Therapeutic potential of boosting NAD^+ levels in aging and age-related bone loss

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NMN

NMNAT1

Abstract

Aging is a natural, slow, and continuous process happening in all organisms. Age-related bone loss is a common occurrence that affects almost all populations. Bone loss seems to start around the age of 40 and continues throughout life, accelerating after the age of 60. Osteoporosis is a form of bone loss observed due to aging in individuals and associated with osteoporotic fractures in the elderly population. Trabecular bone undergoes deterioration earlier than the cortical bone with aging. With advancing age, bone mineral density is decreased, and bone microstructure deteriorates which increases the risk of fragility fractures. The focus of our study is to understand age-related bone loss and explore the effect of Nicotinamide Mononucleotide (NMN) on the aging of bone. NMN is one of the intermediates and /or precursors of the synthesis of Nicotinamide Adenine Dinucleotide (NAD^+). NAD^+ is one of the significant coenzymes in many enzymatic reactions and a major co-substrate for 3 classes of enzymes that have a crucial role in the body. NAD^+ level is decreased as the organism ages. The decreased level of NAD^+ is associated with a number of biochemical reactions leading to many pathophysiological changes in the body. Several studies have demonstrated the boosting of NAD^+ levels have been proved beneficial in many diseases. The age-related bone loss has not studied yet.

There is a significant gap in our understanding of the potential relationship between NAD^+ levels and bone health. Limited research has been conducted to explore the impact of NAD^+ on age-related bone loss and overall bone density. More comprehensive studies are needed to investigate whether NAD^+ plays a role in influencing bone health and if interventions aimed at boosting NAD^+ levels could have positive effects on preventing bone loss or osteoporosis.

In our study, we concentrated on age-related bone loss and supplementation of NAD^+. The administration of NAD^+ in the form of Nicotinamide Mononucleotide (NMN) has been studied in young (12 weeks) and aged (56 weeks) mice groups along with a transgenic aged mice group. We had 2 additional aged mice groups for them NMN was orally supplemented for 8 weeks in the form of two

concentrations such as 0.5mg/ml and 2.0mg/ml. The aged mice group served as our control group, and because we needed to know the difference between aged and young bone structure, we also included a young mice group in our study. In order to assess age-related bone loss in mice with and without NMN, we conducted an *in vivo* study. For this purpose, we evaluated trabecular bone microstructure parameters by Micro CT, bone mechanical strength by 3-point bending, collagen structure and orientation by polarized light microscopy, elemental analysis in the form of calcium and phosphorous present in the bone and finally we did basic histological staining assessment.

Our study especially Micro CT showed bone loss due to aging in the aged bone. The aged bone showed the trabecular bone parameter as the trabecular number and the ratio of bone volume to total volume decreased when compared to the young mice bone. The NMN treatment did not show any increase in BMD and bone volume. We assessed the bone strength in the 3-point bending and noted that transgenic mice and NMN treated exhibited higher strength than the aged mice same results were observed in the polarized light microscopy. The elemental analysis did not show any prominent increase in the phosphorus or calcium content with NMN treatment in the bone.

In conclusion, the study presented in this dissertation has shown that aging is associated with a bone loss which is in accordance with the available literature. The NMN treatment is retaining the collagen structure and retaining some extent of mechanical loading though not significant. We are proposing that the long-term NMN treatment might help to reduce bone loss leading to healthy aging and our study provides insight into boosting NAD^+ levels in relation to the therapeutics in age-related bone loss and other bone diseases.

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List of Publications

Publications during MPhil candidature, but not related to the work contained in this thesis are enlisted below

Co-author for the review “How are aging and osteoarthritis-related?” Shital Wakale, Xiaoxin Wu, **Yogita Sonar**, Antonia Sun, Xiwei Fan, Ross Crawford, Indira Prasadam* published in Q1 journal “Aging and Disease” with an impact factor of 9.968

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List of Conferences

- **Centre for Biomedical Technologies Inaugural Conference (CBT-QUT)**

Thursday 11 and Friday 12 November 2021

I was one of the selected students to present my research at the CBT conference for **Rapid Fire Presentation** (3 Min)

- **2021 International Forum for Tissue Engineering and Regenerative Medicine Australia-China Centre for Tissue Engineering and Regenerative Medicine (ACCTERM- QUT)**

Friday, November 26th, 2021

ORGANISERS:

Queensland University of Technology (QUT)

Guangzhou Medical University

- **Centre for Biomedical Technologies Conference (CBT-QUT)**

Thursday 10 and Friday 11 November 2022

I was one of the selected students to present my research at the CBT conference for **Poster Presentation**

List of Abbreviations

NAD^+ Nicotinamide adenine dinucleotide

NMN Nicotinamide mononucleotide

NA Nicotinic acid

NR Nicotinamide riboside

NMNAT Nicotinamide mononucleotide adenyl transferase

NaMN Nicotinic acid mononucleotide

NAMPT Nicotinamide phosphoribosyltransferase

NAM Nicotinamide

PARPs Poly ADP-ribose polymerase

ATP Adenosine triphosphate

DNA Deoxyribose nucleic acid

mtDNA Mitochondrial deoxyribose nucleic acid

TCA Tricarboxylic acid

Tb N Trabecular number

Tb Th Trabecular thickness

Tb Sp Trabecular spacing

BV/TV Bone volume to the total volume ratio

BMD Bone mineral density

BS/BV Bone surface to bone volume ratio

ROI Region of interest

SEM Scanning electron microscope

EDS Electron dispersive spectroscopy

EDTA Ethylenediaminetetraacetic acid

AIF Apoptosis-inducing factor

ADPR Adenosine diphosphoribose

cADPR Cyclic adenosine diphosphoribose

Acknowledgements

“Education is not the learning of Facts, its rather the training of the mind to think”

Albert Einstein

This famous quote from Albert Einstein validates my MPhil journey! My research journey was truly amazing because I learned not only scientific research but also many other aspects of life.

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Chapter 1 : Introduction and Aims

Research Problem

Age-related bone loss is one of the major concerns of fractures in elderly population. Osteoporosis is characterized by a loss of bone mass and microarchitecture, as well as an increased risk of fragility fractures. Osteoporotic fractures in the aged population are a major health concern around the world.

Bone performs mechanical and homeostatic functions, such as protecting internal organs, permitting locomotion and load bearing, and acting as a home for marrow as well as a reservoir for calcium homoeostasis. These functions deteriorate with age, and bone becomes more fragile leading to osteoporosis and less capable of performing mechanical functions, and calcium stores are frequently depleted. Osteoblasts are the cells responsible for the new bone formation and osteoclast cells are responsible for the aged bone resorption. The process of formation of new bone and replacement of aged bone is called bone remodelling which is highly coordinated cyclic process in response to the stimuli to damage to the bone and variables in the mechanical load. Bone is a dynamic tissue that undergoes an incessant process of resorption and remodelling throughout life. Figure 1 shows the process of bone remodelling. When this delicate balance is disturbed by various factors, one of them is aging, the osteoclastogenesis predominates resulting in no formation of new bone ultimately leading to a bone loss (Almeida, 2012). As a result, aging and osteoporosis are inextricably linked. Individuals begin to lose cancellous bone mass in their third decade, while cortical bone begins to decline after the age of fifty. Women lose bone at a more rapidly after menopause, indicating that estrogen deficiency has a negative impact on bone mass and contributes to the acceleration of skeletal involution with age. The critical pathogenetic mechanism causing age-related skeletal fragility is impaired bone formation, which is caused by a dysfunction of osteoblasts (Almeida, 2012).

As trabecular bone is the primary load bearing bone in vertebrates and indicates the signs of bone loss earlier than cortical bone, it is interesting to study trabecular bone and aging. According to several studies, aging causes the bone to deteriorate in terms of structure and function, including cortical thinning, increased cortical porosity, trabeculae thinning, and loss of trabecular connectivity. These all lead to reduced bone quality and eventually bone strength. The insufficient number of osteoblast leading to bone fragility is the critical cause of the pathogenetic

mechanism of aging in bone (Almeida, 2012). A study on age-related bone loss found that, particularly in female aged mice, increased cortical diameter, decreased cortical thickness, reduced trabecular BV/TV, and cortical porosities were linked to a sharp decline in osteocyte dendritic number and cell density (Tiede-Lewis et al., 2017).

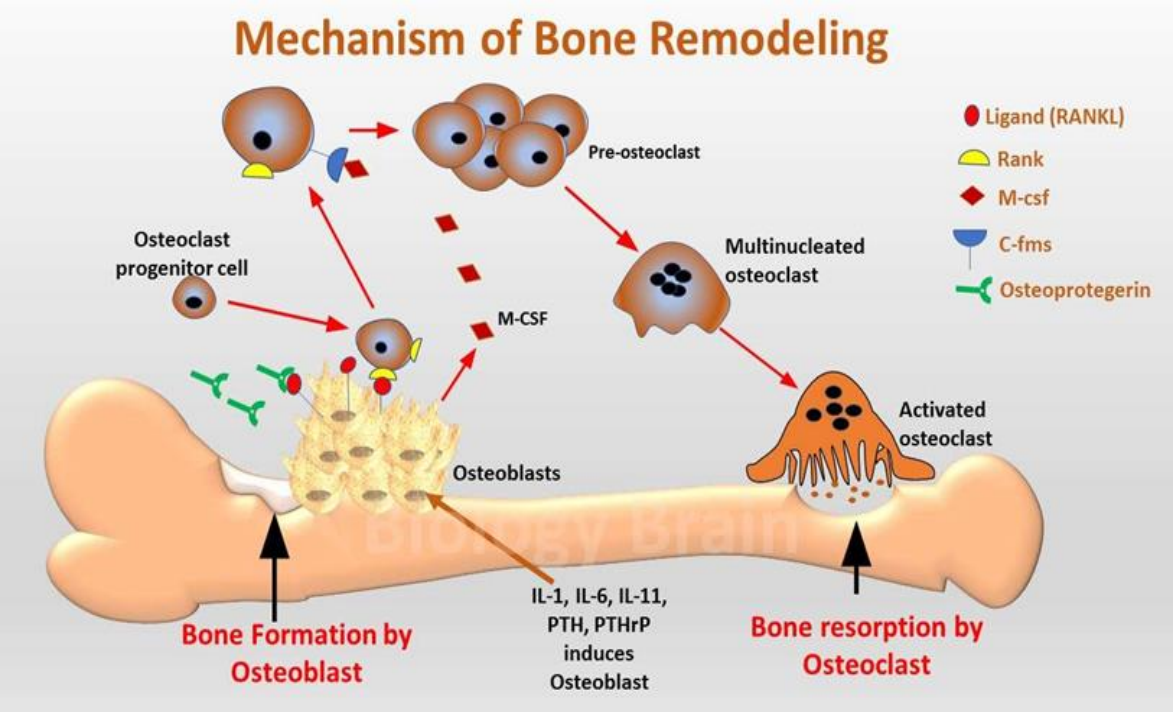

Figure 1. Bone remodelling

Reprinted from (https://www.biologybrain.com/bone-remodeling-process-steps/)

Encouraged by the issue of bone loss and aging, the overall aim of the thesis was to address the question, 'how age-related bone loss can be reduced resulting healthy aging'?

Nicotinamide adenine dinucleotide (NAD^+) is a versatile molecule present in the body that plays a crucial role in the regulation of almost all major biological processes. The amount of NAD^+ in the body diminishes with age. Preclinical experiments to increase

NAD^+ levels for longevity and a variety of aging conditions have already started (A. J. Covarrubias et al., 2021). The various precursors and intermediates of NAD^+ synthesis such nicotinamide mononucleotide (NMN), nicotinamide riboside (NR) boosting in the therapeutics has been studied in several disease models. The fact that NAD^+ levels in osteoblast progenitors decline with age strongly suggests that NAD^+ is a major target of ageing in osteoblastic cells (Kim et al., 2021).

Liang et. al., discovered that aluminium exposure reduced bone intracellular NAD+ levels, BMD, and bone structural characteristics, which were noticeably improved by NMN supplementation. These findings suggested that NMN therapy successfully increased intracellular NAD^+ levels and provided protection from aluminium's bone-damaging effects. The above studies shows that NAD^+ plays a crucial role in bone health. Osteoporosis is a major health issue globally affecting the aged population. Our study will fill the knowledge gap where the age-related bone loss and NMN supplementation is warranted.

Even though much research has been done on NAD^+ and its health advantages, NMN supplementation regarding bone aging has yet to be investigated.

This specific research proposes to answer the key research question: What are the effects of NMN on the aging of bone?

In order to evaluate this research question, further we proposed the following sub questions to answer the age-related bone loss.

The question can be addressed by investigating the following sub-questions:

1) If the age-related bone loss can be reduced using NMN?

2) If the transgenic mice exhibited the anti-aging bone phenotype?

Thesis Structure

This thesis comprises of 5 chapters. **Chapter 1** introduces the topic with the key research question. **Chapter 2** offers the extended literature review about aging and NAD^+. **Chapter 3** is dealing with the material and methods we used to characterize the bone at microstructure level and tissue level. **Chapter 4** is all about the results obtained after our *in vivo* experiments where bone and NMN treatment was evaluated. **Chapter 5** is describing the summary and conclusion with limitations and future perspective regarding bone loss and NMN treatment. Chapter 5 is followed by the supplementary information and bibliography.

Thesis Format

This thesis was written according to the guidelines of Queensland University of Technology's "Thesis by Monograph" format.

Chapter 2 : Literature Review

Introductory Statement

Aging is associated with the impact of the accumulation of numerous molecular and cellular damage over time. Aging is associated with many pathophysiological conditions leading to a variety of cancers, neurodegenerative diseases, cardiac disorders, musculoskeletal diseases, and various organ-specific diseases. Nicotinamide Adenine Dinucleotide (NAD^+) plays a vital role in the process of aging. NAD^+ is one of the most essential and remarkable molecules present in all living cells. NAD^+ is a vital co-factor that assists to facilitate numerous biological processes, including metabolism, DNA repair and gene expression (Magni et al., 2004; Keisuke Yaku et al., 2018). Recent research has shown that NAD^+ levels decrease with aging and that weakened NAD^+ metabolism promotes several aging-related diseases, including metabolic and neurodegenerative disorders as well as a number of cancers. (K. Yaku et al., 2018). Osteoporosis, the bone loss is a musculoskeletal disease due to aging predisposing fractures in the aged population. **The focus of our research is to study the effect of NAD^+ on the aging of bone.**

Literature Review

The efficient functioning, homoeostasis maintenance, and repair capabilities of cells and tissues are significantly influenced by energy metabolism (Sautchuk & Eliseev, 2022). Energy metabolism involves a number of metabolic organs and tissues, including the liver, islets, fat, muscle, and bone. Metabolomic studies have revealed that metabolic pathways change during the pathological progression of osteoporosis, providing significant proof for bone's metabolic role in endocrinology (Zhou et al., 2021). The balance between bone formation and bone resorption is subsequently upset by dysregulation of energy metabolism in bone cells. Additionally, reports suggest that bone homoeostasis is impacted by metabolic diseases (Yang et al., 2020). NAD^+ is a coenzyme that plays a central role in various metabolic processes, including those that involve energy production, utilization, and regulation. NAD^+ serves as a coenzyme in a variety of redox reactions in the majority of energy production pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, and fatty acid oxidation (K. Yaku et al., 2018). As a coenzyme, NAD^+ levels have a direct effect on the activity of metabolic enzymes in these pathways. Several enzymes in the mitochondrial energy production pathway, in particular, use NAD^+ in their redox reactions (Aksoy et al., 2006).

What is NAD^+?

Nicotinamide Adenine Dinucleotide (oxidized form NAD^+) is a critical cellular component in all organisms, from bacteria to the most complex multicellular organisms. In 1906, NAD^+ was first discovered by Harden and Young as a coenzyme in the fermentation of yeast (Reiten et al., 2021; Xie et al., 2020). After that in 1936, Warburg pointed out that NAD^+ is required for redox reactions (Rajman et al., 2018). After the discovery of NAD^+, it has been significantly studied all through the last century. By 1960, it was believed that NAD^+ had undergone all of the exciting research; however, a ground-breaking study published in 1963 showed that NAD^+ was also a co-substrate (Rajman et al., 2018). NAD^+ is present in all living organisms, and it is one of the most essential and remarkable molecules present in all living cells. NAD^+ is required by more than five hundred enzymatic reactions in the body playing a key role in the major biological processes. NAD^+ is an essential cofactor that aids in the mediation of many biological processes, including metabolism, DNA repair, and gene expression (Braidy & Liu, 2020; Cantó et al.,

2015). **In all 4 Nobel Prizes contributed the NAD/NADP-related inventions** (Reiten et al., 2021). NAD^+ is a small ubiquitous hydrophilic cofactor that plays a crucial role in enormous cellular reactions. About 3g of NAD^+ is present in the average person and it is constantly in the state of synthesis, degradation, and recycling (Rajman et al., 2018). NAD^+ is essential for cell survival, signalling, transcriptional regulation, and calcium homoeostasis (Braidy & Liu, 2020; Reiten et al., 2021). NAD^+ is present in the nucleus, mitochondria, and cytoplasm. NAD^+ presence is quite sectionalized within the cell. Mitochondria possess higher concentrations of NAD^+ than the cytosol (McReynolds et al., 2020). Diet plays a key role in aging as several compounds previously linked to longevity in experimental model organisms have been confirmed to play a role in cellular nutrition sensing pathways (Khaidizar et al., 2021b). NAD^+ may help organisms leading to a healthier and longer life. NAD^+ is becoming a key metabolite in the study of ageing, nearly 90 years after its discovery, and NAD^+ level decline is a well-established characteristic of many age-related diseases (Anthony J. Covarrubias et al., 2021). The NAD^+ field is developing quickly and has become one of the most exciting fields of study in the biomedical sciences. NAD depletion pathways plays a key role in age related diseases which had been demonstrated in various studies.

Chemical structure of NAD^+ is shown in Figure 2.

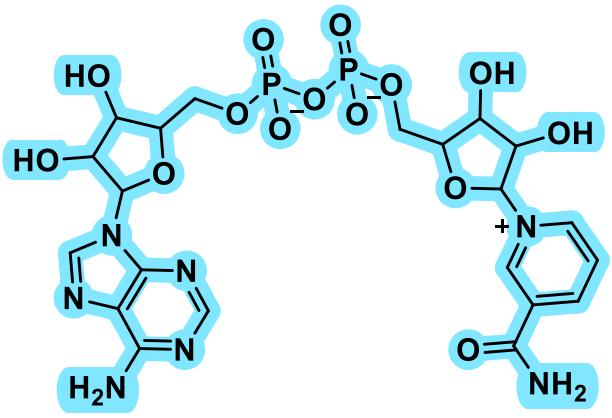

Figure 2. Chemical structure of nicotinamide adenine dinucleotide (NAD^+)

NAD^+ Homeostasis

The NAD^+ is continually synthesized, catabolized and recycled in the cell to maintain the steady cellular NAD^+ levels in different subcellular compartments including the cytoplasm, nucleus, mitochondria and Golgi body (Lautrup et al., 2019). NAD^+ is not only a coenzyme for several crucial reactions such as glycolysis, fatty acid beta-oxidation, tricarboxylic acid cycle etc but it also serves as co-substrates for many enzymatic reactions which regulate numerous important biological processes (Arenas-Jal et al., 2020). When acting as a coenzyme NAD^+ catalyzes the cellular redox reactions and reduced them to NADH. NAD^+ is constantly consumed by sirtuins (SIRTs), poly (ADP-ribose) polymerases (PARPs), ADPR-cyclase, CD38 and CD157 in various bodily processes by playing a critical role as these NAD-consuming enzymes are known to mediate several necessary cellular processes (Johnson & Imai, 2018) such as gene expression, DNA repair, and energy metabolism.

NAD^+ Biosynthetic Pathways

There are various pathways to the synthesis of NAD^+ which involve the five major precursors and intermediates including tryptophan, nicotinamide, nicotinic acid, nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN).

The pries-Handler pathway uses nicotinic acid, a type of niacin such as vitamin B3 which is converted into NAD^+ via three steps. Nicotinamide mononucleotide adenyl transferase (NMNAT) enzyme plays a key role in this pathway by transferring nicotinic acid mononucleotide (NaMN) into nicotinic acid adenine dinucleotide (NAAD) in the presence of ATP. Ultimately NAAD is converted into NAD. NMNAT is found in three distinct forms: NMNAT1 in the nucleus, NMNAT2 in the cytosol and Golgi, and NMNAT3 in the cytosol and mitochondria. NMNAT1 is one of the most important enzymes among the three NMNATs, which is encoded by the gene nmnat1. It plays a crucial enzymatic role in preserving NAD^+ levels in the body. NMNAT1 is localized in the nucleus and the study showed that the deletion of nmnat1 genes in mice results in embryonic lethality (Keisuke Yaku et al., 2018). It indicates the physiological importance of this enzyme. Additionally, it has been reported that Nmnat1 interacts with SIRT1, which is a key element in aging and longevity.

In the **De Novo pathway** of NAD^+ synthesis, the dietary tryptophan, an amino acid, occurs in the Kynurenine pathway (KP). De Novo synthesis follows through multiple enzymatic steps ultimately producing nicotinic acid mononucleotide (NaMN) hence linking with the Preiss-handler pathway (Verdin, 2015) to produce NAD.

