NOTTINGHAM TRENT UNIVERSITY

NOVEL TECHNIQUES TO DETERMINE THE EFFECTS OF EXERCISE ON BONE TURNOVER

By

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ABSTRACT

Understanding bone (re)modelling in health and disease and developing interventions (*e.g.*, exercise) to prevent the weakening of bone tissue across the lifespan is vital to preserve or improve bone health. While changes in the mineralised compartment of bone can be determined over prolonged periods (*e.g.*, months/years), there are a lack of robust methods to study the short-term (days-weeks) physiology of bone collagen turnover, a key aspect of bone strength. As such, there is little understanding of the bone responses to acute interventions, such as exercise.

Indirect measures of bone formation and resorption can be made from the blood by certain biomarkers, but the utility of these markers to determine responses to acute and prolonged exercise interventions is unclear. The first study of this programme of work, reported in Chapter 3, was a systematic review and individual participant data meta-analysis investigating the mean responses and inter-individual variability of reference bone (re)modelling markers (P1NP and β-CTX-1) to a prolonged, continuous running bout in healthy adult males. This study determined that, when measured by reference bone (re)modelling markers, a single running bout does not elicit short-term bone responses in adult healthy males. The utility of this approach to measure short-term changes in bone (re)modelling in response to exercise is not well established and there is a need for alternative methods for studying the dynamic physiology of human bone turnover.

One of these alternative approaches might come from directly measuring bone protein synthesis *in vivo*, which can be carried out using stable isotopically labelled tracers, generally regarded as the gold standard in determining protein fractional synthetic rates, and can be performed on bone if a tissue sample can be collected. As such, in the next two studies, described in Chapters 4 and 5, a bone collagen extraction method and a new deuterium oxide (D_2O) tracer method able to quantify bone collagen synthesis were developed and optimised using a rodent model, showing future applicability to human investigations.

Using the D_2O method, different rates of bone collagen synthesis were determined across specific loaded bone sites (femur diaphysis, proximal tibia, mid-shaft tibia, distal tibia) in a rat model. Bone collagen synthesis rates were greater at the femur diaphysis than at the tibial mid-shaft and the proximal tibia showed greater synthesis compared to the tibial mid-shaft and distal tibia. Furthermore, the following studies, reported in Chapters 6 and 7, investigated the effects of age, phenotype, sex and running training on bone collagen synthesis in young and old rats. Age comparisons determined that older rats had slower bone collagen synthesis than young rats at the proximal tibia. Phenotype, sex and running training effects differed depending upon the site of measurement, showing that running training increased bone collagen formation at the mid-shaft and distal tibia. As an exploratory study, the last study (described in Chapter 8), examined the expression of bone remodelling and collagen genes and their associations with bone collagen formation, providing evidence that the expression of osteocalcin, osteoprotegerin and transforming growth factor β genes was linked to bone formation.

Collective evidence from this thesis highlights that bone responses are site-specific and that the complex structure of bone should be carefully considered when studying bone (re)modelling responses to loading. The geometry (*i.e.*, size and shape) and microarchitecture (*i.e.*, trabecular and cortical internal structure) of bone is not homogenous across long bones and modelling and remodelling processes are tailored to specific areas of the bone surface to optimise bone adaptations.

Key words: bone, bone turnover, bone metabolism, bone remodelling, bone markers, stable isotopes, deuterium oxide, running, collagen, gene expression.

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LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
ACTB	Actin-beta gene
AGEs	Advanced glycation end products
ANOVA	Analysis of variance
APE	Atom percent excess
B2M	Beta-2-microglobulin gene
Bglap	Osteocalcin gene
BMC	Bone mineral content
BMD	Bone mineral density
BMP-2	Bone morphogenetic protein 2
BMPs	Bone morphogenic proteins
Bone-ALP	Bone specific alkaline phosphatase
BW	Body weight
C	Carbon
c-FMS	Colony stimulating factor 1 receptor
$Ca_{10}(PO_4)_6(OH)_2$	Calcium hydroxyapatite
cDNA	Complementary DNA
CI	Confident intervals
CLIA	Chemiluminescence immunoassay
COL1A1	Collagen type 1 alpha 1
COL1A2	Collagen type 1 alpha 2
COL5A1	Collagen type 5 alpha 1

CrI	Credible intervals
Ct	Cycle threshold
CV	Coefficient variation
D	Deuterium
D_2O	Deuterium oxide
DHLNL	Dihydroxylysinornorleucine
Dix5	Distal-less homebox 5
DKK	Dickkopf
DKK-1	Dickkopf-related protein 1
DNA	Deoxyribonucleic acid
Dpd	Deoxypyridinoline
DXA	Dual-energy X-ray absorptiometry
ECLIA	Electro-chemiluminescence assay
EEFG1	Elongation factor 1-gamma gene
ELISA	Enzyme-linked immunosorbent assay
FEM	Femur diaphysis
FGF	Fibroblast growth factor
FSR	Fractional synthesis rate
GC	Gas chromatography
GLP-1	Glucagon-like peptide-1
GSK-3β	Glycogen synthase kinase-3β
H	Hydrogen
HCl	Hydrochloric acid
HLNL	Hydroxylysinonorleucine
HRT	High responders to endurance running training

IBSP	Integrin-binding sialoprotein gene
ICTP	Carboxy-terminal crosslinking telopeptide of type 1 collagen
IDP	Individual participant data
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin-1
IL-6	Interleukin-6
IRMS	Isotope-ratio mass spectrometry
LC	Liquid Chromatography
LOD	Limit of detection
LOX	Lysyl oxidase
LRP-5	Low-density lipoprotein receptor-related proteins 5
LRP-6	Low-density lipoprotein receptor-related proteins 6
LRT	Low responders to endurance running training
M-CSF	Macrophage colony-stimulating factor
mRNA	messenger ribonucleic acid
MS	Mass spectrometer/spectrometry
MS/MS	Tandem mass spectrometry
N	Nitrogen
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
NTX	Amino-terminal crosslinking telopeptide of type 1 collagen
O	Oxygen
OC	Osteocalcin
OPG	Osteoprotegerin
Osx	Osterix

P1CP	Carboxy or C-terminal propeptide of type 1 procollagen
P1NP	Amino or N-terminal propeptide of type 1 procollagen
PGE2	Prostaglandin 2
PICOS	Population, Intervention, Comparator, Outcomes and Study Design
pQCT	Peripheral quantitative computed tomography
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analysis
PTH	Parathyroid hormone
PYR	Pyridinoline
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPL13	Ribosomal protein L13
rRNA	Ribosomal ribonucleic acid
Runx2	Runt-related transcription factor 2
SD	Standard deviation
SOST	Sclerostin
SOX-9	Transcription factor
T-DIS	Tibial distal epiphysis-metaphysis
T-MID	Tibial mid-shaft diaphysis
T-PRO	Tibial proximal epiphysis-metaphysis
TGF-β	Transforming growth factor β
TNF-α	Tumour necrosis factor-α

TNFRSF11B	Osteoprotegerin gene
TRAP5b	Tartrate-resistant acid phosphatase isoenzyme 5b
VDR	Vitamin D receptor
VO_{2max}	Maximum rate of oxygen consumption
β-CTX-1	Carboxy or C-terminal telopeptide of type 1 collagen
μCT	X-ray micro-computed tomography

CHAPTER 1:

GENERAL INTRODUCTION

Bone is a multifunctional, heterogeneous, dynamic tissue that undergoes constant (re)modelling. Bone has metabolic and endocrine functions and a structural function linked to anatomical and mobility factors. In this regard, bone needs to be strong to support mechanical loading but light enough to facilitate mobility (Seeman & Delmas, 2006). Bone tissue is comprised of an inorganic or mineral component (made up of hydroxyapatite), an organic component (largely made up of type 1 collagen proteins) and water (Kenkre & Bassett, 2018; Seeman & Delmas, 2006). Bone continues to turnover throughout an individual's lifetime by the mechanisms of bone resorption and formation, which are mediated by major bone cells called osteoclasts, osteoblasts, and osteocytes. These cellular mechanisms orchestrate bone modelling (construction) and remodelling (reconstruction) and are responsible for the changes and adaptations of the skeleton (Seeman & Delmas, 2006).

Bone mass peaks during the second decade of life and, thereafter, bone mass reduces with age, with women experiencing greater bone loss after menopause (Hendrickx, Boudin, & Van Hul, 2015). This age-associated bone loss is thought to be caused by disruptions to the balance between bone formation and resorption, and can lead to impaired bone strength and disease (*e.g.*, osteoporosis), and ultimately bone fracture (*i.e.*, fragility fracture) (Hendrickx, Boudin, & Van Hul, 2015). Osteoporosis is a major health concern worldwide in our current society, where the number of older people is increasing very rapidly and, therefore, the number of people suffering from this condition and its socioeconomic impact are growing (Hernlund *et al.*, 2013). As such, the development of favourable interventions that prevent bone loss and optimise bone health across the lifespan are urgently warranted.

One such intervention is exercise, given the positive relationship between mechanical loading and the skeleton. It is widely accepted that mechanical stimuli produces a bone adaptive response (Frost, 1987), although research investigating the mechanisms by which this happens is still ongoing (Hart *et al*., 2017; Stewart *et al*., 2020). Nonetheless, exercise that conveys high-impact, multi-directional movement patterns and unaccustomed loads, is widely accepted as providing a strong osteogenic stimulus (*i.e*., stimulation of bone formation) (O'Connor, Lanyon, & MacFie, 1982; Lanyon & Rubin, 1984; Rubin & Lanyon, 1985; Turner & Robling, 2003). These responses are less clear in endurance exercise with

repetitive load cycles (*e.g.*, running) or non-weight bearing exercise (*e.g.*, swimming, cycling) (Hind, Truscott, & Evans, 2006; Scofield & Hecht, 2012). Besides the effects of mechanical loading and exercise, clinical research studies have investigated the effects certain drugs, such as teriparatide, denosumab, and alendronate (Hodsman *et al*., 2005; Seeman *et al*., 2010) and nutrients, such as calcium, magnesium, or vitamin D (Palacios, 2006) on bone mass and structure. Such interventional research studies and clinical practitioners typically use imaging techniques, such as dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT), to measure the long-term (*e.g.*, months/years) bone changes, and for the assessment of musculoskeletal diseases, such as osteoporosis.

Changes in bone mass are not, however, the only factor that accounts for bone strength, with DXA-derived areal bone mineral density only relating to about two-thirds of the bone's strength (Ammann & Rizzoli, 2003). Other factors in the non-mineral compartments of bone, are equally important (Burr, 2002a) and turnover at faster rates. While the mineralised matrix provides stiffness, which gives bone the ability to resist deformation and thereby making loading possible; the bone organic extracellular matrix, made up of collagen, provides ductility and flexibility (Bouxsein, 2005). Bone flexibility is key to enable bones to absorb energy by deforming – to shorten and widen when compressed and to lengthen and narrow in tension (Seeman & Delmas, 2006). Although the mineral component of bone has traditionally been the predominant focus of bone health and strength, mounting evidence indicates that collagen is a vital factor in providing underlying strength to the bone (Viguet-Carrin, Garnero, & Delmas, 2006). The rate of bone collagen turnover may be important in determining bone strength because it influences the pattern of mature/immature collagen crosslinking in bone, which is important for bone quality and bone strength (Burr, 2002a; Viguet-Carrin, Garnero, & Delmas, 2006; Bouxsein, 2005). Little is known about how bone collagen turnover responds to potentially favourable interventions (*e.g.*, exercise and/or diet) to improve bone strength due to the lack of robust analytical approaches to measure it.

Specific biochemical markers of bone formation or resorption, named bone (re)modelling markers, can be measured in the blood and urine (Seibel, 2000; Vasikaran *et al*., 2011). These markers emerged during the late 20th century as a new non-invasive approach to measure bone turnover, with the focus on understanding bone diseases such as osteoporosis and Paget's disease (Delmas, 1990; Epstein, 1988; Russell *et al*., 1981). Since then they have expanded the spectrum of analyses used in the clinical assessment of skeletal pathologies and the research of short-term (hours/days) responses to potential interventions and, unlike more static measures of bone mass and structure, bone biomarkers can provide some information on the short-term metabolic status of the bone (Seibel, 2000; Vasikaran *et al*., 2011). In the clinical field, for example, there has been a great interest in the use of bone (re)modelling markers for the diagnosis and treatment of osteoporosis and osteoarthritis (Rousseau & Delmas, 2007; Wheater *et al*., 2013) and the prediction of fracture risk (Johansson *et al*., 2014; Tian *et al*., 2019). In the research field, these markers are commonly used to investigate bone responses to pharmacological, exercise and nutrition interventions. Caution should be taken when interpreting the responses of bone (re)modelling markers, however, since they have yet to be validated against direct measures of bone collagen synthesis or breakdown (Babraj *et al*., 2005a) and have a number of important limitations. For example, concentrations of these markers may be influenced by processes from other tissues (Seibel, 2000; Smith & Rennie, 2007), are not able to detect changes in bone turnover at the tissue level (*i.e*., are not site specific) and, therefore, do not indicate where on the skeleton the resorption or formation is taking place. Bone (re)modelling markers are sensitive to lifestyle factors such as exercise, diet, and sleep status and their measurement also has high variability caused by pre-analytical (*e.g.*, sample collection, handling, and storage requirements) and analytical (*e.g.*, within and inter laboratory variation, assay reproducibility) factors. Given the variability and validity of bone (re)modelling markers, there is a need for alternative, robust approaches that enable the measurement of short term (*e.g*., days/weeks) changes in bone turnover. Such methods might come from directly measuring bone protein synthesis *in vivo*, which can be carried out using stable isotopically labelled tracers, although this requires access to bone tissue.

Stable isotope tracer methods are generally regarded as the gold standard in determining fractional synthetic rates of protein synthesis (Wilkinson, 2018). Traditionally, stable isotopes such as carbon (^{13}C) or nitrogen (^{15}N) have been used to label amino acids and "trace" the incorporation into human musculoskeletal tissues, predominantly in skeletal muscle (Brook & Wilkinson, 2020), for the subsequently measurement of *in vivo* protein synthesis rates. Only three studies have determined human bone synthesis using stable isotopically labelled amino acid tracers (Babraj *et al*., 2005a; Smeets *et al*., 2019; Scrimgeour *et al*., 1993). Babraj *et al*. (2005a) is the only study to have determined the effects of a controlled intervention on human bone collagen synthesis *in vivo*. The authors investigated the response of a short-term (120 min) feeding intervention in collagen synthesis in human bone, finding a faster turnover in fed compared to post-absorptive states (Babraj *et al*., 2005a). Amino acid stable isotope tracers require intravenous infusions, which means that studies using them are restricted by time (generally <24 h) and the administration of the tracer needs to be performed in a controlled environment, such as in clinical or research settings (Brook & Wilkinson, 2020). This time restriction makes it challenging to accurately measure low synthesis rates, such as are usually found in human musculoskeletal tissues (Smeets *et al*., 2019). Using a deuterium oxide (D_2O or *heavy water*) stable isotope tracer can overcome this limitation because it can be easily ingested orally, with the deuterium becoming rapidly equilibrated within the body water and intracellular amino acid pools (Dufner & Previs, 2003; Wilkinson *et al*., 2014). Therefore, D_2O can be administered with minimal interference to an individual's normal daily activities and the enrichment in the precursor pool can be maintained over days, weeks, and months (Wilkinson *et al*., 2017), making this tracer more suited to the measurement of slow turnover proteins, such as collagen.

1.1. Aims

In order to address fundamental gaps in the knowledge of the short-term physiology of bone collagen turnover and to help develop safe and practical strategies (*e.g*., exercise, diet) to enhance bone strength and prevent weakening of bone tissue, the aims of this research programme are:

1. To investigate the responses and individual variability of reference bone (re)modelling markers responses to an acute running-based exercise intervention in a population of young adult males, using systematic review and meta-analysis approaches in available individual participant data;
2. To develop a deuterium oxide tracer method with high sensitivity that allows the quantification of bone collagen synthesis *in vivo* using a rodent model;
3. To apply the new D_2O method to examine short-term physiological effects on bone collagen synthesis in a rodent model with different age, phenotype, sex, and running training intervention groups;
4. To use other analytical techniques that can provide information relevant to bone turnover such as the expression of genes that regulate pathways involved in bone (re)modelling and bone collagen formation.

1.2. Objectives

The aims of the thesis were achieved via these objectives:

- Evaluation of the mean and individual responses of reference bone (re)modelling markers after a prolonged, continuous running bout in young adult males (Chapter 3);
- Estimation of the inter-individual variation in bone (re)modelling marker responses and to what degree this variation can be attributed directly to the exercise intervention (*i.e.*, a prolonged, continuous running bout) or to external factors (*e.g.*, circadian variation) (Chapter 3);
- Development and optimisation of a bone collagen extraction method using femur and tibia samples from rat models (Chapter 4);
- Development and optimisation of a deuterium oxide stable isotope tracer method for the quantification of bone collagen synthesis rates *in vivo* in young and old rats with different levels of tracer incorporation (Chapters 4 and 5);
- Assessment of the sensitivity of the D_2O method for the measurement of bone collagen synthesis rates at different bone sites (Chapters 4 and 5);

- Application of the D_2O method to investigate the effects of phenotype, sex, and running training on bone collagen synthesis in young rats (Chapter 6);
- Application of the D_2O method to investigate the effects of age, phenotype, sex, and running training on bone collagen synthesis in old rats (Chapter 7);
- Elaboration of a method for the extraction of RNA from bone samples and investigation of the expression of bone remodelling and collagen genes in young rats across phenotype, sex, and running training groups (Chapter 8).

CHAPTER 2:

REVIEW OF LITERATURE

2.1. Introduction to the review of literature

The first section of this review comprises a brief overview of how bone changes across the lifespan and the implications for bone health. Next, the outer and inner structure of the skeleton, the inorganic (*i.e.*, mineral) and organic (mostly collagen) composition of bone, and their importance for bone strength are discussed. The main differences between the human and the animal skeleton are presented, before moving on to a discussion of the critical underpinning knowledge around the collagen component of bone, including how bone collagen is synthesised, how collagen crosslinks are formed, and the role of collagen on bone strength. The next section describes in depth the process of the bone remodelling cycle and the bone cells, signalling pathways, and regulatory factors involved in that process. The subsequent two sections encompass the effects and mechanisms of bone adaptations in response to loading from mechanical forces and the loading factors that influence those adaptations; and the effects of exercise on bone mass and structure, including how the skeleton adapts differently across ages and with various types of activities. The continuing section summarises the uses and limitations of bone (re)modelling biomarkers as tools to investigate short-term, dynamic bone changes in response to exercise; as well as what interpretations can be attained from studies that have used them. The last section of this review informs about the evolution and application of stable isotope tracers as direct methods to study human metabolism, specifically musculoskeletal metabolism and protein synthesis, with an emphasis on the deuterium oxide tracer.

2.2. Bone changes across the lifespan

Across the lifespan, bone experiences meaningful changes to its mass, structure, and metabolism. Indeed, bone is a dynamic tissue undergoing constant (re)modelling throughout an individual's lifetime, under the processes of resorption and formation (section 2.5., page 23). Bone mass accretion begins during foetal growth and continues throughout childhood, adolescence, and development until the end of the second decade of life, when peak bone mass is achieved (**Figure 1**) (Heaney *et al*., 2000). An individual's peak bone mass refers to the maximum amount of bone accrued during young adulthood and it is usually presented as bone mineral content (BMC) or bone mineral density (BMD). Skeletal

growth is made possible by the action of bone modelling, which allows bones to increase diameter (*i.e.*, periosteal and endocortical apposition) and lengthen (*i.e.*, endochondral ossification) (Parfitt, 1994). Although the major part (60-80%) of an individual's bone mass is determined by non-modifiable factors (*i.e.*, genetics, sex, ancestry) (Guéguen *et al.*, 1995; Weaver *et al.*, 2016), other lifestyle and environmental factors such as diet, physical activity, medications, hormonal contraceptives, smoking and alcohol consumption can also affect bone health (Weaver *et al.*, 2016). Physical activity is probably the principal modifiable factor because bone is sensitive to mechanical loading from exercise (section 2.6., page 34). During growth, bone is more sensitive to loading, stressing the importance of physical activity and exercise early in life (section 2.7.1., page 40).

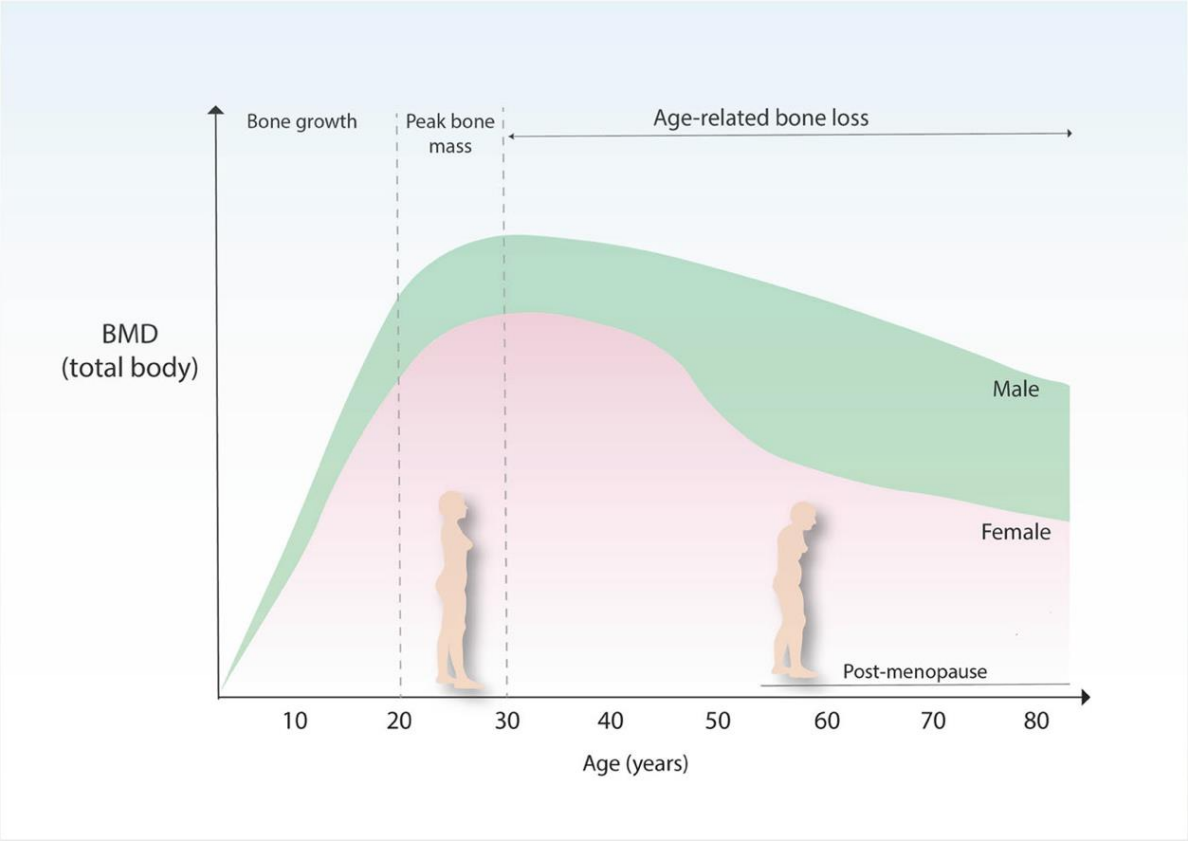


Figure 1. Evolution of bone mineral density (BMD) across the lifespan in males (green) and females (pink). From Santos, Elliott-Sale, & Sale, 2017 with permission.

The acquisition of bone (mineral) mass, quantitatively, follows age and sex specific patterns (**Figure 1**). With puberty and the growth spurt in height, bone mineral accretion increases rapidly, with bone mass peaking shortly after peak hight gain (Bailey *et al*., 1999). Aside from changes in BMD, adaptations in bone structure (*i.e*., size, shape, microarchitecture) and composition (*e.g*., cortical and trabecular bone) also proceed during puberty and development (Weaver *et al*., 2016). Skeletal adaptations, such as increases in cortical density and structural optimisation, can continue into the third decade of life (Weaver *et al*., 2016). These changes increase bone strength and are crucial for maintaining bone health later in life. Sex steroid hormones, including both oestrogens and androgens, are responsible for the sex differences on skeletal size, shape, and mass during growth and they contribute to skeletal homeostasis during adulthood (Manolagas, O'Brien & Almeida, 2013). Males have greater bone mass (*i.e*., BMD) compared to females; a difference that surfaces as sexual maturation progresses (Hendrickx, Boudin, & Van Hul, 2015). With ageing, starting steadily in the late 30s in females and the early 40s in males, BMD starts to decline (**Figure 1**). This decay becomes more rapid in females after the menopause when oestrogen levels decrease (**Figure 1**), putting them at a greater risk of developing low bone mass and related conditions (*i.e*., osteopenia/osteoporosis). Age-related bone loss occurs due to bone resorption being greater than bone formation (Seeman, 2002). Such a decline in BMD may result in compromised bone strength and osteoporosis, with increased risk of fragility fracture (Hendrickx, Boudin, & Van Hul, 2015).

Osteoporosis is a major health concern worldwide in our ageing society. It is estimated that 22 million women and 5.5 million men are living with the disease in the European Union alone (Hernlund *et al*., 2013). Every year, osteoporosis causes more than 8.9 million fragility fractures worldwide, with the number of hip fractures worldwide estimated to increase by more than ~14% by 2050 (Hernlund *et al*., 2013). These fractures also have significant complications, including pain, disability, and even death. The large economic costs of osteoporosis, projected to increase from €37.5 billion in 2010 to €46.8 billion in 2025 (25% increase) in the European Union (EU27) (Hernlund *et al*., 2013), and the large socioeconomic impact of this disease highlights the importance of developing favourable interventions to prevent bone loss and to optimise bone health across the lifespan.

An understanding of the changes in bone mineral mass and structure, and the insights of diseases, such as osteoporosis, are possible thanks to imaging techniques such as dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT). Whilst DXA provides measurements of bone mineral content (BMC) and density (BMD) and it is used to diagnose osteoporosis; pQCT additionally integrates measures of bone geometry and, if high-resolution pQCT, microarchitecture. These imaging techniques, however, do not provide information about the organic or collagenous component of bone, which is also important for bone strength (section 2.4., page 18).

2.3. The human and the animal skeleton

Bone is a multifunctional, heterogeneous, metabolically active tissue. Bone is the source of haematopoietic, mesenchymal, and endothelial stem cells (Taichman, 2005) and a calcium and phosphate reservoir that helps regulate mineral homeostasis (Bonewald, 2007; Bonewald, 2011; Guntur & Rosen, 2012). Bone secretes osteocalcin and fibroblast growth factor 23 (FGF23), hormones that have metabolic and endocrine functions, such as regulating glucose and energy metabolism, appetite, muscle function, and testosterone production (Guntur & Rosen, 2012; Kanazawa, 2017; Mera, Ferron, & Mosialou, 2018; Mosialou *et al*., 2017; Pi & Quarles, 2013). Bone is also the target for hormones and local factors including parathyroid hormone (PTH), oestrogen, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), glucagon-like peptide-1 (GLP-1), other growth factors, adipokines, and cytokines (Brunetti *et al*., 2017; Guntur & Rosen, 2012; Pi & Quarles, 2013).

Importantly, bone has a structural function linked to anatomical and mobility factors. For example, it protects internal organs and it facilitates locomotion and mobility. Hence, bone needs to be strong to support mechanical loading, but light enough so it does not impede mobility (Seeman & Delmas, 2006). The adult human skeleton is comprised of 206 separate bones, which are classified into five groups depending upon their shape. (i) Long bones are cylindrical in shape and longer than they are wide; their function is to support weight and facilitate movement (*i.e*., leverage) and include bones in the

appendicular skeleton (*e.g.*, femur, tibia, fibula, humerus, radius, ulna, metacarpals, phalanges, metatarsals, and phalanges). Long bones are anatomically divided into different parts: the proximal and distal epiphyses and metaphyses, and the diaphyses (**Figure 2**). (ii) Short bones have roughly the same length, width and thickness and are in the carpals of the wrist (*e.g.*, scaphoid, lunate, pisiform, and trapezoid) and tarsals of the ankle joints (*e.g.*, calcaneus, talus, cuboid, cuneiforms); their function is to provide stability and support as well as limited movement. (iii) Flat bones are thin and usually curved (*e.g.*, cranial bones, scapulae, sternum, ribs, and pelvis bones) and provide protection for internal organs and are attachment points for muscles. (iv) Sesamoid bones are small and are embedded in the tendons of the hands and knees, reinforcing, and reducing stress of tendons (*e.g.*, patella). (v) Irregular bones have complex shape and morphology and do not fit into the other classifications, their main function is also to protect organs (*e.g.*, vertebrae, facial bones).

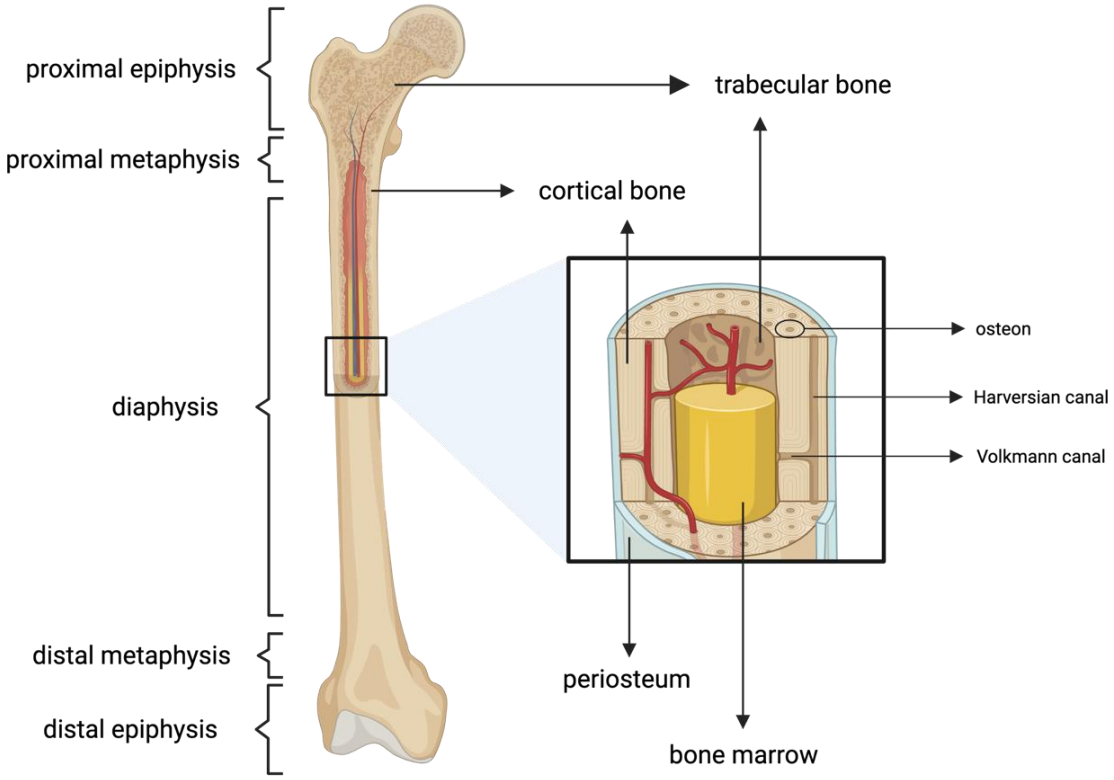


Figure 2. Anatomical parts of a long bone (left side) and internal structure of bone tissue (right side). Created with BioRender.com

2.3.1. Bone internal structure

The external surface of all bones is covered by a membrane called the periosteum (Orwoll, 2003) and the internal structure of bone tissue comprises mineralised bone matrix and extracellular fluid. The inner mineral matrix is composed of three components: the intracortical surface, the endocortical surface and the trabecular surface (Seeman, 2013). Cortical bone, also known as compact bone because of its higher density, forms the superficial layer of all bones. Trabecular bone, also called cancellous or spongy bone for its porosity, is the internal bone tissue and it is predominant in the ends of long bones. All three surfaces contain bone cells responsible for bone remodelling, although the ratio between the internal surface area and the bone mineral matrix influences the access of the mineral matrix to remodelling (section 2.5, page 23) (Robling, Castillo, & Turner, 2006; Seeman, 2013; Seeman & Delmas, 2006). The medullary or marrow cavity, which contains bone marrow, is in the centre of the diaphysis of long bones (**Figure 2**).

