the statistical significance level (alpha) was set to 0.05. The cells of statistical significance are in bold.

Time	VOI	Group	TMD (g/cm ³)	Ct.BV (mm ³)	Ct.Ar (mm ²)	Ct.Th (μm)
Week 2	Baseline	NOM	1.08 ± 0.04	6.84 ± 0.32	1.37 ± 0.06	166.04 ± 19.65
		OVX	1.01 ± 0.05 N	7.13 ± 0.46	1.44 ± 0.09	152.35 ± 15.91 N
		ZOL	1.01 ± 0.04 N	7.36 ± 0.61 N	1.52 ± 0.11 N	156.03 ± 12.84 N
Week 12	Control	NOM	1.09 ± 0.04	7.92 ± 0.54 ↑	1.55 ± 0.09 ↑	254.11 ± 12.27 ↑
		OVX	1.12 ± 0.02 ↑	8.57 ± 0.70 ↑	1.62 ± 0.10 ↑	191.90 ± 9.25 N↑
		ZOL	1.08 ± 0.04 ↑	9.15 ± 0.64 N↑	1.78 ± 0.11 NO↑	181.65 ± 18.13 N↑
	Loaded	NOM	1.11 ± 0.04	8.21 ± 0.41 ↑	1.59 ± 0.08 ↑	258.41 ± 10.66
		OVX	1.10 ± 0.05 ↑	8.98 ± 0.60 N↑	1.67 ± 0.10 ↑	189.67 ± 13.47 N
		ZOL	1.06 ± 0.09	9.16 ± 0.51 N↑	1.76 ± 0.08 N↑	182.29 ± 39.51 N

Appendix Table 4 Spearman's correlation coefficients between SV/BV and the (re)modeling parameters (n=8/group). The data are marked bold for statistical significance (“***” for *p* < 0.01, “*” for *p* < 0.05, and “†” for *p* < 0.15).

	SV/BV - Radius					
	NOM		OVX		ZOL	
MV/BV	0.41	†	0.55	*	0.61	*
MS/BS	0.40	†	0.50	*	0.57	*
MAR	-0.08		0.32		-0.01	
EV/BV	-0.53	*	-0.47	†	0.16	
ES/BS	-0.52	*	-0.49	†	0.14	
MRR	-0.39	†	0.10		0.30	

Appendix Table 5 Spearman's correlation coefficients between SV/BV and the mechanical properties (n=16/group). The data are marked bold for statistical significance (“**” for $p < 0.01$, “*” for $p < 0.05$, and “†” for $p < 0.15$).

	SV/BV - Radius					
	NOM		OVX		ZOL	
F_u	-0.39	*	-0.39	*	-0.54	**
F_y	-0.33	†	-0.33	†	-0.63	**
S	-0.28	†	-0.22		-0.59	**
D_{pre}	0.03		-0.23		0.18	
U_{pre}	-0.31	†	-0.25		-0.40	*
σ_u	-0.33	†	-0.45	**	-0.04	
σ_y	-0.32	†	-0.27	†	-0.06	
E	-0.11		0.05		-0.28	†
ϵ_{pre}	-0.07		-0.30	†	-0.08	
u_{pre}	-0.17		-0.20		-0.14	

Conclusion

In general, the radial samples showed similar tendencies as ulna samples for most of the measured parameters. The lower damage level of radius might be caused by the shielding effects of ulna while receiving external mechanical loadings, and the stronger radial structural properties can be partially due to its thicker cross-section profile. Some site-specific changes have also been identified such as different microstructural responses to ovariectomy. Most of the results and discussions reported in Chapter 7 can be applicable on radius. Further microdamage studies can examine other bone sites to confirm the findings from this dissertation.