The salvage pathway uses nicotinamide mononucleotide (NMN) as an intermediate to convert nicotinamide (NAM; Niacinamide) to NAD^+. After the NAD^+ is consumed by the enzymes such as SIRTs, ARTs and PARPs, NAM is produced as a by-product that acts as a "salvageable precursor" to produce NAD^+. Most of the NAD^+ in the salvage pathway is recycled from NAM, NA, NR, and NMN instead of generated de novo. As a result, the Salvage pathway is the mechanism for recycling NAM into NAD^+. NAM is converted to NMN by the enzyme nicotinamide phosphoribosyltransferase (NAMPT) and then NMNATs convert NMN to NAD^+. The salvage pathway is the main pathway for the synthesis of NAD^+ in mammals. NMNAT1 is one of the most important enzymes among the three NMNAT's, which is encoded by the genes nmnat1. It serves a very important enzymatic role in preserving the body's NAD+ levels.

From the biosynthesis pathways, it is clearly understood that the NAD^+ intermediates play a crucial role in the synthesis and maintaining the levels of NAD^+ at cellular and systemic levels.

Figure 3 indicates the key role of various enzymes and intermediates in the synthesis of NAD and also depicts the interlinking of different pathways.

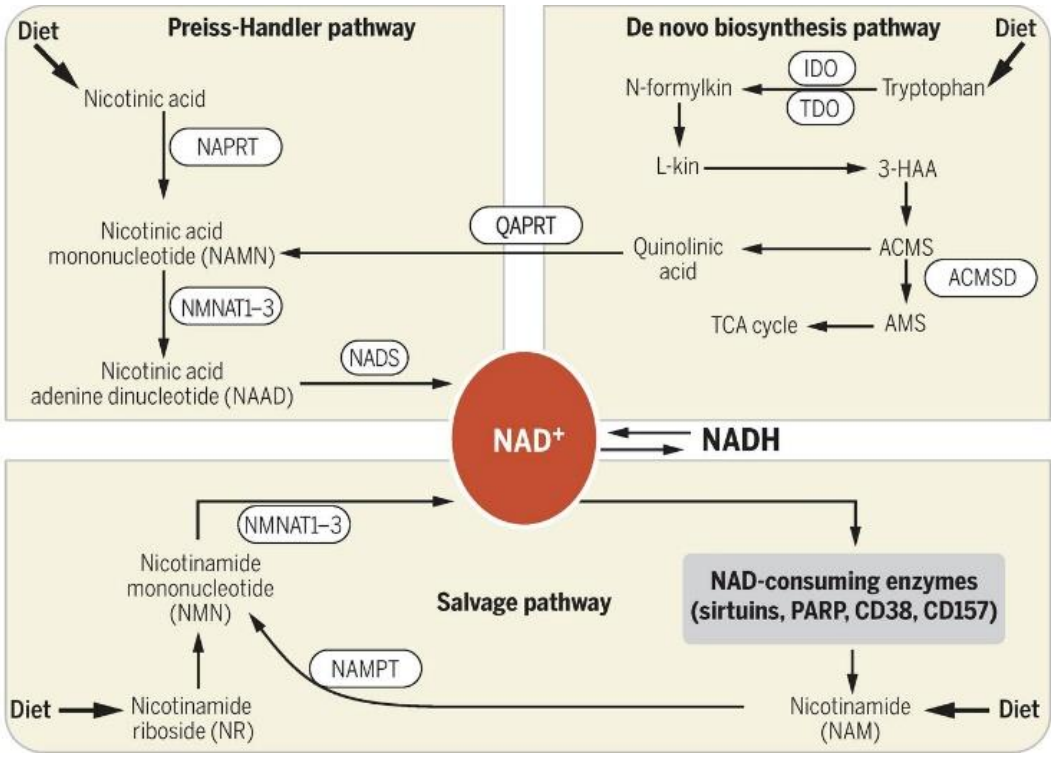

Figure 3. Biosynthesis of NAD⁺

Reprinted from (Verdin, 2015)

Depletion of Cellular NAD⁺ and Aging

As seen in the pathways above, NAD^+ is continuously synthesized, catabolized, and recycled in the cell to maintain the steady cellular NAD^+ levels. NAD^+ is not only a coenzyme for several crucial reactions but it also serves as co-substrates for many enzymatic reactions which regulate numerous important biological processes (Arenas-Jal et al., 2020). Though sirtuins are responsible for the consumption of NAD^+ levels in the body under basal conditions, the rise of NAD^+ levels is greatly connected with sirtuin activation during bodily processes like fasting, exercise and caloric restriction (Anthony J. Covarrubias et al., 2021). PARPs are very well known for their involvement in DNA repair, inflammation, cell death and genotoxic stress. Age-related PARP decline in NAD^+ level mainly depends on the increase in PARP1 activity in the aged tissues indicating the increased NAD^+ consumption (McReynolds et al., 2020). The studies in the animal model have shown that increased DNA

damage demonstrates NAD^+ depletion which was restored by PARP inhibition. (Keisuke Yaku et al., 2018).

Why does NAD^+ decline with aging?

NAD^+ level changes during many physiological processes. Cumulative research suggests that nutrition and environmental stressors have a major impact on intracellular NAD^+ levels (Chini et al., 2017). Several preclinical studies discovered that NAD^+ levels decline in physiological conditions including multiple organ systems such as the heart, kidney, central nervous system, hearing, vision and skeletal muscle (Chini et al., 2021). The delicate balance between NAD^+ synthesis and degradation has long been thought to determine NAD^+ levels, and several studies have indicated that aging is a significant factor in both NAD^+ synthesis and degradation (Keisuke Yaku et al., 2018). **The NAD^+ world concept focuses on NAD's crucial function in linking energy metabolism and aging, a function that is mediated by two important players: the NAMPT enzyme as a "driver" that controls cellular metabolism through NAD^+ biosynthesis, and SIRT1 as a "mediator" that activates survival and aging pathways in response to systemic NAD^+ availability** (Khaidizar et al., 2021a).

The decline of NAD^+ was initially recognised in the mice study as in pancreatic β cells overexpressing SIRT1. After this study, several reported the age-related decline in the NAD^+ observed in worms, flies and mice (Johnson & Imai, 2018). In mice studies, age-associated NAD^+ decline in various tissues and organs causes metabolic dysfunction, neurodegenerative diseases, cardiovascular diseases and cancer (Johnson & Imai, 2018). In old worms NAD^+ levels steadily decrease which causes a shorter lifespan. Age-related NAD^+ decline is seen in various tissues for example muscle, adipose tissue, liver, pancreas, skin, and brain (Mills et al., 2016). Furthermore some studies revealed a decline in NAD^+ levels in the aged human brain and liver (Xie et al., 2020). Figure 4 shows the hallmark of aging with declining NAD^+ levels. There has been a thorough review of the relationship between the ten hallmarks of ageing and NAD^+ (Anthony J. Covarrubias et al., 2021). NAD^+ levels are lowered during aging altering many enzymes with NAD^+ biosynthesis and degradation. NAMPT-mediated NAD^+ biosynthesis is one of the major causes of age-related NAD^+ decline. Reduced Nampt expression at both, mRNA and protein

levels has been demonstrated over-age in a variety of tissues (Johnson & Imai, 2018). A decline in NAD^+ with age can be caused by a number of mechanisms (McReynolds et al., 2020). The most obvious mechanism is an increase in the activity of NAD-consuming enzymes, which may be linked to inflammation (CD38) or DNA damage (PARPs) and SARM1 are important in cellular NAD^+ degradation by consuming the NAD^+. Figure 4 shows the physiological importance of NAD^+ and its relationship with aging.

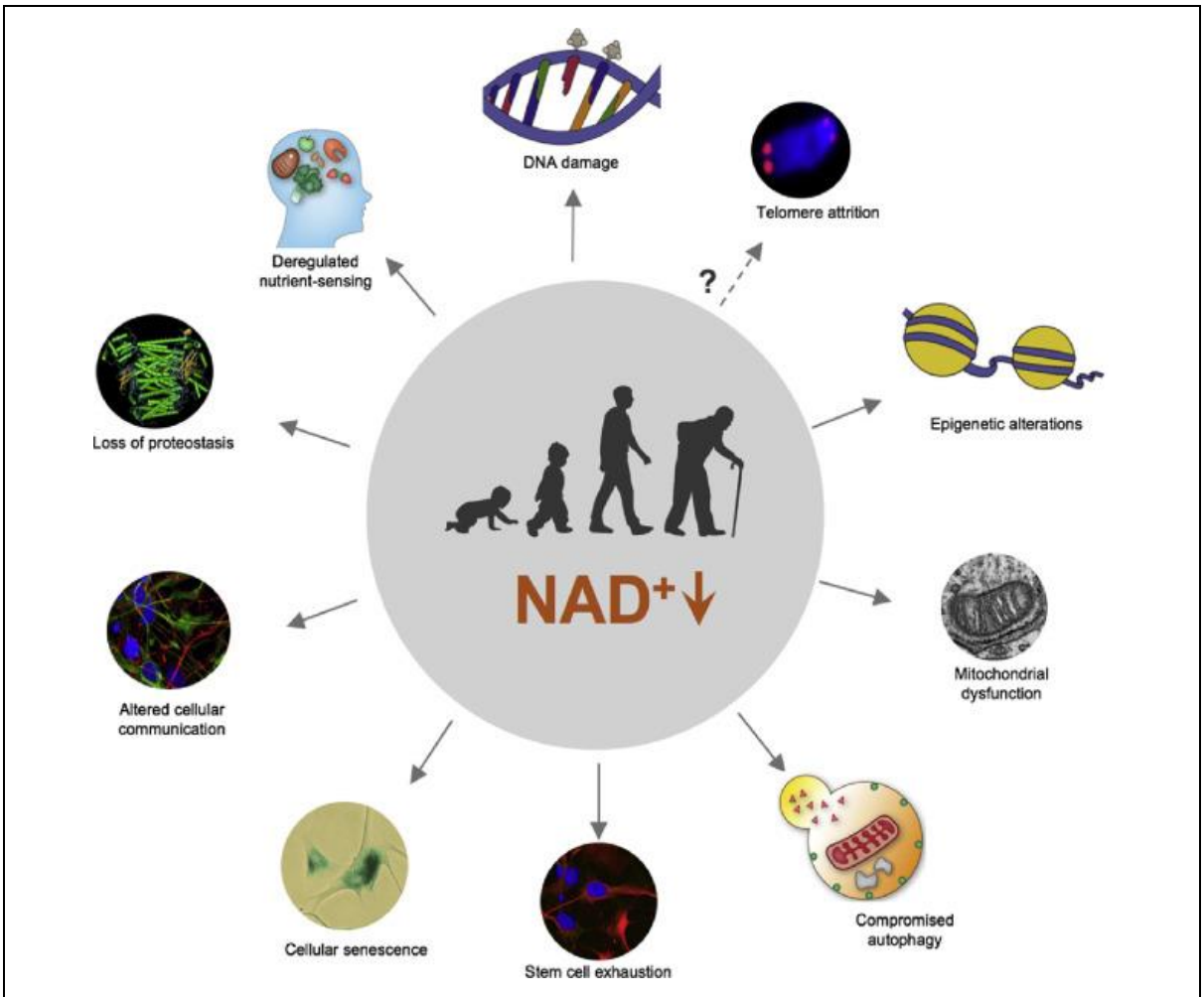

Figure 4. NAD decline and aging

Reprinted from (Aman et al., 2018)

Let us discuss the role of CD38, PARPs and SARM1 in relation to aging and lowering the levels of NAD^+ in the body. Figure 5, below, illustrates the role of NAD^+ consumers in aging.

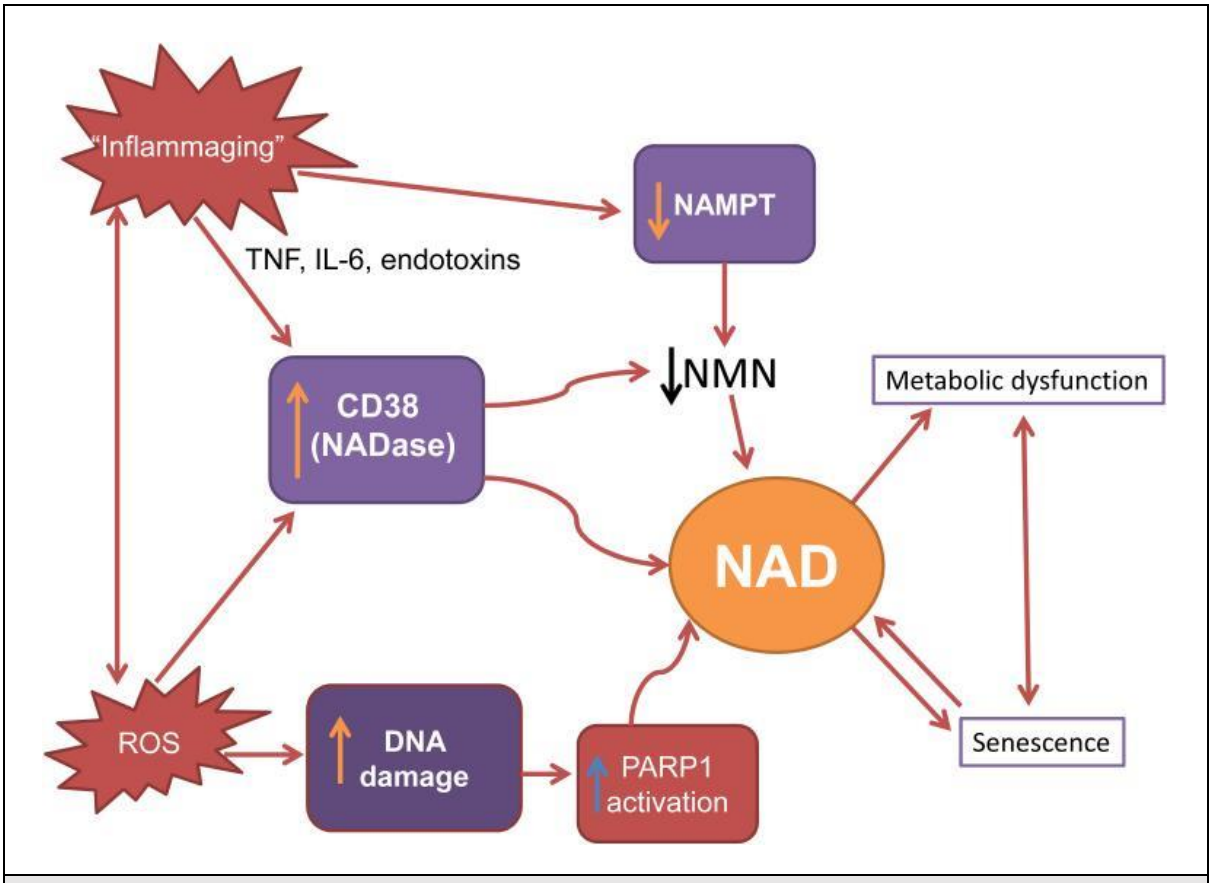

Figure 5. Role of NAD+ consumers in aging

Reprinted from (Chini et al., 2017)

Role of NAD^+ Consuming Enzymes in Aging and Decline of NAD

CD38/CD157

Members of the family of dual-purpose enzymes CD38 and CD157 use NAD^+ to produce the ADP ribose and cyclic ADP-ribose that are essential for calcium signalling. CD38 is a multifunctional protein that functions as both a receptor and an enzyme. As a receptor it stimulates cytokine production and as an enzyme CD38 is extremely expressed throughout the immune system during inflammation. It is also expressed in brain cells such as neurons, astrocytes, and microglia. One of the best

candidates for elucidating an age-related increase in NAD^+ is CD38, the only NAD^+ consumer that has been demonstrated to increase mRNA expression across multiple tissues of aging mice. CD38 and Cd157 (BST1) are glycohydrolases that break down NAD^+ to produce NAM and adenosine diphosphoribose (ADPR). In addition, CD38 hydrolyses cyclic ADPR (cADPR) to ADPR (Rajman et al., 2018). Both enzymes are the catalysts for the hydrolysis of NAD^+ and cADPR which is a Ca^{2+} mobilizing second messenger involved in many cell types. This generated NAM is rapidly recycled to NAD^+ through the salvage pathway. These enzymes have been linked to energy metabolism, cell adhesion and several attributes of immune responses. This has been linked to human diseases such as Parkinson's, ovarian cancer, and leukemia (Rajman et al., 2018). Therefore, CD38 plays a role in both the direct consumption of NAD^+ and the destruction of NMN, which would otherwise act as a precursor in the synthesis of NAD^+ (McReynolds et al., 2020). CD38 expression increases in the spleen, liver, and adipose tissue with age, resulting in lower NAD^+ levels and mitochondrial dysfunction. Increased expression of CD38 plays a significant role in inflammaging and neurodegenerative diseases. It would be an interesting insight to inhibit the CD38 enzyme activity to treat metabolic and aging diseases.

PARPs

Poly ADP-ribose polymerase (PARPs) are the 17 members of enzymes present in the mammalian body which has a crucial role single and in the recruitment and activation of a number of proteins involved in DNA repair which includes double-strand break repair in the helical structure of DNA (Strømland et al., 2021). The PARPs also play a vigorous role in inflammation and cell death along with influencing the circadian rhythm, neuronal function, endoplasmic reticulum stress and metabolism (Cantó et al., 2015). Out of 17 PARPs, most of the research has been done on PARP1 and PARP2 which are responsible for most of the PARP activity in the cell which is activated by DNA strand break followed by the role in response to DNA damage and are the major consumers of NAD^+ in the nucleus. PARPs are associated with age-related NAD^+ decline. DNA damage is related to the enormous NAD^+ consumption. PARP1 acts as a NAD^+ responsive signalling molecule and has been broadly associated with the aging process (Anthony J. Covarrubias et al., 2021). PARP hyperactivation causes cell death via mitochondrial

apoptosis-inducing factor (AIF) and ATP depletion via disrupted glycolysis (Strømland et al., 2021). A genuine PARP has two functions. 1- to transfer the first ADP-ribose moiety from NAD^+ to a recipient protein, particularly lysine/glutamate residues, and 2- to add additional ADP-ribose units to the preceding ones to lengthen the poly (ADP-ribose) chains in a sequential manner (Reiten et al., 2021). PARPs have traditionally been shown to play a two-fold roles in the cells, resulting in either induction of cell death or DNA repair (Cantó et al., 2015). NAD^+ deficiency is seen in animal models with increased DNA damage, which can be repaired by inhibiting PARP and knock out PARP mice showing the increased NAD^+ levels. (McReynolds et al., 2020). As demonstrated, the inhibition of PARP produces DNA damage in cells and induces cancer initiation so the delicate balance between promotion and inhibition of the DNA repair processes by PARP is crucial to preventing aging phenotype *in vivo* (Keisuke Yaku et al., 2018).

SARM1

SARM1 is a modular protein containing catalytic Toll/interleukin-1(IL-1) receptor (TIR) domain which has the role to hydrolyse the NAD^+. SARM1 is involved in axonal or Wallerian degeneration by possessing intrinsic NAD^+ cleavage activity and produces NAM, ADP ribose and cyclic ADPR (McReynolds et al., 2020). SARM1-associated NAD^+ depletion plays a key role in the early stages of age-related neural diseases such as Parkinson's disease, Alzheimer's disease etc (Strømland et al., 2021). It has been demonstrated in the mice model that SARM1 activation rapidly lowers the NAD^+ pool while the inhibition or deletion of the sarm1 gene protects the animal against axonal degeneration by improving the survival rate in the axonopathy mice model and reducing the diabetic peripheral neuropathy (McReynolds et al., 2020). Hence, targeting SARM1 appears to be a promising target for treating neuropathies, and the role of SARM1 in age-related NAD^+ decline needs to be investigated further, as there has not been much research done in this area.

Consumption by SIRTUINS

The sirtuin family in mammals consists of seven genes and proteins, namely SIRT1 to SIRT7, which have different subcellular localizations. SIRT 1 and SIRT 6 in the nucleus, SIRT 7 in the nucleolus, SIRT3, SIRT4 and SIRT5 in mitochondria and SIRT1, SIRT2 and SIRT5 localized in cytosol (Anthony J. Covarrubias et al., 2021; Reiten et al., 2021). Sirtuins are NAD-dependent deacetylases (Chen et al., 2021)

converting NAD^+ to NAM and 2/3-o-acetyl-ribose in the process of removing acyl groups from lysine residues of target proteins (Reiten et al., 2021). Fasting, DR, DNA damage, and oxidative stress are examples of dietary and environmental changes to which sirtuins must adapt. In general, their activation activates nuclear transcriptional programs that increases mitochondrial oxidative metabolism, improve metabolic efficiency, and increase the associated resistance to oxidative stress. (Imai & Guarente, 2014). Sirtuins' role is a key factor in the health span and longevity mechanisms. Nucleus localized SIRT1, SIRT6 and SIRT7 are associated with DNA repair whereas mitochondrial localized SIRT3, SIRT4 and SIRT5 and nuclear SIRT1 are involved in mitochondrial homeostasis. Among all, SIRT1 is the most studied sirtuins and it plays a critical role in the various cell metabolism like tumours, oxidative stress, apoptosis, aging, and inflammation. Sirtuins are constantly active in our cells and particularly under the basal conditions SIRT1 and SIRT2 are responsible for the major consumption of total NAD^+. The rise of NAD^+ level is greatly connected with sirtuin activation during bodily processes like fasting, exercise and caloric restriction (Anthony J. Covarrubias et al., 2021). SIRT1 and SIRT6 are involved in the regulation of the circadian clock by a transcription factor and downstream circadian transcriptome (Anthony J. Covarrubias et al., 2021). Nicotinamide phosphoribosyltransferase (NAMPT), a crucial enzyme in NAD^+ synthesis salvage pathway along with SIRT1 regulates the circadian clock by providing a feedback loop which results in circadian oscillation of NAD^+ levels. Impaired SIRT1-PGC-α signalling is seen in aged mice in response to aging effect decline in mitochondrial biogenesis leading to low NAD^+ levels (Strømland et al., 2021). Findings showing that modest insulin resistance, obesity, and hepatic steatosis are metabolic and age-related issues that can be avoided in mice by overexpressing SIRT1. This reaffirmed the close relationship between sirtuins and metabolism (Cantó et al., 2015).