2.3.1.1. Cortical bone

Cortical bone is contained between the periosteum and the endocortical surface and it is primarily found in the cortex of bones and diaphysis of long bones. Cortical bone tissue comprises 70% of mineralised bone matrix volume and 30% of extracellular volume (Techawiboonwong *et al*., 2008). The mineralised cortical bone is organised in a cylindrical manner forming osteons with Harversian canals placed in the centre and perpendicular Volkmann canals (**Figure 2**) (Seeman, 2013; Seeman & Delmas, 2006). These two canals form the extracellular matrix that provide most of the cortical porosity and also form the intracortical area where bone remodelling is initiated (Han *et al*., 1997; Seeman, 1997; Seeman, 2013). Cortical bone has a large volume of mineralised matrix, but a smaller internal surface area, which limits the access of the mineralised matrix to being remodelled (Seeman, 2013). At a structural level, cortical bone is well organised, dense, rigid, and smooth, with the mineralised bone and collagen fibrils arranged in the direction of usual mechanical load (Augat & Schorlemmer, 2006; Carnelli *et al*., 2013; Burr, 2002a; Currey, 2003; Szulc *et al*., 2006). These characteristics provide cortical bone with the capability to maintain the strength and stiffness of the skeleton and to sustain sudden, high impact forces (Augat & Schorlemmer, 2006; Hart *et al*., 2020).

2.3.1.2. Trabecular or cancellous bone

Trabecular bone is encapsulated by the endocortical surface and forms the internal structure of bone. Trabecular bone tissue is predominately located in weight-bearing bones (*e.g.*, proximal and distal epiphyseal and metaphyseal areas of long bones, vertebrae), comprising 30% of mineralised matrix volume and 70% of extracellular volume, which contains red bone marrow (Seeman, 2013). The porosity of trabecular bone is achieved by its three-dimensional network of trabeculae (*i.e.*, small beams forming a lattice shaped structure) (Ben-Zvi *et al.*, 2017) (**Figure 2**). This porous configuration of trabecular bone facilitates the bone remodelling that is initiated in the mineralised trabeculae, with a high surface area and the reduced mineral matrix volume (Seeman, 2013; Techawiboonwong *et al.*, 2008). The trabeculae structures are predominantly organised in the same direction plane as the mechanical loads placed upon them and are able to deform and absorb energy from mechanical loading and sustain cyclical low-grade forces (Cazenave *et al.*, 2021; Currey, 2003; Hart *et al.*, 2020; Huiskes *et al.*, 2000).

2.3.2. Bone composition and strength

Bone is a composite tissue that comprises an inorganic (or mineral) component (made up of calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]), an organic component (largely made up of type 1 collagen proteins) and water (Seeman & Delmas, 2006). The mineral phase of bone contributes to bone stiffness and allows mechanical loads to be sustained, whereas the collagen matrix determines the toughness of bone by providing ductility and flexibility (Bouxsein, 2005; Burr, 2002b; Viguet-Carrin, Garnero, & Delmas, 2006). Hence, both components are fundamental parameters that influence bone strength (*i.e.*, ability to resist fracture) (Viguet-Carrin, Garnero, & Delmas, 2006).

2.3.2.1. Bone mineral (inorganic) phase

Traditionally, bone mass refers to the bone mineral matrix and has been the most studied determinant of skeletal health. In clinical and research fields, bone mass is reported as BMC and BMD and it is measured using imaging techniques, such as DXA. It is not, however, only the quantity (or density) of

bone mineral that influences bone strength, with measurements of BMD via DXA only explaining about two-thirds of bone strength (Ammann & Rizzoli, 2003). The three dimensional imaging devices pQCT and high-resolution pQCT allow the measurement of the material and structural properties of bone, enabling the examination of bone shape, size and distribution, as well as bone mass and analysing trabecular and cortical bone parameters separately (Hart *et al*., 2020; Stagi *et al*., 2016). The geometry and microarchitecture (*i.e*., how the mineral bone is distributed) are also crucial for bone strength, explaining up to 70% of the variation in bone strength (Ammann & Rizzoli, 2003; Cole & Van Der Meulen, 2011; Seeman & Delmas, 2006). For example, bone strength in the diaphysis of long bones increases with a greater diameter, greater cortical thickness and more ellipticity (Ammann & Rizzoli, 2003; Wang *et al*., 2009); whilst the size and spatial distribution of the trabeculae determine the structural contribution of trabecular bone morphology and porosity (Cole & Van Der Meulen, 2011).

2.3.2.2. Bone organic phase

90% of the organic bone matrix is comprised of type 1 collagen, with the remaining 10% being comprised of non-collagenous proteins, such as osteopontin and osteocalcin. Alterations of collagen properties, such as content, maturity and crosslinking can affect the mechanical properties of bone (Cole & Van Der Meulen, 2011; Burr, 2002b; Viguet-Carrin, Garnero, & Delmas, 2006). The rate of bone collagen turnover may also be important in determining bone strength because it influences the pattern of mature/immature collagen crosslinking in bone (Bouxsein, 2005; Burr, 2002a; Viguet-Carrin, Garnero & Delmas, 2006). This thesis focuses upon the bone collagen matrix and its turnover, which are further described in section 2.4. (page 18).

2.3.3. The animal skeleton

Although the anatomical characteristics of mammalian skeletons, including humans, are similar; bone is a mechanically optimised tissue that dynamically adapts its composition and organisation to accommodate functional and mechanical demands (Bagi, Berryman, & Moalli, 2011). Therefore, there are several crucial differences between species that need consideration when conducting skeletal

research that seeks to be applicable to humans. Given that a great part of skeletal research has been conducted in animals and given that this is also the case in the studies reported in Chapters 4, 5, 6, 7 and 8 of this thesis, some of these relevant differences are addressed below.

Mechanical loading is a key factor for determining the physical characteristics of the skeleton (section 2.6., page 34), including the dimension, morphology (*i.e*., geometry) and structure of trabecular and cortical bone. The mechanical load between humans (bipeds) and animals (quadrupeds) is different, and some adaptations, such as the geometry and the internal structure of human long bones, are not seen in quadrupedal animals (Bagi, Berryman, & Moalli, 2011; Seeman & Delmas, 2006). These differences also exist within animal species. For example, rodents lack Haversian canals and secondary osteons due to minimal intracortical remodelling; whilst larger animals (*e.g*., dogs, pigs, sheep and non-human primates) have similar secondary osteon structures to humans (Bagi, Berryman, & Moalli, 2011; Felder *et al*., 2017; Taguchi & Lopez, 2021). However, the use of rodents, and particularly the use of rat models that ensure homogeneity between animals, is very suitable for conducting research regarding the physiology of long bones and spinal bones (Bagi, Berryman, & Moalli, 2011). Rodent models can also be modelled to better represent human skeleton characteristics (Macedo *et al*., 2019).

Different life expectancies and sexual and skeletal maturation rates are also important factors to consider within animal skeletal research, since these factors can influence bone (re)modelling. Skeletal maturity occurs at 16-32 weeks in small mammals, at 12-40 months in large mammals, at 7-10 years in non-human primates, and at ~25 years in humans (Taguchi & Lopez, 2021). Unlike humans, bone acquisition and longitudinal bone growth in mice continues longer after sexual maturity at 6-8 weeks (Jilka, 2013); and in rats, the growth plate may remain open until 25 months (Martin, Ritman, & Turner, 2003). The bone remodelling period varies between species, with rodents having a 7-18-fold faster remodelling rate than humans (Jilka, 2013). Another difference is that human bone is ~60% mineralised, whilst animal skeletons are ~40% mineral (Seeman & Delmas, 2006). Bone density also differs between humans and animals, with canine and porcine bone tissue being the most similar to humans (Aerssens *et al*., 1998).

2.4. Bone collagen

Collagen is the most abundant protein in the human body and it is present in the extracellular matrix of connective tissues such as tendon, skin, muscle, and bone. Whilst many types of collagens have been identified, the collagen found in these tissues comprises mainly four types (*i.e*., collagen types 1, 2, 3, and 5) and has a fibrillar structure (Viguet-Carrin, Garnero, & Delmas, 2006). Collagen fibrils provide structural integrity to these tissues and the basis to resist tensile, shear, or compression forces; and they form a network that supports muscle fibres and allow the transmission of forces through tendons and ligaments during movement (Viguet-Carrin, Garnero, & Delmas, 2006). In bone, collagen provides toughness by increasing its ductility and ability to absorb energy (Bouxsein, 2005; Seeman & Delmas, 2006) and is also the foundation for the deposition of hydroxyapatite crystals, which mineralises the collagen fibrils (Crockett *et al*., 2011).

2.4.1. Bone collagen synthesis and crosslinking

Approximately 95% of all bone collagen is type 1 collagen (Epstein & Munderloh, 1975); other types, such as collagen type 5, are present at low levels (Niyibizi & Eyre, 1989). Type 1 collagen molecules are composed of three polypeptide chains (two α1 and one α2 chains) that form a triple-helix structure (Depalle *et al*., 2015; Myllyharju & Kivirikko, 2004) (**Figure 3**). The polypeptide chains have a defined sequence consisting of Glycine-X-Y, where X and Y can be any amino acid, but usually are proline and hydroxyproline (Brodsky & Persikov, 2005) (**Figure 3**). This specific sequence of amino acids, with glycine in every third position being the smallest amino acid, allow the polypeptide to twist and form the triple-helix configuration with glycine in the centre position and the other amino acids on the external positions (Viguet-Carrin, Garnero, & Delmas, 2006). The triple-helix structures are flanked on each end by short N- and C-terminal propeptide domains, assembling as soluble molecules known as procollagens (Lamande & Bateman, 1999; Myllyharju & Kivirikko, 2004) (**Figure 3**).

Procollagens are synthesised in the endoplasmic reticulum of the cell (*e.g*., osteoblasts) and secreted into the extracellular matrix via transport vesicles (Myllyharju & Kivirikko, 2004). The biosynthesis of

procollagen comprises various post-translational modifications occurring inside the endoplasmic reticulum, including the conversion of proline residues into their trans form, the hydroxylation of proline residues (crucial for folding and secretion of procollagens), and the formation of hydroxylysine (Lamande & Bateman, 1999; Viguet-Carrin, Garnero, & Delmas, 2006). Molecular chaperones, such as HSP47, a collagen-binding protein, are also required for the folding and assembly of procollagens (Lamande & Bateman, 1999). Once procollagen is secreted into the extracellular matrix, propeptides C and N are removed by C and N proteinases, eliciting spontaneous self-assembly of collagen fibrils (Myllyharju & Kivirikko, 2004) (**Figure 3**). Lastly, collagen fibrils that have been formed in the extracellular environment are stabilised over time by further post-translational modifications that allow immature and mature crosslinking (Viguet-Carrin, Garnero, & Delmas, 2006).

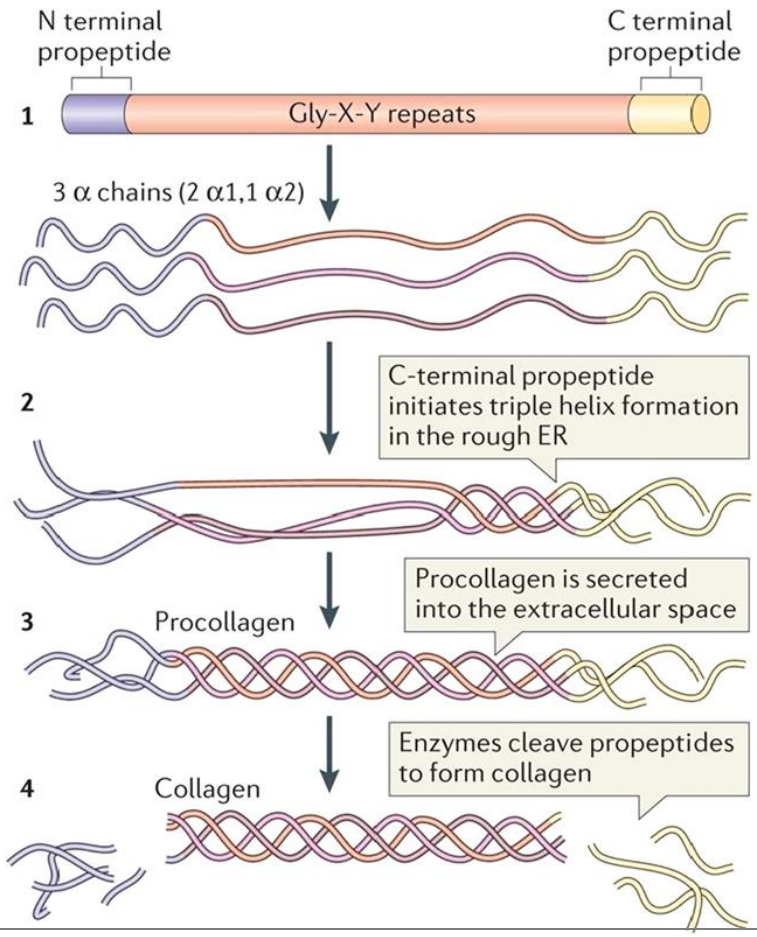


Figure 3. Collagen structure and formation. (1) The standard collagen molecule has amino-(N) and carboxy-(C) terminal propeptide sequences. (2) Three α-chains are intracellularly assembled into a triple helix in the rough endoplasmic reticulum. (3) Procollagen is secreted into the extracellular space. (4) Procollagen is converted into collagen by cleavage of the N- and C-propeptides. Obtained with permission from Springer Nature publishing (Mouw, Ou, & Weaver, 2014).

Collagen crosslinking can be enzymatic and non-enzymatic. Enzymatic crosslinking, activated by lysyl oxidase (LOX), is generally considered the regular process for the formation of healthy collagen and it has positive effects on its material properties (Burr, 2002a; Garnero, 2012; Saito & Marumo, 2010). The enzyme LOX acts on the extracellular collagen fibrils binding to specific lysine and hydroxylysine residues in the telopeptides initiating collagen crosslinking (Knott & Bailey, 1998). Firstly, divalent molecules dihydroxylysinornorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) are formed by the action of LOX creating immature crosslinks (Garnero, 2012; Saito & Marumo, 2010). These entities then react to another telopeptide aldehyde group and are transformed to mature trivalent crosslinks pyrdinoline, deoxypyridinoline, and pyrrolic analogues (Garnero, 2012; Saito & Marumo, 2010). The conversion of immature to mature crosslinks is a continuous independent process, however, the relative amounts of mature and immature crosslinks are influenced by bone turnover rates and mineralisation (Viguet-Carrin, Garnero, & Delmas, 2006). Compared to other collagenous tissues, bone contains a significant amount of immature crosslinks, where mineralisation appears to have an effect on enzymatic crosslinks maturity (Saito & Marumo, 2010).

Non-enzymatic crosslinking processes can be induced by the formation of advanced glycation end products (AGEs), or by spontaneous racemisation and isomerisation of the aspartyl acid or asparagine residues with the C-telopeptide (Garnero, 2012) and their accumulation can result in deteriorated bone mechanical properties. Non-enzymatic glycation-induced crosslinks involve the reaction between an aldehyde group of a sugar, such as glucose, and the ε-amino group of collagen-bound hydroxylysine or lysine (Lapolla, Traldi, & Fedele, 2005). This reaction forms a glucosyl-lysine via Schiff base adduct formation, which undergoes further reactions to form an Amadori product. The Amadori adduct can then undergo additional reactions with amino acids (*e.g.*, lysine or arginine) in adjacent collagen molecules to form AGEs crosslinks (Bailey, Paul, & Knott, 1998; Garnero, 2012). Unlike enzymatic cross-links, which are confined in the terminal domains, AGEs crosslinks can be found at numerous locations along the length of the collagen molecule (Gautieri *et al*., 2014). There exist many sources and pathways that can lead AGE forming in bone collagen, and most factors that regulate the formation of AGEs remain unknown (Depalle *et al*., 2015). The accumulation of AGEs-crosslinks alters the

collagen structure and thereby adversely affects the mechanical properties of the collagen matrix (Vashishth, 2007). AGEs are present in slow turnover proteins and connective tissues including skin, tendon, cartilage, and bone (Viguet-Carrin, Garnero, & Delmas, 2006); and they accumulate with age and disease, for example, osteoporotic bone has more AGEs than healthy bone (Saito *et al*., 2006; Saito, Fujii, & Marumo, 2006). The formation of AGEs-crosslinks is influenced by bone turnover and the degree of glycation and oxidation reactions regulated by glycaemic control, oxidative stress, ageing, and AGEs receptor (Saito & Marumo, 2010).

With ageing, racemisation, and isomerisation reactions on aspartyl acid or asparagine residues occur in tissues with a low metabolic turnover, such as bone (Viguet-Carrin, Garnero, & Delmas, 2006). Racemisation consists of the spontaneous conversion of the L-enantiomeric form into the D-form, and isomerisation consist of the transferal of the peptide bound between aspartic acid residues and the adjacent amino from the α-carboxyl group to the β- or γ-carboxyl groups (Garnero, 2012). Racemisation and isomerisation are processes that occur in ageing proteins and disrupt protein regulation and function, leading to structural alterations of collagen molecules (Garnero, 2012; Viguet-Carrin, Garnero, & Delmas, 2006).

2.4.2. Collagen and bone strength

While the bone mineral matrix provides stiffness, bone collagen provides tensile strength, ductility (post-yield) and flexibility (Bouxsein, 2005; Burr, 2002a) yielding greater effects on the overall toughness of bone tissue (Wang *et al*., 2002), which are all determinants of bone strength. Bone strength is defined as the ability to resist fractures and it is influenced by bone mineral quantity and density and tissue material properties (*i.e*., bone quality). Bone quality includes bone shape, size and microarchitecture, degree of mineralisation, microdamage accumulation, and the intrinsic properties of the organic phase (*e.g*., collagen crosslinks) (Seeman & Delmas, 2006).

Bone remodelling (section 2.5., page 23) affects these variables and may be associated with modifications to collagen crosslinks (Saito & Marumo, 2010). Diseases such as osteogenesis imperfecta also demonstrate the importance of bone collagen properties for bone quality and strength (Rauch & Glorieux, 2004). Osteogenesis imperfecta is caused by mutations in the COL1A1 and COL1A2 genes, which encode the α1 and α2 chains of type 1 collagen; these genetic abnormalities result in defects in the collagen structure, resulting in impaired bone material properties, bone fragility and increased risk of fracture (Grabner *et al*., 2001; Rauch & Glorieux, 2004). Evidence has shown that the of the Sp1 polymorphism in the COL1A1 gene can increase risk of fracture independently of BMD (Mann *et al*., 2001). This fact suggests that there is a direct effect of collagen, independent of mineral mass, on bone quality and strength (Burr, 2002a).

Whilst the role of collagen as a determinant of bone strength is well-defined, the specific molecular mechanisms that provide mechanical properties to bone collagen continue to be investigated. Some studies have suggested that collagen content (Bailey & Knott, 1999) and collagen fibril orientation (Martin & Boardman, 1993) may be important for bone strength. Most studies, however, have focused upon the effects of post-translational modifications (*i.e*., crosslinking) on the mechanical properties of collagen. Collagen crosslinking directly influences tensile strength and post-yield mechanical properties of the bone tissue. Both divalent immature crosslinking, predominant in bone collagen, and trivalent mature enzymatic crosslinking are important for maintaining these properties (Saito & Marumo, 2010; Viguet-Carrin, Garnero, & Delmas, 2006). The ratio of immature/mature crosslinks can alter bone strength, with a decrease in immature crosslinks related to reduced bone strength (Saito *et al*., 2006). Hence, the rate of bone collagen turnover may also be important in determining bone strength because it influences the ratio of mature/immature bone collagen crosslinking (Bouxsein, 2005; Burr, 2002a; Viguet-Carrin, Garnero & Delmas, 2006).

Contrary, non-enzymatic crosslinking (*e.g*., AGEs-crosslinks) are associated with negative effects on bone material properties (Garnero, 2012; Saito & Marumo, 2010), as described in the previous section (page 18). AGEs-crosslinks degrade collagen structure and are associated with skeletal fragility

(Vashishth, 2009; Willett, Pasquale, & Grynpas, 2014). Enzymatic and non-enzymatic crosslinking affect the mineralisation process and microdamage formation; impaired enzymatic crosslinking and/or increased non-enzymatic crosslinks underlie decreases in bone mechanical properties and bone strength/toughness associated with ageing, osteoporosis, and diabetes mellitus (Osterhoff *et al*., 2016; Saito & Marumo, 2010). Additionally, some studies suggest that defective collagen crosslinking affects bone cell differentiation (Ida *et al*., 2018; Turecek *et al*., 2008), and that AGEs directly influence bone cell function (Alikhani *et al*., 2008; Dong *et al*., 2011), thereby emphasising the potential effects of crosslinking on bone remodelling.

2.5. Bone (re)modelling

Bone is a dynamic tissue that is constantly (re)modelling. Throughout life, the skeleton is "constructed" and "reconstructed" by the processes of bone modelling and remodelling (Seeman & Delmas 2006). In the bone modelling process, bone resorption and formation occur independently at separate skeletal sites leading to major changes in bone size and architecture (Kenkre & Bassett, 2018). Bone modelling begins early in skeletal development and it is mostly completed by skeletal maturity, but modelling can still occur in adulthood such as in an adaptive response to mechanical loading and exercise (Bass *et al.*, 2002; Krahl *et al*., 1994; Santos, Elliott-Sale, & Sale, 2017) or in circumstances such as fracture repair or disease (Ubara *et al*., 2003; Ubara *et al*., 2005).

In bone remodelling, bone formation is coupled to preceding bone resorption; hence, over time, the total volume and structure of the bone remain unchanged and bone mass is preserved (Kenkre & Bassett, 2018). The close coordination between bone resorption and formation ensures that structural integrity is maintained while allowing up to 10% of the skeleton to be replaced each year (Manolagas, 2000). Bone remodelling is an essential lifelong process that occurs continuously to (i) maintain levels of homeostasis of essential minerals (*i.e*., calcium, phosphate), by releasing them into the blood if needed; (ii) adapt to mechanical loading maintaining the integrity of the skeleton; and (iii) repair skeletal damage to prevent fracture (Burr, 2002b; Crockett *et al*., 2011; Frost, 2003).

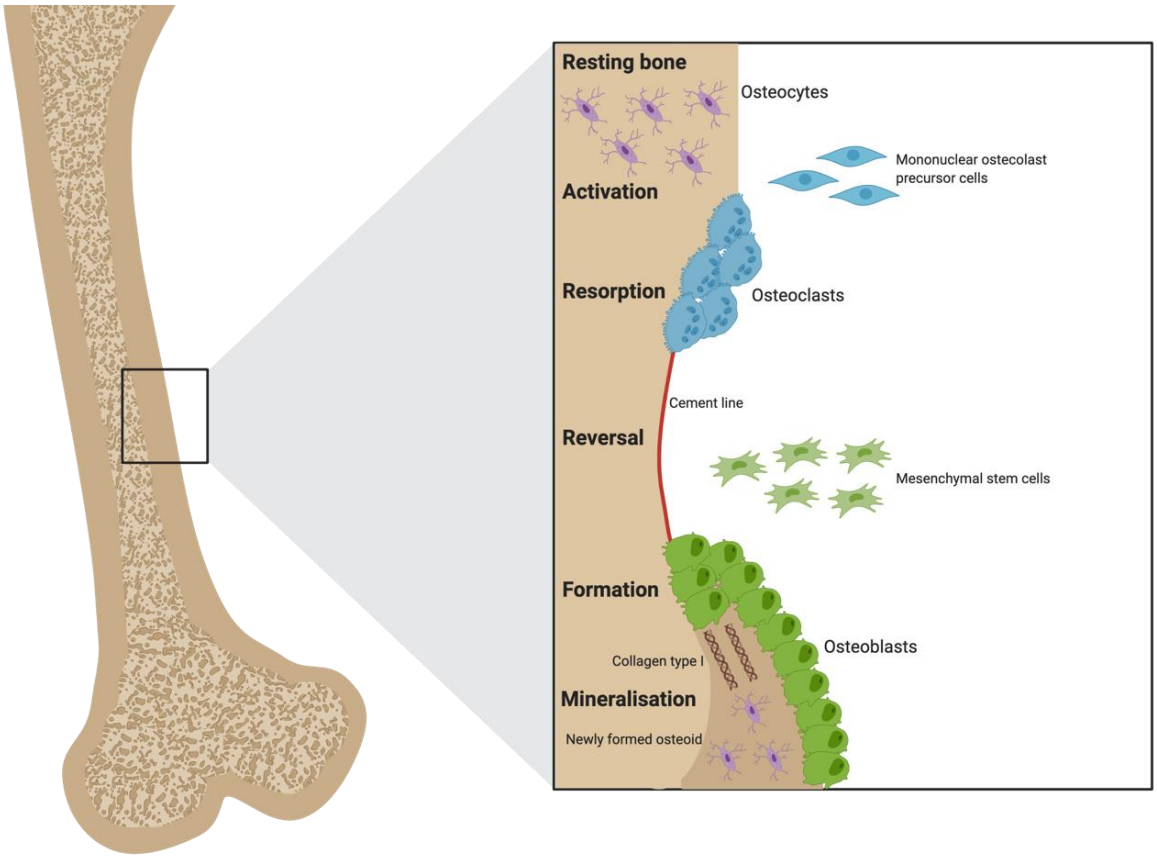


Figure 4. Schematic diagram of the bone remodelling cycle at a cortical site, showing the bone multicellular units at the different phases of activation, resorption, reversal, formation, and termination (or quiescence). Created with BioRender.com

The remodelling cycle takes place in bone multicellular units and it is mediated by major bone cells called osteoclasts, osteoblasts and osteocytes. Bone remodelling is a highly regulated and synchronised process orchestrated by osteocytes, which regulate osteoclastic resorption and osteoblastic bone formation (**Figure 4)**. Kenkre & Bassett (2018) defined the remodelling cycle with five overlapping steps that occur over 120-200 days: activation, resorption, reversal, formation and termination.

2.5.1. Osteoclasts

Osteoclasts are large multinucleated cells derived from the monocytes/macrophage family (Boyle, Simonet, & Lacey, 2003; Schaffler *et al*., 2014), which mediate bone resorption. Osteoclast differentiation is initiated by macrophage colony-stimulating factor (M-CSF), which binds to its receptor colony stimulating factor 1 receptor (c-FMS), stimulating proliferation and differentiation of hematopoietic osteoclast progenitor cells into mononuclear osteoclast precursor cells (Crockett *et al*., 2011; Proff & Römer, 2009). In combination, the receptor activator of nuclear factor kappa-B (RANK) binds with its ligand (RANKL), released by osteoblasts, on the mononuclear osteoclast precursors cell surface (Boyle, Simonet, & Lacey, 2003), leading to mature osteoclast differentiation by the fusion of multiple mononuclear osteoclast precursors into one giant osteoclast cell. The binding of RANKL to RANK continues in mature osteoclasts, which is essential for their activation, function, and survival, ultimately facilitating bone resorption. In inflammatory diseases, such as rheumatoid arthritis, osteoclastogenesis may also be induced by immune cells (Takayanagi, 2012). In contrast, osteoblasts, and other tissues, such as bone marrow, heart, liver, and kidneys, express osteoprotegerin (OPG). Decoy receptor OPG is important for regulating bone resorption (discussed below in section 2.5.5., page 30). OPG binds to RANKL and blocks its binding to RANK, which prevents osteoclastic resorption activity (Boyce & Xing, 2007; Udagawa *et al*., 2000).

Osteoclasts are attracted to bone sites that require resorption (*e.g*., old or damaged bone) and they attach to the bone surface, where a resorption compartment named a lacuna is formed. Osteoclasts secrete hydrochloric acid and enzymes (*e.g.*, cathepsin K, matrix metalloproteinases) that dissolve the mineral component of bone and break down collagen fragments forming a lacuna (Teitelbaum, 2007). Osteoclast activity is directly or indirectly regulated by various hormones, cytokines, and growth factors.

2.5.2. Osteoblasts

Osteoblasts are bone formation cells that derive from the proliferation and differentiation of mesenchymal stem cells available in the bone marrow, but may also be derived from bone lining cells and chondrocytes (Manolagas, 2000; Matic, *et al*., 2016; Yang *et al*., 2014). Osteoblast differentiation is activated by the Wnt-β-catenin pathway, bone morphogenic proteins (BMPs), and transcription factors such as SOX-9, runt-related transcription factor 2 (Runx2), Osterix (Osx), and distal-less homebox 5 (Dix5) (Crockett *et al*., 2011; Heo, Lee, & Kim, 2017; Long, 2012; Nakashima *et al*., 2002). Wnt signalling is negatively regulated by secreted proteins, sclerostin (SOST) and members of the Dickkopf (DKK) family synthesised by osteocytes (Hartmann, 2006; Marie, 2009). Hedgehog protein signalling, Notch and fibroblast growth factors (FGF) signalling are also involved in the regulation of osteoblastogenesis (Long, 2012).

Osteoblasts create new bone by the formation of organic bone matrix. Osteoblasts secrete type 1 collagen, non-collagenous proteins, and proteoglycans; and they deposit hydroxyapatite, which contains calcium and phosphate that mineralises the collagen (Manolagas, 2000). Osteoblasts express the enzyme alkaline phosphate and polyphosphate kinase and bone regulating hormones, such as PTH and 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) (Crockett *et al*., 2011; Proff & Römer, 2009). Osteoblasts also produce RANKL and OPG, which regulate osteoclastogenesis and osteoclast activity.

Besides their role in bone formation, osteoblasts are also important for regulating osteoclastogenesis and osteoclast activity, since they produce RANKL and OPG (Proff & Römer, 2009). Osteoblasts have three possible fates: 1) some osteoblasts (10-30%) become embedded into the mineralised bone matrix and differentiate into osteocytes, 2) some transform into bone lining cells or 3) some die via apoptosis (Crockett *et al*., 2011; Kenkre & Bassett, 2018).

2.5.3. Osteocytes

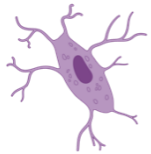
Osteocytes comprise 90-95% of the total adult bone cell population and are, thus, the most abundant types of bone cells. They are formed when mature osteoblasts become embedded in the mineralised

bone matrix and differentiate into osteocytes. Osteocytes exhibit long dendritic branches that inter-connect and allow communication with each other via the canalicular system, forming a cellular network that includes bone lining cells on bone surfaces, endothelial cells, components of bone marrow, and blood vessels (Bonewald, 2011; Palumbo *et al*., 1990).