Mitochondrial Dysfunction and Aging in Relation to NAD

Mitochondria are known as the powerhouse of the cells producing ATP, carrying out a variety of functions for cellular energy metabolism. Cellular processes like amino acid catabolism, ketone body formation, urea cycle, heme biosynthesis and calcium storage are examples in which mitochondria play a crucial role in regulation. For the energy production (ATP) NAD^+ acts as a cofactor and plays a regulatory role in the

energy metabolism pathways including glycolysis, fatty acid oxidation, tri- carboxylic acid (TCA) cycle and oxidative phosphorylation (Cantó et al., 2015; Stein & Imai, 2012; Keisuke Yaku et al., 2018). NAD^+ accepts two electrons and a proton from substrates in the multi-step TCA cycle where NAD^+ is reduced to NADH. Mitochondrial NADH is oxidized by donating an electron to Complex I of the electron transport chain (ETC). Thus NAD^+ and NADH are needed by the TCA cycle and ETC, the ideal NAD/NADH ratio is essential for the efficient mitochondrial function (Stein & Imai, 2012). More than forty serious illnesses and health problems, including type 2 diabetes, cancer, Alzheimer's disease, and other neurodegenerative diseases, are now known to be correlated with mitochondrial dysfunction. NAD^+ plays a key role in the maintenance of mitochondrial function (Figure 6) and age-associated NAD^+ decline causes mitochondrial dysfunction which is a hallmark of aging (Xie et al., 2020). The average quality of the mitochondrial pool in each cell degrades with age because each cell contains multiple mitochondria and mitochondrial damage builds up over time. This is consistent with the energy homoeostasis and mitochondrial dysfunction that are hallmarks of a number of age-related diseases (Fakouri et al., 2019). As discussed earlier, there is an interesting link between sirtuins SIRT1, SIRT3 and mitochondrial function. The life span in worms was increased by inhibiting NR and PARP via activation of the mitochondrial unfolded protein response UPT^{mt} by sir2,1 (Verdin, 2015). Multiple mammalian species have shown that mitochondrial function declines with aging. Previous research on isolated mitochondria from human muscle biopsies or rodent muscles supports the existence of an intrinsic, aging-related mitochondrial defect linked with ATP production and studies using permeabilized muscle fibres show impaired mitochondrial function in elderly humans (Prolla & Denu, 2014). Mitochondrial dysfunction causes cell senescence which is a complex stress response of the cell in which the cell's proliferation activity is arrested (Wiley et al., 2016). Though the role of mitochondrial sirtuins and mtDNA and NAD^+ levels are linked and play a major role in aging, the mechanism of age-associated mitochondrial dysfunction is not fully understood. Reduced NAD^+ levels cause mitochondrial degradation in skeletal muscle by producing a pseudohypoxic state that impairs nuclear-mitochondrial communication, a process that has been found to be controlled by SIRT1 and hypoxia-inducible factor 1 (HIF-1) (Gonzalez-Freire et al., 2015).

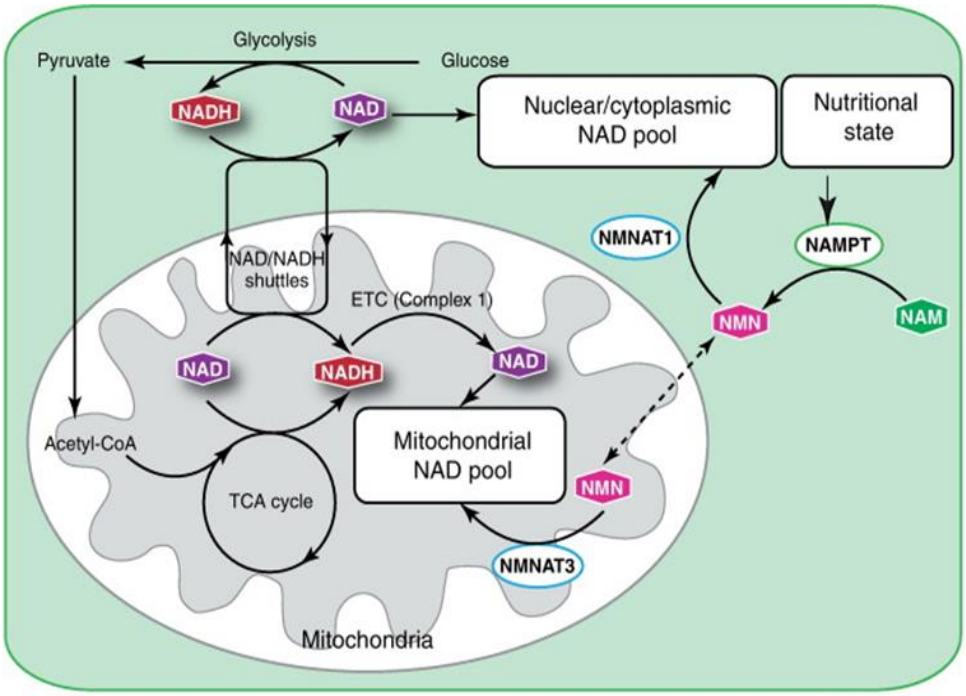

Figure 6. Mitochondrial metabolism

Reprinted from (Stein & Imai, 2012)

Role of NAMPT in Declining NAD^+ levels

The slower rate of NAD^+ production has been linked to age-related NAD^+ decrease. The salvage pathway is the major pathway of biosynthesis of NAD^+ in mammals and lowered NAMPT expression identified during aging is recognized as a key factor. In aged mice, NAMPT expression is reduced at both the mRNA and protein levels in multiple tissues (Strømland et al., 2021). NAMPT plays a vital role in regulating intracellular NAD^+ levels. NAMPT influences cellular metabolism, mitochondrial biogenesis, and responses to inflammatory, oxidative, proteotoxic, and genotoxic stress by influencing the activity of NAD^+-dependent enzymes such as SIRTUINS and PARPs through its NAD^+-biosynthetic activity (Chini et al., 2017). NAMPT-mediated NAD^+ biosynthesis is reduced or inhibited due to chronic inflammation and oxidative stress which are associated with aging (Nadeeshani et al., 2021).

Therapeutic Potential of NAD^+ In Aging Diseases

In several models, cellular NAD^+ levels are essential for cell survival, and NAD^+ may be a factor in longevity assurance (Braidy & Liu, 2020). Age-associated NAD^+ decline is reported in various tissues across several species including humans (Aman et al., 2018). Researchers and drug developers are investigating three main approaches to increasing NAD^+ levels: NAD^+ precursor supplementation, activation of NAD^+ biosynthetic enzymes, and inhibition of NAD^+ degradation (Rajman et al., 2018).

As seen earlier NAD^+ intermediates play a significant role in the synthesis, recycling, and catabolism of NAD^+ constantly in the body. Nicotinamide mononucleotide (NMN) and Nicotinamide riboside (NR) are the extensively studied NAD^+ intermediates in rodent models.

NAD^+ Intermediates / Precursors

Nicotinamide Mononucleotide (NMN)

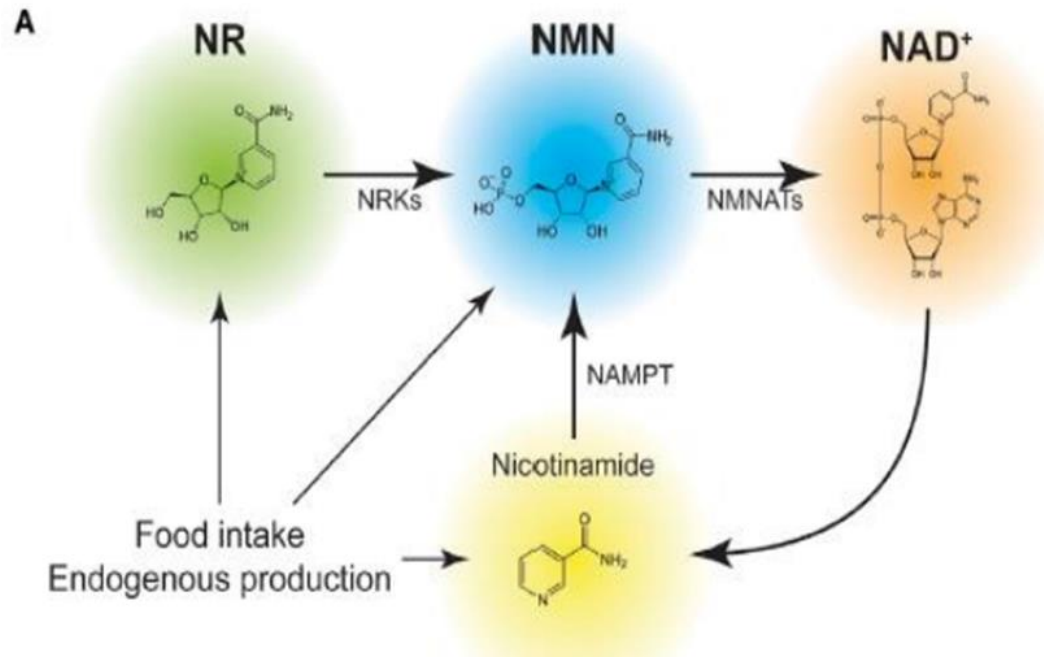

Figure 7.NMN structure

Reprinted from (Yoshino et al., 2018)

NMN is one of the intermediate and a precursor in the synthesis of NAD^+. NMN is localized in the nucleus, mitochondria, and cytoplasm of the organisms. NMN is naturally found in fruits and vegetables such as edamame, broccoli, cucumber, avocado, cabbage and tomato and animal source include raw beef, and shrimp. Figure 7 shows the chemical structure on NMN. NMN is synthesized from nicotinamide (vit. B3) and 5'- phosphoribosyl-1-pyrophosphatase (PRPP) by NAMPT in mammals. Moreover, NMN can be synthesized by NR via NR kinases (NRKs) mediated phosphorylation reaction. NMN is ultimately converted into NAD^+ by NMNATs. According to the research, the administration of NMN has numerous health benefits for both animals and humans. Myriad studies have shown that NMN boosts anti-aging effects in various cells and tissues in in vitro animal models. Earlier NMN was known to be only a precursor in the biosynthesis of NAD^+ but recently a lot of pharmacological activities triggered by elevated NAD^+ levels in the body in response to the anti-aging effect have come into the focus of attraction. The association between NMN and the occurrence of Alzheimer's disease, obesity, cerebral and cardiac diseases, and age and diet-induced type 2 diabetes has been largely studied (Nadeeshani et al., 2021).

NMN treatment has been reported to improve cardiac muscle contractility, reduced cell death and mediate protection against heart diseases (Braidy & Liu, 2020). Many studies revealed that NMN supplementation is effective for reducing age-related metabolic disorders and slowing down the aging physiological phenotypes (Johnson & Imai, 2018). It is important to note that clinical trials of NAM have been started in a variety of cancer patients, and that NMN treatment enhances insulin secretion and sensitivity in age and diet-induced diabetes by restoring NAD^+ biosynthesis (Xie et al., 2020).

Nicotinamide riboside (NR)

NR is a member of the vitamin B3 family and naturally occurred in fruits, vegetables, milk, and meat. NR has recently emerged as one of the most extensively researched NAD^+ precursors, owing to the numerous potential health benefits mediated by increased NAD^+ content in the body (Mehmel et al., 2020). A significant phase clinical trial of NR has been registered for a number of pathologies, such as infection, neoplasms, and various diseases associated with ageing (Xie et al., 2020). Dietary administration of NR seems to be beneficial in cold tolerance, endurance, and

energy expenditure. NR boosting has beneficial effects by decreasing UPR^{mt} in cardiovascular failure mice and by improving mitochondrial function in several muscle disorders mice (Xie et al., 2020). NR enhances liver health through mitochondrial unfolded response, decreases inflammation by reducing the NLRP3 activity and reduces the risk of fibrosis development (Yoshino et al., 2018). NR is a promising therapeutic approach to improve overall liver health.

Nicotinamide (NAM) and Nicotinic Acid (NA)

NAM and NA are the precursors in the biosynthesis of NAD^+. These uncharged molecules are quickly diffusing across plasma enabling the NAD^+ biosynthesis in most tissues *in vivo*. NAM is converted into NA by gut bacteria (Xie et al., 2020). In obese and type 2 diabetes mice, NAM therapy results in significant metabolic improvements.

Due to a decrease in the availability of the methyl group, long-term or high doses of NAM have been linked to adverse effects in the body, including the development of fatty liver. NA tends to have flushing activity mediated by the binding of nicotinic acid to the GPR109A receptor (Chandrasekaran et al., 2019). So, in the conclusion, NMN and NR are the only promising molecules in the therapeutic use of boosting NAD^+ levels in the body. Figure 8 represents the physiological and pharmacological importance of boosting NAD^+ levels.

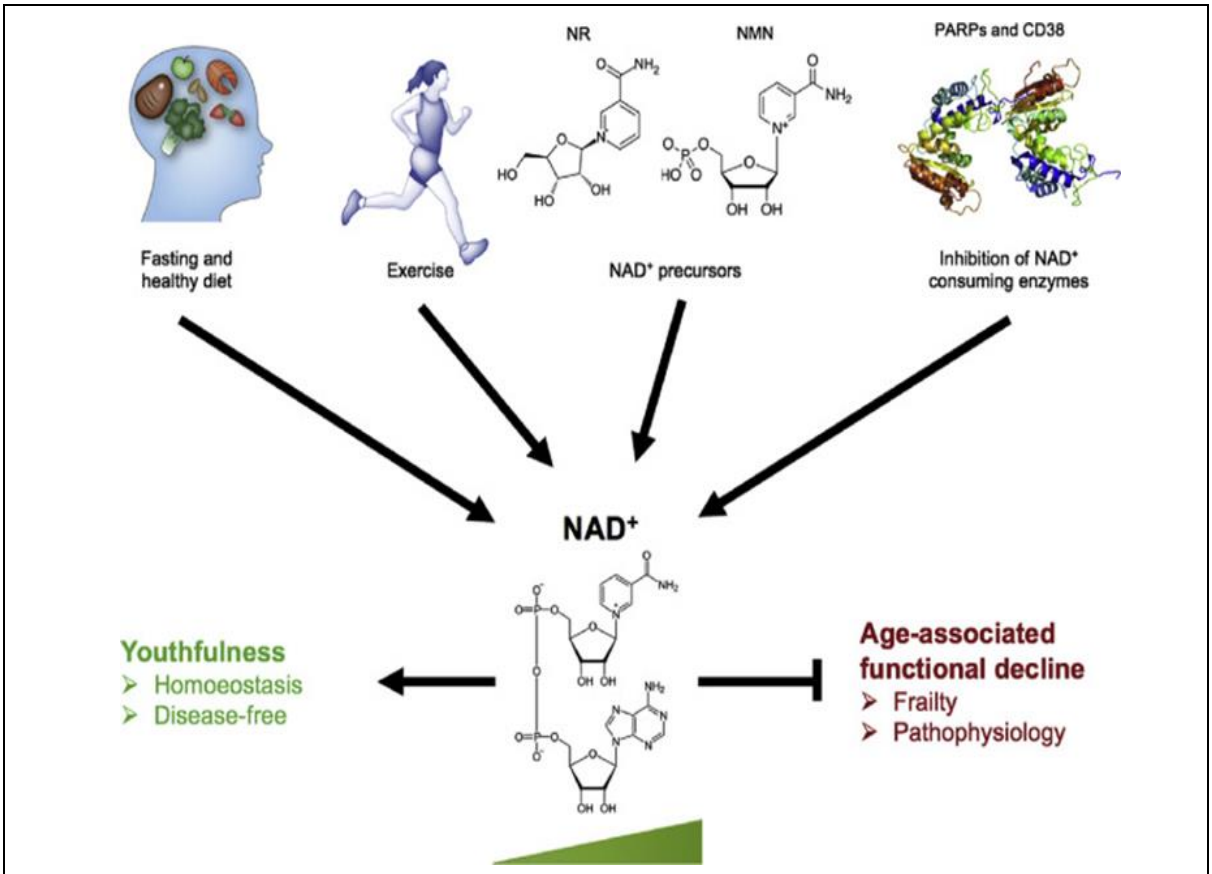

Figure 8. Physiological and pharmacological approaches for boosting NAD+ levels

Reprinted from (Aman et al., 2018)

Aging and Bone

Bone is heterogeneous tissue also known as osseous tissue is a type of connective tissue. Bone is a composed structure comprised of inorganic mineral crystals, organic (extracellular) matrix, lipids, cells, and water. The mineral crystals are hydroxyapatite, and its content depends upon the food ingestion by the animal. The organic component is made up of collagen, primarily type I collagen, as well as other collagen types and a variety of non-collagenous proteins. Bone serves as a home for marrow and a reservoir for calcium homeostasis, as well as providing mechanical and homeostatic functions that protect the organs while permitting movement and load bearing.

Bone Remodelling in Aging

There are many types of cells in the bone mainly originating from mesenchymal cells. Chondrocytes are responsible for the deposition and subsequent formation of the growth plate. Osteoblasts stimulate new bone formation. Osteoblasts are derived from the bone marrow stem cells (BMSCs) in response to the WNT signalling. This is the major pathway that regulates bone mass. Sclerostin acts as an inhibitor for the WNT signalling pathway reducing bone mass and resulting in osteoporosis (Tiede-Lewis et al., 2017). Osteocytes are mature osteoblasts and are especially important for sensing mechanical loading. Osteoblasts are found in the mineralized matrix and are long-lived cells. Mature osteoblasts are responsible to deposit minerals and form the collagen-enriched bone matrix supported by the multifaceted osteocytes. the osteoblasts are the most copious cell type with a vast canalicular network, and they play a key role in maintaining bone mass. Osteocyte signalling molecules have a positive and negative effect on bone remodelling (Pignolo et al., 2021).

Osteoclasts are multinucleated large cells with a hematopoietic origin. They are responsible to resorb the bone matrix which is regulated by extracellular signals secreted by the osteoblasts and osteocytes and is aided by progenitors such as bone marrow monocytes or macrophage precursors. Maria Almeida et. al. has done extensive work regarding the bone aging mechanism. One of their researchers proposes that “A decrease in NAD^+ contributes to the loss of osteoprogenitors and bone mass with aging” (Kim et al., 2021) which is a very critical implication for studying the effect of NAD^+ on various aspects of bone.

Mechanism and Pathways of Age-related Bone Loss

Age-related bone loss is a complex and heterogenous pathophysiology in combination with intrinsic and extrinsic factors. Figure 9 denoting the intrinsic and extrinsic factors in the bone loss. Aging, in conjunction with intrinsic and extrinsic factors, hastens the loss of bone mass, which predisposes to fractures. Intrinsic factors include genetics, peak bone mass accumulation in youth, cellular component alterations, hormonal, biochemical, and vasculature status. Nutrition, physical activity, comorbid medical conditions, and drugs are examples of extrinsic factors (Demontiero et al., 2011).

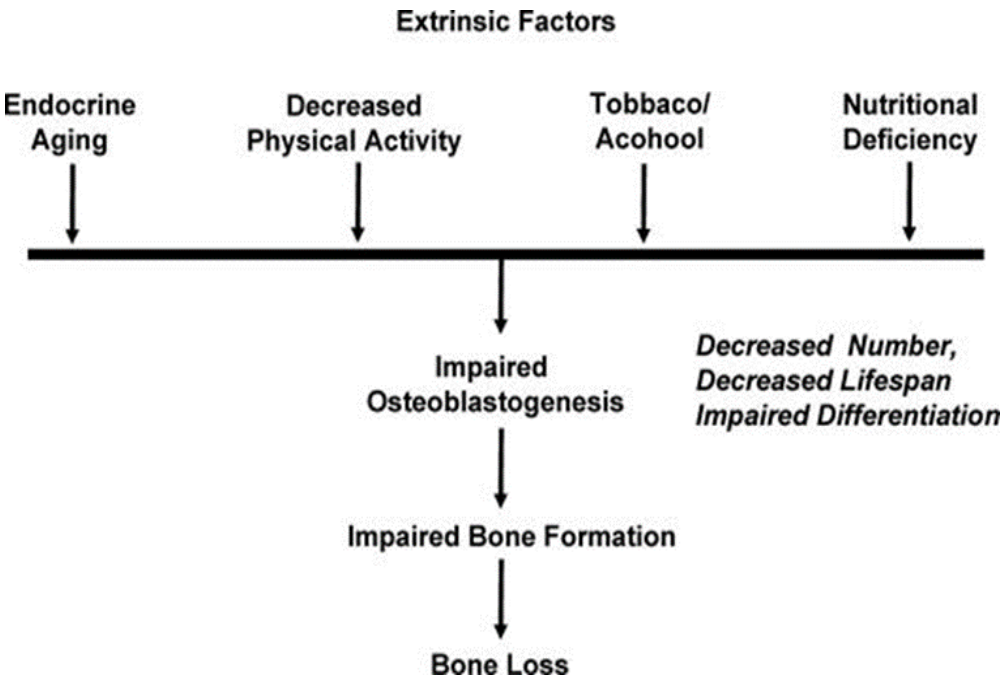

Figure 9. Extrinsic factors are involved in the bone loss

Reprinted from (Marie & Kassem, 2011)

Various signalling pathways and molecules play a vital role in age-related bone loss. One of the key signalling in the bone formation is Wnt signalling which is significant for bone regeneration and homeostasis. Wnt signalling plays a crucial role in the osteogenic differentiation of MSCs, showing that variances in the Wnt signalling pathway may lead to failures of appropriate bone formation. The canonical / Wnt pathway mediates signalling through the stabilization of β catenin which serves as an intracellular signalling molecule in the pathway (Kim et al., 2013). Dysfunction in the β catenin signalling leads to various malignancies. Indirectly the study shows the role of the Sirt1/FoxO/ β catenin pathway in osteoblast progenitors influences bone aging (Kim et al., 2021).