There is evidence that supports the role and function of osteocytes as mechanosensitive cells that orchestrate bone (re)modelling (section 2.6.1.1., page 35), controlling both bone resorption and bone formation (Bonewald, 2011). Osteocytes have a mechanoreceptor function that senses the direction and magnitude of mechanical strain and can respond to mechanical loading (Bonewald, 2007; Vatsa *et al*., 2008). Osteocytes can also transduce these bone-loading signals to regulate the action of osteoclasts and osteoblasts. Through signalling pathways, osteocytes control the migration of osteoclasts and osteoblasts to the places that need remodelling (Bonewald, 2011; Crockett *et al*., 2011). Osteocytes sense changes to mineral homeostasis and secrete the phosphate regulator Fibroblast Growth Factor 23 (FGF23) (Bonewald & Wacker, 2013). Osteocytes produce RANKL, required for osteoclastogenesis, and secrete SOST and Dickkopf-related protein 1 (DKK-1), negative regulators of Wnt signalling, limiting osteoblast formation. Mechanical loading inhibits secretion of SOST and DKK-1, therefore increased loading translates into greater bone formation (Moester *et al*., 2010).

The disruption of the osteocyte canalicular system caused by microdamage of the bone matrix can stimulate osteocyte apoptosis, which leads to the recruitment of osteoblast and osteoclast progenitor cells and activate bone remodelling in a site-specific manner (Atkins & Findlay, 2012; Chen, Senda, & Kubo, 2015; Lin *et al*., 2009; Tatsumi *et al*., 2007). Osteocyte apoptosis under pathological conditions such as osteoporosis and osteoarthritis, has a link to resorption-associated skeletal fragility and bone loss (Ru & Wang, 2020).

Table 1. Summary of bone cells involved in the bone remodelling process.

Cell type	Description	Major Roles	Key signalling pathways
Osteoclasts 	Bone-resorbing cells. Multinucleated cells derived from the monocytes/macrophage.	Dissolve bone mineral and break down collagen by the secretion of hydrochloric acid and enzymes including cathepsin K	• Differentiation is initiated by M-CSF and promoted by RANKL binding to its receptor RANK on precursor cells. • Osteoclastogenesis and osteoclast activity is directly or indirectly regulated by OPG, PTH, IL-1, IL-6, TNF-α, 1,25-$(OH)_2$D, TGF-β and BMP-2.
Osteoblasts 	Bone-formation cells. They are derived from differentiation of mesenchymal stem cells and may also be derived from bone lining cells and chondrocytes. Osteoblasts have three possible fates: they can become osteocytes, bone lining cells, or die via apoptosis.	Synthesise type 1 collagen, non-collagenous proteins, and proteoglycans; and regulate matrix mineralisation.	• BMPs transcription factors such as SOX-9, Runx2 are key for osteoblast differentiation. • Osteoblasts produce RANKL and OPG, which regulate osteoclastogenesis and osteoclast activity and express the enzyme alkaline phosphate and polyphosphate kinase and bone regulating hormones such as PTH and 1,25-$(OH)_2$D. • Osteoblastogenesis is controlled by the Wnt-*β*-catenin pathway. Wnt signalling is negatively regulated by secreted proteins, SOST and members of the DKK family, which are synthesized by osteocytes. • Hedgehog protein signalling, Notch and FGF signalling are also involved in the regulation of osteoblastogenesis.
Osteocytes 	Most abundant adult bone cells (90-95% total population). Formed when mature osteoblasts become embedded in the mineralised bone matrix. They exhibit long dendritic branches inter-connected in an intracellular network connecting osteocytes, bone lining cells and bone marrow cells and blood vessels.	Mechanoreceptors and transducing cells. Osteocytes are sensitive to mechanical strain and transduce bone-loading signals to regulate the action of osteoclasts and osteoblasts and initiate remodelling in a site-specific fashion.	• Osteocytes regulate mineral homeostasis and secrete the phosphate regulator FGF23. • Osteocytes produce RANKL (required for osteoclastogenesis) and secrete SOST and DKK-1 (negative regulators of Wnt signalling, limiting osteoblast formation). • Osteocyte secretion of SOST and DKK-1 is inhibited by mechanical loading, thus increased loading results in a local increase in bone formation.

1,25-$(OH)_2$D, 1,25-dihydroxyvitamin D; BMP-2, bone morphogenetic protein 2; BMPs, bone morphogenetic proteins; DKK-1, Dickkopf-related protein 1; DKK, Dickkopf-related proteins; FGF, fibroblast growth factors; FGF23, fibroblast growth factor 23; IL-1, interleukin-1; IL-6, interleukin-6; M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; PTH, parathyroid hormone; RANK, receptor activator nuclear of factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; Runx2, runt-related transcription factor 2; SOST, sclerostin; SOX-9; transcription factor; TGF-β, transforming growth factor β; TNF-α, tumour necrosis factor-α.

2.5.4. The bone (re)modelling cycle

2.5.4.1. Activation

Lining cells separate from underlying bone, forming a canopy over the site that requires remodelling. Osteoclast precursor cells are activated, multiple mononuclear cells fuse to form multinucleated osteoclasts, which bind to the bone matrix and form a sealed area around the resorption pit that is isolated from the surrounding bone (Hauge *et al*., 2001). Targeted remodelling is only initiated when it is required and targeted to the removal of a specific zone of damaged or old bone (Burr, 2002a). Osteocytes are the cells that produce the initiating signal and use their extensive intercellular network to send the signal to other cells. In contrast, non-targeted remodelling refers to stochastic bone remodelling (*i.e*., not site-dependent) (Burr, 2002a), which is activated in response to changes in hormones such as PTH that regulate mineral homeostasis (Kenkre & Bassett, 2018).

2.5.4.2. Resorption

Osteoclasts adhere to the bone surface and form a sealed area creating the secretory surface area. Osteocytes send protons into the resorbing compartment to dissolve the bone mineral. The collagen-rich bone matrix is degraded by proteases, such as cathepsin K (Delaissé *et al*., 2003). This process takes approximately two weeks in total (Kenkre & Bassett, 2018). The resorption phase is concluded by the programmed cell death of osteoclasts, which avoids excess bone resorption from occurring (Xing & Boyce, 2005).

2.5.4.3. Reversal

The exact signal that couples bone resorption to succeeding formation is still not fully understood although two key actions occur. First, cells from osteoblastic lineage remove unmineralised collagen, secondly non-collagenous mineralised matrix (cement-line) is deposited. This cement-line facilitates osteoblasts' adherence (Zhou, Chernecky, & Davies, 1994). Other signalling pathways may include factors such as bone morphogenetic protein 2 (BMP-2), transforming growth factor β (TGF-β), and

insulin-like growth factor 1 (IGF-1) (Martin & Sims, 2015). The reversal phase has a duration of approximately four to five weeks (Kenkre & Bassett, 2018).

2.5.4.4. Formation

Osteoblasts synthesise and secrete type 1 collagen producing new osteoid matrix. Osteoid is then mineralised, a process that is influenced by osteoblasts, although its regulation is not completely understood. The mineralisation process is achieved by the local release of phosphate, which is generated by phosphatases present in osteoblast-derived matrix vesicles within the osteoid, in addition to the calcium present in the extracellular fluid (Crockett *et al*., 2011). These two factors result in the nucleation and growth of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] deposited amongst collagen fibrils (Crockett *et al.*, 2011). Mineralisation is controlled by the systemic regulation of calcium and phosphate and by local inhibitors of mineralisation, such as pyrophosphate and osteopontin (Kenkre & Bassett, 2018). The ratio of inorganic pyrophosphate to phosphate, which is determined by the activity of alkaline phosphatase and ectonucleotide pyrophosphatase, is a key regulator of mineralisation (Anderson, 2003; Cui *et al*., 2016)

2.5.4.5. Termination or quiescence

Once mineralisation is finalised, osteoblasts that do not undergo apoptosis become resting bone lining cells on the newly formed bone surface or differentiate into osteocytes. Osteocytes are key for the termination of remodelling via the secretion of inhibitors of bone formation and the Wnt signalling pathway, such as SOST and DKK-1 (Bonewald, 2011; Crockett *et al.*, 2011).

2.5.5. Signalling pathways of bone remodelling

In health, bone resorption and bone formation are tightly coupled during the remodelling cycle. In order to achieve this balance, two key signalling pathways control osteoclastic bone resorption and osteoblastic bone formation: RANK/RANKL/OPG and Wnt/β-catenin (Boyce & Xing, 2007; Kenkre and Bassett, 2018). These two pathways have a local regulatory role in determining the balance and

timing of bone resorption and formation that occur within the remodelling cycle at multiple, distinct anatomic sites simultaneously; and are key for the bone adaptations in response to mechanical loading.

2.5.5.1. RANKL/RANK/OPG signalling pathway

When the RANKL/RANK/OPG signalling pathway was identified in the 1990s, it signified a breakthrough in the understanding of osteoclast differentiation and activity. RANKL binding to its receptor RANK, which takes place in osteoclast precursor cells, mediates further osteoclast differentiation into multi-nucleated cells, activation, and longevity (Boyce & Xing, 2007; Kong *et al.*, 1999; Yasuda *et al*., 1998). OPG is secreted by osteoblasts and osteocytes and acts as decoy receptor for RANKL. It binds to RANKL, preventing it to bind to RANK and, therefore, inhibiting osteoclastic bone resorption. The ratio of RANKL to OPG is crucial for the regulation of bone resorption and is controlled by various systemic factors, including interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), 1,25-$(OH)_2$D, PTH, oestrogen, and BMP-2 (Boyce *et al*., 2012).

2.5.5.2. Wnt/β-catenin signalling pathway

The canonical Wnt/β-catenin signalling pathway is a key regulator of osteoblastic bone formation. Wnt ligands bind into co-receptors low-density lipoprotein receptor-related proteins 5 or 6 (LRP-5, LRP-6) and Frizzled genes, which results in the inhibition of glycogen synthase kinase-3β (GSK-3β) phosphorylation of β-catenin and, thus, the accumulation of β-catenin in the cytoplasm. Then, the β-catenin enters the nucleolus and activates target gene transcription that leads to osteoblast proliferation and differentiation, which facilitates bone formation (Baron & Kneissel, 2013; Case & Rubin, 2010). The Wnt signalling pathway and subsequent bone formation, are negatively regulated by SOST and DKK-1 proteins (secreted by osteocytes) binding to LRP-5 and LRP-6 and, thus, preventing the Wnt ligand association (Kawano & Kypta, 2003; Williams, 2014). In the termination phase, newly formed osteocytes embedded in the bone matrix express Wnt antagonists indicating the end of bone formation (Crockett *et al*., 2011).

2.5.6. Endocrine and paracrine regulatory factors of bone remodelling

There are a number of different factors, endocrine and paracrine, involved in the regulation of the bone remodelling cycle; these are summarised in the tables below.

Table 2. Endocrine regulatory factors of bone remodelling.

Endocrine Factors	Effects on bone remodelling
PTH	Depending upon the duration or magnitude of exposure, PTH can have opposite effects on bone remodelling. - Continuous secretion of PTH stimulates bone resorption by increasing RANKL and inhibiting OPG, which facilitates osteoclastogenesis (Locklin *et al*., 2003; Silva *et al*., 2017). - Intermittent PTH signalling reduces the expression of Wnt inhibitors SOST and DKK-1, resulting the increase in canonical Wnt signalling and subsequently osteoblastogenesis, target gene expression and bone formation. Hence, intermittent administration of PTH is used as an anabolic agent for the treatment of osteoporosis (Crockett *et al.*, 2011; Kenkre & Bassett, 2018).
Vitamin D and VDR	Vitamin D ($1,25(OH)_2D$) is important for the regulation of intestinal calcium and phosphate absorption, both substrates for bone mineralisation (Poole & Reeve, 2005). Evidence suggests that $1,25\text{-}(OH)_2D$ and vitamin D receptor (VDR) may affect the regulation of bone mineralisation (Korvala *et al.*, 2010; Yamamoto *et al*., 2013). The $1,25\text{-}(OH)_2D$/VDR system has been shown to stimulate osteoclastogensis *in vitro*, when high doses of $1,25\text{-}(OH)_2D$ are administered and bind to the VDR in osteoblasts increasing the production of RANKL (Goltzman, 2018). In contrast, the $1,25(OH)_2D$/VDR system also presents direct anabolic and anti-resorption effects on bone via its action in mature osteoblasts and osteocytes. The effects of vitamin D on bone remodelling may depend upon the state of calcium balance and the stage of osteoblast differentiation, which may determine the $1,25(OH)_2D$ response.
Calcitonin	Calcitonin receptors are expressed on osteoclasts, and calcitonin inhibits osteoclastic bone resorption (Zaidi *et al*., 2002). For this reason, calcitonin was used for the treatment of bone disorders such as osteoporosis, although it is no longer considered an appropriate treatment. The physiological role of osteocalcin remains uncertain, but it is involved in the regulation of calcium homeostasis (Brown, 2000).
Thyroid hormones	Thyroid hormones have a direct effect on bone formation by stimulating osteoblast differentiation and mineralisation. Thyroid hormone deficiency lengthens the bone remodelling cycle and leads to increased bone mass. But the direct effects of thyroid hormones on osteoclasts are not well established (Delitala, Scuteri, & Doria, 2020). On the other hand, hyperthyroidism decreases the duration of the bone remodelling cycle, resulting in the uncoupling of osteoblastic and osteoclastic activity, and consequently, in bone loss (Bassett &Williams, 2016).

$1,25\text{-}(OH)_2D$, 1,25-dihydroxyvitamin D; DKK-1, Dickkopf-related protein 1; OPG, osteoprotegerin; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor kappa-B ligand; SOST, sclerostin; VDR, vitamin D receptor.

Table 3. Paracrine regulatory factors of bone remodelling.

Endocrine Factors	Effects on bone remodelling
Insulin-like growth factor 1	Insulin-like growth factor 1 (IGF-1) is released from the liver in response to growth hormone. Its role in bone remodelling is to stimulate both osteoblastic bone formation and osteoclastic bone resorption. IGF-1 controls osteoblasts differentiation from mesenchymal stem cells and osteoclasts differentiation and activity by upregulating RANKL (Crockett *et al*., 2011). Nonetheless, IGF-1's effect on osteoblastic bone formation prevails, producing a small net increase in bone mass (Olney, 2003). When growth hormone is deficient, bone resorption predominates leading to low BMD and increased fracture risk (Barake *et al*., 2018).
Glucocorticoids	Glucocorticoids have a negative effect on bone formation. They reduce the maturation, lifespan, and function of osteoblasts and increase osteoclastic bone resorption activity (Mitra, 2011). In addition, indirect effects of glucocorticoids such as the suppression of production of IGF-1 or growth hormone, are related with the pathogenesis of glucocorticoid-induced osteoporosis (Chotiyarnwong & McCloskey, 2020)
Sex hormones	Oestrogens and androgens promote the acquisition of bone mass during puberty and are responsible for the differences between the female and male skeleton (Almeida *et al*., 2017). In addition to their influence on skeletal growth, sex hormones help to maintain bone mass during adult life by slowing the rate of bone remodelling and preserving the balance between resorption and formation (Almeida *et al*., 2017). - The major role of oestrogen in the skeletal system is as a regulatory hormone that, acting through oestrogen receptor-α, inhibits bone resorption by reducing osteoclast number and function and increasing osteoclast apoptosis (Nakamura *et al*., 2007). Oestrogen has a direct effect on the release of IGF-1 from the liver (Almeida *et al*., 2017), and it maintains bone formation and limits bone remodelling by lengthening osteoblast and osteocyte lifespans (Khosla, Oursler, & Monroe, 2012; Krassas & Papadopoulou, 2001). Thus, reduced levels of oestrogen in postmenopausal women result in osteoclastic bone resorption in relation to osteoblastic bone formation, and subsequent bone loss (Krum & Brown, 2008; Nakamura *et al*., 2007). - Androgens, like oestrogen, stimulate bone formation and inhibit bone resorption (Clarke & Khosla, 2009). Oestrogen and androgen deficiency results in an increase in bone (re)modelling and uncoupling of the remodelling cycle with resorption outweighing formation (Manolagas, O'Brien, & Almeida, 2013). - Aromatase is important for the skeletal system because it converts androgens to oestrogens and oestrogen levels, and not androgen levels, are critical for bone mass in ageing men (Santen *et al*., 2009).
Growth factors	Transforming growth factor β (TGF-β) and BMPs are both present in the bone matrix and they stimulate the expression of Runx2, osteoblast transcription factor required for osteoblast differentiation (Bruderer *et al*., 2014). Aside, TGF-β has a complex role on coupling bone resorption and formation, having opposite effects on osteoclast formation. These two opposing actions are: (i) the indirect negative effect on osteoclastogenesis by supressing RANKL expression by osteoblasts; (ii) a favourable effect on osteoclast formation by attracting osteoclast precursor cells to the surface of the bone (Quinn *et al*., 2001; Tang *et al*., 2009).
Cytokines	Cytokines, IL-1, interleukin-6 (IL-6) and TNF-α, can stimulate osteoclastogensis. Conversely, other cytokines, such as IL-4 and gamma interferon, prevent osteoclast formation (Kenkre & Bassett, 2018). In individuals with oestrogen deficiency, such as postmenopausal women, IL-6 and TNF-α cytokines are elevated, leading to an increased expression of RANKL and subsequent increase in osteoclastogenesis and bone resorption (Bertolini *et al*., 1986; Roodman, 1993). IL-6, however, stimulates mesenchymal progenitor cells, which support osteoblasts differentiation and survival.

BMD, bone mineral density; BMPs, bone morphogenetic proteins; IGF-1, insulin-like growth factor 1; *IL-1, interleukin-1; IL-4, interleukin-4; IL-6, interleukin-6; RANKL, receptor activator of nuclear factor kappa-B*

2.6. Mechanical loading

The skeleton has the ability to adapt to the mechanical loading caused by mechanical stress from muscle contractions, impact loading, and gravitational forces (Burr, 1997; Goodman, Hornberger, & Robling, 2015). These stresses generate an osteogenic (*i.e*., stimulation of bone formation) response and bone adaptations are produced via bone modelling and remodelling (section 2.5., page 23), which ultimately lead to changes in bone mass and architecture. This ability of bone to adapt to mechanical stimuli was first documented by Wolff in 1892 and was expanded by Frost's *Mechanostat Theory* (Frost, 1987). Frost (1987) proposed the existence of several mechanical thresholds that control or "switch on" bone (re)modelling producing bone adaptations (*e.g*., when bone is exposed to greater mechanical strains bone formation occurs and below a certain threshold bone is resorbed). Conversely, lack of loading due to, for example, physical disability, bed rest, or hypogravity environments, causes rapid bone loss (Blaber *et al.*, 2014; Nagaraja & Risin, 2013).

To determine the effect of mechanical forces on bone (re)modelling, it is important to understand the mechanisms underlying how the bone can respond to physical demands and adapt to its external environment. Advances in research have enhanced the study and knowledge of this process named mechanotransduction (Duncan & Turner, 1995; Jaalouk & Lammerding, 2009; Stewart *et al*., 2020; Yavropoulou & Yovos, 2016). However, the complex cellular processes and signalling pathways involved on this process are not yet fully understood and comprise the basis of current research.

2.6.1. Mechanotransduction

Mechanotransduction is the process by which mechanical forces are transformed into cellular interactions and biochemical signals (Jaalouk & Lammerding, 2009; Stewart *et al*., 2020). This is a complex process that can be considered in four distinct phases (**Figure 5**): phase 1, mechanical coupling (how mechanical loading causes deformation of bone cells); phase 2, biochemical coupling (how bone cell deformation is converted into intracellular signalling pathways); phase 3, signal transmission (how

the biochemical signal is transmitted from the sensor cell to the effector cell); and phase 4, effector response (how the effector cell's response to the signal leads to new bone formation).

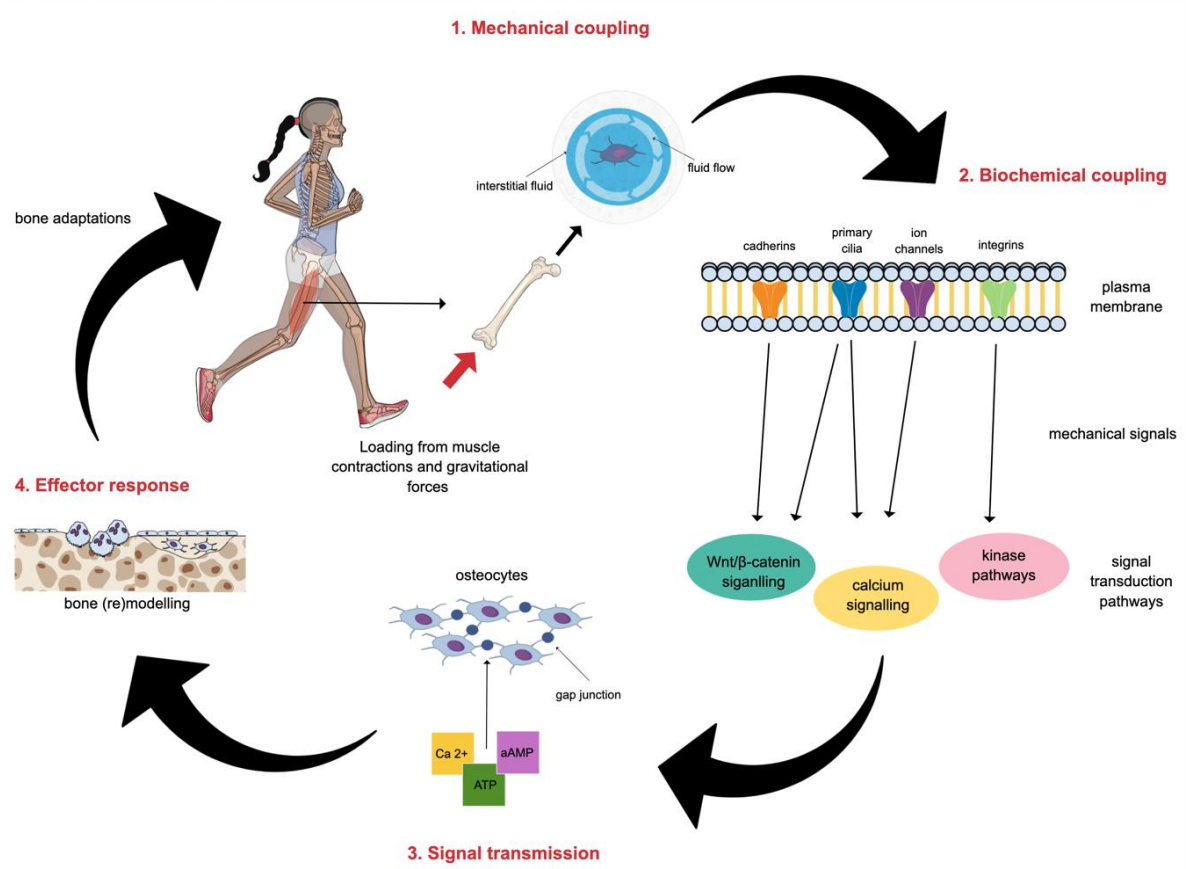


Figure 5. Diagram representing the mechanotransduction process phases: (1) mechanical coupling: mechanical loading causes deformation of bone cells; (2), biochemical coupling: bone cell deformation is converted into intracellular signalling pathways; (3) signal transmission: biochemical signals are transmitted from the sensor cells to the effector cells; and (4) effector response: the effector cells' response to the signal leads to bone remodelling and bone formation. Adapted from Stewart *et al.*, 2020.

2.6.1.1. Mechanical coupling

Bone cells, such as mesenchymal stem cells, osteoblasts, and osteocytes are tightly coupled to their extracellular environment and are mechanosensitive (Stewart *et al.*, 2020). Osteocytes have a key role in detecting mechanical loading and generating biochemical responses intracellularly. They are distributed throughout the entire bone and interconnected via the lacuno-canalicular network, which is surrounded by interstitial fluid. Bone loading stimuli result in the interstitial fluid being "squeezed"

over the osteocytes, and this fluid flow generates stress across the cell membrane (Rochefort, Pallu, & Benhamou, 2010; Yavropoulou & Yovos, 2016). Mechanical loading also activates osteoblasts and mesenchymal stem cells, which are bone formation cells, via different pathways (Stewart *et al*., 2020).

2.6.1.2. Biochemical coupling

The process by which bone cells detect changes to its external environment and subsequently generate a biochemical signal requires the detection of mechanical forces by mechanoreceptors in the cell membrane, which are capable of detecting alternations in external and internal forces (Yavropoulou & Yovos, 2016). Mechanosensors exist in nearly every cell type and are essential for the maintenance of many mechanically stressed tissues, such as muscle, bone, cartilage, and blood vessels (Jaalouk & Lammerding, 2009). Several mechanosensors have been identified as crucial in recognising mechanical forces acting upon bone cells, including integrins, ion channels, primary cilia, and cadherins (Stewart *et al*., 2020). The stimulation of these mechanosensors triggers intracellular signalling cascades, including protein kinase pathways, calcium signalling, and Wnt/β-catenin signalling (Goodman, Hornberger, & Robling, 2015; Stewart *et al*., 2020; Yavropoulou & Yovos, 2016). Once activated in the bone, these pathways have two essential endpoints: (i) the generation of osteogenic biochemical signalling molecules within the sensor cell, producing an autocrine biochemical response; and (ii) the generation of biochemical signalling molecules, which are subsequently released from the sensor cell to act on a target or effector cell (Jaalouk & Lammerding, 2009; Stewart *et al*., 2020).

2.6.1.3. Signal transmission

The transmission of biochemical signals between sensor and effector cells occurs through a number of mechanisms that can be classified as direct or indirect (Duncan & Turner, 1995; Schaffler *et al*., 2014; Stewart *et al*., 2020). Direct mechanisms include membrane-to-membrane contact between cells via gap junctions (communication channels that connect adjacent cells) (Schaffler *et al*., 2014; Stewart *et al*., 2020). Small ions such as calcium, adenosine triphosphate and cyclic adenosine monophosphate (cAMP) can be transported through gap junctions into adjacent cells and activate signalling pathways

in neighbouring cells (Genetos *et al*., 2007). Evidence shows that bone can behave similarly to a neural network through these gap junctions, by disseminating mechanical stimuli across the cellular network and amplifying the biochemical signal from the sensor cells (Stewart *et al*., 2020; Turner *et al*., 2002). The indirect mechanisms relate to the transmission of signalling factors from sensor cells to specific receptors on effector cells and inducing an osteogenic response, for example through paracrine signalling (Schaffler *et al*., 2014). Paracrine mediators include prostaglandin 2 (PGE2), prostacyclin and IGF-I (Stewart *et al*., 2020). Autocrine signalling has also a crucial role in mechanotransduction (Harter, Hruska, & Duncan, 1995). PGE2 has a role in both paracrine (by acting on osteoblasts to stimulate alkaline phosphatase and collagen synthesis) and autocrine (by increasing levels of calcium and cAMP intracellularly) signalling capacities (Duncan & Turner, 1995; Stewart *et al*., 2020).

2.6.1.4. Effector response

Following the action of osteogenic signalling factors, the cellular response ultimately manifests in a tissue response (*i.e*., new bone formation). It has been suggested that it typically takes 3-5 days following mechanical stimuli before bone formation occurs (Duncan & Turner, 1995), which may indicate the time required for the cascade of mechanotransduction processes described herein to take place. The response of bone to mechanical forces explains why loading through weight-bearing exercise can increase BMD (Kohrt, Barry, & Schwartz, 2009). Furthermore, loading factors, such as magnitude, frequency, and strain rate, affect the cellular response and are very important for determining bone (re)modelling responses and the adaptation of the skeleton to its external loading environment (Yavropoulou & Yovos, 2016).

2.6.2. Loading factors

The nature of the applied load on the skeleton is also important for subsequent bone adaptations. Not only the magnitude of the strains, but other factors such as the strain rate, strain distribution, strain frequency, number of loading cycles, and resting periods also influence bone responses to mechanical loading (Hart *et al*., 2017).

2.6.2.1. Stress and strain magnitudes

Bone receives stresses of different intensities from applied forces that generate strains (structural deformation) of varying magnitudes. The magnitudes of strain produced by muscular contractions and gravitational load are the centre of the *Mechanostat Theory* (Frost, 1987); whereby, if the strain magnitude exceeds the minimum effective strain threshold, bone formation and changes in bone architecture occur in order to meet mechanical demands (Ehrlich & Lanyon, 2002; Frost, 2003; Hsieh & Turner, 2001; Sugiyama, Yamaguchi, & Kawai, 2002). Strain magnitude, however, is not the only factor related to bone adaptation and is not always linked to bone formation (Ehrlich & Lanyon, 2002; Wallace *et al*., 2014).

2.6.2.2. Strain rate and distribution

Strain rate and strain distribution exemplify the temporal and spatial characteristics of produced strains (Hart *et al*., 2017). Human and animal studies have pointed to the influence of strain rate in bone formation independently of strain magnitude (Burr, Robling, & Turner, 2002; Judex & Zernicke, 2000; Mosley & Lanyon, 1998); with dynamic strains, rather than static, resulting in bone adaptations and osteogenesis (Rubin & Lanyon, 1985; Turner & Robling, 2003). Unusual and irregular strain distribution, influenced by the location, direction and gradient of strains, is also positively associated with osteogenesis (Hart *et al*., 2017).

2.6.2.3. Strain frequency

Strain frequency refers to the number of applied strain cycles per second and is expressed in hertz. The frequency of strain yield on bone has an effect on the osteogenic response, where increased loading frequency enables the stimulation of bone formation at lower strain magnitudes by reducing the minimum effective strain required for osteogenesis (Hart *et al*., 2017; Hsieh & Turner, 2001; Judex & Zernicke, 2000). The inverse relationship between strain magnitude and frequency (Cullen, Smith, & Akhter, 2000; Hsieh & Turner, 2001; Judex *et al*., 2003) explains the osteogenic effects of loading

regimens (or exercises) with different strain magnitudes. For example, both high impact exercise (high magnitude, low frequency strains) and whole-body vibration (low magnitude, high frequency strains) can produce beneficial bone adaptations (Judex *et al*., 2007; Judex & Zernicke, 2000; Marin-Puyalto *et al*., 2018; Ward *et al*., 2004).

2.6.2.4. Strain volume and loading cycles

Although many combinations of strain magnitude, rate, and frequency can cooperate to create an effective osteogenic stimulus, bone adaptation is not associated with strain volume (the strain combination duration for a given loading cycle). Specifically, increases in strain volume do not produce proportional increases in bone formation (Burr, Robling, & Turner, 2002; Robling, Castillo, & Turner, 2006). Further, the bone responsiveness to mechanical loading eventually declines, which indicates a suppression of mechanosensitivity. Animal studies have determined that ~95% of mechanosensitivity is reduced after only ~20 to 40 loading cycles, without an osteogenic benefit beyond ~100 loading cycles within equivalent strain environments (Burr, Robling, & Turner, 2002; Rubin & Lanyon, 1984; Umemura *et al*., 1997). The relationship between strain volume and mechanosensitivity is fluid because it is influenced by strain magnitude and frequency (Hart *et al.*, 2017). The existence of a saturation point beyond a given number of loading cycles has important implications for the design of mechanical loading and exercise regimens to improve bone health through loading-induced bone adaptations. In line with this, a study in young adult women showed significant improvements in bone mass after only 10 jumps a day three times a week for 6 months (Kato *et al*., 2006).

2.6.2.5. Resting periods

Rest periods are necessary following prior loading bouts in order to restore mechanosensitivity (Raab-Cullen *et al*., 1994; Umemura, Sogo, & Honda, 2002). The restoration of mechanosensitivity occurs rapidly, at least initially, until it reaches an inflection point, beyond which only osteogenic improvements follow (Robling, Burr, & Turner, 2001). Specifically, resting periods ranging from ~15 seconds to ~4 hours have been shown to increase bone formation outcomes by ~65% to 100%; whilst

there is no significant advantage beyond ~8 to 10 hours (Hart *et al.*, 2017). Furthermore, studies have determined that ~98% of mechanosensitivity is restored ~24 hours following a loading event (Robling, Castillo, & Turner, 2006). Therefore, the inclusion of rest periods between several loading bouts or sessions would potentially increase the anabolic responses of bone induced by mechanical loading.