Another key factor includes hormones, which play a significant role in skeletal aging. Parathyroid hormone is playing a key role in association with calcium and vitamin D deficiency. Hyperthyroidism increases osteoclastic activity and bone resorption leading to cortical bone loss. Another hormone contributing to skeletal aging is sex hormones. Several studies demonstrated bone health and deficiency of gonadal

hormones. Various signalling pathways participate in this mechanism. Estrogens are found to be a key player in both males and females by suppressing RANKL production by osteoblastic cells and T-lymphocyte (Demontiero et al., 2011). Biopsy and MRI studies of animal models and humans demonstrated the bone marrow fat accumulation in the aged bone. Mesenchymal cells are differentiated into adipocytes involving the recruitment of MSCs, the release of the proper amount of growth factors and activation of lineage-specific transcription. The bone marrow adipogenesis appears to be lipotoxic on osteoblast. Studies have proposed that autophagy is another possible pathway playing a vital role in bone aging. This pathway maintains the healthy and long-lived cells by destroying the damaged cell organelle and may have a positive impact on the osteocyte (Tiede-Lewis & Dallas, 2019). The cellular senescence pathway is also one of the key players in aging. Reactive Oxygen Species (ROS) levels increase with age by causing cell death in osteoblasts and osteocytes furthermore promoting the osteoclast differentiation activity leading to damaging bone (Pignolo et al., 2021).

Bone loss is caused by three key age-related mechanisms. The first and most serious issue is trabecular bone loss. Trabecular bone loss is caused by trabecular thinning and, especially in early postmenopausal women, alteration of the trabecular microstructure and loss of trabecular components. The second mechanism that contributes to bone loss is a decrease in cortical bone, which is mostly caused by increasing porosity from an increase in resorption cavities as well as an accumulation of incompletely closed osteons with ageing. Continued nett resorption at the endocortical surface is the third process (Chen et al., 2013). The below figure (Figure 10) describing the molecular mechanism in bone loss.

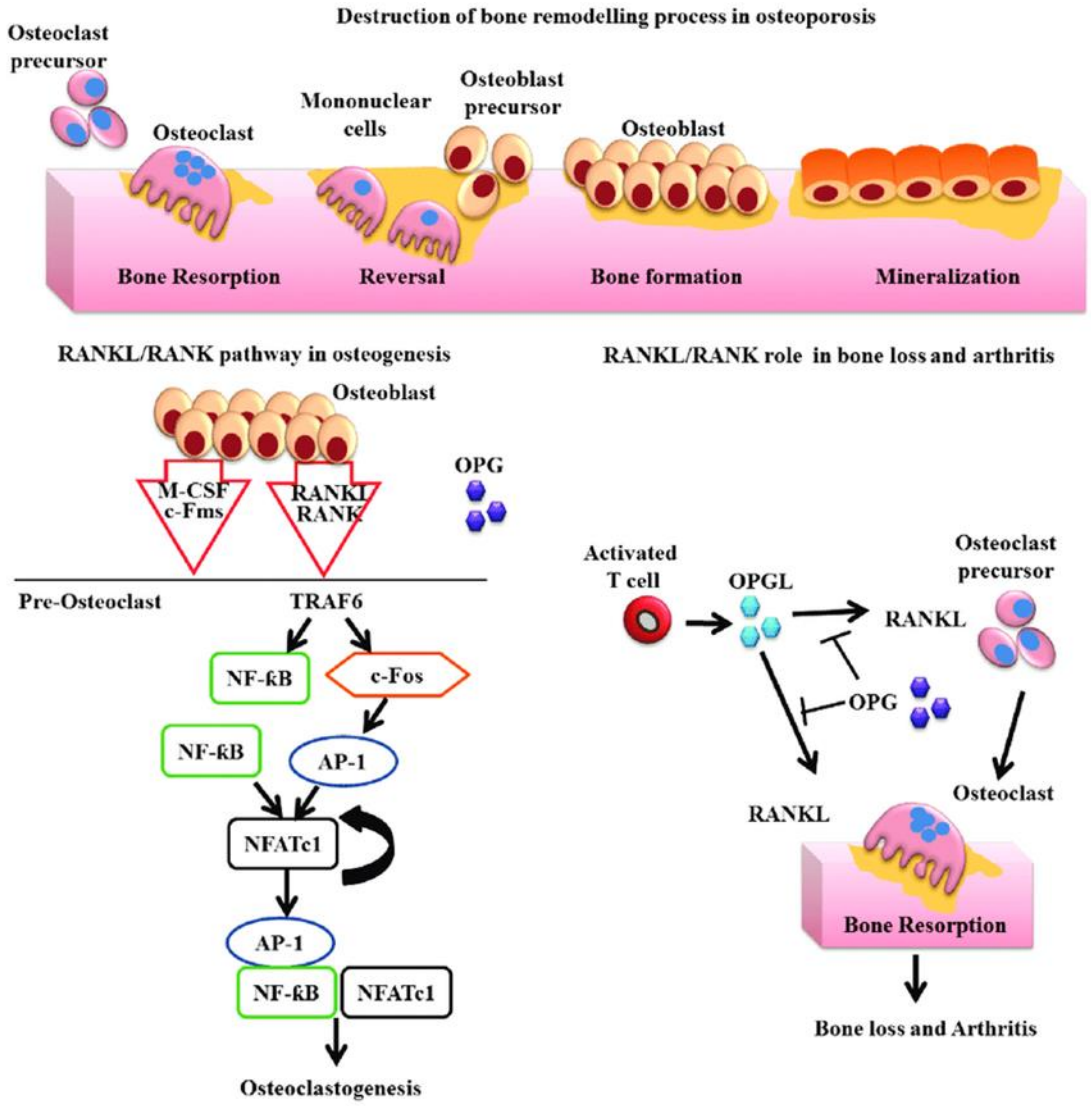

Figure 10. The molecular mechanism involved in bone loss

Reprinted from (Idrus et al., 2018)

Chapter 3 : Materials and Methods

Animal Model

For this study, we received C57 BL/6 different age and NMN treatment mice samples from our collaborator from the University of New South Wales (UNSW). C57 mice were divided into the following groups:

(i) **Young mice** 3 months (12 weeks)

(ii) **Aged mice** 14 months (56 weeks)

(iii) **Aged mice** 14 months (56 weeks) **fed with 0.5mg/l NMN** daily for one month

(iv) **Aged mice** 14 months (56 weeks) fed with **2.0mg/l NMN** daily for one month

(v) **Aged wild type** 14 months (56 weeks)

(vi)**Transgenic mice (NMNAT1**) 14 months (56 weeks)

As previously stated, NMNAT1 is one of the most important enzymes, but it has not been studied in relation to bone aging, hence more research on this topic is required. We have 56 weeks or 14-month-old NMNAT1 transgenic mice, which are encoded by the nmnat1 gene. NMNAT is a stress response protein that has been implicated in numerous biological processes, yet the effects of NMNAT on ageing have yet to be studied (Liu et al., 2018). The transgenic mice used in thus study expressed the NAD^+ biosynthetic enzymes NMNAT1 or NMNAT3 constitutively hence NAD^+ was always present in those mice. The NMNAT group design opted from our collaborators study (Bertoldo et al., 2020).

Figure 11a) shows the experimental model for our study and Figure 11b) shows the mice age and human age comparison.

For each experiment, we used **n=3** femurs from each group.

Ethics

We received the euthanized mice from our collaborator Dr Lindsay Wu from UNSW. We solely used tissue from the samples, and QUT shared usage ethic approval number is AE TU 2022-6579-11893.

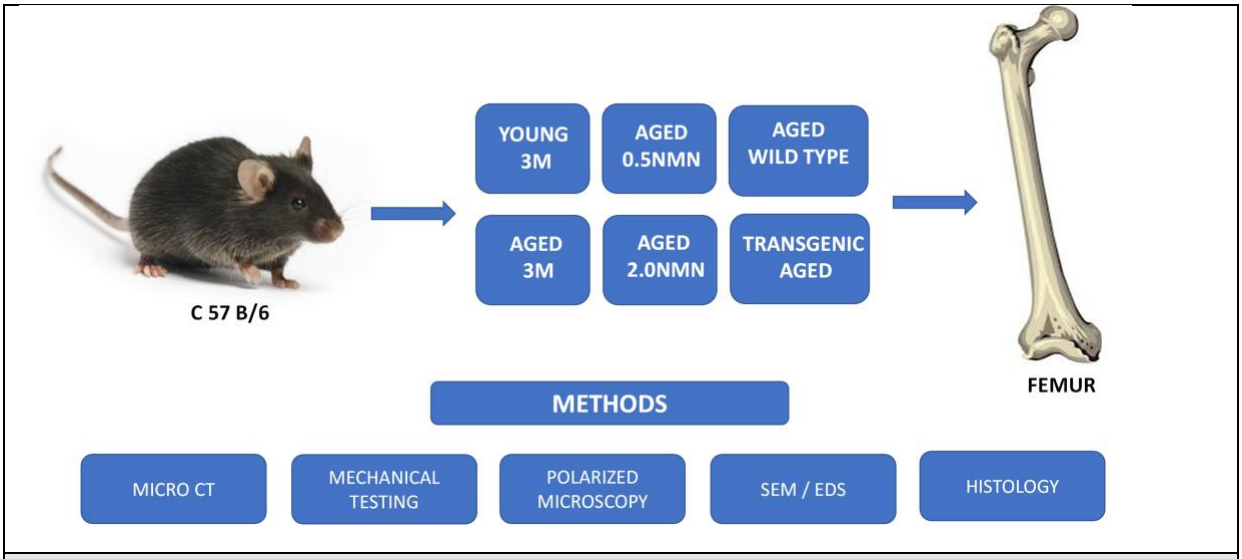

Figure 11. a) Experimental model

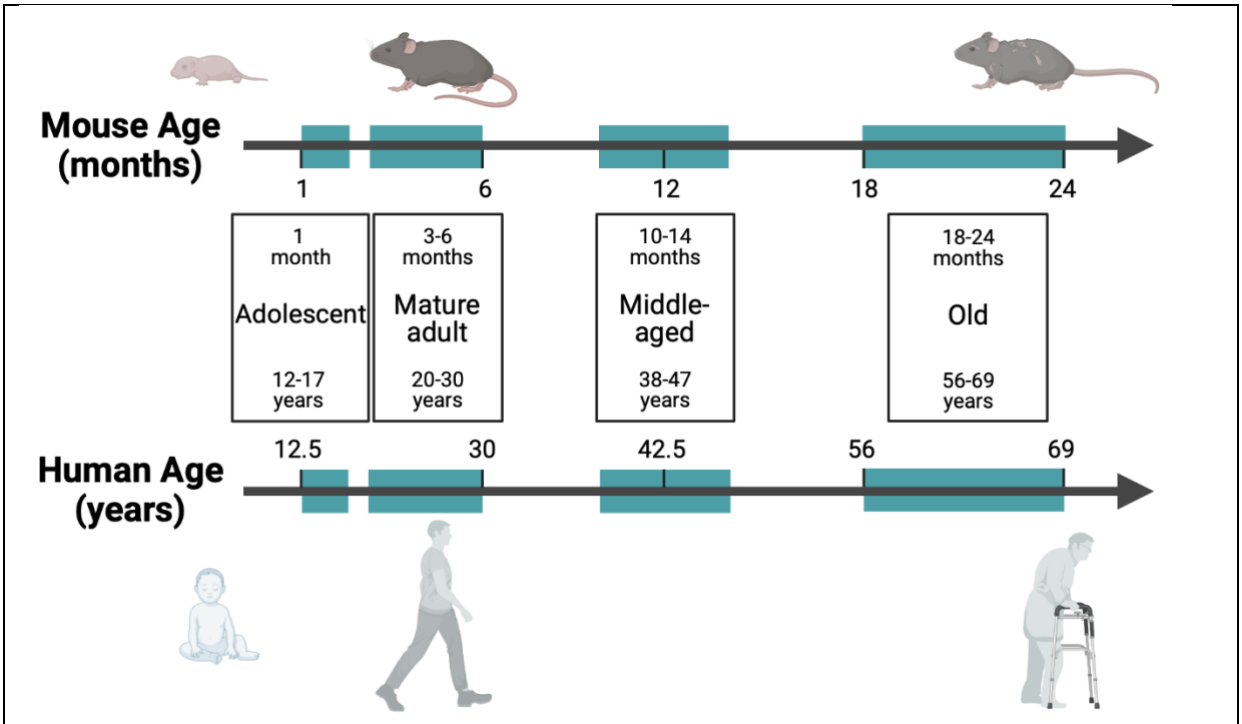

Figure 11. b) Mice age and human age comparison

(Reprinted from (McWain et al., 2022)

Micro-CT

In the small animal study paradigm, the Micro CT technique is the "gold standard" for evaluating bone morphology and microarchitecture. It has been used as a standard imaging technique for the measurement and visualization of bone structure. Trabecular bone is highly porous form of bone tissue organized into a network of interconnected plates and rods which are called as trabeculae. There are cavities in the network of trabeculae which are filled with bone marrow. Trabecular bone is the main load bearing bone in the human skeleton and in the process of osteoporosis or bone loss trabecular bone affects rapidly than cortical bone there is an imbalance of bone regeneration and resorption.

To assess bone loss, we first performed a Micro CT scan on all the mice's bones to determine the quantitative trabecular microstructure parameters.

The dissected femurs were cleaned nicely and washed with PBS solution twice. Three mice femur for all groups except 2.0 mg/l NMN mice for which we had two bone samples were scanned by cone-beam micro-computed tomography (µCT50, SCANCO Medical AG, Brütisellen, Switzerland). Samples were carefully oriented (proximal up, distal down), wrapped in ethanol-soaked foam padding and placed in ø14 mm tubes containing ethanol. Each tube was stacked with three samples separated by foam. Scanning was done in ethanol at an energy of 55 kVp and a current of 145 µA. A 0.1 mm aluminium filter was used during scanning. The integration time was set at 600 ms, once averaged, resulting in a sample time of 0.6 s. Scans of 1 mm height (equivalent to 227 slices) for each region of interest (proximal, mid-shaft and distal) were taken at an isotropic voxel size of 4.4 μm^3. All images were first exported as DICOM files for 3D visualization. The images were then analyzed using the Scanco evaluation program. Bone properties like bone mineral density, bone volume to total volume ratio, trabecular number, and trabecular spacing, trabecular thickness were analyzed using Scanco bone evaluation software. The reconstructed images obtained from Micro-CT accurately represent bone microstructure for quantitative evaluations. Micro-CT50 assessed age-related bone loss and microstructural changes in the bone in all our study groups. The 3D pictures from the region of interest, particularly the proximal,

midshaft, and distal sections of the femurs, were analyzed. The bone volume (BV) / total volume (TV), bone marrow density (BMD), trabecular number, trabecular spacing, and trabecular thickness were evaluated. Further we used Drishti software to reconstruct the 3D images for illustration purpose and which gave us clear idea of scanned bone in 3D view. Figure 12 is the image of Micro CT machine.

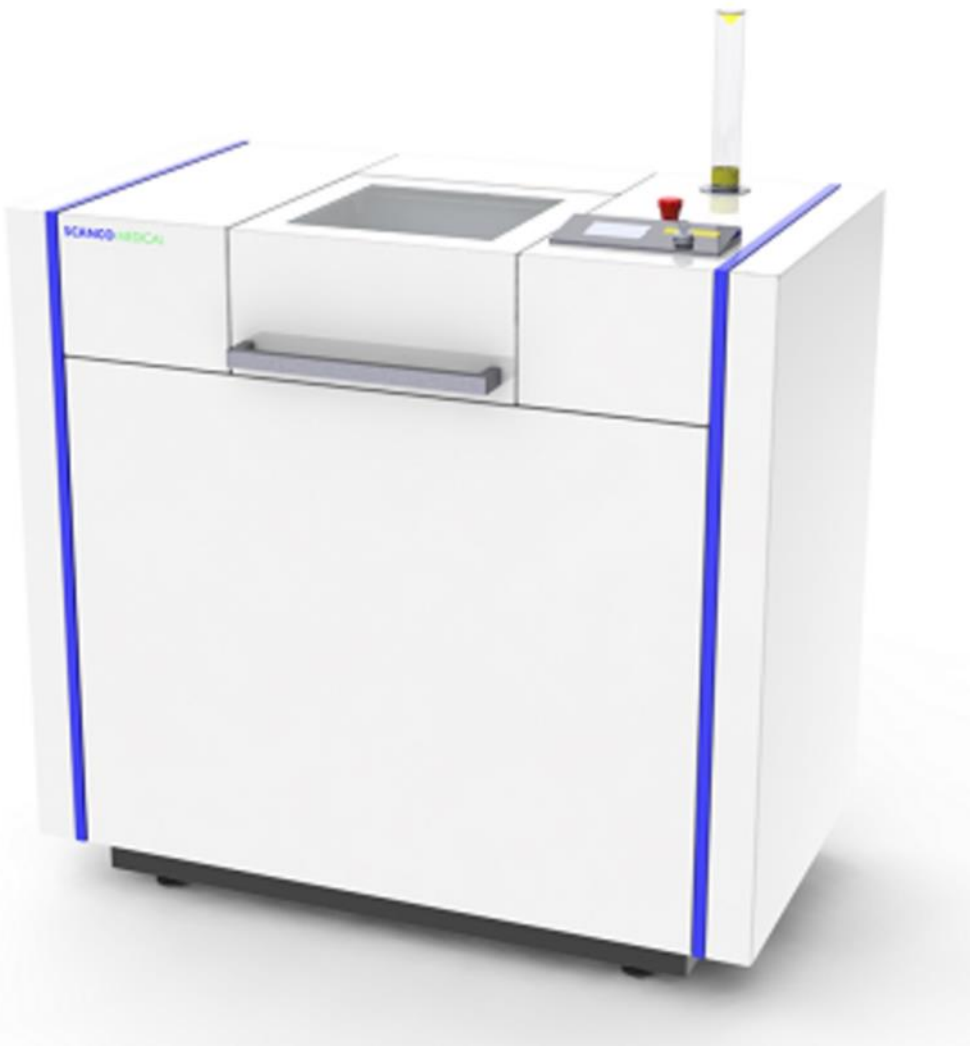

Figure 12. Micro CT Instrument

Reprinted from (Internet Source)

Mechanical Testing

The measuring of the mechanical characteristics of small animal bones has attracted people's interest. Animal models, particularly mice, allow researchers to naturally achieve or genetically design a skeletal phenotype linked to disease, as well as to conduct destructive fracture tests on the bone to assess the consequent change in mechanical characteristics. The bone's mechanical properties at macro or micro to nano level consist of strength, stiffness or elastic modulus which are influenced by the hierarchical structure of the bone. There are various mechanical tests available to perform on the bone such as tensile test, compression test and bending test to analyse the mechanical properties of bone. The bending test is commonly used to assess the mechanical properties of rodents and other small animal bones. There are two types of bending tests are available such as 3-point bending and 4-point bending. The entire bone is loaded in bending until fracture during the bending test (Oksztulska-Kolanek et al., 2016). All biomechanical tests are predicated on a significant link between the load applied to a structure and the resulting displacement, and these parameters are recorded during the test.

For our study we performed 3-point bending of the freshly removed femurs without any muscle or skin remnants from all our study groups (n=3). The samples were always kept moist with PBS solution. In the 3-point bending test, we used a custom-made miniature Trent, using a 30 kN Instron machine in which the whole bone was positioned onto two supports and a single - pronged loading device was applied to the opposite surface at a point precisely in the middle of the two supports. Before testing, the machine was calibrated using a standard weight. The point of fracture in the 3-point bending test was standardized by always placing the femur similarly in the testing machine. A supporter with 2 loading points, 10 mm apart, as shown in Figure 13, was used on the stage of the testing machine. A press head compressed the middle of the femoral shaft at a constant speed of 0.2 mm/s until fracture occurred.