2.7. Exercise and bone mass and strength

Physical activity and exercise have long been regarded as lifestyle factors that influence bone health (Weaver *et al*., 2016), given the positive relationship between mechanical loading and the skeleton described in the previous section. Weight-bearing exercise is generally considered to be beneficial for bone health and is associated with improvements in BMD, particularly at the load bearing sites (Bass *et al*., 2002; Hind & Burrows, 2007; Kelley, Kelley, & Kohrt, 2013; Kemmler *et al*., 2018; Marques, Mota, & Carvalho, 2012; Zhao, Zhao, & Xu, 2015). Improvements in bone strength, however, can occur without necessary increases in BMD, when bone modelling and remodelling adaptations to external loads produce changes in the shape and structure of bone (*e.g*., ellipticity, cortical thickness, periosteal and endosteal circumferences) (Ammann & Rizzoli, 2003; Cole & Van Der Meulen, 2011; Seeman & Delmas, 2006; Wang *et al*., 2009).

Changes in BMD and bone architecture, however, require months or years and are indicative of chronic bone adaptations. Hence, studies typically use imaging techniques, such as DXA and pQCT, to measure these changes in the mineral compartment of bone. Understanding the short-term (*e.g*., hours, day) physiology of bone adaptations requires the analysis of dynamic changes in bone (re)modelling, which has been investigated by using bone biochemical markers of bone resorption and formation, known as bone (re)modelling markers (Dolan *et al.*, 2020a), detailed in section 2.8. (page 43).

2.7.1. Exercise and bone adaptations across the lifespan

Evidence from cross-sectional and longitudinal studies indicate that weight-bearing exercise and participation in organised sport during childhood and adolescence generate significant osteogenic

adaptations (Bailey *et al*., 1999; Baxter-Jones *et al*., 2008; Behringer et al., 2014; Greene *et al*., 2012; Gunter, Almstedt, & Janz, 2012; Khan *et al*., 2000; Weidauer *et al*., 2012; Wang *et al*., 2009). Exercise-induced adaptations during growth, particularly during peripubertal development, are highly beneficial for maximising peak bone mass and bone strength by enhancing bone formation on the periosteal surface; and can translate to long-term advantages by slowing down future bone loss associated with ageing or the menopause (Wang *et al*., 2009; Warden *et al*., 2007; Weaver *et al*., 2016).

Meta-analyses studies indicate that exercise interventions involving weight-bearing activities, such as hopping and jumping and/or resistance training alone or as part of a multi-modal program, can improve bone health parameters in adult, pre- and post-menopausal (Kelley, Kelley, & Kohrt, 2013a; Kelley, Kelley, & Kohrt, 2013b; Zhao, Zhao, & Xu, 2015), and older (Marques, Mota, & Carvalho, 2012) populations. Although the effects are smaller than those shown in cross-sectional studies in younger or athletic populations, many trials have reported modest benefits (*i.e*., 1-3%) from exercise by preventing loss or promoting gains of BMD in adult (Beck *et al*., 2017) and older (Allison *et al*., 2013; Allison *et al*., 2015) populations. Exercise training can enhance bone strength independently of changes in BMD by adaptations in the bone structure and non-mineral compartment, although only a limited number of studies have examined the effects of exercise on bone structure and strength, with results being inconclusive (Polidoulis, Beyene, & Cheung, 2012). It is also unknown if greater exercise stimulus (*e.g*., high-load resistance exercise) may promote further enhancements to BMD in these populations (Beck *et al*., 2017). Weight-bearing exercise is recommended for the prevention and management of osteoporosis by different authorities (Beck *et al*., 2017; Weaver *et al*., 2016) and experts (Daly *et al*., 2019; Sinaki *et al*., 2010).

2.7.2. Bone adaptations to different exercise types

Whilst the best exercise regimen (*i.e*., type, intensity, duration, and frequency) to optimise bone responses is still not well defined, animal and human research suggests that exercise that conveys dynamic, high-impact, rapid, multi-directional movement patterns and unaccustomed loads, is likely to produce a strong osteogenic stimulus if adequate load intensity is achieved (O'Connor, Lanyon, &

MacFie, 1982; Lanyon & Rubin, 1984; Rubin & Lanyon, 1985; Turner & Robling, 2003). Further, short bouts separated by periods of rest are more effective because they allow restoration of mechanosensitivity (Raab-Cullen *et al*., 1994; Robling, Burr, & Turner, 2001; Umemura, Sogo, & Honda, 2002). Research in athletic populations in support of this evidence shows that individuals who participate in high-impact activities, such as gymnastics, volleyball, powerlifting, or racket and team sports usually present higher BMD than sedentary controls (Alfredson, Nordström, & Lorentzon, 1997; Fredericson *et al*., 2007; Tournis *et al*., 2010; Tsuzuku, Ikegami, & Yabe, 1998; Tveit *et al*., 2015) and/or athletes participating in low impact sports (Heinonen *et al*., 1995; Maïmoun *et al*., 2013; Taaffe *et al*., 1995).

For individuals who participate in weight-bearing endurance sports, such as running, and non-weight bearing sports, such as cycling and swimming, the evidence is not as clear (Scofield & Hecht, 2012). Endurance runners are reported to have greater BMD (total and/or regional) than controls (Duncan *et al*., 2002; Feldman *et al*., 2012; Kemmler *et al*., 2006; Stewart & Hannan, 2000; Wilks *et al*., 2009) and cyclists, swimmers and triathletes (Duncan *et al*., 2002; Rector *et al*., 2008; Scofield & Hecht, 2012; Stewart & Hannan, 2000). Some studies have reported that cyclists and swimmers have lower BMD compared to matched controls (Scofield & Hecht, 2012). When endurance running is compared to other weight-bearing, high-impact exercise, such sprinting, gymnastics, or team-sports, however, runners consistently show lower BMD (Duncan *et al*., 2002; Fredericson *et al*., 2007; Nichols *et al*., 2007; Taaffe *et al*., 1997). Some cross-sectional studies show that runners (Hind, Truscott, & Evans, 2006), particularly young females with menstrual irregularities (Pollock *et al*., 2010) or restrained dietary intakes (Barrack, Rauh, & Nichols, 2008), can present low BMD at the lumbar spine. Indeed, recent research has highlighted the site-specific effects of mechanical loading on the lower extremities compared to non-loading sites (*i.e*., lumbar spine) in runners (Herbert *et al*., 2021). Furthermore, endurance athletes (Bennell *et al*., 1996; Neidel *et al*., 2019; Scofield & Hecht, 2012), including runners (Barrack *et al*., 2014; Barrow & Saha, 1988; Hutson *et al*., 2021; James, Bates, & Ostering, 1978; Johnston *et al*., 2020; Kelsey *et al*., 2007; Milner *et al*., 2006) have been reported to be at high risk of suffering stress fractures injuries (as a result of cumulative sub-fracture threshold loading and potential

inadequate energy availability levels). In effect, bone health can be negatively affected in athletes engaging in sports that emphasise a lean physique or require high energy expenditures, where there is greater prevalence of low energy availability (Ackerman *et al*., 2011; Mountjoy *et al*., 2018).

2.8. Bone (re)modelling marker responses to exercise

2.8.1. Uses of bone (re)modelling markers

Bone (re)modelling markers are proteins, enzymes, or other molecules secreted by bone cells and by-products of bone formation or resorption that are released into the circulation and subsequently excreted in urine. During the last decade, the number of experimental studies using commercially available assays for the analysis of these markers has been increasing and several are commonly used in the research and clinical fields (**Table 4**). Other bone markers that are less common and their functions or utilities are the matter of some debate, and these include: DKK-1, sclerostin, OPG/RANKL ratio, hydroxyproline, hydroxylysine, bone sialoprotein, and cathepsin K. Although measuring bone markers in urine has a clear advantage from the sample collection standpoint compared to the invasive venepuncture associated with blood samples, urinary marker concentrations must be corrected for creatinine, which adds variability (Hlaing & Compston, 2014; Vasikaran *et al*., 2011) and it is not always appropriate in studies with exercise interventions given the effects of exercise on creatinine (Refsum & Strömme, 1974). Blood-derived bone (re)modelling markers, however, can also have variability and present important limitations that need to be taken into consideration when conducting research investigations and using them in clinical settings. Two blood bone (re)modelling markers have been designated by The International Osteoporosis Foundation as the reference markers for the assessment of bone (re)modelling, given their smaller biological variability, higher specificity to bone metabolism, and better utility for the prediction and monitoring of osteoporosis compared with the other markers (Vasikaran *et al*., 2011). These reference markers are N-terminal propeptide of type 1 procollagen (P1NP), as a marker of bone formation, and C-terminal telopeptide of type 1 collagen (β-CTX-1), as a markers of bone resorption.

Table 4. Commonly used bone (re)modelling markers. Adapted from Banfi *et al*., 2010; Dolan *et al*., 2020a; Hlaing & Compston, 2014; and Vasikaran *et al*., 2011.

Abbreviation	Biomarker full name	Biological material	Main function
Bone formation			
Bone-ALP	Bone specific alkaline phosphatase	Serum	Enzyme attached to the surface membrane of osteoblasts, which is a by-product of osteoblast activity and plays an important role in bone mineralisation.
P1CP	Carboxy or C-terminal propeptide of type 1 procollagen	Serum and EDTA plasma	By-product of type 1 collagen synthesis derived from its precursor procollagen, which contains carboxy-terminal extensions that are released into the bloodstream.
P1NP	Amino or N-terminal propeptide of type 1 procollagen	Serum and EDTA plasma	By-product of type 1 collagen synthesis derived from its precursor procollagen, which contains amino-terminal extensions that are released into the bloodstream.
Bone resorption			
Pyr	Pyridinoline	Serum and urine	Pyr is a molecule involved in the crosslinking of collagen peptides and released into the bloodstream as a result of the breakdown of collagen from various tissues.
Dpd	Deoxypiridinoline	Serum and urine	Ddp is a molecule involved in the crosslinking of collagen peptides and released into the bloodstream as a result of the breakdown of bone collagen.
ICTP	Carboxy-terminal crosslinking telopeptide of type 1 collagen	Serum	ICTP is secreted into the blood stream during osteoclastic hydrolysis of bone collagen, generated by matrix metalloproteinases
NTX-1	Amino-terminal crosslinking telopeptide of type 1 collagen	Serum, plasma, and urine	NTX-1 is fragmented from type 1 collagen by cathepsin-K during osteoclastic-mediated degradation of bone collagen.
CTX-1	Carboxy-terminal crosslinking telopeptide of type 1 collagen	Serum, EDTA plasma, and urine	CTX-1 is secreted by osteoclasts during the hydrolysation of bone collagen fibrils. The alpha aspartic acid converts to beta, forming β-CTX-1, as measured in blood. Urine CTX-1 can be measured as α-CTX-

			1 (non-isomerised) or β-CTX-1 (isomerised).
TRAP5b	Tartrate-resistant acid phosphatase (isoenzyme 5b)	Serum	Osteoclast-derived enzyme containing sialic acid. It may reflect osteoclast number instead of osteoclast activity.
Bone remodelling			
OC	Osteocalcin	Serum and urine	Non-collagenous protein secreted by osteoclasts. Total OC is comprised of active (undercarboxylated, not hydroxyapatite-binding) and inactive (carboxylated, hydroxyapatite-binding) osteocalcin. These two OC forms have different roles, Although OC has been habitually used as a bone formation marker, it seems that total OC rather indicates general bone metabolism (however, it is not a bone-specific indicator).

2.8.2. Limitations of bone (re)modelling markers

There are a number of issues that limit the utility of bone (re)modelling markers or at least the interpretations that can be made from their use, such as variability derived from pre-analytical and analytical factors (Hannon & Eastell, 2000; Hlaing & Compston, 2014; Seibel, 2000) and lack of specificity (Dolan *et al.*, 2020a; Vasikaran *et al.*, 2011). As such, the design and interpretation of research studies using these markers to investigate the bone responses to exercise should be done with caution and an understanding of their limitations.

2.8.2.1. Pre-analytical factors

These factors include biological uncontrollable and controllable sources of variability. Uncontrollable biological factors relating to the population in whom the blood or urine samples are collected include age, sex, menopausal status, pregnancy and lactation, oral contraceptives, disease (*e.g.*, thyroid disorders, diabetes, renal and liver diseases), drugs (*e.g.*, corticosteroids, hormonal replacement therapy,

anticonvulsants, heparin, gonadotropin-releasing hormone agonists, anabolic agents, antiresorptive agents), fractures, reduced mobility or immobility, geography, and ethnicity (Hannon & Eastell, 2000; Hlaing & Compston, 2014; Vasikaran *et al*., 2011). Controllable biological factors include exercise (acute effects, up to 72 h after strenuous exercise, and potentially chronic effects), menstrual cycle (*i.e*., small changes during the luteal phase), nutrition (*e.g*., calcium intake), fasting status (especially important for specific markers such as β-CTX-1), and seasonal (*i.e*., small decreases over winter months), and circadian (extremely important, particularly for bone resorption markers, which have greater variation in their circadian rhythms) factors (Hannon & Eastell, 2000; Hlaing & Compston, 2014; Vasikaran *et al*., 2011). The remaining controllable factors for consideration relate to how the sample is collected, handled, and stored. For example, some markers are sensitive to thermo-degradation, UV radiation, hemolysis, freeze-thaw cycles and freezer temperature (Hlaing & Compston, 2014; Seibel, 2000; Szulc *et al*., 2017).

Researchers should appreciate these uncontrollable factors and study designs should take into consideration controllable factors and standardise the sampling procedures to minimise variability (*e.g*., standardising diet and exercise prior to collecting samples, taking samples in a fasted state, controlling, replicating and reporting the time when the samples are collected, standardising and following best practices between the collection and analysis of samples). Recommendations for standardised procedures to reduce pre-analytical variability of controllable factors in β-CTX-1 and P1NP measurements have been made by the National Bone Health Alliance (Szulc *et al*., 2017).

2.8.2.2. Analytical factors

As chemical analytes, markers of bone (re)modelling have analytical sources of variability resulting from assay reproducibility factors (expressed as a coefficient of variation or CV), and intra-individual and inter-laboratory variations. Multiple manual and automated assays, such as RIA, ELISA, ECLIA, CLIA; exist for each marker. Each assay has a different analytical CV and established individual reference ranges, albeit not always appropriately developed and not always in a population-specific manner (Hu *et al*., 2013; Morris *et al*., 2017; Vasikaran *et al*., 2011). Because of the inter-assay and

inter-laboratory variability (Bauer *et al*., 2012; Schafer *et al*., 2010), comparing data between studies, and particularly between laboratories, can be problematic, including when using the reference markers β-CTX-1 and P1NP (Bhattoa *et al*., 2021; Jørgensen *et al.*, 2017).

Authorities and organisations, such as The International Osteoporosis Foundation, the International Federation of Clinical Chemistry and Laboratory Medicine, and the National Bone Health Alliance, have recognised the need for the appropriate characterisation of reference ranges of bone (re)modelling markers, as well as the standardisation of commercial assays and analytical methods for the measurement of bone (re)modelling markers, specifically β-CTX-1 and P1NP (Bauer *et al*., 2012; Morris *et al*., 2017; Vasikaran *et al*., 2011). There is a collaborative project between these authorities and commercial reagent manufacturers aiming to harmonise and standardise reference materials and measurement procedures for β-CTX-1 and P1NP blood analytes and enable them to be widely and interchangeably used in clinical and research settings (Bhattoa *et al*., 2021).

2.8.2.3. Specificity

Many of the bone markers reported are not, however, bone-specific, meaning that their measurement may not be indicative of changes in bone (re)modelling (Dolan *et al*., 2020a; Vasikaran *et al*., 2011) and careful interpretation is required. For example, some markers that are by-products of collagen metabolism (*e.g*., P1NP, Pyr, and ICTP) could be affected by the metabolism of collagen from other tissues. Others, such as OC (Lombardi *et al*., 2015), have extra-skeletal functions or are affected by other body functions. Furthermore, bone (re)modelling markers are systemic and do not indicate bone changes at a specific site of the skeleton, which is critically important for exercise research because bone responds to mechanical loads (*e.g*., from exercise) in a site-specific manner (Bass *et al*., 2002; Judex, Gross, & Zernicke, 1997; Kannus *et al*., 1994).

2.8.3. Interpreting bone (re)modelling markers responses to exercise

Bone (re)modelling markers are the only available *in vivo* technique to evaluate the immediate bone response to exercise (or other nutritional and pharmacological stimuli) in humans. Controlled, acute exercise interventions have been used to investigate these responses, but have used a variety of designs and protocols, and usually included other variables (*e.g.*, nutritional interventions), making it difficult to make general conclusions. Intensity, duration, and the type of the exercise seem important for eliciting a response in bone markers. For example, studies using low intensities and/or shorter exercise protocols do not generally show bone markers responses to interventions (Dolan *et al*., 2020a). Increases in β-CTX-1 have been reported after acute exercise interventions including treadmill running (Scott *et al.*, 2012a), cycling (Barry *et al*., 2011; Guillemant *et al*., 2004), and jumping (Rantalainen *et al*., 2009). Changes in P1NP are less consistent, with only a few studies reporting increases after a prolonged running bout (Scott *et al*., 2011; Scott *et al*., 2012) and football sessions (Bowtell *et al*., 2016). A recent systematic review and meta-analysis in this area (Dolan *et al*., 2022) concluded that a single exercise session produces very small effects in bone (re)modelling markers responses and that bone resorption markers, including CTX-1, are more responsive to acute exercise compared to bone formation markers. The largest increases in CTX-1 were reported within 2 hours post-exercise consisting of a bout of long-duration cycling (Dolan *et al*., 2022). Nonetheless, Dolan *et al*. (2022) noted that the results of their meta-analysis have low certainty and they identified high within-study variability due to lack of standardisation and lack of inclusion of non-exercise control groups.

Research investigating longer-term bone adaptations to exercise training using bone (re)modelling markers is inconclusive. Longitudinal exercise intervention studies in healthy adult populations have consistently reported increased resting levels of bone formation markers after prolonged exposure to exercise training (Adami *et al*., 2008; Alp, 2013; Ardawi, Rouzi, & Qari, 2012; Erickson & Vukovich, 2010; Fujimura *et al*., 1997; Hu *et al*., 2011; Kim *et al*., 2009; Kim *et al*., 2015; Lester *et al*., 2009; Lutz *et al.*, 2012; Tajima *et al*., 2000). In some studies, these changes were reported alongside improvements in BMD (Adami *et al*., 2008; Ardawi, Rouzi, & Qari, 2012; Kim *et al*., 2015; Lester *et al*., 2009). All types of exercise, including low impact (Alp, 2013; Kim *et al*., 2015), mixed training (Adami *et al*.,

2008; Ardawi, Rouzi, & Qari, 2012; Lester *et al*., 2009), resistance training (Fujimura *et al*., 1997; Hu *et al*., 2011; Tajima *et al*., 2000), and higher-impact activities (Erickson & Vukovich, 2010; Lutz *et al.*, 2012), have generated positive changes in bone formation markers (P1NP and/or bone-ALP). In terms of bone resorption markers, they appear to be less responsive to longer-term exercise training. Most studies reported no change following an exercise training program (for a review, please see Dolan *et al*., 2020a), although a few studies in older populations have reported decreases in bone resorption markers (Alp, 2013; Basat, Esmaeilzadeh, & Eskiyurt, 2013; Roghani *et al*., 2013). In contrast, cross-sectional studies in athletic populations reported that athletes involved in high-impact sports (*i.e*., gymnastics, football, decathlon) show increases in both bone resorption and formation markers (Courteix *et al*., 2007; Karlsson *et al*., 2003; Maïmoun *et al*., 2008), which may indicate greater bone turnover overall. Athletes participating in lower-impact sports (*i.e*., swimming, horse-racing, cycling) tend to show decreases in bone formation markers (Creighton *et al.*, 2001; Maïmoun *et al*., 2004) or increases in bone resorption markers (Dolan *et al*., 2012; Mcveigh *et al*., 2015), which would suggest a bone metabolic profile favouring bone loss. Other studies, however, have reported no differences in bone (re)modelling markers between controls and young gymnasts (Maïmoun *et al*., 2013; Tournis *et al*., 2010), or between controls and master speed/power athletes (Nowak *et al*., 2010), despite showing adaptations in BMD and/or bone structure. It is worth considering, however, that studies in this area have used heterogeneous study designs and populations, and a mixture of bone formation and resorption markers, with only some including both reference markers (β-CTX-1 and P1NP) (Adami *et al*., 2008; Ardawi, Rouzi, & Qari, 2012; Lutz *et al.*, 2012; Maïmoun *et al*., 2013; Tournis *et al*., 2010).

2.8.4. The future of bone (re)modelling markers

Knowledge in this area is based upon studies with various study designs (*e.g*., populations and exercise interventions) and different levels of standardisation and control, principally around the choice of markers and the collection of samples, which adds uncertainty when drawing conclusions from collective data (for a review, please see Dolan *et al*., 2020a). Although research shows that bone (re)modelling markers respond to both acute and chronic exercise stimuli, there are many gaps in the understanding of the external factors and mechanistic pathways that regulate bone responses to different

types of exercise. Meta-analytic evidence (Dolan *et al*., 2022) indicates, however, that acute responses are small, time-limited, and influenced by the characteristics of the exercise bout; and therefore, make it difficult to extract conclusions from bone (re)modelling markers that will help understanding the short-term bone metabolic adaptations to exercise. While some longer-term adaptations to exercise training (*i.e*., increases in bone formation markers) agree with the concept of the bone remodelling cycle (section 2.5., page 23), bone markers data do not support chronic adaptations in the mineral compartment, such as BMD and bone architecture, and the generally accepted idea that high-intensity, high-impact activities/sports are required to induce an osteogenic response.

These observations may indicate that bone (re)modelling markers are not necessarily predictive of changes in bone mass and geometry and that, although these questions remain unanswered, bone markers may not be able answer them because they do not allow the measurement of adaptations at specific bone sites. Furthermore, the effects that weight-bearing (*e.g*., running) and non-weight bearing (*e.g*., cycling) endurance exercise with repetitive load cycles elicit on bone metabolism are not clear, and factors other than external mechanical loading, such as nutrition and energy availability, exercise intensity and duration, and resting status influence these effects.

Dolan *et al*. (2020a) emphasised the importance of interpreting bone (re)modelling biomarkers beyond increases, decreases, or no changes in isolated markers, and instead consider the context and magnitude of the observed changes within the complexity of the bone (re)modelling process. The challenges for future research include the definition of the physiological relevance of the changes and responses of these biomarkers to interventions, and their role and practical application in contributing towards the development of strategies to protect or enhance bone health, including their predictive ability and relationship with bone mineral chronical adaptations. Appropriate and harmonised use of bone (re)modelling markers (Bauer *et al*., 2012; Bhattoa *et al*., 2021; Vasikaran *et al*., 2011) and meta-analytic research that includes assessments of the quality of studies (Dolan *et al*., 2022) are steps in the right direction to appreciate the full potential of these biomarkers.

2.9. Stable isotope tracer techniques

In contrast to the indirect and limited measures of bone (re)modelling markers, direct and site-specific measurements of bone metabolism (*i.e.*, formation) can be conducted by methods using stable isotopically labelled tracers. Since their discovery and application in the 1930s, stable isotope tracer techniques have been used by scientists to study animal and human metabolism. The use of tracers has also been implemented in other areas such as forensics, geology, art, and archaeology. Novel methodologies using stable tracers have been widely developed over the past decades alongside improvements in mass spectrometry technology (the equipment used to measure stable isotopes). These advances have increased the sensitivity of stable isotope measurements and have made it possible to accurately detect changes in metabolism from the whole body, at a tissue-specific level, and even at an individual protein level (Rennie, 1999; Wilkinson, 2018), making stable isotope tracer techniques powerful and useful tools in physiology and metabolism research.

2.9.1. Measurement of stable isotopes

Stable isotopes are types of atoms of a chemical element. Different isotopes of the same element can be common, stable, or radioactive isotopes, but all have the same chemical characteristics and are functionally identical (*i.e.*, are in the same position in the periodic table). Stable isotopes have the same number of protons and electrons but differ in mass because they have one or more additional neutrons in the atomic nucleus than the common isotopes. For example, for the ^{12}Carbon (^{12}C), ^{14}Nitrogen (^{14}N), ^{16}Oxygen (^{16}O), and ^{1}Hydrogen (^{1}H) common isotopes, their heavier stable isotopic equivalents are ^{13}C, ^{15}N, ^{18}O, and ^{2}H or deuterium (D). Although stable isotopes are naturally occurring, they are significantly less abundant than common isotopes.

Stable isotopes are considered to behave identically to their more common equivalents and, therefore, they can be incorporated into organic compounds (*e.g.*, amino acids, fatty acids, glucose) and used within biological systems as “labelled” compounds. Once introduced into a system, the metabolic fate of the labelled compounds can be “traced” and detected (because of their mass difference). Therefore,

using stable isotopes as tracers can provide dynamic measures of the metabolism of a biological system or a combination of systems *in vivo*. Contrary to radio-isotope tracers (such as ^{14}C or ^{3}H) stable isotopes are non-radioactive, which makes them ideal to study human metabolism because they are generally very safe, and the amounts used are small and do not affect normal metabolic processes (Wilkinson *et al*., 2017).

Their difference in mass (extra neutrons) allows stable isotopes to be detected using mass spectrometry analytical techniques, such as gas (GC) and liquid (LC) chromatography. Mass spectrometer (MS) machines can separate whole molecules or atoms, based upon their mass and/or charge. Molecules/atoms undergo ionisation as they pass through a magnetic and/or electrical field under vacuum, a process that separates them into ions. Ions of different mass/charge hit the detector at different velocities or trajectories, which makes them distinguishable. GC-MS and LC-MS instrumentation has improved significantly in accuracy and precision from early designs, especially by the introduction of tandem mass spectrometry (MS/MS) (Wilkinson, 2018). Besides, isotopic abundance detection levels can be improved from ~0.1 atom per cent excess (APE), which is equivalent to having 0.1% more heavy isotopes than normal in the system, to ~0.0005% when GC/LC is combined with combustion or pyrolysis isotope ratio mass spectrometry (GC-combustion-IRMS and GC-pyrolysis-IRMS) (Wilkinson, 2018). This increase in sensitivity is particularly important for the measurement of metabolic pools that turnover at slow rates (*e.g*., musculoskeletal tissues) (Brook & Wilkinson, 2020). The choice of the tracer and study design are usually dictated by the analytical sensitivity of the available mass spectrometry equipment (Brook & Wilkinson, 2020). For example, advances in GC-pyrolysis-IRMS included novel designs that allowed good separation and precision measurements of ^{2}H using He as the carrier gas (Begley & Scrimgeour, 1996), which has, in turn, influenced the widespread use of deuterated stable isotopes, such as deuterium oxide.

Stable isotope tracers are typically substrate specific, although there are some exceptions (see section 2.9.3.). As such, stable isotopically labelled amino acids are used to study amino acid and protein metabolism, labelled fatty acids are used on fat metabolism, and labelled glucose is used on

carbohydrate metabolism and storage. These tracers have been, and are, used across a myriad of areas in the biological sciences and have led to important discoveries. In the exercise physiology field, *in vivo* studies using stable isotope tracer techniques have been key to investigate substrate utilisation and oxidation (Friedlander *et al*., 2007; Phillips *et al*., 1996; Romijn *et al*., 1993), energy expenditure (Hunter *et al*., 2000; Hunter *et al*., 2015; Plasqui *et al*., 2019; Sjödin *et al*., 1994; Stein *et al*., 1987), and metabolic responses of musculoskeletal tissues to exercise (Biolo *et al*., 1995; Burd *et al*., 2011; Kumar *et al*., 2009a; Miller *et al*., 2005; Miller *et al*., 2007; Pennings *et al*., 2011; Phillips *et al*., 1999). Stable isotope tracers are considered a useful and informative tool in contemporary research and they will likely underpin future advances in metabolic research and maintain their relevance within biological sciences (Wilkinson, 2018).

2.9.2. Musculoskeletal protein synthesis and stable isotope tracers

Since the start of the 21st century, the regulation of musculoskeletal tissues, specifically skeletal muscle, has been an essential part of research in the areas of metabolism and exercise physiology. Stable isotopes have been a fundamental tool for providing information on musculoskeletal metabolism and the responses of these tissues to exercise and nutrition (Wilkinson, 2018; Wilkinson *et al*., 2017), with this research being vital to understand and find strategies to mitigate the effects of ageing, disease, and disuse in the musculoskeletal system.

Stable isotopically labelled amino acid tracers, predominantly [1,2–$^{13}C_2$]-leucine or [ring–$^{13}C_6$]-phenylalanine, are commonly used to investigate whole body and muscle protein metabolism *in vivo*. Amino acid tracers are typically administered intravenously with a continuous infusion over a time period (hours) or a flooding dose of highly labelled tracer (Wilkinson, 2018; Wilkinson, Brook, & Smith, 2021). Using amino acid tracers, protein metabolism can be measured indirectly by calculating arteriovenous balance and with direct incorporation methods, which require access to tissue sampling (*e.g*., via surgical muscle biopsies) (Brook & Wilkinson, 2020). Once inside the body, labelled amino acids enter intracellular amino acid pools of muscle cells, where they are incorporated into newly

synthesised proteins within the muscle. By obtaining a sample of muscle tissue the amount of incorporated labelled amino acids over time can be measured, which, together with the measurement of the tracer enrichment in the precursor pool (*i.e.*, the amino-acyl-tRNA) or a proxy surrogate (*e.g.*, intracellular amino acid pool), enable the determination of fractional synthesis rates (FSRs) (Rennie *et al.*, 1982; Wilkinson, 2018). Direct incorporation methods, also named precursor-product approaches, estimating FSRs are considered the current gold standard for studying muscle protein synthesis (Wilkinson, 2018). Importantly, muscle tissue consists of different types of protein fractions (*i.e.*, myofibrillar, collagen, and mitochondrial), which can be isolated when measuring protein synthesis. Indeed, reporting protein synthesis determined specifically for one, or more of these fractions, is preferable (Brook & Wilkinson, 2020).

The use of stable isotope amino acid tracer techniques in combination with the precursor-product approach has provided the foundations of the current understanding of skeletal muscle protein metabolism, including the dynamic balance between muscle protein synthesis and muscle protein breakdown (Atherton & Smith, 2012; Kumar *et al.*, 2009a) the key role of nutrition (particularly protein and leucine ingestion) on regulating muscle protein synthesis (Rennie *et al.*, 1982; Smith *et al.*, 1998; Tang *et al.*, 2009; Witard *et al.*, 2014), the effects of resistance exercise and the posterior enhanced "anabolic window" of muscle protein synthesis (Biolo *et al.*, 1997; Bukhari *et al.*, 2015; Burd *et al.*, 2010; Miller *et al.*, 2005; Wilkinson *et al.*, 2018), or the anabolic resistance associated to ageing (Cuthbertson *et al.*, 2005; Guillet *et al.*, 2004; Kumar *et al.*, 2009b; Smith et al., 2012; Volpi *et al.*, 2000). Several studies have used stable isotopes to determine rates of synthesis of human tendon collagen *in vivo* (Babraj *et al.*, 2005a; Dideriksen *et al.*, 2017; Doessing *et al.*, 2010; Hansen *et al.*, 2009; Hansen *et al.*, 2013; Miller *et al,*. 2005; Miller *et al.*, 2007; Smeets *et al.*, 2019), using continuous infusions and/or flooding doses of typically [^{15}N]-proline, [1–^{13}C]-proline, [^{13}C]-leucine, or [ring- $^{13}C_6$]-phenylalanine. A smaller number of studies measured synthesis rates of ligament (Babraj *et al.*, 2005b) and bone (Babraj *et al.*, 2005a; Scrimgeour *et al.*, 1993) collagen, using [^{15}N]- and [^{13}C]-labelled proline and alanine, and [ring- $^{13}C_6$]-labelled phenylalanine. The increased difficulty of obtaining samples from these tissues, particularly bone, is probably the reason why there is such limited information about the

dynamic changes of these tissues. More recently, a new study collected samples from muscle, tendon, ligament, cartilage, and bone in individuals undergoing knee elective replacement surgery and compared synthesis rates across tissues, showing slower synthesis rates at the femoral and patellar bone compared to the *vastus lateralis* muscle (Smeets *et al*., 2019).