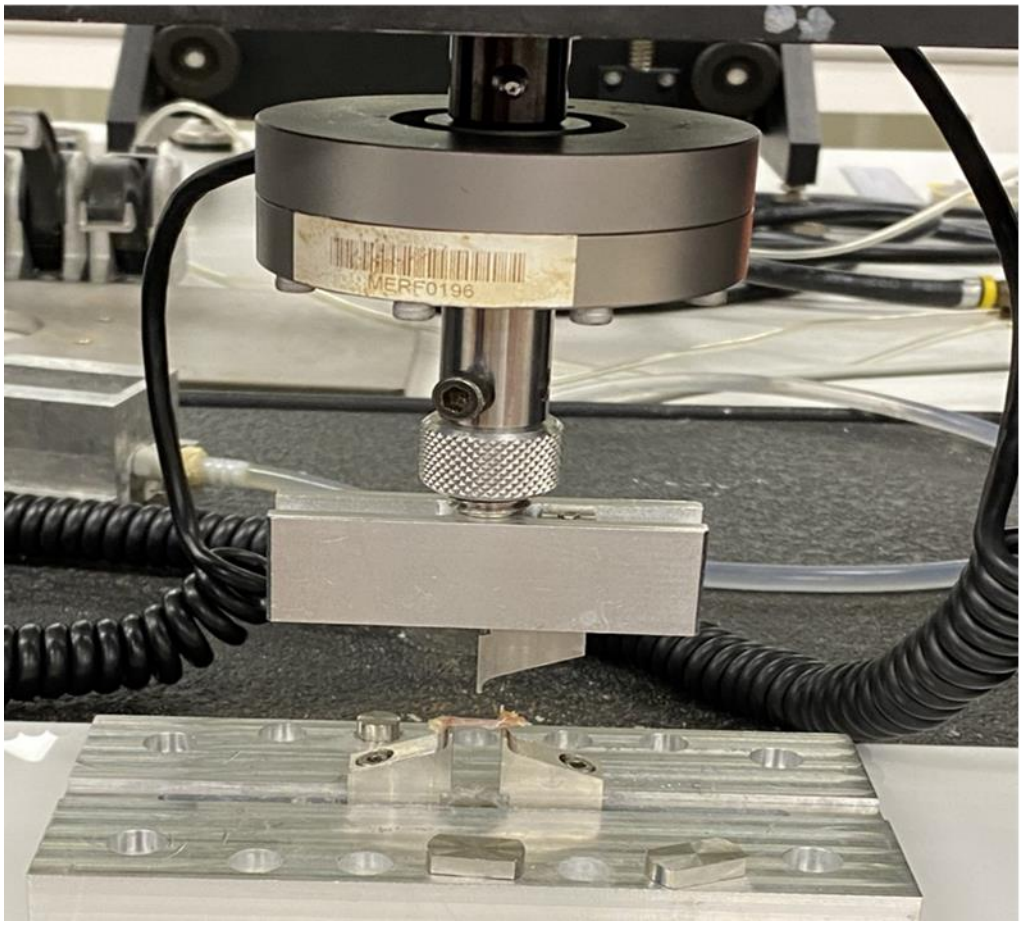

Figure 13. 3-point bending test of the mice femur in 30kN Instron Machine (Actual experiment photo)

Polarized Light Microscopy (PLM)

Bone is a mineralized tissue that supports the body structures and maintains mineral homoeostasis in bodily fluids. It is made up of organic extracellular matrix (ECM) and inorganic minerals. Type I collagen is the most abundant ECM component in bone accounting for total 90% of total protein content. Type I collagen's biochemical and biophysical properties are known to influence cell behaviour, such as survival, proliferation, and differentiation. The previous study suggested that characterising collagen crosslinking in bone may be useful to gain insight into both bone cellular activities and mechanical properties.

ECM staining is especially useful in studying tissue remodelling, particularly in connective tissue pathologies. Several studies have shown that picrosirius red staining is especially useful for observing collagen network abnormalities in connective tissue. The collagen appears different colours under polarised light with picrosirius staining in areas where collagens I, II, and II are located. Collagen type I was yellow, orange, or red, whereas collagen type III was green. Collagen type II is found in cartilage, and chondrosarcomas vary in colour depending on the tissue and species. However, there is always a clear distinction between collagen types I and III in terms of colour and morphology.

Changes in the collagen structure of bone may contribute to brittle ness due to the shift in the cross-linking profile of the collagen present. Investigations of collagen fibre orientation in bone provided the important insight of the bone structure and composition changes as per our study groups of NMN treatment and aging. So next we assessed the bone quality in terms collagen structure.

For polarized light microscopy (PLM), all specimens, n=3 per group were fixed with 4% paraformaldehyde for 24 hours followed by decalcification of al specimens by 10% EDTA. Specimens were in EDTA around 4 weeks. EDTA was changed every alternate day. Decalcification was confirmed by poking a needle into the bone. After decalcification, the specimens were embedded in paraffin using standard protocol. Serial 5µm sections cut on a Leica RM2145 rotary microtome (Leica Microsystem, Richmond Hill, Ontario, Canada). The serial sections were stained with Picrosirius red by using standard protocol. Sections were then mounted for observation under polarized light microscopy (Nikon LV100ND Polarized). Pictures of the same areas

were taken under the same conditions (exposure time) before and after 90^0 stage rotation and compared.

Energy Dispersive X-ray Spectroscopy (EDS)

As we saw collagen structure of the mice femurs, we then performed the elemental analysis of bone to see the calcium and phosphorous content of the bones. Calcium and phosphorous are the main constituents of hydroxyapatite crystals of the bone and due to aging these 2 micronutrients decreases succumbing bone to the bone loss. The phosphorous is present in the form of inorganic phosphates. For proper bone mineral acquisition and preservation throughout life, calcium and inorganic phosphate make up a crucial combination (Bonjour, 2011 #352). The elemental composition of a sample or a small area of interest on a sample can be determined using the EDS technique. Inside a scanning electron microscope, a sample is exposed to an electron beam during EDS (SEM). For our study, the bone samples were prepared using following method. The femurs from each group were fixed in 4% PFA at 4^0 C for 24 hours. Samples were dehydrated through ascending concentrations of ethanol (70%, 80%, 90%, 100%) at 4°C over a 3-week period, placed in a transitional solvent, xylene, at ambient temperature for 8 hours, then infiltrated with increasing concentrations of methyl methacrylate resin (pre-infiltration and infiltration solutions) at 4°C for 7 days each. Samples were placed in Technovit 9100 resin embedding solution (9:1 Solution A: Solution B), placed under negative pressure (0.1mbar) for 5 minutes to remove air, and then polymerised at -20°C for 5-7 days. Please refer Table 1 and 2 for the resin solution details for how much ingredients were used to make the resin blocks.

Resin Solutions

STOCK Solutions	Basic Solution (filtered) – Methyl methacrylate	PMMA Powder	Hardener 1	Hardener 2	Regulator
WORKING Solutions					
Pre-Infiltration	200ml		1g		
Infiltration	250ml	20g	1g		
Stock Solution A	500ml	80g	3g		
Stock Solution B	50ml			4ml	2ml

Table 1. Resin solution preparation process

Technovit 9100 Resin Embedding Protocol

Reagent	**Amount**	**Date Prepared**	**Comment**
Solution A	270ml	11/06/2021	
Solution B	30ml	11/06/2021	
	300ml		

Table 2. Amount of resin solution used to prepare final resin for our experiment

1. Washed all glassware, cylinders, flea, embedding moulds, etc with 100% ethanol.
2. Allowed to dry before use.
3. Labelled all embedding moulds.
4. Placed samples into moulds and photograph.
5. Started the timer and combined Solution A & Solution B.

6. Mixed embedding medium in a clean glass beaker, completely covered in alfoil, in fume hood for 5 mins.
7. Carefully added a solution to embedding moulds so the orientation/location of samples was maintained.
8. Lifted samples to ensure resin was beneath the sample and to remove any trapped air bubbles.
9. Placed moulds and xs resin into the Citovac.
10. Evacuated, then held at vacuum for 5 mins before venting.
11. Removed samples from Citovac.
12. Lifted samples to remove any trapped air. Topped up with resin.
13. Repositioned samples within moulds.
14. Seal lids.
15. Transferred in clip lock container to BTM freezer in Q519.
16. Recorded time was taken from commencement of resin mixing to freezer 17 minutes.
17. Decanted fresh infiltration solution from sample pots into the USED Infiltration bottle (on the lower shelf of the resin fridge) and refrigerated again.
18. Filled in usage forms on the fridge: Sol A, Sol B, Used Infiltration
19. Filled in the Citovac logbook.
20. Cleaned all glassware, cylinders, flea, etc with 100% ethanol.
21. Placed on sink drainer to dry.

Once the blocks were ready, further we cut the resin blocks in the suitable size and polished them to prepare the samples for the scanning electron microscopy and electron dispersive spectroscopy. The sample size after polishing was 14mm thick and 30mm wide. **All This work was enabled by use of the Central Analytical Research Facility (CARF) at the Queensland University of Technology (QUT).**
We decided to measure the quantity of major mineral from the femur, so we determined the calcium and phosphorous from the young, aged, 0.5 NMN, 2.0 NMN and transgenic mice femur. Our resin embedded samples were coated with 10mm of carbon and images were acquired using TESCAN MIRA3 scanning electron microscope with 80 mm2 ThermoFisher Scientific UltraDry EDS detector. Element analysis was performed by Pathfinder Microanalysis software using the Spectral Imaging function using 15 kV with beam intensity of 15.

Histological Staining

Staining and Sectioning

All This work was enabled by use of the Central Analytical Research Facility (CARF) at the Queensland University of Technology (QUT).

Histopathology routinely employs **hematoxylin and eosin** staining techniques to identify various cell types and tissues, which reveal details about the distribution, morphology, and structure of cells in a tissue sample. H and E stain is the combination of two stains namely hematoxylin which stains nuclei a purple blue and eosin which stains ECM and cytoplasm pink with other structures staining in various shades, hues, and combination of these colours.

Safranin-O staining is also known as basic red 2 is a biological stain used in histology and cytology. It can be used to identify cartilage, mucin, and mast cell granules, among other things. Safranin-O is frequently used to distinguish between chondrocytes produced from human and rodent mesenchymal stem cells. In some staining techniques, safranin is used as a counterstain, turning all cell nuclei red.

n=3 femurs per group were processed for the decalcification. The freshly isolated femurs were cleaned off the soft tissue and then fixed in 4% paraformaldehyde for 24 hours. Rinsed thoroughly with PBS and kept in the 10% EDTA for four weeks on the shaker. The EDTA was changed every alternate day. After four weeks the decalcified bones were embedded in paraffin by using a standard protocol from our group. Serial 5µm sections cut on a Leica RM2145 rotary microtome (Leica Microsystem, Richmond Hill, Ontario, Canada). These sections were further used for hematoxylin-eosin (H and E) and safranin O staining following the standard staining protocol from our group.

Statistical analysis

Statistical difference of the results for Micro CT and EDS was tested using two-way ANOVA analysis (4 groups) followed by Tukey's multiple comparisons of femur ROI for study groups and two-way ANOVA analysis (2 groups) followed by Sidak's multiple comparisons of femur ROI for study groups. Mechanical testing analysis using Unpaired t-test, significance is defined as $p<0.05$

All analyses were performed using GraphPad Prism 9, and $p \leq 0.005$ is significant. All data are presented as mean ± SD.

Chapter 4 : Results

Micro CT Results

Micro CT result showed that aging was associated with changes in bone trabecular parameter whereas NMN treatment did not show profound changes in all the trabecular parameters

Micro CT analysis of our study groups showed changes in the femoral structure with aging. There is a significant change in terms of Tb N, Tb Sp and BV/TV of aged and young mice. The Tb N and BV/TV is decreased, and Tb Sp increased in the aged mice when comparing with young mice femur. As per the literature the aged mice femur showed aging effect in our results as well. We assessed the femur at proximal, midshaft and distal areas. Reconstructed images of our region of interest (ROI) were shown in the Figure 14. By using Drishti software. The data obtained after scanning each bone has been presented in the table format. Please refer tables at the end of the thesis in the supplementary information section. Supplementary table 1 to supplementary table 12 specified the Micro CT scanning data n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group trabecular parameters were measured at the proximal, midshaft and distal of each study group.

The respective results as below

Trabecular Number (Tb N)

The trabecular number at the proximal end was decreased in the aged mice and NMN treated groups than in the young mice where p value is $\leq$ 0.005.

There was no change in the midshaft of any of the groups including NMN treatment.

The trabecular number at the distal was increased in the young mice than in the aged mice and NMN treated mice.

There was no change at the proximal and distal end of the wild type and transgenic mice in terms of the trabecular number instead we could observe the increased Tb number in the transgenic mice at the midshaft than the wild type Tb number.

Please refer Fig 15-a for the trabecular number graph.

The trabecular number was increased at the proximal and distal end of the young mice compared to aged and NMN treatment groups. Midshaft had no change in any of the study groups including NMN treatment.

Moreover, the midshaft of transgenic mice showed increased Tb number than the wild type mice but not any change at the proximal and distal end.

Trabecular Thickness (Tb Th)

There was no change in the trabecular thickness at the proximal end of the femur of any study groups.

The trabecular thickness of young mice was greater than in NMN treatment groups at the midshaft and lower in young mice at the distal end compared to other mice groups.

There was no change in the wild type and transgenic mice trabecular thickness at any region of interest.

Please refer Fig 15-b for the trabecular thickness graph.

Tb Th was not changed at the proximal end of any study group. It had been seen to increase in the midshaft of young mice than in other animals and decreased at the distal of young mice than in the other study groups. The wild type and transgenic mice comparison did not show any change in the Tb Th.

Trabecular Spacing (Tb Sp)

Trabecular spacing increased in all the aged mice study groups than in the young mice at the proximal, midshaft and distal end.

Wild type and transgenic mice comparison there was no change at the proximal end but midshaft and distal end showed increased Tb spacing in transgenic mice.

Please refer Fig 15-c for the trabecular spacing graph.

Tb Sp is increased in all the aged mice than in the young mice in all three regions. Tb Sp exhibited high in the transgenic mice than in WT mice at the midshaft and distal end.

Bone Volume / Total Volume (BV/TV)

BV/TV was higher in young mice than in all the other groups of aged and NMN treatment mice groups, but an increase has been seen in BV/TV at the distal end of transgenic mice than the wild type mice.

Please refer Fig 15-d for the bone volume to total volume ratio graph.

BV/TV was higher in young mice at the proximal and distal end than in all the aged and treated animals. BV/TV was slightly increased in aged mice than all other groups at the midshaft only.

Bone Mineral Density (BMD)

BMD was increased in all the three groups namely aged, 0.5 NMN and 2.0 NMN mice than in the young mice in the three ROIs.

There was no change in the BMD comparing wild type to the transgenic mice in any region of the bone.

Please refer Fig 15-e for the bone mineral density graph.

Bone Surface / Bone Volume (BS/BV)

The BS/BV was higher in the young femur at the distal end of the bone as compared to aged and NMN treatment groups, but there was no change in the midshaft of any young, aged or NMN treatment either. The NMN treatment groups did not show any increase in the BS/BV than aged group. BS/BV was higher in the distal of the young femur than the aged femur.

Please refer Fig 15-f for the bone surface to bone volume ratio graph.

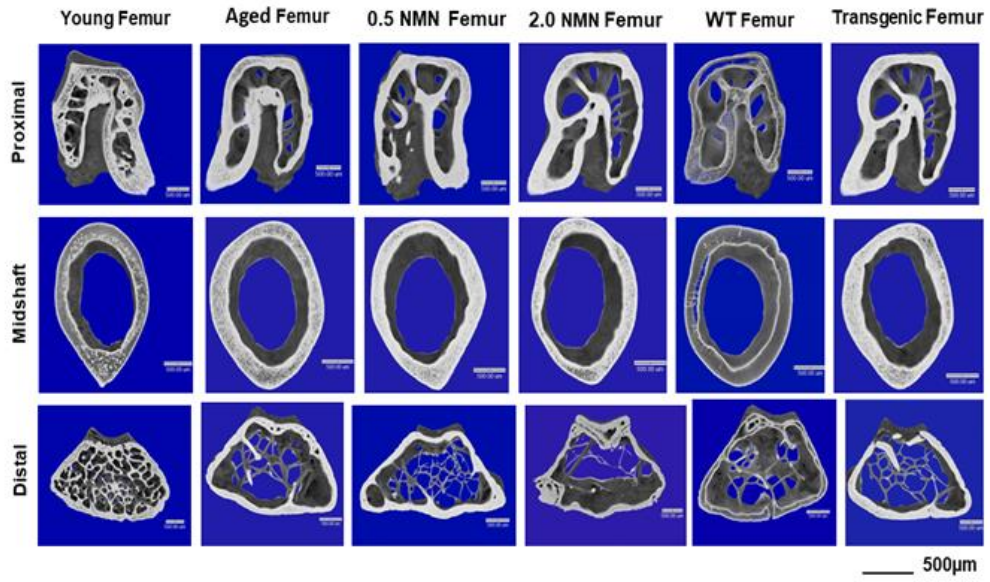

Figure 14. Representative image of Micro CT reconstruction of femur at proximal, midshaft and distal areas done for all the study group animals

The images were obtained by using 3D software Drushti to reconstruct the images to visualize the trabecular parameters at proximal, midshaft and distal part of the mice femur. 1mm bone at each region was analysed by trying to be as consistent as possible while selecting region of interest manually.

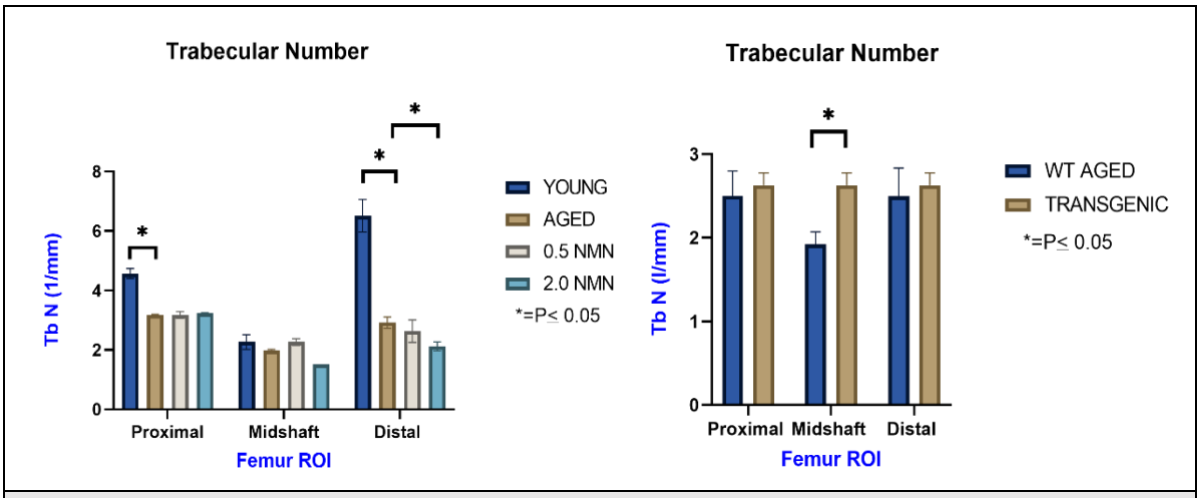

Figure15. a) Trabecular number graph

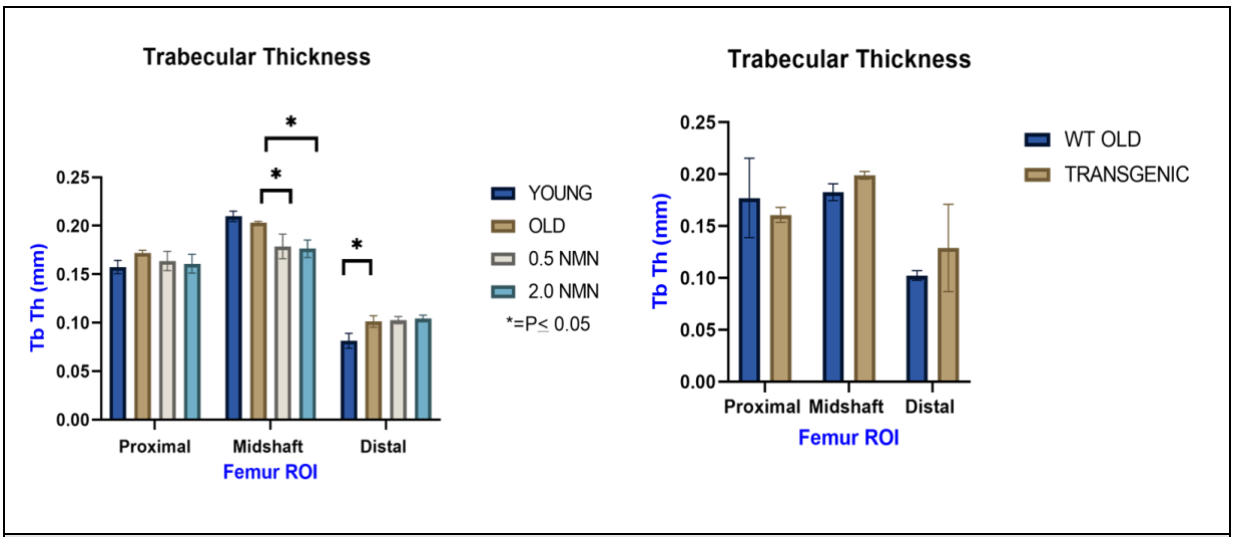

Figure 15. b) Trabecular thickness graph

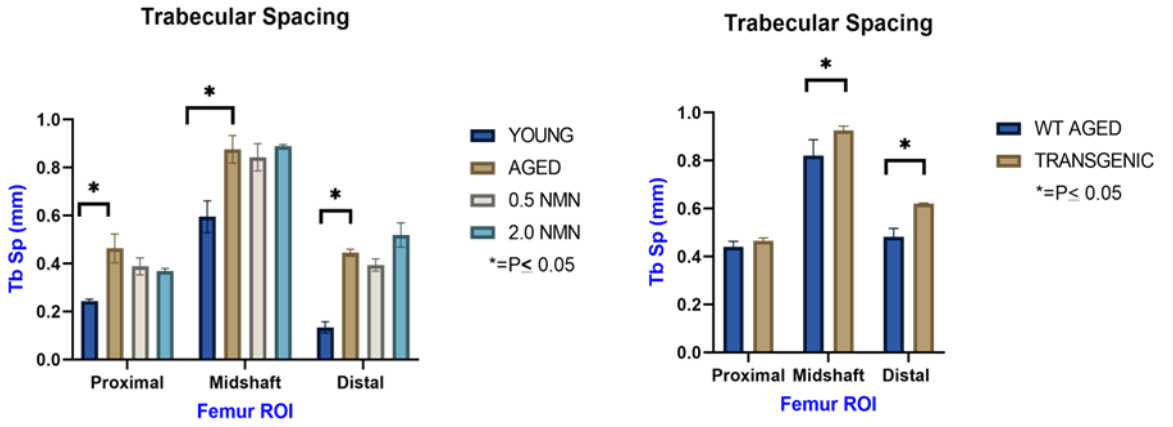

Figure 15. c) Trabecular spacing graph

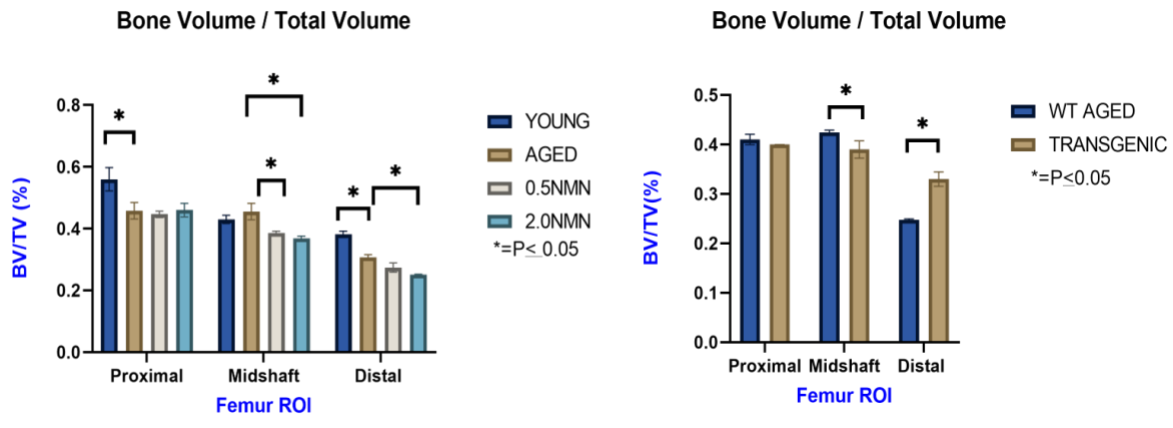

Figure 15. d) Bone volume to total volume ration graph

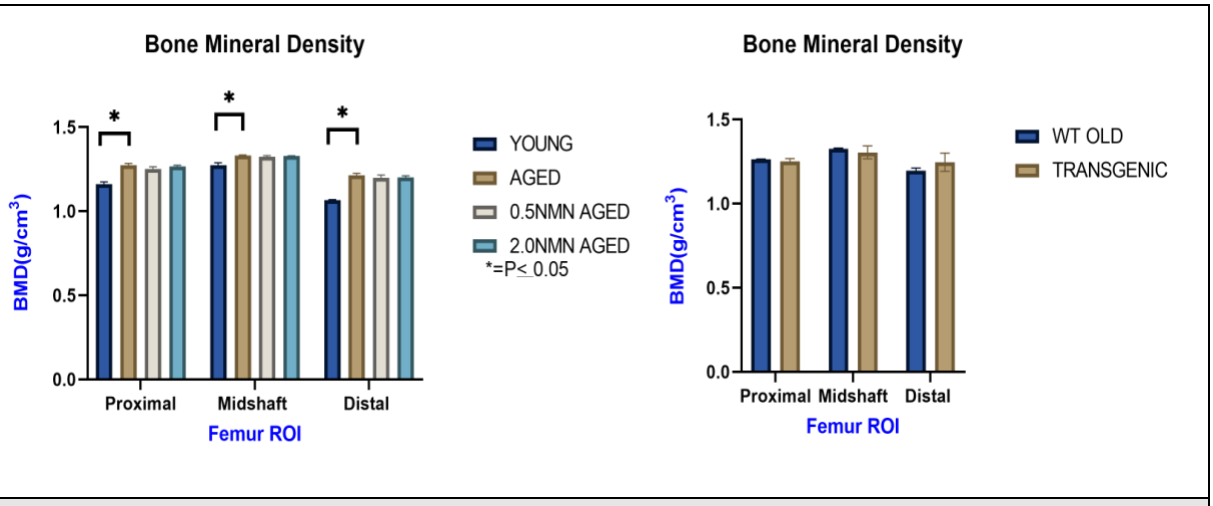

Figure 15. e) Bone mineral density graph

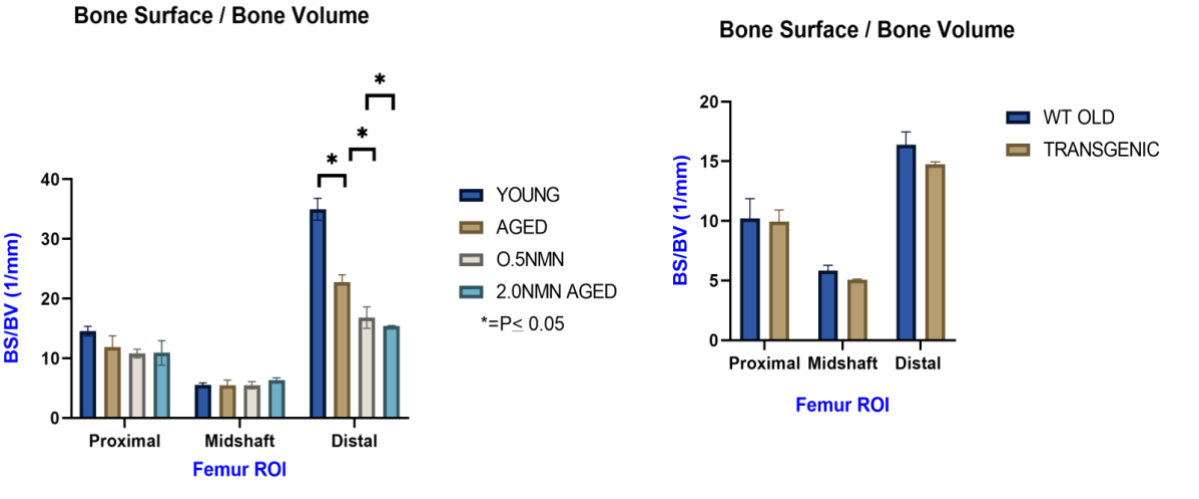

Figure 15. f) Bone surface to bone volume ratio graph

Figure 15. (a-f) shows the trabecular parameter analysis for young, aged, NMN treatments and transgenic mice

The above graphs from a-f show Micro CT quantitation of trabecular bone parameters such as trabecular number (Tb N), trabecular thickness (Tb Th), trabecular spacing (Tb Sp), bone volume / total volume (BV/TV), bone mineral density (BMD) and bone surface / bone volume (BS/BV). Data are mean± SEM, * = p≤ 0.05, ANOVA/Tukey's, / Sidak's n= 3 for all the groups except 2.0 NMN mice n=2

Mechanical Testing Results

The transgenic mice exhibited the higher strength than the wildtype mice whereas the NMN treatment also had the positive effect though not significant

After studying the trabecular parameters in Micro CT, the three-point bending technique was used to assess the mechanical property such as fracture point of the mice femur. A constant amount of force was applied to the midpoint of the femur to determine the maximum load that could be applied to the femur until fracture or breakage occurred. Figure 16 a) is the experimental set up for the mechanical testing of the mice femur.

We wanted to investigate the mechanical property of the bone to evaluate the strength of the bone if the NMN treatment show any change in the mechanical properties of the bone. From the graph (Figure 16-b) we observed that the aged bone fracture point was occurred earlier than the 0.5NMN and 2.0NMN group. It means aged bone attended the fracture point easily with less force needed. There was an increase in the strength of the NMN treated bone. Though the ANOVA analysis demonstrated no significant difference in the values of fracture point but the individual force for the fracture point was bit higher for the NMN treatment bones (Table 13 in the supplementary information). Transgenic bone well exhibited increase in the strength when compared with the wild type aged femur as shown in the 3-point bending transgenic mice graph (Figure 16 b). From the ANOVA analysis using Unpaired t-test for the transgenic mice, it was clearly demonstrated the significance was defined as $p \leq 0.05$ and P value was 0.0035 which denotes the transgenic mice femur exhibited anti-aging phenotype in terms of mechanical testing or the strength of the femur. Please refer table 13 and 14 for the mechanical testing data.

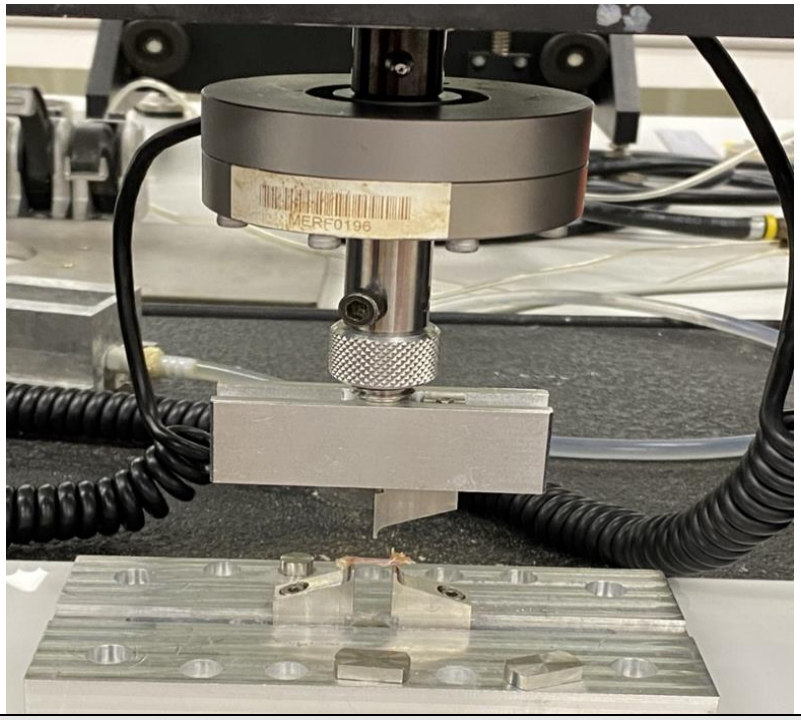

Figure 16. a) 3-point bending test of the mice femur in 30kN Instron Machine (Actual experiment photo)

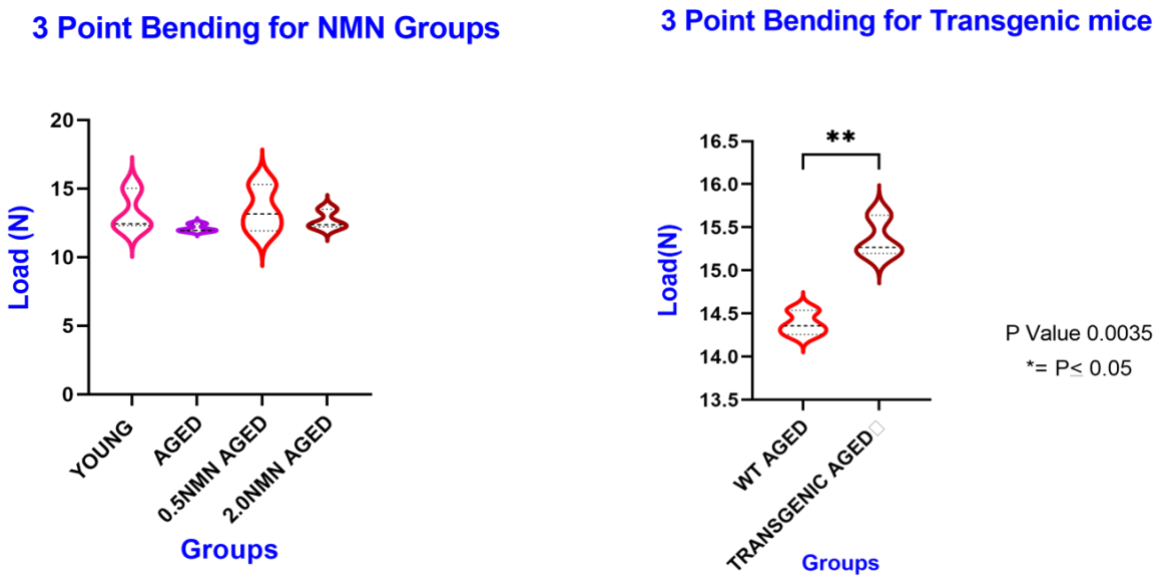

Figure 16. b) 3 point bending graphs for all study groups

* Indicates the significant difference. The P value is ≤ 0.05 and n=3. Group comparison by ANOVA analysis followed by Tukey's test for NMN treatment and Unpaired -T test for transgenic mice to wild type group comparisons

Polarized Light Microscopy Results

NMN treated femur and transgenic femur showed the uniform orientation of collagen fibres giving NMN femur and transgenic femur a strong structure

Type I collagen is the most abundant ECM component in the bone which is indicated by red orange colour under the polarized light with picrosirius red staining.

After studying Micro CT and mechanical testing of mice femur further we evaluated the collagen structure in the mice femurs. Images taken under polarised light microscopy of NMN-treated bones, specifically 2.0 NMN femurs and transgenic mice femurs, revealed uniformly bright contrast throughout cortical bone thickness, indicating uniform collagen orientation along the bone long axis within the cortical bone. The femur of the aged mice, in contrast, displayed darker and patchier contrast, indicating that the collagen fibbers' orientation along the long bone axis was not particularly prominent.

From the Figure 17 we could clearly demonstrate that collagen I was abundant in 2.0 NMN and transgenic mice femur than the aged femur and with no collagen orientation abnormalities were seen. The aged femur showed distorted collagen orientation showing the aging effect in the aged femur. The NMN treatment was restoring the collagen structure.

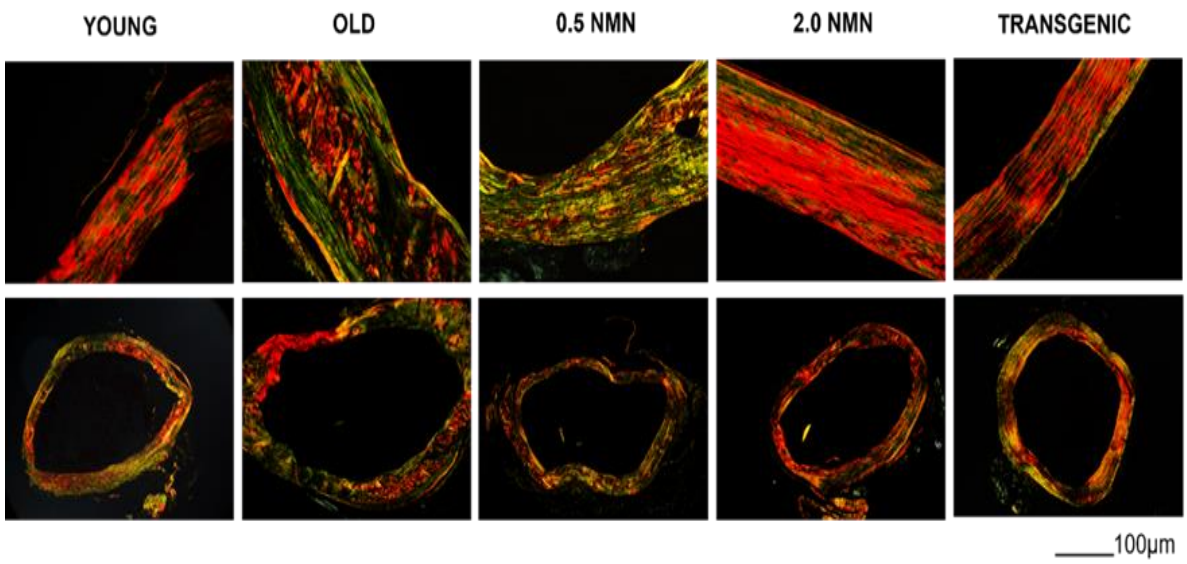

Figure 17. Representative images of Polarised Light Microscopy done for all the study groups

Deterioration of collagen fibre preferential orientation in the femur of all study groups mice. Images taken with a polarised light microscope of longitudinal sections around the midshaft of the young, aged, and transgenic mouse femurs (n=2). Birefringence images representing collagen orientation taken under similar conditions

Energy Dispersive X-ray Spectroscopy (EDS) Results

When comparing the calcium and phosphorus content of NMN-treated bone to aged bone, there was no discernible difference.

The EDS study was carried out to evaluate the important minerals of our study femurs. We could find out the phosphorous content was lower in the young mice than all the aged mice femur. The calcium content of the young mice was higher in the than all the aged mice femurs. Elemental analysis representation of scanning electron microscope for each group is shown in Figure 18. The graph in the Figure 19 showed the representation of the peak attend during the elemental analysis of the calcium and phosphorous. From the obtained data it had been indicated that there was not significant difference in the phosphorous and calcium contents of aged mice and NMN treatment mice groups which is represented in the form of graph in Figure 20. The NMN treatment didn't help in rising the Ca and P content in the bone.

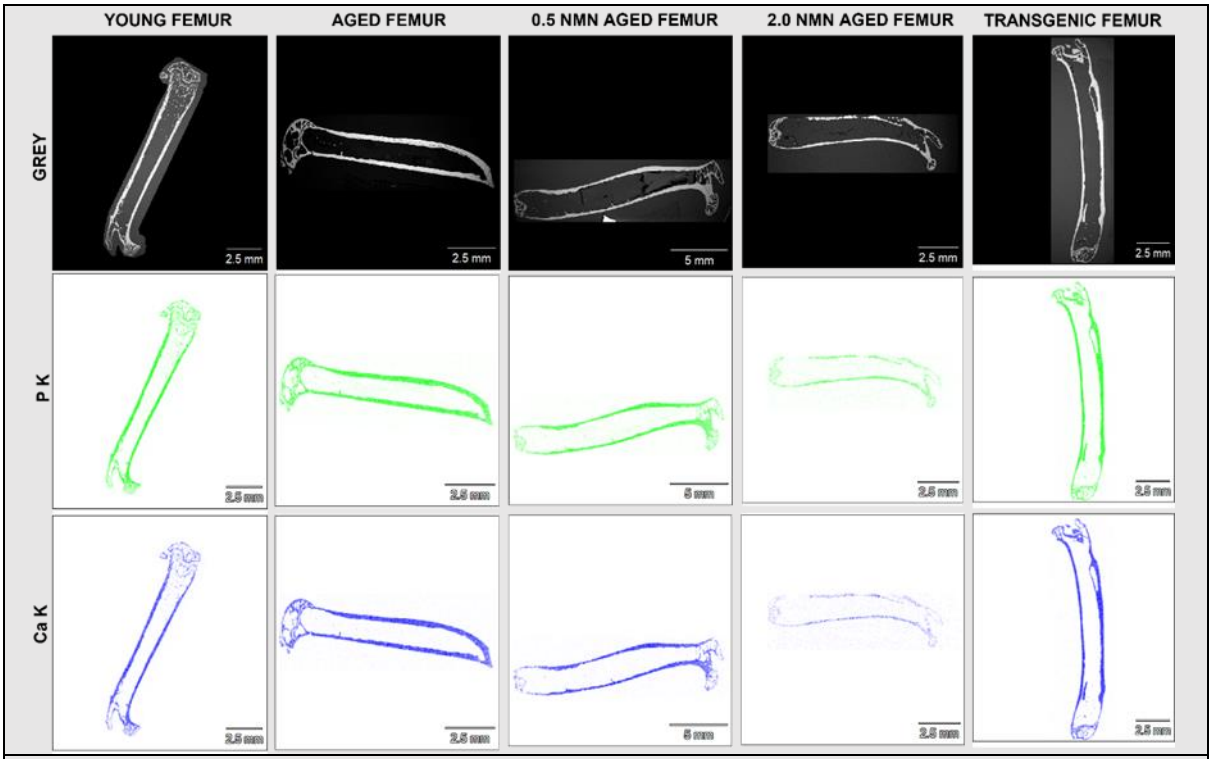

Figure18. Representative images from the SEM-EDS for each study group (n=2)

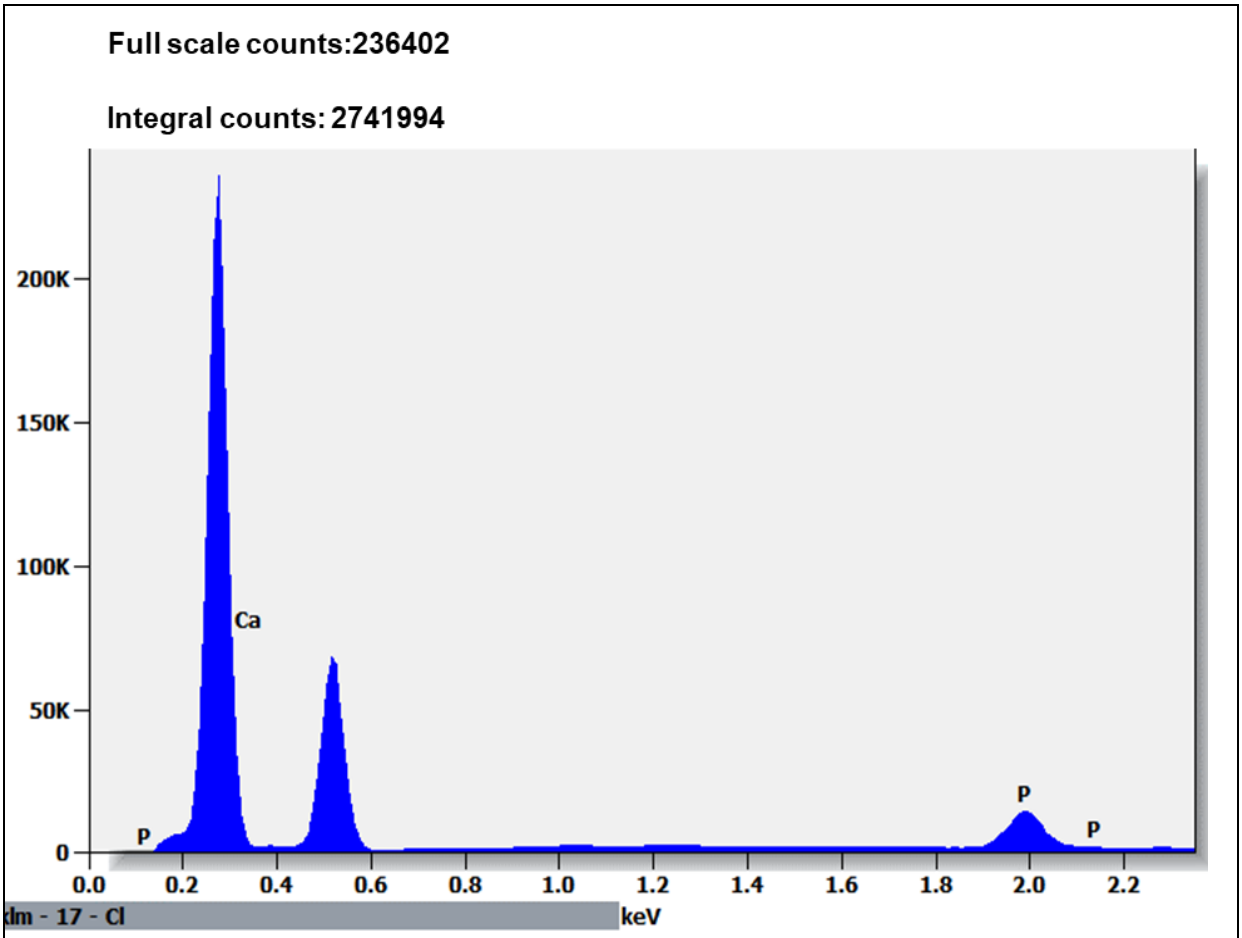

Figure 19. Representative graph of phosphorous and calcium content across all the study groups