Whilst amino acid stable isotope tracers provide vital information in musculoskeletal research, they have several limitations. As such, the requirement of intravenous infusions via cannulation creates a time restriction (generally <24 h) and the need for a controlled environment (Brook & Wilkinson, 2020). This time restriction makes it challenging to accurately measure synthesis rates of slow turnover proteins, such as bone collagen (Brook & Wilkinson, 2020; Wilkinson, Brook, & Smith, 2021). Moreover, short-term muscle protein synthesis may not compare to long-term changes in muscle mass and function (Atherton *et al*., 2015; Mitchell *et al*., 2014). All of these issues highlight the relevance and benefits of approaches that would allow longer-term measures of musculoskeletal protein synthesis (Dufner & Previs, 2003; Wilkinson, Brook, & Smith, 2021). In fact, after the newest advances in analytical instrumentation (*i.e*., GC-pyrolysis-IRMS) permitting the high-precision measurement of hydrogen and oxygen stable isotopes (Hilkert *et al*., 1999; Ripoche *et al*., 2006), a new wave of research using deuterium oxide (^2H_2O or D_2O) tracers in this field has emerged since the early 2000s.

2.9.3. Deuterium oxide tracer

Deuterium oxide (^2H_2O), also commonly known as D_2O or *heavy water*, is a stable isotope tracer that was first used in the late 1930s, with its benefits and applications being more fully recognised around the mid 1990s following advances in analytical instrumentation (*i.e*., gas chromatography–pyrolysis–isotope ratio mass spectrometry or GC-pyrolysis-IRMS, and liquid chromatography–tandem mass spectrometry or LC-MS/MS) (Wilkinson *et al*., 2017).

D_2O tracers can overcome some of the common limitations related to the use of substrate-specific stable isotope tracers mentioned above. D_2O can be easily administered by drinking *heavy water* as a single

bolus (Macdonald *et al*., 2013; Wilkinson *et al*., 2014; Wilkinson *et al*., 2015) or in the form of regular doses (Brook *et al*., 2015; Decaris *et al*., 2015; Robinson *et al*., 2011), with the deuterium becoming rapidly equilibrated within the body water (Dufner & Previs, 2003). This allows the D_2O tracer to be administered and maintained outside the controlled laboratory setting, in free-living conditions with minimal interference to an individual's normal daily activities. Because of the relative slow turnover of the body, the enrichment in the precursor pool is easily maintained over weeks and months (Brook *et al*., 2015; Robinson *et al*., 2011; Wilkinson *et al*., 2014; Wilkinson *et al*., 2015), and hence allowing the measurement of chronic and cumulative metabolic rates. Furthermore, unlike with substrate-specific tracers, deuterium can be incorporated into different substrates (*e.g*., fatty acids, glucose, amino acids, nucleotides, metabolites) at stable C-H positions during *de novo* synthesis. As such, a single D_2O tracer can be used to monitor the metabolism of lipids, glucose, proteins, DNA/RNA, and other systems simultaneously (Brook *et al*., 2017; Dufner & Previs, 2003) (**Figure 6**).

The investigation of lipid metabolism has underpinned the application of D_2O tracer methods since the 1980s (for a review see Wilkinson, 2018), demonstrating its utility for measuring lipogenesis, triglyceride synthesis, and cholesterol metabolism (Castro-Perez *et al*., 2011; Diraison, Pachiaudi, & Beylot, 1997; Strawford *et al*., 2004; Turner *et al*., 2003). In protein metabolism, although D_2O was first used for the measurement of murine protein turnover by Hans Ussing in 1941 (Ussing, 1941), the interest and application of this tracer in *in vivo* human research has been established only in recent decades (Previs *et al*., 2004; Wilkinson, Brook, & Smith, 2021). In only a few years, D_2O tracer methods have been validated against gold standard measurements of human muscle protein synthesis using [$^{13}C_6$]phenylalanine (Wilkinson *et al*., 2014; Wilkinson *et al*., 2015) and these approaches have been widely applied in the human muscle metabolism research field. Most amino acids can be labelled by deuterium at their alpha C positions, although the non-essential amino acids alanine, glycine, glutamate, and glutamine can be labelled at multiple C positions (Busch *et al*., 2006; Herath *et al*., 2011). Therefore, non-essential amino acids, particularly alanine, are widely used in studies in this field. By isolating the protein of interest and measuring the amount of deuterium incorporated at the C positions in the protein-bound amino acids, the FSR of protein can be quantified (Dufner & Previs, 2003). The

amount of deuterium labelling is usually low because of the slow turnover rate of the majority of protein pools. The level of deuterium incorporation depends on how much and for how long the D_2O tracer is provided for the enrichment of the body water pool, which in humans is typically restricted to <2% (Wilkinson, 2018). For this reason, the improvement in instrumentation sensitivity is specially important for the measurement of slow metabolic pools, such as musculoskeletal tissues, and slow turnover proteins, such as collagen, and nucleotides (Wilkinson, Brook, & Smith, 2021).

A number of studies have shown the applicability of D_2O tracer techniques in a range of interventions (*e.g.*, nutrition, exercise, drugs, immobilisation) measuring human skeletal muscle protein synthesis during short (1-8 days) (Damas *et al*., 2016; Kilroe *et al*., 2020; Wilkinson *et al*., 2014) and longer (3-6 weeks) (Brook *et al*., 2015; Franchi *et al*., 2015; Groennebaek *et al*., 2018; Sieljacks *et al*., 2019) periods, and in different populations (*e.g*., across sexes and age groups) (Bell *et al*., 2015, Brook *et al*., 2016; Gharahdaghi *et al*., 2019; Murphy *et al*., 2016; Scalzo *et al*., 2014). Studies on protein synthesis in rodent muscle (Gasier *et al*., 2009; Kobak *et al*., 2021; Langer *et al*., 2018), bone (Busch *et al*., 2006; Cross *et al*., 2020; Do *et al*., 2006; Jeong *et al*., 2005) and other tissues/pools (Busch *et al*., 2006; Cross *et al*., 2020; Dufner *et al*., 2005) have also developed methods using D_2O tracers. Collectively, these studies continue to contribute towards the knowledge and understanding of musculoskeletal protein metabolism. In Chapter 4 (page 93), studies that have used D_2O for the measurement of bone synthesis are discussed further.

There is a growing interest in the application of D_2O tracers to measure slower turning over pools, such as dynamic nucleotide metabolism to quantify DNA and RNA turnover (Busch *et al*., 2007; Voogt *et al*., 2007) and, since the 2010s, researchers have used D_2O to track the synthesis of DNA (Robinson *et al*., 2011) and RNA (Brook *et al*., 2017). Furthemore, studies combining D_2O with novel proteomics techniques have been able to measure the turnover of individual proteins (Price *et al*., 2012; Shankaran *et al*., 2016), and combining D_2O with metabolomics has the potential to provide rates of flux through different individual pathways simultaneously (fluxomics) (Wilkinson *et al*., 2017; Wilkinson, 2018).

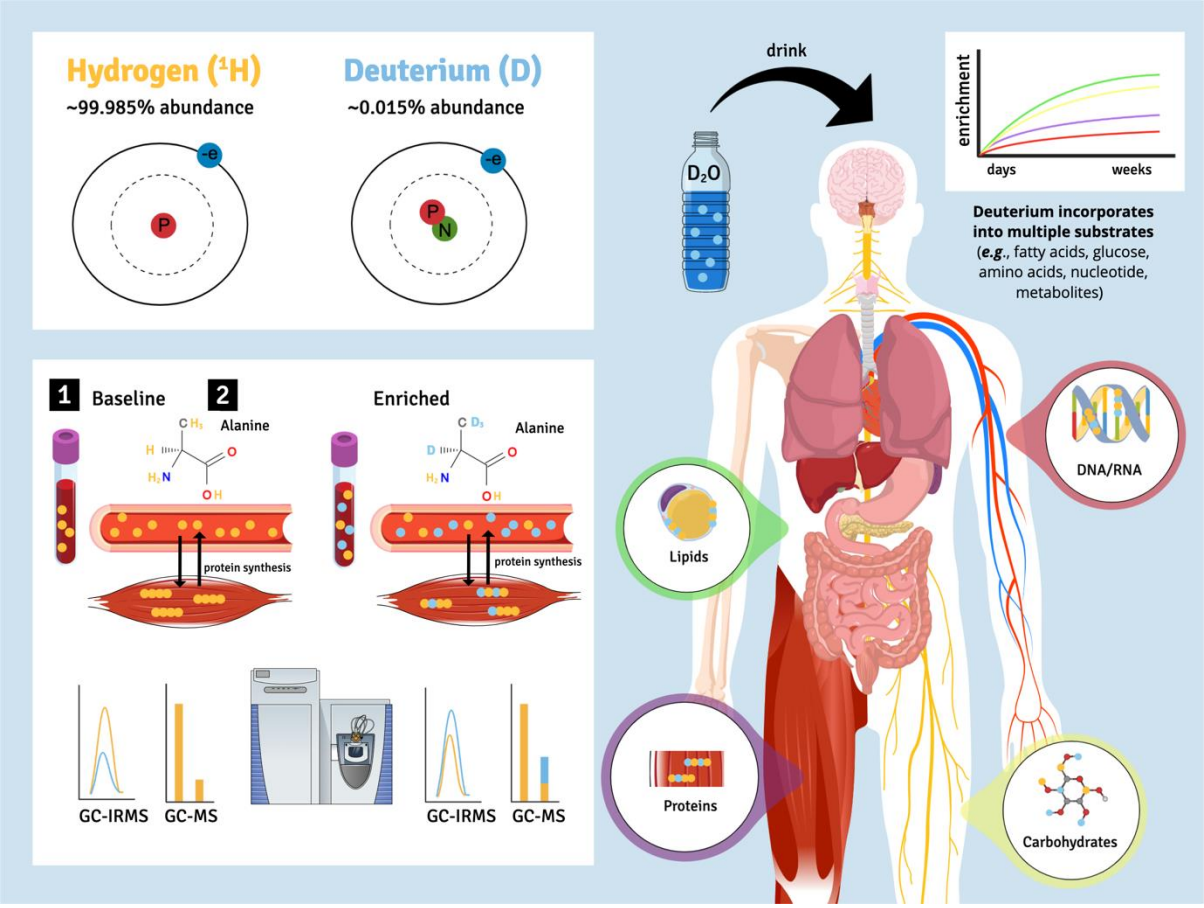


Figure 6. Schematic diagram representing the chemical structure of Hydrogen (^{1}H), in yellow, and Deuterium (^{2}H or D), in blue, highlighting their differences in mass and relative abundance (top left corner); the incorporation of deuterium, upon oral administration of deuterium oxide (D_2O) tracer, into multiple biological substrates in the body such as fatty acids (green), glucose (yellow), amino acids (purple), and nucleotides (red), enabling the measurement of protein, carbohydrate, lipid, and DNA/RNA metabolism (right side); and an example of how the rate of synthesis of new protein can be analysed by collecting a blood and a muscle sample before (baseline) and after (enriched) ingesting the D_2O tracer, using mass spectrometry techniques such as GC-MS (measuring the molecular mass and the abundance of that molecule containing heavier, labelled isotopes, such as deuterium) and GC-IRMS (combusting the whole molecule and measuring if the ratio between the lighter (hydrogen) and heavier (deuterium) isotopes is increased more than it occurs naturally, with the resulting difference representing the incorporation of heavier isotopes (deuterium) over a specific timeframe (bottom left). Adapted from Brook *et al.*, 2017 and Wilkinson *et al.*, 2017.

2.10. Summary of the literature review

This review focused upon key areas of the literature that provide relevant context to this programme of work. It was established that non-modifiable and lifestyle factors affect bone health across the lifespan and highlighted the importance of strategies to improve/maintain bone mass and strength. The dynamic and mechanosensitive characteristics and the heterogenous and complex structure of bone tissue were

described. It was recognised that weight-bearing exercise is generally osteogenic, which has been predominantly determined by long-term bone adaptations of the mineral compartment of bone; and that the understanding of the short-term bone responses to exercise is limited and nuanced, and it is restricted by the current methodological approaches (*i.e.*, bone (re)modelling markers). Several unanswered questions, gaps in knowledge, and emerging areas of research were identified in this review and will be covered in the studies described herein. These gaps include the short- and long-term effects of endurance running (which entails a repetitive and cyclic loading pattern) on bone, the acute responses of bone (re)modelling markers to a bout of exercise, and the developing utility of stable isotope tracer techniques to study the short-term (days/weeks) physiology of bone.

CHAPTER 3:

P1NP AND β-CTX-1 RESPONSES TO A PROLONGED, CONTINUOUS RUNNING BOUT IN HEALTHY ADULT MALES: A SYSTEMATIC REVIEW AND INDIVIDUAL PARTICIPANT DATA META-ANALYSIS

3.1. Introduction

As described in Chapter 2, section 2.7. (page 40), weight-bearing exercise is generally considered to be beneficial for bone health and is associated with improvements in bone mineral density (BMD) and bone architecture, particularly at load bearing sites (Hind & Burrows, 2007; Kelley, Kelley, & Kohrt, 2013; Marques, Mota, & Carvalho, 2012; Santos, Elliott-Sale, & Sale, 2017). In contrast to longitudinal and cross-sectional studies examining chronic bone adaptations, investigating the short-term (*e.g.*, hours, days, weeks) physiology of bone and how a single bout of exercise affects bone (re)modelling is key to understanding the specific mechanisms by which bone responds to exercise. In turn, this understanding would contribute towards determining the most effective regimen of exercise – type, duration, frequency – for improving bone health. Dynamic and acute changes in bone (re)modelling can be investigated by using biochemical markers of bone formation and resorption. The established reference marker for bone formation is N-terminal propeptide of type 1 procollagen (P1NP) and the reference marker for bone resorption is C-terminal telopeptide of type 1 collagen (β-CTX-1) (Vasikaran *et al*., 2011). Please see section 2.8. (page 43) for more details on bone (re)modelling markers.

The effects of prolonged, continuous running exercise on bone (re)modelling markers are interesting because, although running produces greater gravitational loading compared to other low-impact activities, such as cycling (Rector *et al*., 2008), it also has a repetitive loading cycle and has been associated with a relatively high prevalence of stress fracture injury (Arendt *et al*., 2003; Scofield & Hecht, 2012). Low BMD is prevalent in endurance runners, particularly at non-loaded sites (Hind, Truscott, & Evans, 2006), where the beneficial effects of mechanical loading do not counteract the potential negative influences associated with endurance exercise (Scofield & Hecht, 2012). Examining the bone (re)modelling responses to running exercise bouts are a logical approach to further investigate the effects of this common type of exercise on bone, although almost all studies that have measured bone (re)modelling markers before and after a running bout were not designed to directly answer this question. Instead, most studies investigated bone (re)modelling responses to acute running under different conditions (*e.g.,* nutrition interventions, different exercise intensities or training status of

participants) and, therefore, did not include a comparison to a non-exercise control group. Some observations from the data reported in the available studies, however, can be made.

The bone resorption marker, β-CTX-1, concentrations decreased from immediately to 4 hours following a ≥60 min running bout at intensities ranging from 55 to 75% VO_{2max} (Sale *et al*., 2015; Scott *et al*., 2011; Townsend *et al*., 2017). Feeding before (Scott *et al*., 2012) and feeding after (Townsend *et al*., 2017) a running bout showed an amplifying effect on the decline of these concentrations. In most studies, there are no evident changes in β-CTX-1 levels for the 1-4 days after a running bout (Sale *et al*., 2015; Scott *et al*., 2011; Scott *et al.*, 2012; Townsend *et al*., 2017), although one study showed increased levels of β-CTX-1 over the 1-4 days after a running bout compared to a non-exercise control group (Scott *et al.*, 2010). Responses appear to be more consistent across studies with respect to the bone formation marker, P1NP. Increased P1NP levels compared to baseline have been shown during and immediately after a ≥60 min running bout at intensities ranging from 55 to 75% VO_{2max} (Sale *et al*., 2015; Scott *et al*., 2011; Scott *et al.*, 2012; Townsend *et al*., 2017), returning to baseline levels 1-2 hours post-exercise, although nutritional interventions might elevate P1NP again at 3-4 h post exercise (Townsend *et al*., 2017). Only in one of these studies P1NP elevations were sustained over the 1-4 days after running (Sale *et al*., 2015). The differences in exercise intensity and feeding/nutrition status among these studies needs further consideration and may explain some of the inconsistencies across studies. With the information from single studies, usually without including a control (non-exercise) group, it is difficult to establish if inter-individual variability or circadian rhythms have affected the results.

Better understanding of the underlying factors responsible for the inter-individual variability and the discrepancies across studies in the bone (re)modelling marker responses to prolonged running can help to improve study designs and evaluate the robustness of exercise interventions. There are several methods that have been proposed for describing and quantifying inter-individual variation (Atkinson & Batterham, 2015; Hecksteden *et al*., 2015; Swinton *et al*., 2018). These methods are generally based upon the modelling of three sources of variation in observed changes in scores across an intervention, including measurement error, biological variability, and intervention response variation. Measurement

error refers to stochastic fluctuation of observed measurements around a hypothetical true underlying value and is comprised of instrumentation noise (*e.g.*, derived from assays used for the measurement of bone (re)modelling markers) and biological noise (*e.g.*, caused by factors such as markers' circadian rhythms, nutrition intake, sleep, or resting status). The estimation of the typical variation in observed scores derived from measurement or biological error can be quantified through the variation in scores in control conditions (by adding a control group). For example, when investigating the responses of bone (re)modelling markers to an exercise intervention, the estimation of typical variation would allow quantification of the degree to which the observed changes were affected by factors external to the intervention itself, such as measurement error caused by laboratory assays or circadian rhythms within the markers. Quantifying these factors using non-exercise control groups, can allow estimation of the degree to which the intervention itself may contribute toward the observed variation.

Currently, the degree to which variation in observed changes in bone (re)modelling markers after exercise are caused by different variation sources and how much inter-individual variability exists is not known. Whilst obtaining accurate estimates of these variability assessments is difficult for single studies, meta-analytic approaches can counteract limitations of small effects, noisy measurement outcomes and small sample sizes. Individual participant data (IPD) meta-analyses, however, aside from providing even better estimates of mean responses, also allow for the assessment of effects at the participant level by using the raw data from selected studies (Riley, Lambert, & Abo-Zaid, 2010; Stewart *et al*., 2015), and, thus, can determine inter-individual variation and provide a more accurate estimate of the mean response.

The aims of this study were to (i) evaluate the mean responses of P1NP and β-CTX-1 after a prolonged, continuous running exercise bout in healthy adult males, (ii) estimate the inter-individual variability in bone (re)modelling marker responses and (iii) determine to what degree any inter-individual variability was caused by factors directly related to the prolonged, continuous running bout (herein termed the intervention response), such as timing relative to the exercise.

3.2. Methods

The present study forms a separate arm of a continuing body of work completed collaboratively between Universidade de São Paulo (Eimear Dolan), Robert Gordon University (Paul Swinton), Nottingham Trent University (Rita Civil, Craig Sale, Kirsty Elliott-Sale, Ian Varley, and Lívia Santos), and University of Nottingham (Philip Atherton). The initial body of work included two systematic reviews and meta-analyses that investigated the (a) acute and (b) chronic effects of exercise on bone (re)modelling markers. The study reported herein was designed by RC to investigate a small section, within the first part (a) of this body of work, in more detail.

The protocol for this review included all items described in the checklist of Preferred Reporting Items for Systematic Review and Meta-Analysis of Individual Participant Data (PRISMA-IPD) (Page *et al*., 2021; Stewart *et al*., 2015). The protocol for this review was pre-registered on the Open Science Framework before analysis (https://osf.io/y69nd).

3.2.1. Eligibility Criteria

The PICOS (Population, Intervention, Comparator, Outcomes and Study Design) approach was used to guide the determination of eligibility criteria for this study.

3.2.1.1. Population

Studies that included young (18-35 years old), healthy (*i.e*., non-smokers, injury free and not taking medication from any condition known to affect bone metabolism), active males were considered for inclusion. Differences in training status are unlikely to influence the responses of bone (re)modelling markers after a running exercise bout (Scott *et al*., 2010) and, therefore, participants included healthy males who were active (*i.e*., recreationally) or endurance trained (*e.g*., runners, triathletes). Only male participants were included because most studies in this area have focussed upon young, healthy, adult male populations. Studies in healthy active females are lacking on this topic and this disparity is considered in the discussion section.

3.2.1.2. Intervention

The term 'intervention' in this IPD meta-analysis was taken to mean a prolonged, continuous running bout, regardless of whether or not this was the focus of the original studies from which the data were extracted. Studies were considered for inclusion if they included blood sample collections and analyses of P1NP and β-CTX-1 markers at baseline, before, during, and after prolonged, continuous treadmill running at an intensity of ≥65% VO_{2max} (or equivalent) and with a duration of 60-120 min.

This study focused on one type of exercise, running, which is a weight-bearing impact exercise with a repetitive loading cycle. Therefore, studies were only included in this review if they involved a continuous treadmill running-based exercise bout to control for mechanical loading across studies. Furthermore, because exercise intensity can modulate the responses of bone (re)modelling markers (Dolan *et al*., 2020a; Scott *et al*., 2011), the studies included in this review had similar running interventions limited to a continuous running bout with an intensity of ≥65% VO_{2max} and a duration between 60 and 120 min.

Given that bone (re)modelling markers are acutely responsive to nutritional interventions (Sale *et al*., 2015; Townsend *et al*., 2017) and circadian rhythms (Diemar *et al*., 2022; Qvist *et al*., 2002), studies included in this analysis were restricted to those in which the assessment took place in the morning, with a baseline sample after an overnight fast and taken in the morning. Equally, feeding before, during, and after a bout of exercise can affect P1NP and β-CTX-1 responses to an exercise bout (Sale *et al*., 2015; Scott *et al*., 2012; Townsend *et al*., 2017). Therefore, studies were only considered if all blood samples, or at least in one group/trial, were collected in a fasted state or consuming a non-caloric placebo (*e.g*., drink made with artificial sweeteners).

Diet and chronic nutritional factors, such as energy availability (Papageorgiou *et al*., 2017), macronutrient composition of diet (Heikura *et al*., 2020) and calcium intake (Guillemant *et al*., 2004; Haakonssen *et al*., 2015) may affect bone (re)modelling marker concentrations, although there are no clear guidelines on how to control these variables in the days leading up to experimental studies.

Thereby, this factor did not limit the inclusion of studies in this review but was considered in the assessment of methodological quality as part of the risk of bias evaluation.

3.2.1.3. Comparator

Bone (re)modelling markers measured during and after the running bout were compared to the baseline measurement. Data from all available time-points on each study were included in the analysis, including the baseline blood sample, blood samples collected during the day after baseline (before, during, and after running), and blood samples collected in the morning of the days after the running intervention.

To quantify the typical variation caused by factors outside the running intervention, such as instrumentation noise derived from analysis of the markers and biological noise derived from circadian rhythms, data from control conditions (resting/non-exercise) are required. In many studies that had measured bone (re)modelling markers before and after an exercise bout, the effect of exercise *per se* was not the focus of their intervention; and, therefore, most studies did not include a non-exercise control group. Studies that did not fulfil the exercise intervention criteria but fulfilled the rest of the inclusion criteria and included a control/non-exercise group (with fasted samples collected during the hours and days after baseline) were also considered for inclusion, and only the data from these control groups were included in the analysis.

3.2.1.4. Outcomes and Prioritisation

The primary outcome was the difference of bone (re)modelling markers between baseline and during and after a single bout of prolonged, continuous running. Only data from the reference markers P1NP and β-CTX-1 (Vasikaran *et al.*, 2011) were included in this analysis. There are various commercially available assays for the measurement of these two markers and, although differences between assays exist (Bhattoa *et al*., 2021), the assays used were not a limiting factor for including studies in this analysis. The provision of information regarding the assays used (*e.g.*, type, brand, coefficient

variation), however, was considered in the assessment of risk of bias and this limitation is included in the discussion.

3.2.2. Study design

This analysis comprised data extracted from experimental studies whereby blood samples had been taken pre and post a prolonged, continuous running bout for the assessment of the bone (re)modelling markers (P1NP and β-CTX-1) response. The included studies were investigations designed to compare the bone (re)modelling responses to exercise under different conditions, including, for example, different nutrition interventions, exercise intensities or participant training statuses. Control data were obtained from studies that collected multiple blood samples during the hours prior to the exercise or included a control group, where participants did not exercise, and measured P1NP and β-CTX-1 in resting conditions. A baseline sample and subsequent samples taken at different time-points were reported in these investigations.

3.2.3. Search strategy and study selection

Studies were identified by RC directly from the list of included articles in a recent systematic review and meta-analysis on the bone (re)modelling marker response to acute exercise interventions (Dolan *et al*., 2020b). For further details of the protocol, including eligibility criteria, search strategy, study selection and data extraction, of this meta-analysis please refer to Dolan *et al*., (2020b). In summary, seven electronic databases were used to source the material: MEDLINE, Embase, Cochrante CENTRAL, Sport Discus, PEDro, LILACS, and IBEC; and were supplemented by citation screening of all selected studies and relevant reviews and book chapters. This search was last updated in May 2022. Additionally, data from a study included in a PhD thesis (from the university's research group) that fulfilled the inclusion criteria was considered for inclusion (Varley, 2014).

The searches conducted by ED (Dolan *et al*., 2020b) in the abovementioned databases used free-text terms including bone AND (exercise OR physical activity) AND (biomarkers OR turnover OR

remodelling OR formation OR resorption). A combination of free-text and database-specific subject headings were used for a more comprehensive assessment of all available studies. Only peer-review studies published in scientific journals were considered for inclusion. Searches were limited to human studies, and no restrictions were placed on either date of publication or language. The search results from each database were downloaded as a .ris file, then uploaded to a systematic review management software (covidence.org) and deduplicated using the automatic option provided by the software. If a duplicate record was not detected using this automatic option, it was manually removed during the screening process.

The selection strategy undertaken by Dolan *et al.* (2020b) consisted of a three-stage selection process performed independently by two members of the review team that included title/abstract screen, full-text screen/full-text appraisal, and filtering the results using the pertinent eligibility criteria. At the end of each screening stage, the independent screeners assembled, and discrepancies were resolved (with a third party invited if required). During the full-text screen and review stages, the reasons for exclusion were classified as one or more of the following: (1) inappropriate population, (2) inappropriate intervention, (3) inappropriate comparator, (4) inappropriate outcome, (5) inappropriate study design, and (6) others.

The list of articles selected for inclusion in the investigation by Dolan *et al*. (2020b), was subsequently screened by RC to identify studies that met the eligibility criteria for the current study. The search strategy and study selection process is illustrated using a modified version of the PRISMA-IPD search flow diagram (**Figure 7)**.

3.2.4. Data extraction and items

Data extraction was done by RC. A spreadsheet (**Appendix A**) and codebook (**Appendix B**) were developed using Microsoft excel. Data were first extracted into the spreadsheet (according to the eligibility criteria detailed above). The following data were extracted in the spreadsheet directly from the publications of the selected studies: study details (authors; year; study design); participant

characteristics (final *n*; training status; age; height; weight; BMI); exercise characteristics (duration; intensity; total work [duration*intensity]); sampling conditions (time of day; diet and exercise standardisation/control before, during and after the intervention, sample handling, assay type); and, if appropriate, intervention group (*e.g.*, higher/lower intensity, placebo).

Anonymised, individual participant raw data were collected from each publication when available (*e.g.*, supplemental material), or directly from study authors, who were contacted via email, with a maximum of two email attempts over a period of 1 month. Individual participant data were collected only from the study-groups that fulfilled the inclusion criteria (*e.g.*, placebo/fasted groups, higher exercise intensities trials). Individual participant data were entered into the codebook. Numerical data were transformed to the same units when included in the codebook (*e.g.*, cm, kg, min); for P1NP and β-CTX-1 data ng·ml^{-1} was used.

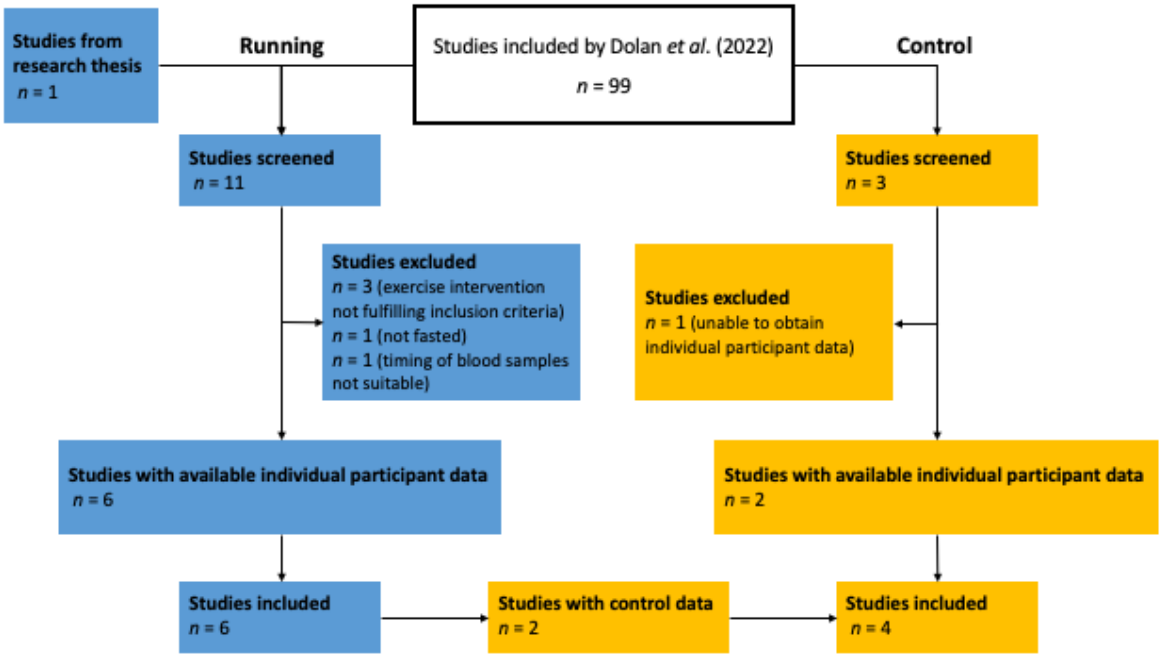


Figure 7. Selection of studies flow diagram. Studies including running data (blue) and studies including control data (orange).

3.2.5. Risk of bias assessment in individual studies

The risk of bias assessment contributed to highlighting specific areas that need to be addressed by future investigations on this topic to improve the quality of future evidence. The risk of bias for each individual study was independently assessed in duplicate by two members of the research team (RC and CS) using a modified version of the Downs & Black (1998) checklist (**Appendix C**). This tool was selected because it provides a comprehensive assessment of the methodological quality of both randomised and non-randomised trials in healthcare research and has been validated as a tool to evaluate the quality of reporting as well as the internal and external validity (Downs & Black, 1998). The original tool was modified by removing some items that were considered unnecessary for this review, either because they were only relevant to longitudinal interventions or because they related to the quality of reporting on factors that were not deemed to bias the outcomes of interest. Additionally, some items relevant for this analysis were added, such as the adequate control of blood sample collection and handling, the inclusion of washout periods between crossover trials, the information regarding fasting and timing of blood sampling, the correction for exercise-induced shifts in plasma volume, the details regarding the time of the day when baseline samples were collected, and the standardisation/monitoring of nutrition and physical activity before the experiment. This tool was not used to exclude any eligible studies.