The graph displays the peak levels of calcium and phosphorus that were found during the elemental analysis of the study groups

Groups		P		Ca		Total
		Weight % Error	Atom % Error	Weight % Error	Atom % Error	
Young	Femur 1	23.13 ±138.79	28.03 ±168.15	76.87 ±3382.24	71.97 ±3166.88	100.00
	Femur 2	32.62 ±0.33	42.65 ±0.44	54.11 ±0.43	54.67 ±0.43	100.00
Aged	Femur 1	34.81 ± 0.32	40.86 ± 0.37	65.19 ± 0.50	59.14 ± 0.45	100.00
	Femur 2	35.56 ± 0.28	41.66 ± 0.33	64.44 ± 0.40	58.34 ± 0.36	100.00
0.5 NMN	Femur 1	34.73 ± 0.29	40.78 ± 0.34	65.27 ± 0.42	59.22 ± 0.38	100.00
	Femur 2	36.57 ± 0.30	42.73 ± 0.35	63.43 ± 0.43	52.27 ± 0.38	100.00
2.0 NMN	Femur 1	35.60 ± 0.38	41.70 ± 0.44	64.40 ± 0.63	58.30 ± 0.57	100.00
	Femur 2	32.52 ± 0.49	41.41 ± 0.62	57.55 ± 0.69	56.64 ± 0.68	100.00
Transgenic Femur	Femur 1	33.15 ± 0.25	39.09 ± 0.29	66.85 ± 0.34	60.91 ± 0.31	100.00
	Femur 2	34.81 ± 0.32	40.86 ± 0.37	65.19 ± 0.50	59.14 ± 0.45	100.00

Table 3. Quantitative results of EDS

The table denotes the quantitative results of phosphorous and calcium content in the young, aged, NMN treatment and transgenic mice bone shown in terms of percentage with ± error

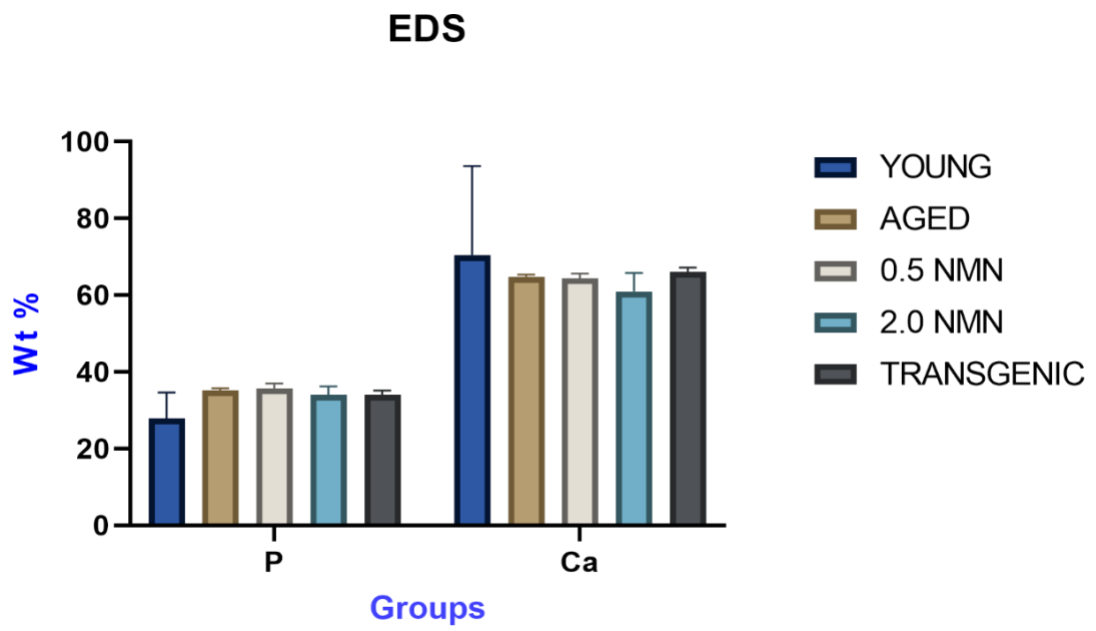

Figure 20. SEM-EDS graph

The graph showing the phosphorous and calcium content of young, aged, NMN treatments and transgenic mice

Histological Staining Results

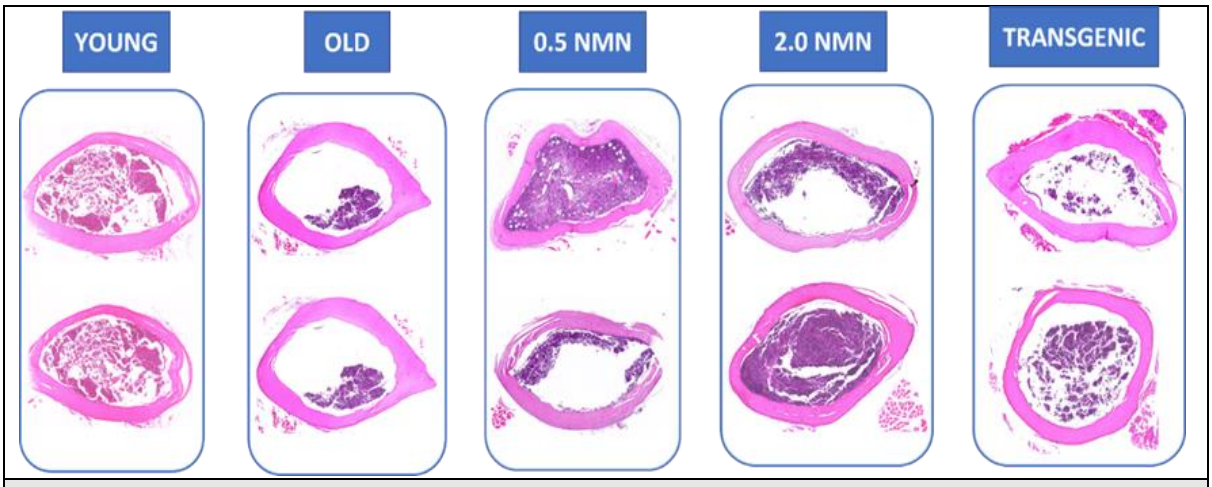

Figure 21. Hematoxylin and Eosin (H and E) staining for all study groups cortical bone

Representative of H and E-stained sagittal sections of young, aged, NMN treatments in mice femur at the cortical bone. Scale bar **500µm**

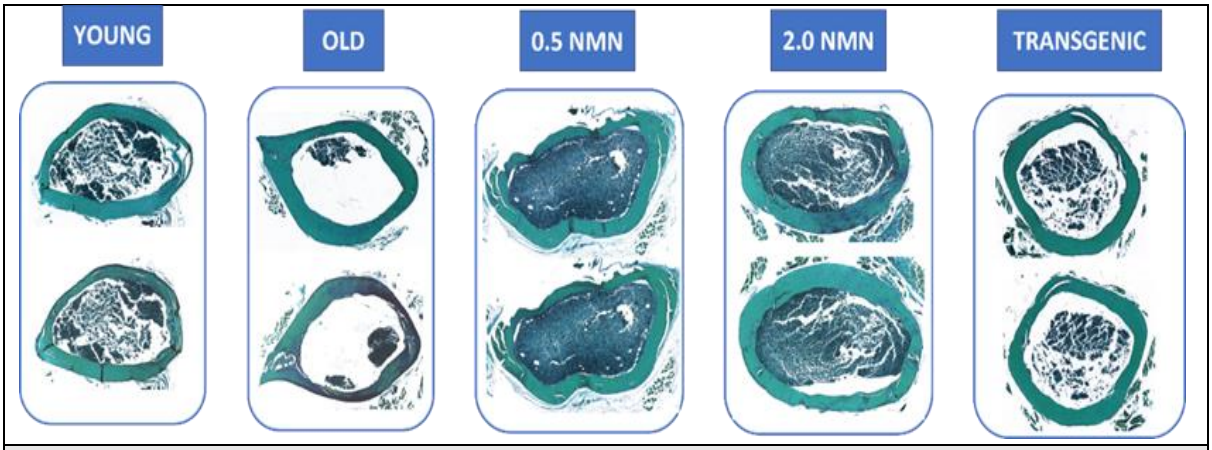

Figure 22. Safranin O staining for all study groups cortical bone

Representative Safranin O-stained sagittal sections of young, aged, NMN treatments in mice femur at the cortical bone. Scale bar **500µm**

Further we assessed the study group femurs at tissue level. For this study we stained the sections of cortical bone.

The H and E staining and Safranin O staining (Figure 21and Figure 22) of cross sections of the long femur at the cortex showed the distinct cell morphology in the cortex and marrow tissue of NMN treatment and transgenic mice than the aged mice. There was no bone marrow tissue observed in the aged cortical bone.

Chapter 5 : Discussion, Summary, Conclusion and Limitation

Discussion

In this study of age-related bone and boosting of NAD^+, a comprehensive analysis of bone in regards with aging and supplementation of NAD^+ in the form of NMN has been studied. The 12 weeks and 56 weeks old mice were used in this study to perform various experiments to evaluate the bone quality in regards with bone loss. It has been known that aging is a prime factor for the bone loss. Age related bone loss is a complex and heterogenous process and associated with innumerable factors as well. Several factors, including bone microstructural degradation and potential changes in bone material qualities including the composition and degree of collagen cross linking, could contribute to how ageing affects fracture risk. The femur bone is longest and strongest bone in the body. The bone at the proximal, midshaft and distal part of the femur bone has been selected for our study. The proximal bone and distal bone prone to fracture in elderly population due to the low BMD with ageing. The midshaft is the hollow but strongest part of the bone comprising of cortical bone.

Our results showed that the BMD of the NMN-treated mice did not change as compared to aged mice. The obtained result of bone loss and administration of NMN are consistent with a study from researchers where they demonstrated that three months of NMN supplementation was insufficient to increase the bone mass in 12 months old mice (Song et al., 2019). The same kind of results obtained in another study showed that there is a significant enhancement in the bone density and eye function of the mice with long-term treatment of NMN (Mills et al., 2016). Femoral trabecular parameters, collagen structure and mechanical strength are assessed in young, aged, 0.5 NMN, 2.0 NMN and transgenic mice groups. There is a significant difference in the young and aged trabecular parameters including decreased trabecular number and BV/TV in the aged femur than the young femur which is also confirmed in the studies where the researchers showed the degeneration of the canaliculi network in mice model of aging (Tiede-Lewis et al., 2017). Our BMD of aged mice was higher than the young mice confirming the literature where researchers compared the young and aged bone trabecular parameters in rat models of healthy aging where they demonstrated that BMD was increased by 30% in rats of age from 4 to 27 months of age (Duque et al., 2009). In our study we

observed bone mineral density is higher in aged femur than the young femur. BMD is the mineral content or ash content of the bone which is high in growing and development phase of the animal. Our result of BMD is supported due to the middle age of our mice which is analogous to Duque et al. where they proposed that BMD is higher in mice from 4 to 27 months and our mice's age less than 27 months old. They did not attain the aging yet but started showing some symptoms of aging by showing the reduction BV/TV and trabecular number. All these results are in consistent with earlier demonstrated research. The transgenic mice did not show any changes in the trabecular parameters again these results support the age of the mice as they are not completely aged mice.

The NAD^+ supplementation showed positive effects when we compare the results of 2.0 NMN group to the aged group femur in mechanical testing. The mechanical testing results showed that the 2.0 NMN and transgenic mice exhibited more force to fracture the bone than the aged bone. Though statistically mechanical testing results are not significant in our study, but we can justify this one in regard with the concentration of NMN and duration of administration. Most of the studies tried 250 mg / day (Igarashi et al., 2022) and in long term exposure studies 100- 300 mg / day for a year (Mills et al., 2016). In the femurs of 2.0 NMN and transgenic mice we could see the uniform orientation of collagen fibres. For the first time, researchers discovered that the mechanical properties of the collagen network change with age (Wang et al., 2002). The red orange colour indicates the abundance of collagen type I in the 2.0 NMN and transgenic femur. Whereas the distortion of collagen fibres was significantly observed in aged mice. This changes in the collagen structure contributed the brittleness due to the shift in the cross-linking profile of the collagen present in the aged femur. The collagen results and mechanical testing are supporting each other in terms of the strength of the 2.0 NMN and transgenic femur.

The calcium plays a role in the osteoblastogenesis and eventually the BMD and bone mass is affected with the calcium level. Our results did not show increase in the calcium phosphorous content and the BMD in our aged, NMN treatment and transgenic mice were not altered because our mice did not attain the aging yet. It applies to the histological results as well.

As discussed in literature review in chapter 2 of this dissertation, NAD^+ intermediates such NMN, and NR has promising results in boosting cellular NAD^+ levels. The study of age-related decrease in the NAD^+ level and age associated diseases is always attracting the researchers to investigate further. From our results, we could see that there are positive results in the bone strength, but further study must carry out in terms of dosages and duration of the supplementation. Our results can be a guideline in this direction for further studies. Scientists are trying to improve the life expectancy of human beings and contributing towards the longevity of human beings and for that they are trying to prolong the aging. The aging mechanism is still not fully understood as it is a complex process. In our results, it is noteworthy that, in terms of collagen orientation and strength of the bone NMN supplementation has started showing up elevation. NMNAT1 could be an excellent player in the antiaging benefits as our results of transgenic mice and its collagen content and orientation is retained as seen under polarised light in our experiment. Our study of aging and bone loss is trying to contribute towards bone health and subsequently healthy aging with the administration of NAD^+.

Summary and Conclusion

- Our research is the first of its kind to reveal the age-related bone loss and boosting of NAD^+ by the supplementation of NMN, *in vivo* study of mice model
- There was a significant difference between the young and aged trabecular bone parameters as the aged bone showed the signs of aging
- The mechanical property of the NMN treated bone was retained in 3 point bending test up to some extent though not significant and polarised light results showed that the collagen type I structure in the bone was retained due to NMN treatment. Collagen cross-linking and mechanical properties were improved with the NMN treatment
- The transgenic mice exhibited strength in response to the mechanical properties and collagen type I structure
- The trabecular parameters did not show a significant rise in BMD and bone volume etc in the NMN treatment groups or either in transgenic mice femur

- 2.0mg/l NMN supplementation has positive effects on the femur than the 0.5mg/l NMN supplementation in regard to collagen structure
- From all the above results, we can conclude that the bone loss at the microstructure level of trabeculae, the long-term supplementation of NAD^+ in the form of NMN is needed

Limitation and Future Prospective of the Study

Limitation of this study is the short-term supplementation of NMN to the animals. We could see the NMN treatment showed some positive results. As bone remodelling with age is a very slow process, the micro or nano level trabecular parameter changes did not show significant differences. The long-term treatment with proper concentration of NMN would be interesting insight for the therapeutic aspects of NAD^+ in the bone diseases. The clinical trials are already underway for the NMN supplementation, and our study is contributing towards the optimisation of dose and duration of the supplement for the bone health. Previous research of NAD^+ and the link of other diseases including cardiovascular, neurodegenerative, ocular, skin and metabolic diseases has already been studied extensively. Bone related diseases such as osteoporosis and supplementation of NAD^+ need to investigate further.

Our study involved a preliminary investigation into the potential effects of administering NMN over a period of 4 weeks. The primary objective was to observe whether this administration can lead to an increase in NAD levels, which in turn might help mitigate age-related bone loss. NAD is known for its essential role in various cellular processes, including DNA repair and energy metabolism, and its decline with age has been associated with several age-related health issues.

In this initial phase of our research, the main focus was to determine the impact of NMN on NAD^+ levels and whether it can have a positive influence on age-related bone health. This question is particularly important as age-related bone diseases like osteoporosis can significantly impact the overall well-being and quality of life of older individuals.

The outcome of this preliminary study showed some promising results with NMN and its effects on NAD^+ levels and bone health, it could set the stage for more extensive investigations. Exploring the potential of long-term NMN administration to reverse age-related bone diseases could have far-reaching implications for promoting healthy aging and delaying the onset of age-related health concerns.

Several critical factors need to be taken into account when conducting this kind of research. These include carefully determining the appropriate dose, frequency, and duration of NMN supplementation. Striking the right balance between potential benefits and any associated risks or side effects will be pivotal in designing a comprehensive, successful long-term study.

One key strength of this preliminary research lies in its potential to provide a foundational framework for future studies. Building on existing knowledge is a key component of scientific progress, and the findings of this study could serve as a guide for other researchers interested in this topic.

Supplementary Information

TRABECULAR NUMBER												
ROI OF FEMUR	**YOUNG**			**AGED**			**0.5 NMN**			**2.0 NMN AGED**		
Proximal	4.4500	4.6861	5.0426	3.1974	2.6099	3.174	3.1937	3.276	3.0542	3.2103	3.2439	
Midshaft	2.0389	2.2265	2.5286	1.9512	2.0059	6.0471	1.6945	2.1815	2.3426	1.5028	5.236	
Distal	6.1323	6.9049	8.2851	1.9512	3.0557	2.7829	2.9545	2.7202	2.2128	2.0141	2.2245	

Supplementary Table 1. Trabecular Number data collection for NMN treatment via Micro CT

Trabecular Number data collection via Micro CT n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group. Tb N is measured at the proximal, midshaft and distal of each study group.

TRABECULAR NUMBER						
ROI OF FEMUR	**WT OLD**			**TRANSGENIC**		
Proximal	2.5796	2.7523	2.169	2.7019	2.4591	2.7255
Midshaft	1.8402	1.8296	2.0933	2.7019	2.4591	2.7255
Distal	2.1143	2.6931	2.692	2.7019	2.4591	2.7255

Supplementary Table 2. Trabecular Number data collection for transgenic mice via Micro CT

Trabecular Number data collection via Micro CT. n=3 per Wild type Aged and Transgenic Aged group. Tb N is measured at the proximal, midshaft and distal of each study group.

TRABECULAR THICKNESS										
ROI OF FEMUR	**YOUNG**			**AGED**			**0.5 NMN**			**2.0 NMN AGED**	
Proximal	0.1524	0.1544	0.1652	0.1707	0.1697	0.175	0.1584	0.1572	0.1751	0.1539	0.1677
Midshaft	0.2054	0.2157	0.2078	0.2043	0.2015	0.2034	0.1923	0.1766	0.1672	0.1699	0.1827
Distal	0.0903	0.0768	0.0771	0.0948	0.1028	0.1062	0.1039	0.1055	0.0985	0.1020	0.1068

Supplementary Table 3. Trabecular Thickness data collection for NMN treatment via Micro CT

n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group. Tb Th is measured at the proximal, midshaft and distal of each study group.

TRABECULAR THICKNESS						
ROI OF FEMUR	**WT OLD**			**TRANSGENIC**		
Proximal	0.1542	0.1557	0.2213	0.1626	0.153	0.1669
Midshaft	0.1732	0.1872	0.1875	0.2023	0.1996	0.1948
Distal	0.0974	0.1067	0.1027	0.1093	0.1771	0.1002

Supplementary Table 4. Trabecular Thickness data collection for transgenic mice via Micro CT

n=3 per Wild type Aged and Transgenic Aged group. Tb Th is measured at the proximal, midshaft and distal of each study group

TRABECULAR SPACING									
ROI OF FEMUR	**YOUNG**			**AGED**			**0.5 NMN**			**2.0 NMN AGED**	
Proximal	0.2355	0.2528	0.2424	0.4338	0.5315	0.4231	0.3642	0.3716	0.429	0.3765	0.3603
Midshaft	0.6589	0.5996	0.5252	0.9165	0.8348	0.006*	0.7795	0.8624	0.8872	0.8939	0.886
Distal	0.1559	0.1356	0.1094	0.4549	0.3855*	0.4341	0.3759	0.4118	0.5351*	0.5547	0.4826

Supplementary Table 5. Trabecular Spacing data collection for NMN treatment via Micro CT

n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group. Tb Sp is measured at the proximal, midshaft and distal of each study group.