3.2.6. Statistical analysis

Individual participant data meta-analyses were conducted to quantify the responses of P1NP and β-CTX-1 during, immediately after, and following exercise. Responses were quantified based upon estimates of the mean difference, the standard deviation (SD) of the difference, and the proportion of participants exhibiting an increased response. All models were conducted using a Bayesian framework with random intercepts to account for systematic variation across individual studies. Change scores relative to baseline were calculated for each participant on an absolute scale ($ng \cdot ml^{-1}$). Visual exploration of the data identified the existence of heteroscedasticity, with a positive relationship between baseline values and change scores. Therefore, a parameter accounting for heteroscedasticity was included in all models. Default priors were used for all parameters, including weakly

informative Student-t and half Student-t distributions with 3 degrees of freedom for coefficients and variance parameters.

Distributional random effects models, with the baseline value entered as a predictor for model error were conducted for both control and exercise data. Standard deviation of the change scores were compared between exercise and control. Where estimates showed a mean difference for the exercise group and greater standard deviation of changes score, proportion of positive response was estimated for the exercise group by calculating the amount of the distribution (mean difference plus additional standard deviation of the difference) above zero. Inferences from all analyses were performed on posterior samples generated using the Hamiltonian Markov Chain Monte Carlo method (five chains, 100,000 iterations and 50,000 warmup). Interpretations were based on the median value (0.5-quantile), credible intervals (CrI) and subjective probabilities calculating from the proportion of the posterior sample that exceeded the relevant value selected. Analyses were performed by PS using the R wrapper package brms interfaced with Stan to perform sampling.

3.2.7. Updates from the pre-registered protocol

In the pre-registered protocol (https://osf.io/y69nd), a combined approach of aggregate data and individual participant data meta-analyses was proposed; however, because individual participant data were obtained from all running studies, the aggregate analysis was deemed unnecessary for the purpose of this investigation. The pre-registered protocol indicated that the statistical model would include the estimation of variability ratio (ratio of standard deviation of inter-individual difference scores relative to measurement error values); this estimation was not included in the statistical approach due to the finding that measurement error values were often as large as variation in the intervention.

3.2.8. Definition of terms

To ensure the clearness of the notions described in this study, key analytical and statistical terms utilised herein are defined in the table below (**Table 5**).

Table 5. Definition of key terms.

Term	Definition
Intervention	Systematic modification (*e.g*., diet, drug treatment, therapeutic technique) included in a research study designed to cause a change in the true score of the variable of interest. The intervention of interest of this study was the running exercise bout.
Measurement error	Processes that cause an observed score on a measurement to be different from the underlying "true score"; comprises instrumentation noise and biological noise.
Instrumentation noise	Measurement error derived from the measurement tool (*e.g*., bone biomarkers assays).
Biological noise	Measurement error derived from biological factors (*e.g*., circadian rhythms, nutrition status, sleep).
Typical variation	The variation occurring in a measurement caused by measurement error (*i.e*., instrumentation and biological noise).
Proportion of response	Estimate of the proportion of participants expected to respond to an intervention such that the underlying change is not due to measurement error or biological variation.

3.3. Results

3.3.1. Data collection and estimations

3.3.1.1. Running data

From the selected studies by Dolan *et al*. (2020b), five studies were subsequently selected for inclusion in the analysis of the current study (Lehrskov *et al*., 2020; Sale *et al*., 2015; Scott *et al*., 2011; Scott *et al*., 2012; Townsend *et al*., 2017). In addition, one study from a PhD thesis of the university's group was also included (Varley, 2014). In total, six studies with a running bout intervention were included (**Table 6**). Individual participant data were collected from blood samples measuring P1NP and β-CTX-1 markers at baseline and at all available time-points on each study during and after the running bout (*i.e*., 20 min during running, 30-40 min during running, 30 min post-running, immediately after, 1 h post-running, 2 h post-running, 3 h post-running, 4 h post-running, 24 h post-running, 48 h post-running, 72 h post-running, and 96 h post-running). For time-points 30 min and 4h post-running, data were only available from one study and, therefore, were not included in the analyses.

3.3.1.2. Control data

From the six studies included, two (Lehrskov *et al*., 2020; Scott *et al*., 2012) collected multiple blood samples (*i.e*., 1-3 samples) in resting (control) conditions before the running bout. Three additional studies (Alkahtani *et al*., 2019; Evans *et al*., 2020; Scott *et al*., 2010), which fulfilled all inclusion criteria except the intervention characteristics, but included a control non-exercise group were also identified; however individual participant data was only obtained, and thereby included, from two of these studies (Evans *et al*., 2020; Scott *et al*., 2010) (**Table 6**). For the total of these four studies, individual participant data were obtained from blood samples collected at baseline and during a 1-2.5 h period (hourly) and 24-96 h (daily) after the baseline sample in the control conditions/group. These data were used to estimate the hourly and daily P1NP and β-CTX-1 mean difference and SD of the difference in control (resting) conditions.

Table 6. List of studies included in the analysis.

Study	Running/control data	Bone (re)modelling markers	Assay used
Lehrskov *et al*., 2020	Running and control	β-CTX-1 P1NP	CLIA (IDS) CLIA (IDS)
Sale *et al*., 2015	Running	β-CTX-1 P1NP	ECLIA (Roche) ECLIA (Roche)
Scott *et al*., 2011	Running	β-CTX-1 P1NP	ECLIA (Roche) RIA (Orion)
Scott *et al*., 2012	Running and control	β-CTX-1 P1NP	ECLIA (Roche) RIA (Orion)
Townsend *et al*., 2017	Running	β-CTX-1 P1NP	ECLIA (Roche) ECLIA (Roche)
Varley, 2014	Running	β-CTX-1	ELISA (IDS)
Evans *et al*., 2020	Control	β-CTX-1 P1NP	CLIA (IDS) CLIA (IDS)
Scott *et al*., 2010	Control	β-CTX-1 P1NP	ECLIA (Roche) RIA (Orion)

CLIA, chemiluminescence immunoassay; ECLIA, electro-chemiluminescence assay; *ELISA,* Enzyme-linked immunosorbent assay; *P1NP, amino-terminal propeptide of type 1 procollagen; RIA, radioimmunoassay; β-CTX-1, carboxy-terminal telopeptide of type 1 collagen.*

3.3.1.3. Estimations and comparisons between control and running data

For the running data, the changes in P1NP and β-CTX-1 markers during and after the running bout were evaluated by estimating the mean difference (subtracting each time-point from baseline) and the SD of difference for all time-points. With the control data, the hourly typical variation of P1NP and β-CTX-1 markers was assessed by estimating the mean difference and SD of difference of blood samples taken at rest before a running bout (Lehrskov *et al*., 2020; Scott *et al*., 2012) or in a control group (Evans *et al*., 2020) compared to baseline (**Table 7**). The typical daily variation of P1NP and β-CTX-1 markers was assessed by estimating the mean difference and SD of difference of blood samples taken 24-96 h post-baseline in control (non-exercise) groups (Evans *et al*., 2020; Scott *et al*., 2010) compared to the baseline collected on day 1 (**Table 7**). The running data for the 20 min during running, 30-40 min during running, and 1 h, 2 h, 3 h post-running time-points were compared to the typical hourly variation. The running data for the 24 h, 48 h, 72 h and 96 h post-running time-points were compared to the typical daily variation.

Table 7. Hourly and daily typical variation of P1NP and β-CTX-1 in control conditions.

Marker	Studies	Number of participants and observations	Mean difference [95% CrI]	SD of difference [95% CrI]
Hourly				
P1NP ($ng \cdot ml^{-1}$)	Lehrskov *et al*., 2020; Scott *et al*., 2012; Evans *et al*., 2020	Participants *n* = 27 Observations *n* = 58	0.06 [-7.5 to 5.5]	7.6 [6.8 to 8.5]*
β-CTX-1 ($ng \cdot ml^{-1}$)	Lehrskov *et al*., 2020; Scott *et al*., 2012; Evans *et al*., 2020	Participants *n* = 27 Observations *n* = 58	-0.13 [-0.34 to 0.06]	0.11 [0.11 to 0.12]*
Daily				
P1NP ($ng \cdot ml^{-1}$)	Evans *et al*., 2020; Scott *et al*., 2010	Participants *n* = 22 Observations *n* = 52	-0.39 [-4.3 to 2.9]	5.7 [5.1 to 6.5]*
β-CTX-1 ($ng \cdot ml^{-1}$)	Evans *et al*., 2020; Scott *et al*., 2010	Participants *n* = 22 Observations *n* = 52	-0.03 [-0.54 to 0.30]	0.10 [0.09 to 0.11]*

* Evidence of heteroscedasticity

3.3.2. Typical hourly and daily variation of P1NP and β-CTX-1

The typical hourly and daily variation in P1NP and β-CTX-1 was determined by the mean difference and SD of the difference in control conditions (**Table 7**). There was limited evidence of a mean difference for hourly (0.06 [95% CrI -7.5 to 5.5] ng·ml^{-1}) and daily (-0.39 [95% CrI -4.3 to 2.9] ng·ml^{-1}) P1NP changes, although the SD of difference indicated some inter-individual variability, which was higher in the hourly changes (±7.6 [95% CrI 6.8 to 8.5] ng·ml^{-1}) than in the daily changes (±5.7 [95% CrI 5.1 to 6.5] ng·ml^{-1}). For β-CTX-1, hourly mean differences showed a substantial decrease (0.13 [95% CrI -0.34 to 0.06] ng·ml^{-1}), but the daily β-CTX-1 mean difference was negligible (-0.03 [95% CrI -0.54 to 0.30] ng·ml^{-1}). The SD of the difference was consistent between hourly (±0.11 [95% CrI 0.11 to 0.12] ng·ml^{-1}) and daily (±0.10 [95% CrI 0.09 to 0.11] ng·ml^{-1}) typical changes of β-CTX-1. There was consistent evidence of heteroscedasticity with greater change score magnitudes for those with higher baselines.

3.3.3. P1NP and β-CTX-1 responses to a prolonged, continuous running bout

3.3.3.1. Bone formation

There was clear evidence that the levels of circulating P1NP increased during and immediately after the running bout, when the mean differences were 4.2 [95% CrI 0.2 to 8.8] ng·ml^{-1} at 20 min during the running bout, 9.2 [95% CrI 5.3 to 14.3] ng·ml^{-1} at 30-40 min during the running bout, and 12.0 [95% CrI 8.4 to 16.0] ng·ml^{-1} immediately after the running bout (**Table 8**), compared to a 0.06 [95% CrI -7.5 to 5.5] ng·ml^{-1} mean difference in control conditions (**Table 7**). There was a greater variation in the SD of the difference only at 30-40 min during (±8.1 [95% CrI 7.1 to 9.4] ng·ml^{-1}) and immediately (±10.2 [95% CrI 9.3 to 11.3] ng·ml^{-1}) after the running bout (**Table 8**) compared to the hourly variation (±7.6 [95% CrI 6.8 to 8.5] ng·ml^{-1} (**Table 7**). For these three time-points (20 min during, 30-40 min during and immediately after) the proportion of response was estimated as close to 100% (**Table 8**), indicating that close to all participants reported an increase in P1NP levels. From one hour after finishing the running bout and for the next three hours, P1NP returned to "normal" levels, with similar mean differences and SD of difference (**Table 8**) than the typical hourly variation (**Table 7**). Likewise,

for the four days (24-96 h) after the baseline in the running conditions, P1NP mean differences and SD of the difference (**Table 8**) were similar to the typical daily variation (**Table 7**). The proportion of response was not estimated for these time-points due to these similarities (*i.e.*, between the mean difference and SD of the difference of the control and running data). There was evidence of heteroscedasticity across all time-points except at 2 h and 72 h post-running.

Table 8. Responses of P1NP bone formation marker to a prolonged, continuous running bout.

P1NP ($ng \cdot ml^{-1}$)	Mean difference [95% CrI]	SD of difference [95% CrI]	*P* of increased variation	Proportion of response
Hourly				
20 min during running (25 observations / 2 studies)	4.2 [0.2 to 8.8]	6.1 [5.2 to 7.3]*	0.088	1.0 50% CrI [1.0 to 1.0] 1.0 75% CrI [0.99 to 1.0] 1.0 95% CrI [0.54 to 1.0]
30-40 during running (35 observations / 3 studies)	9.2 [5.3 to 14.3]	8.1 [7.1 to 9.4]*	0.700	1.0 50% CrI [0.99 to 1.0] 1.0 75% CrI [0.99 to 1.0] 1.0 95% CrI [0.99 to 1.0]
Immediately after (75 observations / 5 studies)	12.0 [8.4 to 16.0]	10.2 [9.3 to 11.3]*	>0.999	1.0 50% CrI [0.99 to 1.0] 1.0 75% CrI [0.90 to 1.0] 1.0 95% CrI [0.99 to 1.0]
1 hour post-running (75 observations / 5 studies)	1.1 [-3.1 to 5.2]	5.0 [4.5 to 5.6]*	< 0.001	-
2 hours post-running (60 observations / 4 studies)	0.6 [-3.1 to 4.3]	6.1 [5.5 to 6.8]	0.016	-
3 hours post-running (40 observations / 3 studies)	0.6 [-4.6 to 5.7]	4.6 [3.8 to 5.1]*	<0.001	-
Daily				
24 hours post-running (70 observations / 4 studies)	1.4 [-0.5 to 3.5]	5.2 [4.8 to 5.8]*	0.172	-
48 hours post-running (40 observations / 3 studies)	0.6 [-2.3 to 3.7]	5.9 [5.2 to 7.1]*	0.612	-
72 hours post-running (40 observations / 3 studies)	0.5 [-2.8 to 3.9]	7.8 [6.9 to 9.0]	0.999	-
96 hours post-running (30 observations / 2 studies)	-0.4 [-3.9 to 3.0]	5.4 [4.7 to 6.4]*	0.349	-

* Evidence of heteroscedasticity

3.3.3.2. Bone resorption

Although β-CTX-1 blood levels showed a small decrease in mean differences during the running bout and for the four hours after finishing the running bout, the mean differences and SD of the difference

(**Table 9**) were similar to the β-CTX-1 typical hourly variation (-0.13 ± 0.11 ng·ml^{-1}) (**Table 7**). For the four days (24-96 h) after the baseline in the running conditions, β-CTX-1 concentrations only showed trivial differences (**Table 9**), that were similar to the β-CTX-1 typical daily variation (-0.03 ± 0.10 ng·ml^{-1} (**Table 7**). The proportion of response was not estimated for any time-points due to the small mean differences in the running conditions and the similarities in the SD of the difference between running and control conditions. There was evidence of heteroscedasticity across all time-points except for 72 h and 96 h post-running time-points.

Table 9. Responses of β-CTX-1 bone resorption marker to a prolonged, continuous running bout.

β-CTX-1 (ng·ml^{-1})	**Mean difference [95% CrI]**	**SD of difference [95% CrI]**	***P* of increased variation**	**Proportion of response**
Hourly				
20 min during running (25 observations / 2 studies)	-0.09 [-0.55 to 0.37]	0.04 [0.03 to 0.05]*	< 0.001	-
30-40 during running (35 observations / 3 studies)	-0.06 [-0.29 to 0.14]	0.10 [0.08 to 0.11]*	0.563	-
Immediately after (116 observations / 6 studies)	-0.01 [-0.09 to 0.06]	0.13 [0.12 to 0.14]*	0.996	-
1 hour post-running (60 observations / 4 studies)	-0.02 [-0.13 to 0.08]	0.10 [0.09 to 0.12]*	0.244	-
2 hours post-running (60 observations / 4 studies)	-0.08 [-0.18 to 0.01]	0.10 [0.09 to 0.11]*	0.109	-
3 hours post-running (40 observations / 3 studies)	-0.13 [-0.36 to 0.03]	0.11 [0.10 to 0.13]*	0.576	-
Daily				
24 hours post-running (111 observations / 5 studies)	0.01 [-0.02 to 0.04]	0.12 [0.11 to 0.13]*	> 0.999	-
48 hours post-running (81 observations / 4 studies)	0.01 [-0.06 to 0.07]	0.15 [0.14 to 0.16]*	> 0.999	-
72 hours post-running (81 observations / 4 studies)	0.06 [-0.09 to 0.19]	0.32 [0.29 to 0.35]	> 0.999	-
96 hours post-running (30 observations / 2 studies)	-0.03 [-0.27 to 0.24]	0.09 [0.08 to 0.11]	0.311	-

* Evidence of heteroscedasticity

3.3.4. Risk of bias assessment

The assessment of risk of bias via a modified version of the Downs & Black (1998) checklist (**Appendix C**) determined that most studies were classified as high quality, except two studies which were classified as having moderate quality (**Table 10**). This tool had a total of 16 items and maximum score of 20 and was tailored to identify the methodological concerns relevant for this analysis. The most common reasons why studies were downgraded were because of lack of details provided regarding the storage and handling of the blood samples (Evans *et al*., 2020; Scott *et al*., 2010), the lack of specification of the standardisation of the exact time of the day when the fasted morning baseline was collected (Evans *et al*., 2020; Lehrskov *et al*., 2020), and the inadequate or absent standardisation/monitoring of important nutrition and diet variables (Evans *et al*., 2020; Lehrskov *et al*., 2020; Varley, 2014). All running studies (Lehrskov *et al*., 2020; Sale *et al*., 2015; Scott *et al*., 2011; Scott *et al*., 2012; Townsend *et al*., 2017; Varley, 2014) were downgraded because the during and post running data were not corrected for shifts in plasma volume.

Table 10. Risk of bias in individual studies.

Study	Score	Quality
Lehrskov *et al*., 2020	16/20	!
Sale *et al*., 2015	19/20	+
Scott *et al*., 2011	18/20	+
Scott *et al*., 2012	19/20	+
Townsend *et al*., 2017	19/20	+
Varley, 2014	18/20	+
Evans *et al*., 2020	17/20	!
Scott *et al*., 2010	19/20	+

Green circle, high quality; yellow circle, moderate quality

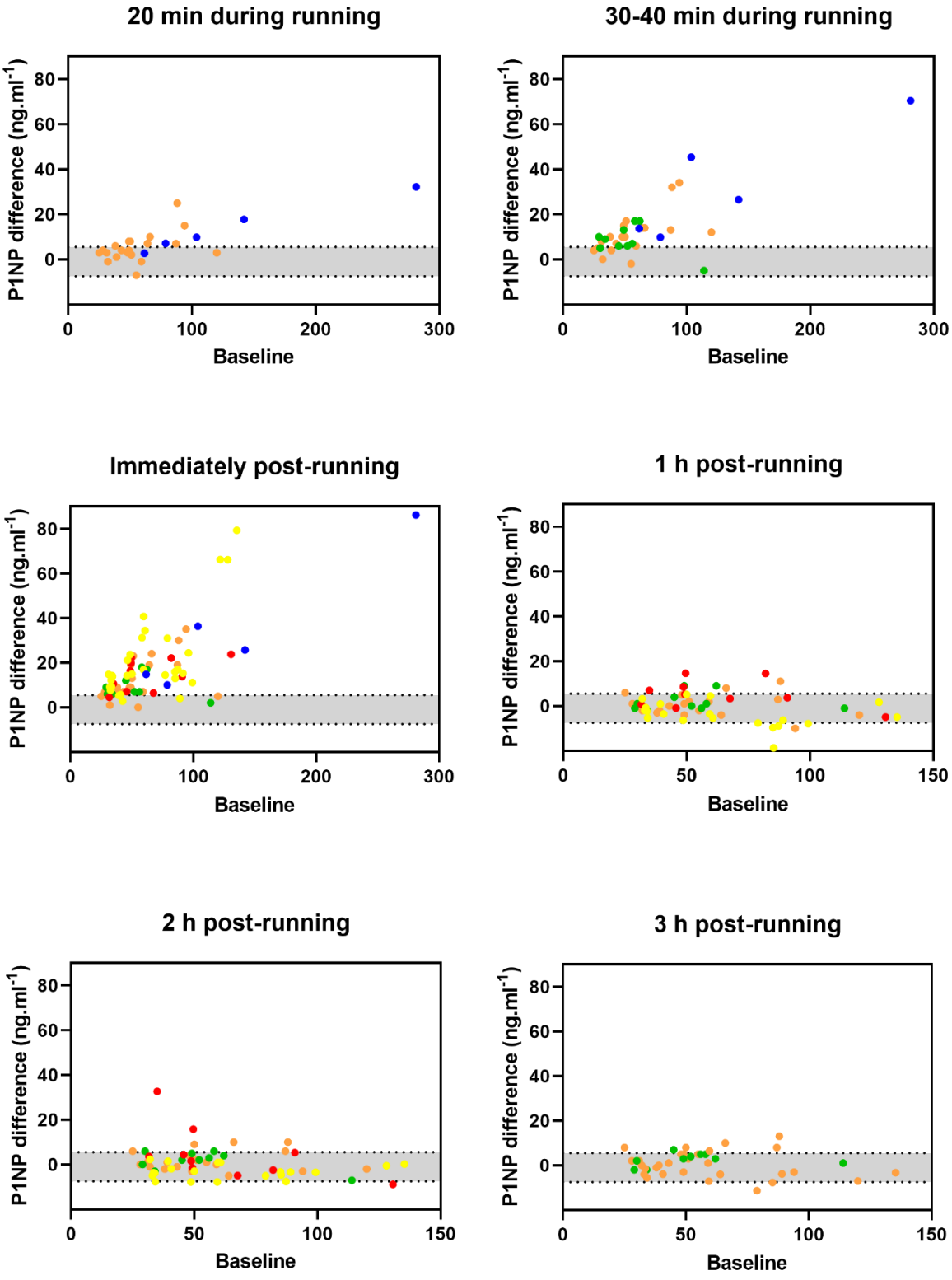


Figure 8. P1NP differences (y axis) from baseline (x axis) at different time-points during and after a continuous, prolonged running bout. Orange: Scott *et al*., 2011; blue: Lehrskov *et al*., 2020; green: Scott *et al*., 2012; red: Sale *et al*., 2015; yellow: Townsend *et al*., 2017. The grey shaded area represents 95% CrI of the mean difference in control conditions (typical hourly variation).

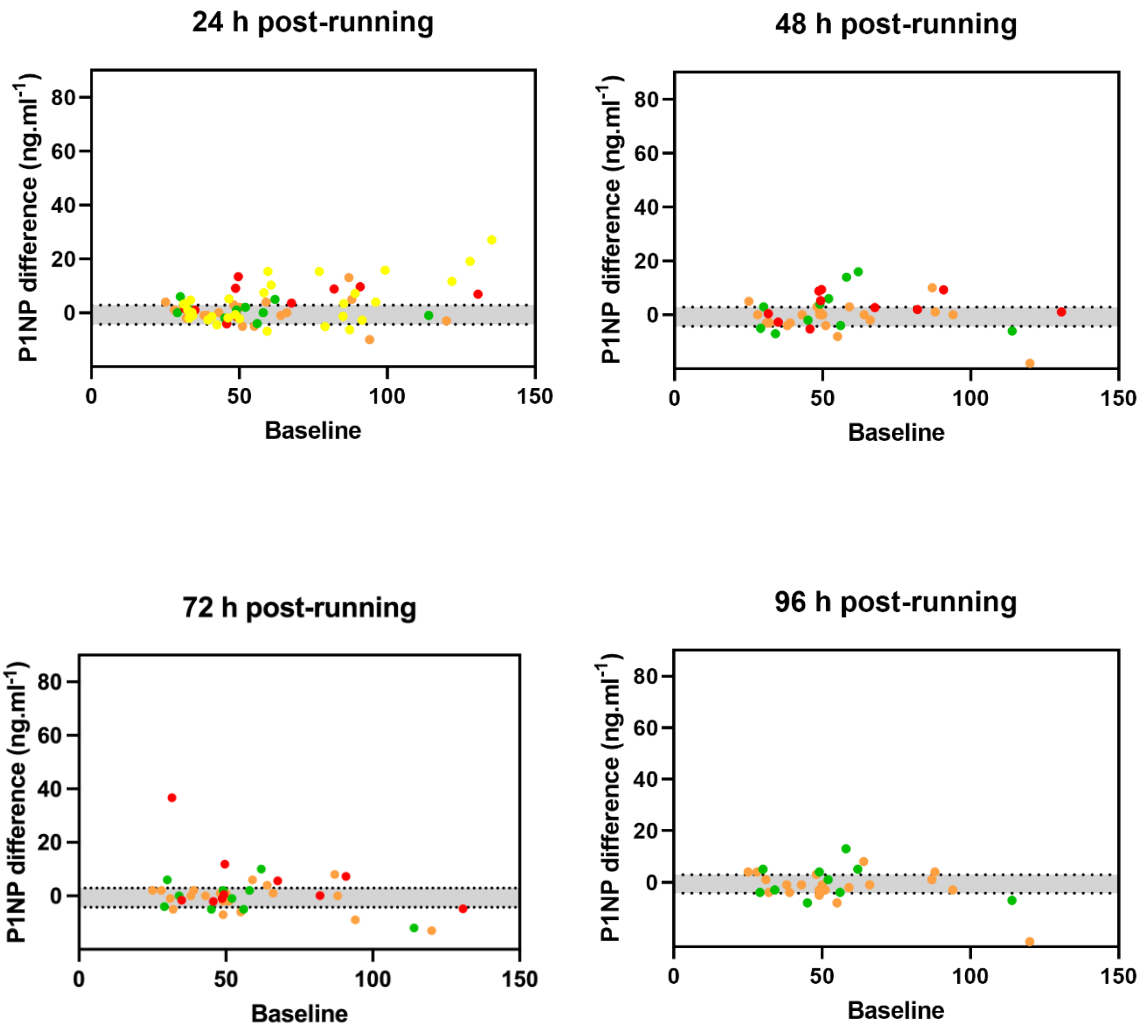


Figure 9. P1NP differences (y axis) from baseline (x axis) at different time-points during and after a continuous, prolonged running bout. Orange: Scott *et al*., 2011; green: Scott *et al*., 2012; red: Sale *et al*., 2015; yellow: Townsend *et al*., 2017. The grey shaded area represents 95% CrI of the mean difference in control conditions (typical daily variation).

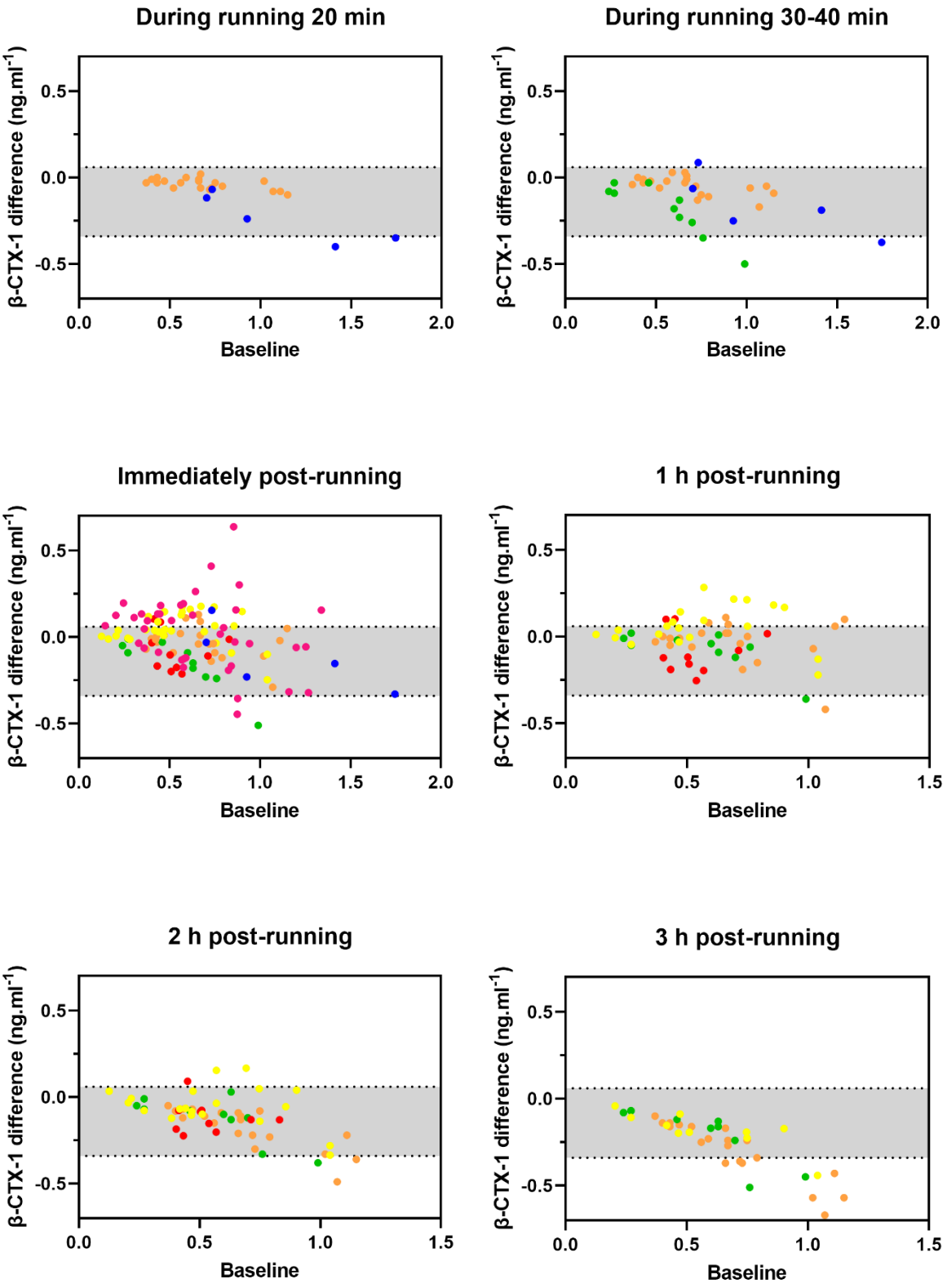


Figure 10. β-CTX-1 differences (y axis) from baseline (x axis) at different time-points during and after a continuous, prolonged running bout. Orange: Scott *et al*., 2011; blue: Lehrskov *et al*., 2020; green: Scott *et al*., 2012; red: Sale *et al*., 2015; yellow: Townsend *et al*., 2017. The grey shaded area represents 95% CrI of mean difference in control conditions (typical hourly variation).

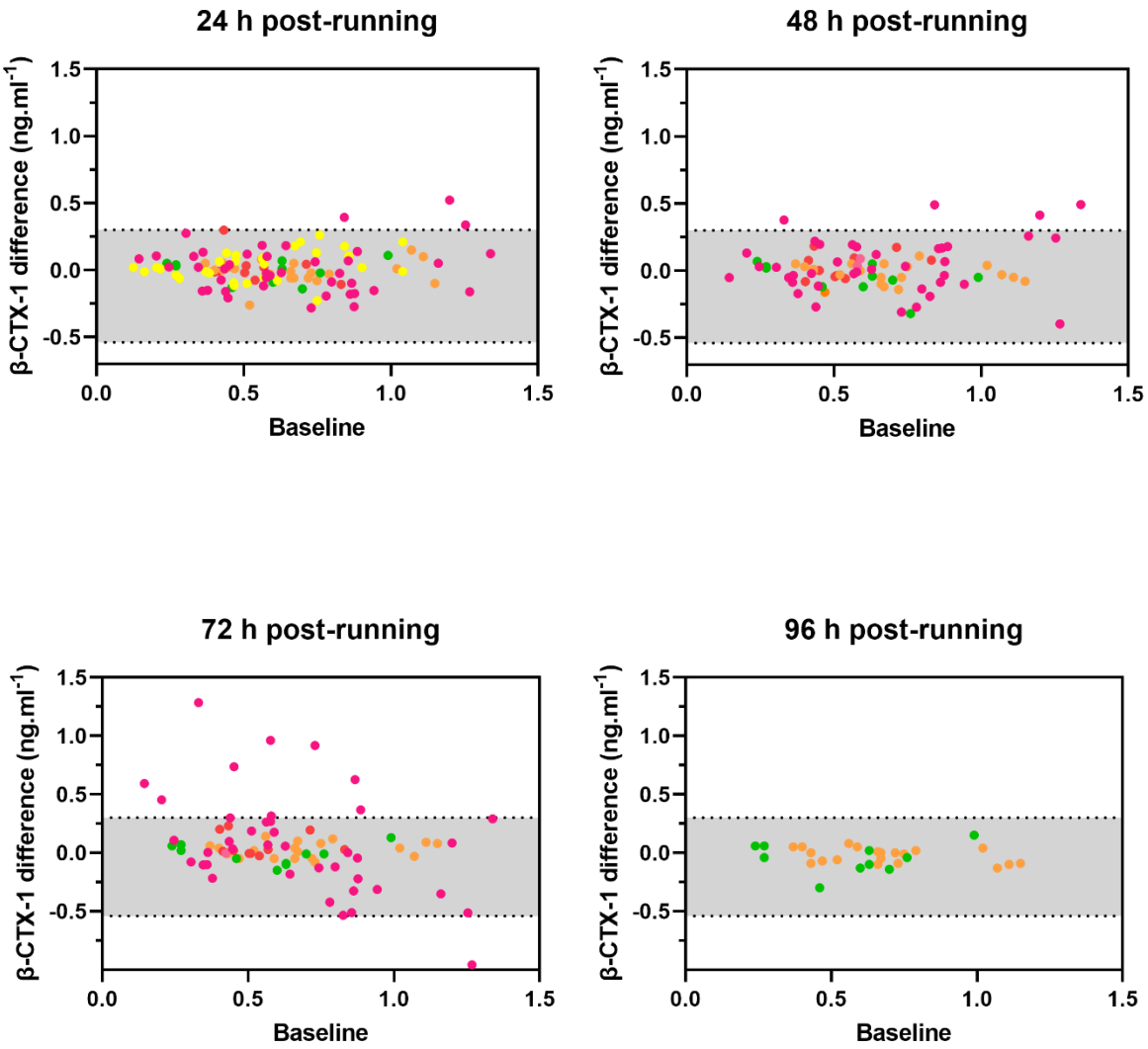


Figure 11. β-CTX-1 differences (y axis) from baseline (x axis) at different time-points during and after a continuous, prolonged running bout. Orange: Scott *et al*., 2011; green: Scott *et al*., 2012; red: Sale *et al*., 2015; yellow: Townsend *et al*., 2017; pink: Varley, 2014. The grey shaded area represents 95% CrI of mean difference in control conditions (typical daily variation).

3.4. Discussion

This individual participant data meta-analysis evaluated the mean responses and inter-individual variability of the bone (re)modelling reference markers P1NP and β-CTX-1 during and after a prolonged, continuous running bout in healthy adult males. The key findings of the study were that: (i) P1NP increased exclusively during and immediately after running and β-CTX-1 showed no meaningful changes linked to running, (ii) the inter-individual variability of P1NP and β-CTX-1 change scores were similar between resting (control) conditions and during and after running, except for P1NP levels during and immediately after the running bout, and, therefore, (iii) there was an overall lack of inter-individual response in P1NP and β-CTX-1linked to running, with reported decreases in β-CTX during the hours after running not being attributable to the running intervention.

The increases in P1NP levels were limited to during and immediately after exercise, although these were consistent across participants (~100%). Greater inter-individual variability compared to the control condition, was also seen during this timeframe, suggesting that these responses were caused by the running intervention. Because these changes were sudden and transient, however, it seems unlikely that they reflect any meaningful increase in bone formation. Very similar results were reported in the meta-analysis by Dolan *et al*., (2022), where they pooled the acute responses of bone (re)modelling markers after different exercise interventions, showing increases in P1NP within 15 minutes of the cessation of exercise. The authors speculated that this transient increase in circulating P1NP could be due to small leaks of P1NP from the connective tissue into the circulation or due to haemodynamic shifts. Indeed, P1NP is not a bone-specific marker and can be affected by the metabolism of collagen from other tissues (Vasikaran *et al*., 2011). Although fluids were provided during the running bout in two studies (Sale *et al*., 2015; Scott *et al*., 2011); shifts in plasma volume were not accounted for any of the running studies included in this review, which could explain the transient higher concentrations. An earlier study (Brahm, Piehl-Aulin, & Ljunghall, 1997) reported similar, sudden and transient, increases in a different bone formation marker (*i.e*., P1CP) in young individuals after a running to exhaustion intervention with a total duration of ~35 minutes, which mirrored changes in plasma volume (showing decreases following the same pattern) and corresponded to increases in haematocrit. They

reported no significant changes in P1CP when correcting for plasma volume shifts (Brahm, Piehl-Aulin, & Ljunghall, 1997).

The stimulation of bone formation in response to exercise may require a longer period; herein, however, there were no changes in P1NP levels for the 1-3 hours and 1-4 days post-running, which indicates that a single prolonged, continuous running bout did not stimulate bone formation, at least up until the fourth day after the running bout. Most studies that have measured bone (re)modelling markers after an acute exercise intervention have only done so for a few days (1-3 days) after the intervention (Clifford *et al*., 2019; Guerriere *et al*., 2018; Herrmann *et al*., 2007; Kouvelioti *et al*., 2018; Kouvelioti *et al*., 2019; Nelson *et al*., 2020) and, therefore, there is no data available on longer-term changes of bone formation markers in response to a single exercise session. In contrast, longitudinal studies in healthy adult populations looking at the chronic responses of bone (re)modelling markers to repeated exercise training of various types have consistently shown increased resting levels of bone formation markers, including P1NP (Adami *et al*., 2008; Alp, 2013; Ardawi, Rouzi, & Qari, 2012; Erickson & Vukovich, 2010; Fujimura *et al*., 1997; Hu *et al*., 2011; Kim *et al*., 2009; Kim *et al*., 2015; Lester *et al*., 2009; Lutz *et al.*, 2012; Tajima *et al*., 2000). Interestingly, this chronic positive response of bone formation markers to exercise training is not consistent when studying athletic populations (Maïmoun *et al*., 2013; Nowak *et al*., 2010; Tournis *et al*., 2010); and it appears to be influenced by the impact level of the sport, with athletes participating in lower-impact sports (*i.e*., swimming, horse-racing, cycling) showing decreases in bone formation markers (Creighton *et al.*, 2001; Maïmoun *et al*., 2004). The responses of bone formation markers to prolonged running training, however, are not clear. Although limited research suggests that long-distance runners have increased bone remodelling, by showing higher osteocalcin levels than controls (Hetland, Haarbo, & Christiansen, 1993; Lee, 2019), no studies have shown the chronic effects of running on bone formation and resorption reference markers.

For circulating levels of β-CTX-1, despite showing small decreases during and in the hours after running, there is no clear evidence that these changes were caused by the running intervention when compared to the control data. Similar reductions in β-CTX-1 were reported in the control (non-exercise)

conditions, and comparable inter-individual variability was shown for the hourly running and hourly control data. Together, these results suggest that the small decreases in circulating β-CTX-1 shown during and in the hours after running were caused by measurement error rather than as a result of the running intervention. These reductions in β-CTX-1 coincide with the circadian rhythm of this biomarker in fasting conditions, peaking in the early morning (around 0300), decreasing during the morning (0500-1100) and reaching their nadir between 1100-1400 (Bjarnason *et al*., 2002; Christgau *et al*., 2000). In contrast, aggregate meta-analytic evidence (Dolan *et al*., 2022) suggests that β-CTX-1 responses to exercise are influenced by the type of exercise, with evident increases shown from 15 minutes to 2 hours after long-duration cycling. Increases in β-CTX-1 could be explained by increases in PTH, triggered by reductions in serum calcium, that subsequently stimulates osteoclastic bone resorption (Wherry, Swanson, & Kohrt, 2022). Although this mechanism seems to agree with the β-CTX-1 increases to cycling interventions (Kohrt *et al.*, 2018), it does not explain the lack of a response reported herein, where increases in PTH were also present in some of the included running studies (Sale *et al*., 2015; Scott *et al*., 2011; Scott et al., 2012; Townsend *et al*., 2017). It is important to note, however, that some studies included in Dolan *et al*. (2022) meta-analysis reported small decreases or no effects in β-CTX-1 after exercise, and that the level of certainty in the outcomes was very low due to the variability in study designs and quality of the studies. As reported previously (Bjarnason *et al*., 2002), nutritional status (*i.e*., feeding) and time of day influences β-CTX-1 levels, and it was acknowledged by the authors that the lack of standardisation of these factors in some of the studies included in the meta-analysis contributed to the high variability in their outcomes (Dolan *et al*., 2022). In all studies included in this individual participant meta-analysis, the running intervention was conducted in the morning and in fasting conditions or consuming a non-caloric placebo, indicating that the observed inter-individual variability was likely caused by other factors, such as differences in analytical assays (Jørgensen *et al*., 2017). For example, β-CTX-1 samples analysed using ELISA methods, as in the Varley (2014) study, seem to yield higher variability in the data (**Figures 10 and 11**, pink dots) compared to others.

For the 1-4 days after the running bout, β-CTX-1 blood levels were also similar to the daily typical variation determined by the control data, indicating that the running intervention did not yield

significant responses to β-CTX-1 circulating levels. Similar results were reported by the Dolan *et al*. (2022) meta-analysis, which included studies with different designs and exercise interventions, although they showed some evidence of increases in β-CTX-1 at 72 hours post-exercise. The lack of a response (*i.e*., increase) in β-CTX-1 shown herein, could be considered as a beneficial outcome for bone adaptations if it is interpreted as the lack of resorption activity that can lead to bone loss. On the contrary, it could be considered, that an initial increase in bone resorption markers is indicative of the activation of the bone (re)modelling cycle (Robling, Castillo, & Turner, 2006), described in Chapter 2, section 2.5. (page 23). In this case, it could be concluded that a single running bout does not stimulate bone remodelling, at least within the next four days. In actual fact, bone (re)modelling is a nuanced process that is continuously ongoing at different stages across different skeletal sites (Kenkre & Bassett, 2018). Bone (re)modelling markers, though, are systemic and do not necessarily represent local bone adaptations/changes, and using this approach in exercise research is a critical limitation because bone responses to mechanical loads are largely site-specific (Bass *et al*., 2002; Judex, Gross, & Zernicke, 1997; Kannus *et al*., 1994). For example, and in relation to the present study, chronic running training has been shown to generate site-specific effects, with greater BMD at the lower extremities compared to non-loading sites (*i.e*., lumbar spine) in runners (Herbert *et al*., 2021; Kemmler *et al*., 2006). Therefore, studies utilising bone (re)modelling markers to investigate the bone responses to acute or short-term exercise interventions will likely be missing key information about the local effects that loading has on the skeleton, which means they need to be interpreted carefully and highlights the need for alternative methods that might be more suitable for this type of research.

3.4.1. Strengths and limitations of the study

Studies included in this meta-analysis were overall classified as high quality (n = 8). It should be noted, however, that the inclusion criteria applied herein was thorough and delimited; and, therefore, low quality studies would likely not have met this criterion. In the Dolan *et al*. (2022) meta-analysis, which had a less restricted inclusion criteria and included a larger number of studies (n = 88), the general quality of the studies was reported as moderate. While a more inclusive criteria would have allowed the inclusion of a greater number of studies and, thereby, more data points; it would have also added more

variability. The aim of this meta-analysis was to investigate the responses of P1NP and β-CTX-1 in very specific conditions by reducing potential sources of variability, such as the type of exercise intervention (*i.e.*, impact level, duration, and intensity, intermittent/continuous), participant characteristics (*i.e.*, age, sex and health status) and study design (*i.e.*, feeding/fasting conditions, time of the day). Removing these sources of variability allowed for a better understanding of the inter-individual variability caused by measurement error (*e.g.*, analytical assays, circadian rhythms).

This individual participant data meta-analysis was not without limitations, including those inherited form the included studies. Although all included studies collected a baseline sample in the morning, the exact time of the day when the fasted baseline sample was taken ranged from 0800-0840 or was not specified (Evans *et al*., 2020; Lehrskov *et al*., 2020). Similarly, the exact time of the day when the running bout began was different across studies. These factors could have impacted the changes in bone (re)modelling markers; particularly β-CTX-1. The circadian rhythm of β-CTX-1 can produce a ~60% daily variation (Diemar *et al*., 2022), peaking in the early morning and declining in the morning hours (Bjarnason *et al*., 2002; Christgau *et al*., 2000). Although fasting can significantly reduce the amplitude of this variation (Diemar *et al*., 2022), changes in β-CTX-1 levels across participants might have been influenced by its defined circadian rhythm pattern, with the natural decline of this marker during these hours being more or less pronounced depending upon the exact time of the day when the blood samples were collected. Additionally, habitual dietary and nutritional factors, such as energy availability, macronutrient composition of the diet and vitamin D and calcium intakes were not controlled in the included studies, and could have affected P1NP and β-CTX-1 baseline levels and potentially their responses to exercise (for a review please see Dolan *et al*., 2020a). For example, increased β-CTX-1 and reduced P1NP levels can be caused by low energy availability (in females) (Papageorgiou *et al*., 2017) and a low-carbohydrate high-fat diet (Heikura *et al*., 2020), while calcium intake can attenuate exercise-induced (*i.e.*, cycling) increases in β-CTX-1 (Guillemant *et al*., 2004; Haakonssen *et al*., 2015).

Differences in training status or habitual training could have influenced the baseline levels of bone (re)modelling markers, since previous research has shown increases in both formation and resorption

markers in athletes involved in high-impact sports (Courteix *et al*., 2007; Karlsson *et al*., 2003; Maïmoun *et al.*, 2008). In a study from Scott *et al*., (2010), however, it was reported that endurance-trained males did not have different baseline levels of P1NP and β-CTX-1 than recreationally active individuals. Hence, it is unlikely that this factor would have affected the results to a large degree, given that all participants included in the analysis were described as recreationally active healthy males, except for participants in one study (Townsend *et al*., 2017) who were trained endurance runners. The baseline level of a marker might be an important variable determining the subsequent response to exercise, and potentially to other interventions as well, as the heteroscedasticity shown in the participant data of this meta-analysis suggested that those participants with higher baselines had greater changes for both markers and across time-points. To better understand this phenomenon and its influence on inter-individual baseline variability, future studies should consider investigating the effects and potential sources (*e.g*., sleep, training, nutrition) of intra-individual variability (Chrzanowski-Smith *et al*., 2020) of these markers.

Importantly, only two of the running studies included a non-exercise control group, which means that the control data used to estimate the typical variation of P1NP and β-CTX-1 in resting conditions was predominantly from different participants (although with similar characteristics). It is, therefore, possible that the inter-individual variability was greater than if all participant control data had been obtained from the same running participants. Nonetheless, even with this discrepancy, the mean differences and variability (*i.e*., SD of the difference) in P1NP and β-CTX-1 were similar between the control and running data. Another factor that could have increased variability in the data is the difference between analytical assays used, which is part of measurement error. In single studies, variability from measurement error comprises the variation (usually quantified as the coefficient of variation) of the instrumentation (*i.e.*, analytical assays used to measure bone biomarkers) and biological noise. In this meta-analysis, the measurement error from the instrumentation includes the variation of various types of assays, which all have different intra- and inter-assay coefficients of variation, generally ranging from 1.4-4.9% (P1NP) and 2.1-5.3% (β-CTX-1) (Bhattoa *et al.*, 2021). Although efforts have been made since the 2010s to standardise and harmonise commercially available assays and methodologies

for the measurement of P1NP and β-CTX-1 (Bauer *et al*., 2012; Morris *et al*., 2017; Vasikaran *et al*., 2011), there is remaining poor agreement between these assays (Bhattoa *et al*., 2021) and the variation caused by the different assay methods needs to be considered when interpreting results. This variation can be critical for exercise research given the overall small responses that bone (re)modelling markers exhibit after acute exercise interventions (Dolan *et al*., 2022).

The current meta-analysis only included studies with a running intervention in healthy adult males because similar studies in female populations are lacking. Early research (Salvesen, Piehl-Aulin, & Ljunghall, 1994) suggests that bone (re)modelling marker (*i.e*., P1CP and osteocalcin) responses to a running intervention (12 minutes of submaximal test followed by 9 minutes of maximal test) may differ between sexes. In contrast, Dolan *et al*. (2022), showed that sex did not influence exercise-associated changes of P1NP and β-CTX-1 markers, although they included studies with very varied exercise interventions. Available literature on this topic in females usually involves post-menopausal populations and include exercise interventions using plyometric/jumping (Nelson *et al*., 2020; Prawiradilaga *et al*., 2020) or activities with lower impact such as walking (Shea *et al*., 2014) or cycling (Rudberg *et al*., 2000). A limited number of studies have investigated the acute responses of reference markers P1NP and/or β-CTX-1 to exercise (*e.g*., jogging, brisk walking with resistance training, football) in young females (Bowtell *et al*., 2016; Nelson *et al*., 2020; Tosun *et al*., 2006), and results are inconsistent for P1NP, but show evidence of increases in β-CTX-1 within 5-15 minutes post-exercise. No studies have directly compared these responses between males and females, meaning that it is not clear if acute exercise, or running specifically, yields different effects on bone metabolism across sexes. Investigating these short-term bone adaptations is important for female runners, who are at high risk of stress fractures (Bennell *et al*., 1996; Grimston *et al*., 1991; Kelsey *et al*., 2007; Milner *et al*., 2006).

3.4.2. Implications for future research

The evidence obtained from this investigation has important implications for past and future research. The majority of studies included in this meta-analysis did not include a control (non-exercise) group

because they were designed to investigate how various factors (*e.g.*, nutrition, exercise intensity) may moderate the bone (re)modelling marker responses to a running bout. Indeed, this study design is habitual within exercise research and only about a quarter of studies looking at acute exercise responses of bone (re)modelling markers included a control group (Dolan *et al*., 2022). Surprisingly, single studies have not previously determined if, by which magnitude and to which direction, bone (re)modelling markers respond to acute exercise interventions within the hours and days after the intervention, in comparison to the typical circadian variation of the markers. The results of this meta-analysis suggest that responses of bone (re)modelling markers during and after running may not indicate short-term bone adaptations, but rather other biological artefacts (*i.e.*, haemodynamic shifts and markers' circadian rhythms), or might be masked by inter-individual variability. It is recommended that non-exercise control groups are included in future studies to quantify the variability of the instrumentation noise (*i.e.*, from assays) and biological noise (*e.g.*, from circadian rhythms) (Swinton *et al*., 2018), and to establish if exercise interventions of different kinds produce an effect on bone (re)modelling markers.

The effects of running on short- and long- term bone adaptations are currently not well understood, because research evidence is somewhat conflicting and appears to be site-specific (Herbert *et al*., 2021; Hetland, Haarbo, & Christiansen, 1993; Kemmler *et al*., 2006; Lee, 2019). Although this investigation suggests that a single bout of running may not affect bone metabolism; it is also plausible that, if there was an effect, circulating bone formation and resorption markers, P1NP and β-CTX-1, were not able to capture these potential bone adaptations. This hypothesis questions the validity of these markers to reflect adaptive responses that lead to changes in bone mass or structure. In fact, bone (re)modelling markers have not yet been validated or linked to a primary reference measurement because there is no alternative reference measurement system available that can act as a higher order standard or gold standard (Bhattoa *et al*., 2021); and it is not clear whether they can predict changes detected by imaging techniques, such as DXA or pQCT (Vasikaran *et al.*, 2011). Two other hypotheses that could explain the lack of responses on P1NP and β-CTX-1 are that (a) the times when the blood samples were collected missed any significant changes, and (b) the running intervention was insufficient or inadequate to generate a response. These hypotheses could be confirmed with further research that (a) includes a

larger number of samples during the hours/days and for a longer period (>4 days) after the running bout, and (b) investigates the effects of different running interventions or other types of exercise in comparison to a control group.

For the time being, research using these markers to investigate the short-term bone responses to exercise interventions need to be interpreted carefully considering this context and the magnitude of responses. Equally, it is important that studies including bone (re)modelling markers adhere to the recommended standardisation guidelines (Bauer *et al*., 2012; Bhattoa *et al*., 2021; Vasikaran *et al*., 2011), control important factors before the intervention (*e.g*., nutrition, sleep, physical activity), clearly report the time of the day of all measures, sampling timing, storage, and handling of the samples, and report the quality control information for the assays used. Future studies should also consider measuring the sleep patterns and habitual activity levels of participants, which might influence markers' baseline levels and projected changes after exercise. Following these recommendations would reduce the inter-individual variability and help when making comparisons with other studies. Given the potentially misleading increases in P1NP during and immediately post-running reported herein, studies should also consider shifts in plasma volume and fluid lost or report both adjusted and unadjusted data for changes in plasma volume. Future research should incorporate female participants and determine if differences exist between sexes or within different menstrual status/phases on the acute responses of bone (re)modelling markers to running or other exercise interventions, which could help understanding why female runners are at higher risk of stress fractures (Barrack *et al*., 2014; Bennell *et al*., 1996).

3.4.3. Summary and conclusions

This individual participant data meta-analysis determined that a prolonged, continuous bout of treadmill running (60-120 min at 65-75% VO_{2max}) does not result in changes in bone (re)modelling, as determined by P1NP and β-CTX-1, in healthy adult males. Whilst there was evidence of a transient increase of P1NP during and immediately after running, this response was likely caused by biological aspects (*e.g*., shifts in plasma volume, leakage from other connective tissues) rather than being reflective of bone

formation. Similar small decreases in β-CTX-1 were shown in control and running data, suggesting that these changes were due to the marker's circadian rhythm and not the running intervention.

CHAPTER 4:

METHOD DEVELOPMENT.

A COLLAGEN EXTRACTION AND DEUTERIUM OXIDE STABLE ISOTOPE TRACER METHOD FOR THE QUANTIFICATION OF BONE COLLAGEN SYNTHESIS RATES *IN VIVO*

The study described in this Chapter corresponds to the following publication (**Appendix D**):

Civil, R., Brook, M., Elliot-Sale, K.J., Santos, L., Varley, I., Lensu, S., Kainulainen, H., Koch, L.G., Britton, S.L., Wilkinson, D.J., Smith, K., Sale, C., Atherton, P.J. (2021). A collagen extraction and deuterium oxide stable isotope tracer method for the quantification of bone collagen synthesis rates *in vivo*. *Physiological Reports*, 2021 May; 9 (10). https://doi.org/10.14814/phy2.14799

4.1. Introduction

From Chapter 2 (sections 2.2. and 2.3., pages 9 and 12), it is clear that practical strategies are required to promote bone health, and to prevent the weakening of bone tissue resulting in disease (*e.g.*, osteopenia, osteoporosis, and osteoporotic fractures). There are a number of modifiable factors that could contribute to bone health, including diet, exercise and physical activity, and pharmacological interventions (Hernlund *et al*., 2013; Weaver *et al*., 2016). As such, there is a need for robust approaches that are able to determine the efficacy of these factors in improving bone health.

The rate of bone collagen turnover is important in determining bone strength (Bouxsein, 2005; Burr, 2002a; Viguet-Carrin, Garnero, & Delmas, 2006), but the responses to potentially favourable interventions (*e.g.*, drugs, exercise, diet) to improve bone strength are poorly defined due to the lack of robust analytical methods for its measurement. Bone (re)modelling markers, discussed in Chapters 2 (section 2.6., page 34) and 3 (section 3.1., page 61), have been employed in this regard, although they have several limitations (*i.e.*, pre-analytical and analytical variability and lack of site specificity) that limit their utility and the interpretations that can be made from them. Therefore, other approaches to measure bone collagen turnover that can overcome some of these limitations are warranted.

Isotopically labelled tracers are generally regarded as the gold standard in determining fractional synthetic rates of proteins and could be used to measure bone collagen synthesis if a tissue sample can be collected. Traditionally, amino acid isotope tracers have been used to measure *in vivo* synthesis of human musculoskeletal tissues, such as muscle and tendon (Wilkinson *et al*., 2017), and, in a limited number of studies, of bone (Babraj *et al*., 2005a; Scrimgeour *et al*., 1993; Smeets *et al*., 2019). Studies that use these amino acid tracers require the preparation of high-cost infusions and venous/arterial cannulation and are restricted by time (generally <24 h), which makes it challenging to accurately measure very low rates of bone collagen synthesis (Wilkinson *et al*., 2014). Furthermore, traditional amino acid tracer approaches are hindered by the heterogeneity of amino acid body pools (Brook *et al.*, 2017). In contrast, deuterium oxide (D_2O or *heavy water*) stable isotope tracers can be easily ingested, with the deuterium becoming rapidly equilibrated within the body water and intracellular amino acid

pools (Wilkinson *et al*., 2014). This allows D_2O to be administered with minimal interference to an individual's normal daily activities, with enrichment in the precursor pool easily maintained over weeks and months (Wilkinson *et al*., 2017), making this tracer more suited to the measurement of slow turnover proteins, such as collagen.

Previous assessments of mixed bone protein synthesis rates using D_2O have been made in rodents, however these required high levels of ^2H body water enrichment (~3-5%) and were performed in growing animals, where bone protein synthesis rates are considerably higher (Busch *et al*., 2006; Cross *et al*., 2020; Do *et al*., 2006; Jeong *et al*., 2005). As such, methods isolating bone collagen protein and using D_2O to measure bone collagen synthesis rates in adult animals' scenarios and in humans, where bone collagen synthesis rates are considerably lower, are lacking. To address this, the aim of this study was to develop and optimise a novel method using a deuterium oxide stable isotope tracer combined with GC-*pyrolysis*-IRMS techniques to quantify low levels of bone collagen turnover *in vivo*, using lower levels (<1%) of ^2H body water enrichment in adult non-growing rodents.

4.2. Methods

Femur and tibia bones were derived from male and female rats, which were selectively bred for yielding low or high responses to endurance running training (Koch, Pollott, & Britton, 2013). The bone samples used herein were opportunistically harvested for this purpose as an addition to other independent investigations already being conducted. Two different cohorts of rats were used in this thesis: a young and an old cohort. The *Young cohort* was used for the initial development of this method.

4.2.1. Animals

4.2.1.1. Artificial selection

Male and female rats from a selectively bred rat model, exhibiting either low (LRT) or high (HRT) responses to endurance running training, and divided into control and trained groups were used for this study.

This rat model was developed at the University of Michigan, USA (Koch, Pollott, & Britton, 2013). A large-scale bidirectional experiment was started in a genetically heterogenous rat population (N/NIH stock) to develop two contrasting lines of LRT and HRT phenotyped rats. This artificial selection was defined on the magnitude of change in running capacity (quantified by maximal treadmill distance in meters [**Δ** DIST]) as a result of an 8-week aerobic treadmill training protocol. This phenotyping protocol was repeated across generations, developing a rat model system that contrasted for low and high response to endurance exercise training (**Figure 12**). After fifteen generations of selection, rats bred as HRT increased maximal treadmill running distance from 646 to 869 m (change, 223 ± 20 m), whereas LRT rats decreased from 620 to 555 m (change, −65 ± 15 m) after completing the same training (Koch, Pollott, & Britton, 2013).

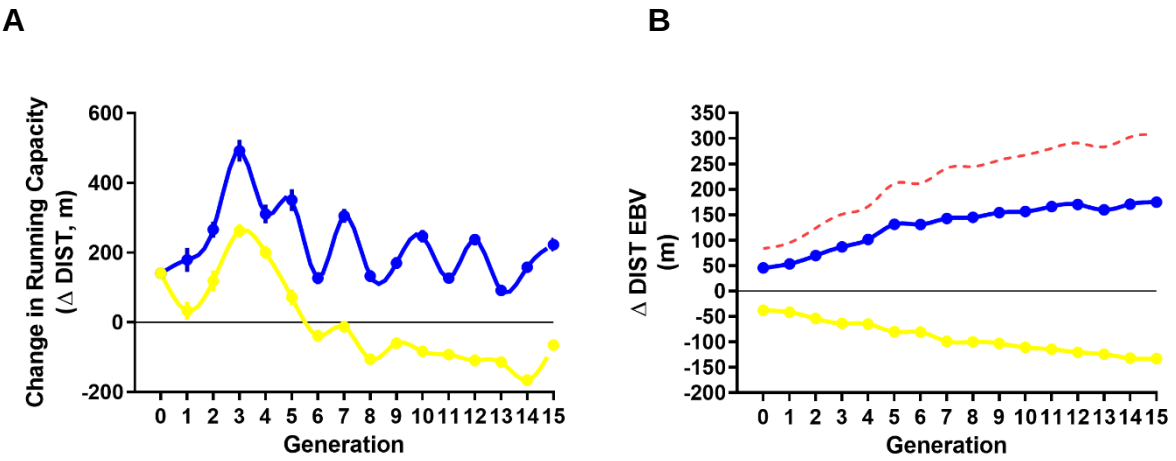


Figure 12. Response to the artificial selection across 15 generations for low and high exercise training capacity. A: phenotypic trend for Δ DIST across 15 generations of selection for low and high response to training. Mean of the founder population is plotted at generation 0 (as denoted by the green box on the figure). B: genotypic trend. Each point represents the mean genetic value for ΔDIST represented as estimated breeding values for each generation. The dashed red line is the difference between the LRT and HRT selected lines. In panels A and B, the LRT phenotype responses are shown with an orange line and the HRT phenotype responses are shown with a blue line. From Koch, Pollott, & Britton (2013) with permission.

4.2.1.2. Young cohort

Rats from generations 17 and 18 of selection for their training response, were sent from Michigan (USA) to Finland (University of Jyväskylä), where the interventions (training intervention and deuterium enrichment) were carried out. These interventions were primarily designed to investigate training responses following high intensity training across sex and phenotype groups. At the end of the interventions, researchers from the University of Nottingham (UK) travelled to Finland to collect the blood samples and femur and tibia bones from this cohort of rats and then, transported them to the Centre of Metabolism, Ageing & Physiology research laboratory (University of Nottingham, UK), where the experiments detailed below (bone collagen protein extraction, isolation, and derivatisation; body water enrichment and deuterated alanine analyses; and fractional synthesis rates calculations) were completed as part of this programme of work.

Forty-six male and female rats were included in the *Young cohort* (**Table 11**). Rats were ~9 months old at the start of the experiment that was carried out at the University of Jyväskylä, Finland. All experiments were approved by the Animal Care and Use Committee of Southern Finland, license number ESAVI-2010-07989/Ym-23, STH 534A (21.9.2010) and complements ESAVI/1968/04.10.03/2011, PH308A (30.3.2011) and ESAVI/722/04.10.07/2013, PH275A (1.3. 2013); and were conducted in accordance with the Guidelines of the European Community Council Directive 86/609/EEC. No additional ethical approval was required for the secondary analysis performed herein.

Table 11. Total number of femur and tibia bones available from the *Young cohort* divided into phenotypes of low (LRT) and high (HRT) responders to endurance running training, a control condition and a training intervention, and male and female.