TRABECULAR SPACING						
ROI OF FEMUR	**WT OLD**			**TRANSGENIC**		
Proximal	2.5796	2.7523	2.169	2.7019	2.4591	2.7255
Midshaft	1.8402	1.8296	2.0933	2.7019	2.4591	2.7255
Distal	2.1143	2.6931	2.692	2.7019	2.4591	2.7255

Supplementary Table 6. Trabecular Spacing data collection for transgenic mice via Micro CT

n=3 per Wild type Aged and Transgenic Aged group. Tb Sp is measured at the proximal, midshaft and distal of each study group

Bone Volume /Total Volume (BV/TV) %												
ROI OF FEMUR	**YOUNG**			**AGED**			**0.5NMN**			**2.0NMN**		
Proximal	0.388	0.5332	0.5864	0.4623	0.429	0.4817	0.4393	0.458	0.4408	0.4443	0.4755	
Midshaft	0.4143	0.4357	0.4387	0.4251	0.4641	0.4761	0.3929	0.3801	0.3818	0.3624	0.3736	
Distal	0.374	0.3888	0.4798	0.2981	0.3132	0.3107	0.2885	0.2749	0.2583	0.2493	0.2517	

Supplementary Table 7. Bone Volume to Total Volume data collection for NMN treatment via Micro CT

n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group. BV/TV is measured at the proximal, midshaft and distal of each study group.

Bone Volume /Total Volume (BV/TV) %						
ROI of FEMUR	**WT AGED**			**TRANSGENIC**		
Proximal	0.4021	0.4064	0.4221	0.3996	0.3997	0.48
Midshaft	0.4254	0.4284	0.4194	0.3706	0.4051	0.3934
Distal	0.2487	0.3728	0.2459	0.2231	0.3401	0.3194

Supplementary Table 8. Bone Volume to Total Volume data collection for transgenic mice via Micro CT

n=3 per Wild type Aged and Transgenic Aged group. BV/TV is measured at the proximal, midshaft and distal of each study group.

Bone Mineral Density (BMD) mgHAcm^3										
ROI OF Femur	**YOUNG**			**AGED**			**0.5 NMN AGED**			**2.0 NMN AGED**	
Proximal	1.147	1.16	1.174	1.271	1.285	1.267	1.239	1.257	1.261	1.261	1.271
Midshaft	1.256	1.286	1.276	1.334	1.329	1.332	1.319	1.331	1.322	1.329	1.328
Distal	1.07	1.061	1.064	1.209	1.227	1.206	1.195	1.217	1.184	1.208	1.193

Supplementary Table 9. Bone Mineral Density data collection for NMN treatment via Micro CT

n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group. BMD is measured at the proximal, midshaft and distal of each study group.

Bone Mineral Density (BMD) mgHAcm^3						
ROI OF FEMUR	**WT OLD**			**TRANSGENIC**		
Proximal	1.263	1.266	1.264	1.238	1.245	1.27
Midshaft	1.325	1.331	1.326	1.261	1.323	1.33
Distal	1.214	1.187	1.188	1.212	1.309	1.218

Supplementary Table 10. Bone Mineral Density data collection for transgenic mice via Micro CT

n=3 per Wild type Aged and Transgenic Aged group. BMD is measured at the proximal, midshaft and distal of each study group.

BONE SURFACE / BONE VOLUME (BS/BV) %												
ROI OF FEMUR	**YOUNG**			**AGED**			**0.5 NMN**			**2.0 NMN AGED**		
Proximal	14.4686	15.3973	13.7924	12.8801	13.0082	9.6963	11.3786	10.9871	9.963	12.3496	9.4614	
Midshaft	5.3285	5.2449	5.9413	4.8176	6.0898	11.0614	5.0449	5.8915	10.2395	6.6126	6.0936	
Distal	30.8526	36.2714	33.6863	23.6294	21.9295	16.3436	16.7811	15.0297	18.63	15.3401	15.4673	

Supplementary Table 11. Bone Surface to Total Volume data collection for NMN treatment via Micro CT

n=3 per Young, Aged, 0.5 NMN Aged group and n=2 for 2.0 NMN Aged group. BS/BV is measured at the proximal, midshaft and distal of each study group.

BONE SURFACE / BONE VOLUME (BS/BV) %						
ROI OF FEMUR	**WT OLD**			**TRANSGENIC**		
Proximal	11.3921	17.1521	9.0369	10.0275	10.8856	8.9866
Midshaft	6.2969	5.4165	5.8315	5.0458	5.1472	5.0378
Distal	15.6151	17.1521	18.1457	14.8943	6.5063	14.5746

Supplementary Table 12. Bone Surface to Bone Volume data collection for transgenic mice via Micro CT

n=3 per Wild type Aged and Transgenic Aged group. BS/BV is measured at the proximal, midshaft and distal of each study group.

Load at the fracture Point (Newton)			
YOUNG	**AGED**	**0.5 NMN AGED**	**2.0 NMN AGED**
12.3	11.95	11.92	12.37
15.02	12.44	13.15	12.22
12.44	11.95	15.31	13.5

Supplementary Table 13. Mechanical testing load data for the NMN treatment

Femur n=3 per Young, Aged, 0.5NMN AGED, 2.0 NMN AGED group. The tables denote the maximum load at which breakage of the femur occurred in 3 point-bending experiments.

Load at the fracture Point (Newton)	
WT AGED	TRANSGENIC AGED
14.36	15.20
14.26	15.27
14.54	15.64

Supplementary Table 14. Mechanical testing load data for the transgenic mice

Femur n=3 per wild-type aged and transgenic aged group. The tables denote the maximum load at which breakage of the femur occurred in 3 point-bending experiments.

Bibliography

Almeida, M. (2012). Aging mechanisms in bone. *BoneKEy reports*, *1*, 102. https://doi.org/10.1038/bonekey.2012.102

Aman, Y., Qiu, Y., Tao, J., & Fang, E. F. (2018). Therapeutic potential of boosting NAD+ in aging and age-related diseases. *Translational Medicine of Aging*, *2*, 30-37. https://doi.org/https://doi.org/10.1016/j.tma.2018.08.003

Arenas-Jal, M., Suñé-Negre, J. M., & García-Montoya, E. (2020). Therapeutic potential of nicotinamide adenine dinucleotide (NAD). *European Journal of Pharmacology*, *879*, 173158. https://doi.org/https://doi.org/10.1016/j.ejphar.2020.173158

Bertoldo, M. J., Listijono, D. R., Ho, W.-H. J., Riepsamen, A. H., Goss, D. M., Richani, D., Jin, X. L., Mahbub, S., Campbell, J. M., & Habibalahi, A. (2020). NAD+ repletion rescues female fertility during reproductive aging. *Cell reports*, *30*(6), 1670-1681. e1677.

Braidy, N., & Liu, Y. (2020). NAD+ therapy in age-related degenerative disorders: A benefit/risk analysis. *Experimental Gerontology*, *132*, 110831. https://doi.org/https://doi.org/10.1016/j.exger.2020.110831

Cantó, C., Menzies, K. J., & Auwerx, J. (2015). NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus. *Cell Metabolism*, *22*(1), 31-53. https://doi.org/10.1016/j.cmet.2015.05.023

Chandrasekaran, K., Anjaneyulu, M., Choi, J., Kumar, P., Salimian, M., Ho, C.-Y., & Russell, J. W. (2019). Chapter Seven - Role of mitochondria in diabetic peripheral neuropathy: Influencing the NAD+-dependent SIRT1–PGC-1α–TFAM pathway. In P. Fernyhough & N. A. Calcutt (Eds.), *International Review of Neurobiology* (Vol. 145, pp. 177-209). Academic Press. https://doi.org/https://doi.org/10.1016/bs.irn.2019.04.002

Chen, Y., Zhou, F., Liu, H., Li, J., Che, H., Shen, J., & Luo, E. (2021). SIRT1, a promising regulator of bone homeostasis. *Life Sciences*, *269*, 119041. https://doi.org/https://doi.org/10.1016/j.lfs.2021.119041

Chini, C. C. S., Tarragó, M. G., & Chini, E. N. (2017). NAD and the aging process: Role in life, death and everything in between. *Molecular and cellular endocrinology*, *455*, 62-74. https://doi.org/10.1016/j.mce.2016.11.003

Chini, C. C. S., Zeidler, J. D., Kashyap, S., Warner, G., & Chini, E. N. (2021). Evolving concepts in NAD+ metabolism. *Cell Metabolism*, *33*(6), 1076-1087. https://doi.org/https://doi.org/10.1016/j.cmet.2021.04.003

Covarrubias, A. J., Perrone, R., Grozio, A., & Verdin, E. (2021). NAD(+) metabolism and its roles in cellular processes during ageing. *Nat Rev Mol Cell Biol*, *22*(2), 119-141. https://doi.org/10.1038/s41580-020-00313-x

Covarrubias, A. J., Perrone, R., Grozio, A., & Verdin, E. (2021). NAD+ metabolism and its roles in cellular processes during ageing. *Nature Reviews Molecular Cell Biology*, *22*(2), 119-141. https://doi.org/10.1038/s41580-020-00313-x

Demontiero, O., Vidal, C., & Duque, G. (2011). Aging and bone loss: new insights for the clinician. *Therapeutic Advances in Musculoskeletal Disease*, *4*(2), 61-76. https://doi.org/10.1177/1759720X11430858

Duque, G., Rivas, D., Li, W., Li, A., Henderson, J. E., Ferland, G., & Gaudreau, P. (2009). Age-related bone loss in the LOU/c rat model of healthy ageing. *Experimental Gerontology*, *44*(3), 183-189. https://doi.org/https://doi.org/10.1016/j.exger.2008.10.004

Fakouri, N. B., Hou, Y., Demarest, T. G., Christiansen, L. S., Okur, M. N., Mohanty, J. G., Croteau, D. L., & Bohr, V. A. (2019). Toward understanding genomic instability, mitochondrial dysfunction and aging. *The FEBS Journal*, *286*(6), 1058-1073. https://doi.org/https://doi.org/10.1111/febs.14663

Gonzalez-Freire, M., de Cabo, R., Bernier, M., Sollott, S. J., Fabbri, E., Navas, P., & Ferrucci, L. (2015). Reconsidering the Role of Mitochondria in Aging. *The Journals of Gerontology: Series A*, *70*(11), 1334-1342. https://doi.org/10.1093/gerona/glv070

Idrus, R., Sainik, N., Ansari, A. S., Mohamed, S., Razali, R., Nordin, A., Saim, A., & Naina-Mohamed, I. (2018). Ficus carica and Bone Health: A Systematic Review. *Sains Malaysiana*, *47*, 2741-2755. https://doi.org/10.17576/jsm-2018-4711-17

Igarashi, M., Nakagawa-Nagahama, Y., Miura, M., Kashiwabara, K., Yaku, K., Sawada, M., Sekine, R., Fukamizu, Y., Sato, T., Sakurai, T., Sato, J., Ino, K., Kubota, N., Nakagawa, T., Kadowaki, T., & Yamauchi, T. (2022). Chronic nicotinamide mononucleotide supplementation elevates blood nicotinamide adenine dinucleotide levels and alters muscle function in healthy older men. *npj Aging*, *8*(1), 5. https://doi.org/10.1038/s41514-022-00084-z

Imai, S., & Guarente, L. (2014). NAD+ and sirtuins in aging and disease. *Trends Cell Biol*, *24*(8), 464-471. https://doi.org/10.1016/j.tcb.2014.04.002

Johnson, S., & Imai, S.-I. (2018). NAD (+) biosynthesis, aging, and disease. *F1000Research*, *7*, 132-132. https://doi.org/10.12688/f1000research.12120.1

Khaidizar, F. D., Bessho, Y., & Nakahata, Y. (2021a). Nicotinamide Phosphoribosyltransferase as a Key Molecule of the Aging/Senescence Process. *International journal of molecular sciences*, *22*(7), 3709. https://www.mdpi.com/1422-0067/22/7/3709

https://mdpi-res.com/d_attachment/ijms/ijms-22-03709/article_deploy/ijms-22-03709.pdf

Khaidizar, F. D., Bessho, Y., & Nakahata, Y. (2021b). Nicotinamide Phosphoribosyltransferase as a Key Molecule of the Aging/Senescence Process. *International journal of molecular sciences*, *22*(7). https://doi.org/10.3390/ijms22073709

Kim, H.-N., Ponte, F., Warren, A., Ring, R., Iyer, S., Han, L., & Almeida, M. (2021). A decrease in NAD+ contributes to the loss of osteoprogenitors and bone mass with aging. *npj Aging and Mechanisms of Disease*, *7*(1), 8. https://doi.org/10.1038/s41514-021-00058-7

Kim, J. H., Liu, X., Wang, J., Chen, X., Zhang, H., Kim, S. H., Cui, J., Li, R., Zhang, W., Kong, Y., Zhang, J., Shui, W., Lamplot, J., Rogers, M. R., Zhao, C., Wang, N., Rajan, P., Tomal, J., Statz, J., Wu, N., Luu, H. H., Haydon, R. C., & He, T.-C. (2013). Wnt signaling in bone formation and its therapeutic potential for bone diseases. *Therapeutic Advances in Musculoskeletal Disease*, *5*(1), 13-31. https://doi.org/10.1177/1759720X12466608

Lautrup, S., Sinclair, D. A., Mattson, M. P., & Fang, E. F. (2019). NAD(+) in Brain Aging and Neurodegenerative Disorders. *Cell Metabolism*, *30*(4), 630-655. https://doi.org/10.1016/j.cmet.2019.09.001

Liu, X., Liu, M., Tang, C., Xiang, Z., Li, Q., Ruan, X., Xiong, K., & Zheng, L. (2018). Overexpression of Nmnat improves the adaption of health span in aging Drosophila. *Exp Gerontol*, *108*, 276-283. https://doi.org/10.1016/j.exger.2018.04.026

Magni, G., Amici, A., Emanuelli, M., Orsomando, G., Raffaelli, N., & Ruggieri, S. (2004). Enzymology of NAD+ homeostasis in man. *Cellular and Molecular Life Sciences CMLS*, *61*(1), 19-34. https://doi.org/10.1007/s00018-003-3161-1

Marie, P. J., & Kassem, M. (2011). Extrinsic Mechanisms Involved in Age-Related Defective Bone Formation. *The Journal of Clinical Endocrinology & Metabolism*, *96*(3), 600-609. https://doi.org/10.1210/jc.2010-2113

McReynolds, M. R., Chellappa, K., & Baur, J. A. (2020). Age-related NAD+ decline. *Experimental Gerontology*, *134*, 110888. https://doi.org/https://doi.org/10.1016/j.exger.2020.110888

McWain, M. A., Pace, R. L., Nalan, P. A., & Lester, D. B. (2022). Age-dependent effects of social isolation on mesolimbic dopamine release. *Exp Brain Res*, *240*(10), 2803-2815. https://doi.org/10.1007/s00221-022-06449-w

Mehmel, M., Jovanović, N., & Spitz, U. (2020). Nicotinamide Riboside-The Current State of Research and Therapeutic Uses. *Nutrients*, *12*(6), 1616. https://doi.org/10.3390/nu12061616

Mills, K. F., Yoshida, S., Stein, L. R., Grozio, A., Kubota, S., Sasaki, Y., Redpath, P., Migaud, M. E., Apte, R. S., Uchida, K., Yoshino, J., & Imai, S.-i. (2016). Long-Term Administration of Nicotinamide Mononucleotide Mitigates Age-Associated Physiological Decline in Mice. *Cell Metabolism*, *24*(6), 795-806. https://doi.org/https://doi.org/10.1016/j.cmet.2016.09.013

Nadeeshani, H., Li, J., Ying, T., Zhang, B., & Lu, J. (2021). Nicotinamide mononucleotide (NMN) as an anti-aging health product – Promises and safety concerns. *Journal of Advanced Research*. https://doi.org/https://doi.org/10.1016/j.jare.2021.08.003

Oksztulska-Kolanek, E., Znorko, B., Michałowska, M., & Pawlak, K. (2016). The Biomechanical Testing for the Assessment of Bone Quality in an Experimental Model of Chronic Kidney Disease. *Nephron*, *132*(1), 51-58. https://doi.org/10.1159/000442714

Pignolo, R. J., Law, S. F., & Chandra, A. (2021). Bone Aging, Cellular Senescence, and Osteoporosis [https://doi.org/10.1002/jbm4.10488]. *JBMR Plus*, *5*(4), e10488. https://doi.org/https://doi.org/10.1002/jbm4.10488

Prolla, Tomas A., & Denu, John M. (2014). NAD+ Deficiency in Age-Related Mitochondrial Dysfunction. *Cell Metabolism*, *19*(2), 178-180. https://doi.org/https://doi.org/10.1016/j.cmet.2014.01.005

Rajman, L., Chwalek, K., & Sinclair, D. A. (2018). Therapeutic Potential of NAD-Boosting Molecules: The In Vivo Evidence. *Cell Metab*, *27*(3), 529-547. https://doi.org/10.1016/j.cmet.2018.02.011

Reiten, O. K., Wilvang, M. A., Mitchell, S. J., Hu, Z., & Fang, E. F. (2021). Preclinical and clinical evidence of NAD+ precursors in health, disease, and ageing. *Mechanisms of Ageing and Development*, *199*, 111567. https://doi.org/https://doi.org/10.1016/j.mad.2021.111567

Sautchuk, R., & Eliseev, R. A. (2022). Cell energy metabolism and bone formation. *Bone Reports*, *16*, 101594. https://doi.org/https://doi.org/10.1016/j.bonr.2022.101594

Song, J., Li, J., Yang, F., Ning, G., Zhen, L., Wu, L., Zheng, Y., Zhang, Q., Lin, D., Xie, C., & Peng, L. (2019). Nicotinamide mononucleotide promotes osteogenesis and reduces adipogenesis by regulating mesenchymal stromal cells via the SIRT1 pathway in aged bone marrow. *Cell Death & Disease*, *10*(5), 336. https://doi.org/10.1038/s41419-019-1569-2

Stein, L. R., & Imai, S.-i. (2012). The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism*, *23*(9), 420-428. https://doi.org/https://doi.org/10.1016/j.tem.2012.06.005

Strømland, Ø., Diab, J., Ferrario, E., Sverkeli, L. J., & Ziegler, M. (2021). The balance between NAD+ biosynthesis and consumption in ageing. *Mechanisms of Ageing and Development*, *199*, 111569. https://doi.org/https://doi.org/10.1016/j.mad.2021.111569

Tiede-Lewis, L. M., & Dallas, S. L. (2019). Changes in the osteocyte lacunocanalicular network with aging. *Bone*, *122*, 101-113. https://doi.org/https://doi.org/10.1016/j.bone.2019.01.025

Tiede-Lewis, L. M., Xie, Y., Hulbert, M. A., Campos, R., Dallas, M. R., Dusevich, V., Bonewald, L. F., & Dallas, S. L. (2017). Degeneration of the osteocyte network in the C57BL/6 mouse model of aging. *Aging*, *9*(10), 2190-2208. https://doi.org/10.18632/aging.101308

Verdin, E. (2015). NAD+ in aging, metabolism, and neurodegeneration. *Science*, *350*(6265), 1208-1213. https://doi.org/10.1126/science.aac4854

Wang, X., Shen, X., Li, X., & Mauli Agrawal, C. (2002). Age-related changes in the collagen network and toughness of bone. *Bone*, *31*(1), 1-7. https://doi.org/https://doi.org/10.1016/S8756-3282(01)00697-4

Wiley, Christopher D., Velarde, Michael C., Lecot, P., Liu, S., Sarnoski, Ethan A., Freund, A., Shirakawa, K., Lim, Hyung W., Davis, Sonnet S., Ramanathan, A., Gerencser, Akos A., Verdin, E., & Campisi, J. (2016). Mitochondrial Dysfunction Induces Senescence with a Distinct Secretory Phenotype. *Cell Metabolism*, *23*(2), 303-314. https://doi.org/https://doi.org/10.1016/j.cmet.2015.11.011

Xie, N., Zhang, L., Gao, W., Huang, C., Huber, P. E., Zhou, X., Li, C., Shen, G., & Zou, B. (2020). NAD+ metabolism: pathophysiologic mechanisms and therapeutic potential. *Signal Transduction and Targeted Therapy*, *5*(1), 227. https://doi.org/10.1038/s41392-020-00311-7

Yaku, K., Okabe, K., & Nakagawa, T. (2018). NAD metabolism: Implications in aging and longevity. *Ageing Res Rev*, *47*, 1-17. https://doi.org/10.1016/j.arr.2018.05.006

Yaku, K., Okabe, K., & Nakagawa, T. (2018). NAD metabolism: Implications in aging and longevity. *Ageing Research Reviews*, *47*, 1-17. https://doi.org/https://doi.org/10.1016/j.arr.2018.05.006

Yang, J., Ueharu, H., & Mishina, Y. (2020). Energy metabolism: A newly emerging target of BMP signaling in bone homeostasis. *Bone*, *138*, 115467. https://doi.org/https://doi.org/10.1016/j.bone.2020.115467

Yoshino, J., Baur, J. A., & Imai, S.-i. (2018). NAD+ Intermediates: The Biology and Therapeutic Potential of NMN and NR. *Cell Metabolism*, *27*(3), 513-528. https://doi.org/https://doi.org/10.1016/j.cmet.2017.11.002

Zhou, R., Guo, Q., Xiao, Y., Guo, Q., Huang, Y., Li, C., & Luo, X. (2021). Endocrine role of bone in the regulation of energy metabolism. *Bone research*, *9*(1), 25. https://doi.org/10.1038/s41413-021-00142-4