	YOUNG							
	High responders to training (HRT)				**Low responders to training (LRT)**			
	Interval running training		**Control**		**Interval running training**		**Control**	
	Male	Female	Male	Female	Male	Female	Male	Female
Femur	*n* = 6	*n* = 6	*n* = 5	*n* = 4	*n* = 5	*n* = 6	*n* = 4	*n* = 4
Tibia	*n* = 6	*n* = 7	*n* = 5	*n* = 5	*n* = 7	*n* = 6	*n* = 4	*n* = 5

4.2.1.3. Training intervention

Rats were single-housed in air-conditioned rooms at an ambient temperature of 21 ± 2°C and relative humidity at 50 ± 10%. Artificial lighting provided light cycles of 12:12-h light-total darkness. Commercially available pelleted rodent diet (R36; Labfor; Lantmän nen, Malmö, Sweden) and tap water (from the municipal water system of Jyväskylä, Finland) were available *ad libitum*. The energy content of the feed was 1,260 kJ·100 g^{-1} (300.93 kcal·100 g^{-1}). The feed contained 18.5% raw protein, 4.0% raw fat, 55.7% nitrogen-free extracts, 3.5% fibre, 6.3% ash, and 12% water. During a 7-week intervention period, rats of both phenotypes were divided into trained and control groups, where rats in the trained groups underwent interval running training on a treadmill. Interval running training consisted of a warm-up for 5 min at 50-60% of maximum speed (individual speed for each rat) followed by a 15 min session that comprised 3x3 min bouts of running at 85-90% maximum speed. With 2 min recovery periods between each bout at 50% maximum speed, repeated three times, with 15° uphill inclination. Training was completed three times per week with 1-day rest between. Rats in the control groups were kept in the same conditions as the exercising rats but were not trained on the treadmill during the 7-week intervention period.

4.2.1.4. Deuterium enrichment

As detailed in section 2.9.3. in Chapter 2 (page, 55), D_2O tracers can be easily ingested and has multiple advantages for the measurement of slow turnover proteins, such as collagen. During the last 3 weeks of the 7-week intervention, rats received a gavage of 7.2 ml·kg BW^{-1} 70% D_2O period and drinking water was enriched to 2% D_2O to maintain body-water enrichment. Body water enrichment was determined from blood samples collected at necropsy and used to represent the average enrichment throughout. Baseline blood (non-enriched) was collected from a different group of rats that did not receive D_2O. Although variability may occur over time, drinking enriched water minimises these effects. Blood samples were collected at necropsy (~5 ml) and plasma was separated by centrifugation and stored frozen at −20°C until analysis.

4.2.2. Bone samples collection and collagen extraction

Forty-eight hours after the last training bout, animals were anesthetised with carbon dioxide and killed by cardiac puncture and thereafter immediately necropsied. Left femur and tibia bones were rapidly exposed, removed, and immediately frozen by complete immersion in liquid nitrogen and were kept frozen at −80°C until analysis.

Figure 13. Standing clamp and bone sample before being cut.

The first bone samples to be analysed were the femur bones from the *Young cohort*. A smaller sample from the femur diaphysis (FEM) was obtained using pestle and mortar. The frozen bone was placed in the mortar and the pestle was used to smash the femur diaphysis and break it into small chunks. This approach, however, did not allow for an accurate control of the bone-site from where the sample was taken from the femur diaphysis, since it depended upon where along the shaft each bone would break more easily. As such, it was next decided to change the approach and standardise the bone sample sites.

It was speculated that different anatomical bone sites might have different turnover rates because of bone heterogeneity (trabecular and cortical bone) and the potential different effects of strains from mechanical loading across long bones. As such, bone samples from three different sites of the tibia were subsequently obtained using an electric hand saw (Dremel 3000 Rotary Tool, USA) and standing clamp that allowed a more accurate cutting of the bone (**Figure 13**). Samples from the tibia bones were obtained from the tibial proximal epiphysis-metaphysis (T-PRO), the tibial mid-shaft diaphysis (T-MID), and the tibial distal epiphysis-metaphysis (T-DIS), each sample was ~20% of the tibia length (**Figure 14**). The weights for FEM, T-PRO, T-MID, and T-DIS were 0.10 ± 0.03 g, 0.30 ± 0.08 g, 0.09 ± 0.04 g, and 0.11 ± 0.02 g.

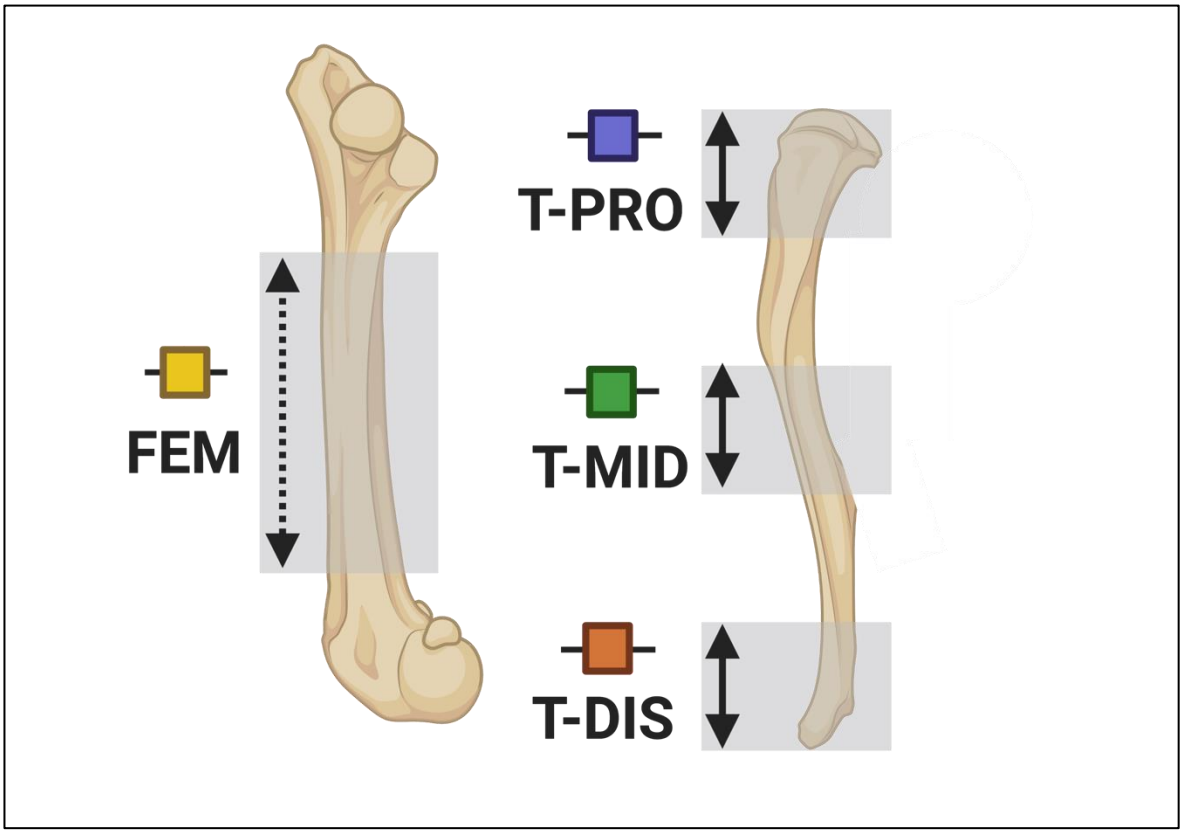


Figure 14. Sampling areas of tibia and femur shown in highlighted in grey. FEM, femur diaphysis (not site-controlled, sample obtained from any site within the grey area); T-PRO, tibia proximal epiphysis-metaphysis; T-MID, tibia mid-shaft diaphysis; T-DIS, tibia distal epiphysis-metaphysis. Created with BioRender.com

Bone samples were transferred into 0.3-0.5 M HCl until samples were completely decalcified and appeared translucent and flexible. This process typically took 10-15 days with the HCl solution being changed every 1-4 days. During the method development process, it was established that ~13 days in 0.5 M HCl solution changed every >2 days and with a vortexing bout before and after each change of solution was the most optimal procedure to decalcify the bone samples. This acidic solution was used to disassociate aldimine crosslinks (*i.e.*, immature collagen), whereas more mature ketoimine crosslinks (*i.e.*, mature collagen) were released by cleavage with pepsin. Any visually remaining connective tissue was manually removed with a sharp scalpel before or during the demineralisation process.

NaOH was used after demineralisation to clean the collagen samples from other tissues/proteins or interstitial and intracellular fluids containing free amino acids in order to have minimal contamination of non-collagen sources such as bone marrow, which would impact the measurement. Bone marrow and alkali soluble proteins were dissolved and removed with a 0.3 M NaOH solution, which was changed ~3 times over 2-5 days. Bouts of vortexing were added during this process to help dissolve and remove the remaining bone marrow and soluble proteins, leaving the bone collagen proteins. The optimal frequency for changing the NaOH solution was ~3 times, keeping the samples in the solution for >1 day and with bouts of vortexing before and after changing the solution. Centrifuging bouts and/or manually cutting the tissue with scissors were other techniques used to help with dissolving and removing bone marrow from particularly difficult samples that did not appear “clean”.

This process was monitored on an individual basis, in a way that each sample was individually inspected and, if needed, the samples spent longer in the HCl and/or NaOH solutions, with extra bouts of vortexing/centrifuging also being added until the mineral and non-collagenous proteins were completely removed. This individual monitoring explains the variability of the timings during the process. Demineralisation of the bone with HCL followed by NaOH is expected to leave behind mature collagen and other fractions, representing >95% of the total collagen, with minimal non-collagen contamination.

4.2.3. Isolation and derivatisation of bone collagen protein

For the isolation and derivatisation of bone collagen protein fractions the protocol from Wilkinson *et al.* (2014), which was used for the preparation of muscle proteins, was adapted with slight modifications. Bone collagen proteins were isolated and released using acid hydrolysis by incubating in 0.1 M HCl in Dowex H^+ resin slurry overnight at 110°C before being eluted from the resin with 2 M NH_4OH and evaporated to dryness. Amino acids were then derivatised as their N-methoxycarbonyl methyl esters. First, femur dried samples from the *Young* cohort were suspended in 60 µl of distilled water and 32 µl of methanol, and following vortex, 10 µl of pyridine and 8 µl of methyl chloroformate were added. Samples were vortexed for 30 s and left to react at room temperature for 5 min. The newly formed N-methoxycarbonyl methyl ester amino acids were then extracted into 100 µl of chloroform. A molecular sieve was added to each sample for ~20 s before being transferred to a clean glass gas chromatography insert, removing any remaining water by size exclusion absorption. Dried tibial samples were derivatised later with this same methodology, but the volume of the solutions were as follows: 120 µl of distilled water, 64 µl of methanol, 20 µl of pyridine, 16 µl of methyl chloroformate, and 200 µl of chloroform. This change in volumes was done in order to optimise mass spectrometry analyses.

4.2.4. GC-MS/MS body water enrichment analyses

Body water enrichment was measured in plasma by incubating 100 µl of each sample with 2 µl of 10 M NaOH and 1 µl of acetone for 24 h at room temperature. Following incubation, the acetone was extracted into 200 µl of n-heptane and 0.5 µl of the heptane phase was injected into a TRACE 1310 Gas Chromatograph connected to TSQ 8000 triple quadrupole GC-MS/MS (Thermo Scientific) for analysis. A standard curve of known D_2O enrichment was run alongside the samples for calculation of enrichment.

4.2.5. GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates

Protein-bound alanine enrichment was determined by pyrolysis-gas chromatography with isotope-ratio mass spectrometry (Delta V Advantage, Thermo Scientific). Bone collagen fractional synthetic rate (FSR) was calculated from the incorporation of deuterium-labelled alanine into protein using the enrichment of body water [corrected for the mean number of deuterium moieties incorporated per alanine (3.7) and the dilution from the total number of hydrogens in the derivative (*i.e.*, 11)] as the surrogate precursor labelling over the 3-week period of D_2O labelling. The equation used was:

$$\mathrm{FSR} = ln\left[\frac{1 - \left(\frac{APEala}{APEp}\right)}{t}\right]$$

where APEala equals deuterium enrichment of protein-bound alanine, APEp indicates mean precursor enrichment over the time period, and *t* represents time (*i.e.*, 3 weeks).

4.2.6. Statistical analyses

In order to demonstrate the efficacy of the method development and show the differences across bone-sites, data from all rats in the *Young* cohort (independently of the sex, phenotype and training groups) were pooled and analysed together. Descriptive statistics were performed for all data sets to check for normal distribution (accepted if $p > 0.05$) using the Shapiro-Wilk test. All data are presented as means ± 1SD. Differences between FSRs of the FEM and T-MID samples were analysed by Wilcoxon matched pairs test. The Kruskal-Wallis test was used to compare T-PRO, T-MID and T-DIS samples. *Post-hoc* analysis was performed using Dunn's multiple comparisons test to determine the differences between each of the tibial sites. The effect size Cohen's *d* was estimated for significant differences between two bone sites, defined as small ($d = 0.20$), moderate ($d = 0.50$) and large ($d = 0.80$). All analyses were performed on GraphPad Prism 8 (La Jolla, CA, USA) except for effect sizes analyses, which were

performed on RStudio (version 1.4.1717) with packages *tidyverse* and *rstatix*. The level of significance was set at $p \leq 0.050$ and all data are presented as mean ± 1SD and 95% confidence intervals [95% CI].

4.3. Results

The average body water enrichment in the young rats was 0.685 ± 0.089 APE (atom percent excess), whilst the average change in the deuterium labelling, expressed as delta per mil deuterium (δ^2H) was FEM 352 ± 38 δ^2H, T-PRO 548 ± 45 δ^2H, T-MID 170 ± 21 δ^2H and T-DIS 83 ± 10 δ^2H (**Figure 15**) the higher the value reflecting the greater incorporation of labelled alanine.

Bone collagen FSRs at the FEM (0.131 ± 0.078 %·d^{-1}; 95% CI [0.106-0.156] %·d^{-1}) were significantly greater ($p < 0.001$; $d = 1.18$; **Figure 16**) than the FSRs at the T-MID (0.055 ± 0.049 %·d^{-1}; 95% CI [0.040-0.070] %·d^{-1}). The highest bone collagen FSRs were at the T-PRO site (0.203 ± 0.123 %·d^{-1}; 95% CI [0.166-0.241] %·d^{-1}) and the lowest at the T-DIS (0.027 ± 0.015 %·d^{-1}; 95% CI [0.022-0.031] %·d^{-1}). The three tibial sites had significantly different FSRs ($p < 0.001$, **Figure 17**). T-PRO was significantly different from T-MID ($p < 0.001$; $d = 1.59$) and T-DIS ($p < 0.001$; $d = 2.01$), but the difference between T-MID and T-DIS was not significant ($p = 0.057$).

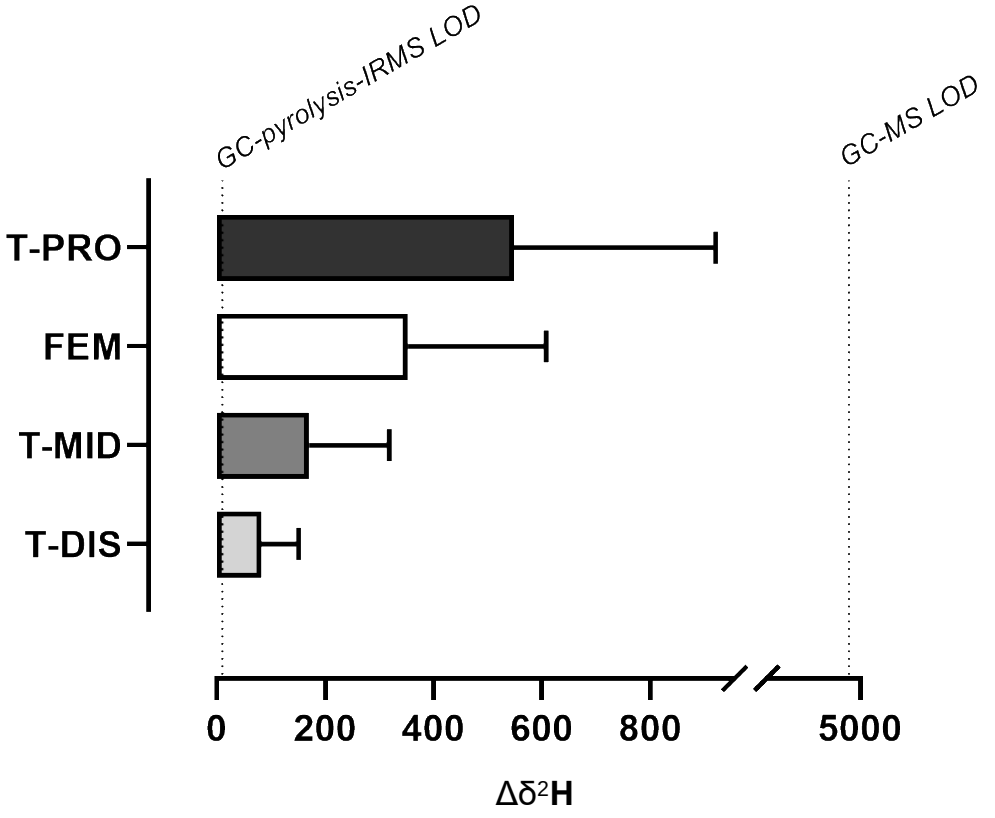


Figure 15. Change in protein-bound deuterium enrichment (Δδ2H) across the proximal tibia (T-PRO), tibial mid-shaft (T-MID), distal tibia (T-DIS) and femur (FEM). GC-pyrolysis-MS limit of detection (LOD) shown as 10 δ2H and GC-MS LOD shown as 4700 δ2H.

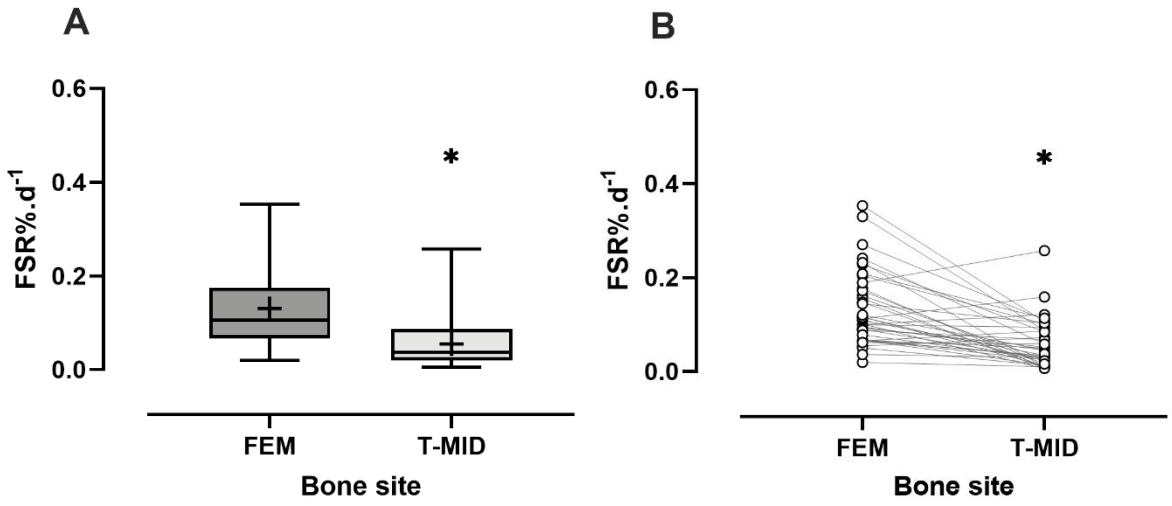


Figure 16. Bone collagen fractional synthetic rates (FSR%·d^{-1}) for the femur (FEM) and the mid-shaft of the tibia (T-MID). A) Data represented as box plots, + represents mean. B) Individual values. * Wilcoxon matched pairs test ($p < 0.001$)

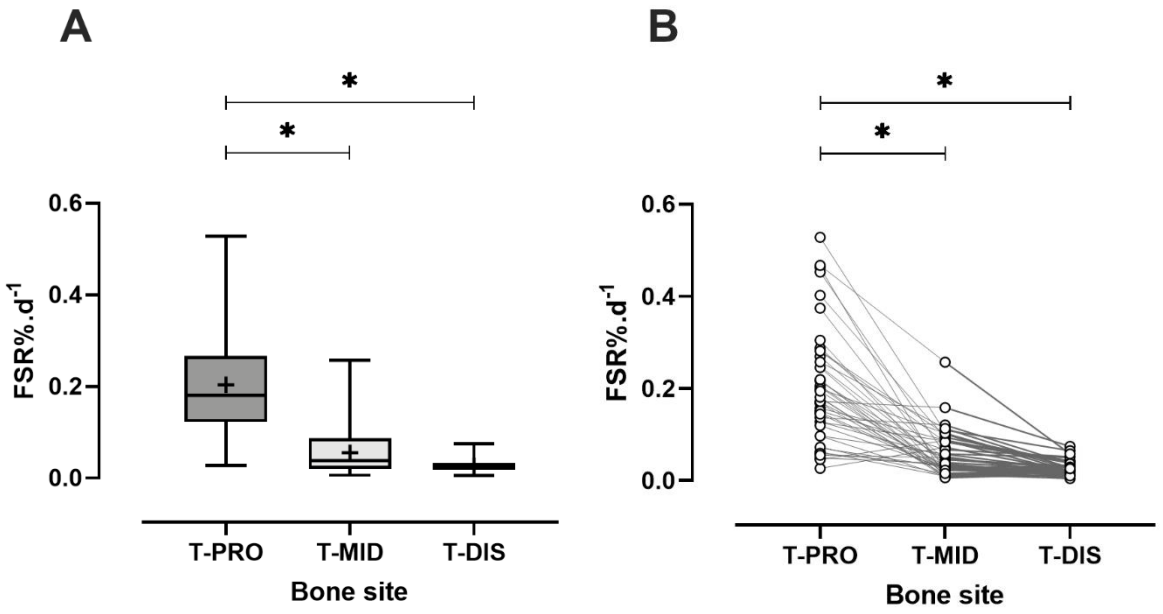


Figure 17. Bone collagen fractional synthetic rates (FSR%·d^{-1}) across the proximal (T-PRO), mid-shaft (T-MID) and distal (T-DIS) sites of the tibia. A) Data represented as box plots, + represents the mean. B) Individual values. * Post hoc Dunn's multiple comparisons test (p <0.001)

4.4. Discussion

This Chapter describes a novel method for the quantification of bone collagen synthesis *in vivo* using a deuterium oxide stable isotope tracer technique. The results show that this method is capable of quantifying bone collagen synthesis *in vivo*, using lower levels (<1%) of ^{2}H body water enrichment over a 3-week period in non-growing adult rodents. By combining the deuterium oxide stable isotope tracer with sensitive GC-*pyrolysis*-IRMS techniques, this method enables the measurement of slow turnover proteins such as collagen, with the potential to determine short-term changes in bone collagen synthesis.

The sensitivity of this method was tested by its ability to detect differences in bone collagen FSRs between the femur and the tibia and across sites along the length of the same bone (tibia). Femoral samples were obtained from the diaphysis of the bone (not site-controlled) and tibial samples were obtained from the mid-shaft diaphysis (corrected by the length of each rat's bone). In addition, samples

from proximal and distal epiphyses-metaphyses were obtained, in order to investigate collagen FSRs across bone regions that have different compositions of trabecular and cortical bone. Bone collagen synthesis rates at the femur diaphysis were significantly greater than at the tibia diaphysis (mid-shaft). Bone collagen synthesis rates were faster at the proximal tibial site than at both the mid-shaft and distal tibia sites. These differences between collagen FSRs across bone sites reported herein underline the limitations in the utility of bone (re)modelling markers that estimate whole body bone turnover. In fact, previous work in humans has pointed to the incongruities between the P1NP biomarker and changes in bone (Babraj *et al*., 2005a) and tendon (Miller *et al*., 2007) turnover. Further, these results highlight the importance of controlling and reporting the bone site used for analysis in future studies.

The potential higher bone (re)modelling rates of trabecular bone, which has a greater internal surface of area and, therefore, it is more metabolically active compared to cortical bone (Robling, Castillo, & Turner, 2006; Seeman, 2013), could explain the differences between bone collagen synthesis among bone sites. The higher FSRs at the proximal site of the rat tibia (composed of more trabecular bone), compared to the mid-shaft and distal tibia (composed of more cortical bone) shown herein may be due to such disparity in bone (re)modelling. Additionally, the potential presence of growth plate in the tibial epiphyses (proximal and distal) (Wilsman *et al*., 2008) may have affected our measurements of bone collagen synthesis. However, this issue will likely be minimised in human studies, where growth plates close in late puberty and the control for the bone site during sampling can be made more easily in larger bones (Kember & Sissons, 1976; Shim, 2015).

Bone collagen FSRs ranged between 0.005 and 0.529 %·day^{-1}, being ~30 fold lower than mean muscle protein synthesis rates measured in this cohort (data not shown). Earlier D_2O studies in rodents have showed active bone synthesis (Do *et al.*, 2006; Jeong *et al*., 2005), with one study reporting rates of ~17 %·week^{-1} femur bone synthesis in young growing mice (Busch *et al*., 2006). A recent study determined skull bone FSRs in growing mice (12 weeks old), with reported rates of ~0.035 %·d^{-1} (Cross *et al*., 2020). In humans, where the obtention of bone tissue is more challenging, only three studies have measured synthesis of human bone collagen *in vivo* by using various amino acid tracers and different

bone protein fractions, which makes comparisons between studies difficult (Babraj *et al*., 2005a; Scrimgeour *et al*., 1993; Smeets *et al*., 2019). To date, no studies have used D_2O as a direct incorporation tracer technique to determine human bone collagen synthesis.

Although this method requires obtention of a bone tissue sample, which would involve invasive procedures, it has many strengths. Compared to bone (re)modelling markers, it allows measurements of site-specific bone collagen synthesis. The use of D_2O has many advantages for determining bone turnover compared to traditional amino acid tracer approaches, where changes in bone synthesis can only be captured in a short timeframe (*i.e.*, over hours). D_2O labelling with continued oral ingestion of *heavy water* can safely maintain body water enrichment for days, weeks, or months (Wilkinson *et al*., 2017). This is especially important for slow turnover proteins, such as collagen obtained from the bone, which may need longer periods of labelling for longer term interventional studies looking at changes in bone synthesis.

The combination of D_2O tracer and GC-*pyrolysis*-IRMS offers a highly sensitive technique for quantifying small changes in δ^2H and therefore bone collagen synthesis *in vivo*. Despite very low FSRs, this will ultimately permit measures of bone collagen synthesis in humans using well tolerated D_2O loading protocols (*i.e.*, 150 ml + 50 ml·week^{-1}). As such, there is great future applicability of this method to human investigations to determine differences in bone turnover between age, sex, health and disease and responses to interventions looking to improve bone health such as exercise, diet, and drugs.

CHAPTER 5:

METHOD DEVELOPMENT.

CAN BONE COLLAGEN SYNTHESIS RATES BE QUANTIFIED WITH LOW LEVELS OF DEUTERIUM INCORPORATION *IN VIVO*?

5.1. Introduction

In Chapter 4, a D_2O GC-*pyrolysis*-IRMS method for the quantification of bone collagen synthesis *in vivo* was developed, tested, and optimised in a young cohort of rats. The method was shown to be sensitive enough to detect small changes in δ^2H and differences in bone collagen synthesis across bone sites. Although D_2O tracer methods have been widely used to study human skeletal muscle protein synthesis since the 2010s (Brook *et al*., 2016; Damas *et al*., 2016; Franchi *et al*., 2015; Fuchs *et al*., 2020; Holwerda *et al*., 2018; Saner *et al*., 2020; Wilkinson *et al*., 2014), there are further challenges when studying bone tissue with this method due to its seeming slower rates of turnover (Smeets *et al*., 2019).

Because the D_2O tracer is ingested (achieving low ^2H body water enrichment levels) as part of this method, rather than being intravenously infused (typically achieving high enrichments), the labelling period of the tracer is an important factor when designing studies aiming to quantify synthesis of slow turnover proteins, such as bone collagen. D_2O tracer methods have not yet been employed to study bone collagen synthesis rates in humans and, therefore, the labelling period required to achieve a sufficient ^2H incorporation into human bone tissue to quantify bone collagen synthesis is not known, even with very sensitive mass spectrometry equipment such as GC-pyrolysis-IRMS. Human bone appears to have slower synthesis rates compared to other musculoskeletal tissues (Smeets *et al.*, 2019), which means that studies in this area will likely require longer periods (*e.g.*, a few days) of D_2O labelling to achieve adequate levels of ^2H incorporation into the tissue.

Optimising the length of the D_2O labelling periods would enable more time and cost-effective experimental designs. It is, therefore, important to investigate whether bone collagen synthesis can be quantified with lower levels of ^2H incorporation into bone proteins with a short labelling period. This question could be easily answered, herein, by using available tibia bones from a population of old rats, which had a shorter D_2O labelling period. Therefore, the aim of this study was to investigate if the method developed in Chapter 4 could be used to quantify bone collagen synthesis with very low ^2H incorporation levels by applying the method in older rats with a 3-day D_2O labelling period.

5.2. Methods

5.2.1. Animals

Bones were derived from the rat model created by Koch, Pollott, & Britton (2013), as described in Chapter 4 (section 4.2.1, page 95). For the initial development of the method, femur and tibia bones from a *Young cohort* of rats were used (Chapter 4). In this Chapter, and for the purposes of extending the development of the method with a lower deuterium incorporation, tibia bones from an *Old cohort* of the same rat model were used.

5.2.1.1. Old cohort

Fifty-five male and female rats that were selectively bred for yielding low (LRT) or high (HRT) responses to endurance running training (Koch, Pollott, & Britton, 2013) were included in the *Old cohort* (**Table 12**). Rats were ~22 months old at the start of the study and were housed in the Unit for Laboratory Animal Medicine at the University of Michigan (USA), where the interventions (training intervention and deuterium enrichment) were carried out. These interventions were all approved by the University of Michigan Institutional Animal Care and Use Committee. Tibia bones and blood samples from this cohort of rats were sent from University of Michigan (USA) to the Centre of Metabolism, Ageing & Physiology research laboratory (University of Nottingham, UK), where the experiments described below (bone collagen protein extraction, isolation, and derivatisation; body water enrichment and deuterated alanine analyses; and fractional synthesis rates calculations) were completed as part of this programme of work. No additional ethical approval was required for this secondary analysis.

Table 12. Total number of femur and tibia bones available from the *Old cohort* divided into phenotypes of low (LRT) and high (HRT) responders to endurance running training, a control condition and a training intervention, and male and female.

	OLD							
	High responders to training (HRT)				**Low responders to training (LRT)**			
	Interval running training		**Control**		**Interval running training**		**Control**	
	Male	Female	Male	Female	Male	Female	Male	Female
Tibia	*n* = 8	*n* = 8	*n* = 6	*n* = 8	*n* = 6	*n* = 8	*n* = 4	*n* = 7

5.2.1.2. Training intervention

All rats were familiarised with treadmill running for one week before they were tested for maximal treadmill running capacity as previously described (Koch *et al*., 2013). Briefly, rats ran on a motor driven treadmill set at a constant grade of 15° and at an initial speed of 10 m·min^{-1}. Speed was progressively increased 1 m·min^{-1} every 2 min until exhaustion. Exhaustion was operationally defined as the third time a rat remained on the shock grid for 2 s. The total distance run in m was calculated from belt speed (m·min^{-1}) and duration (min) of the run.

After the testing period, rats in the trained groups underwent moderate running training on a treadmill 2-3 times per week at 60% of their maximum tested running speed during a 16-week intervention period (37 trials in total). The treadmill speed started at 10 m·min^{-1} on session 1 and increased by 1 m·min^{-1} every other session up to a maximum speed of 21 m·min^{-1}. The duration for each training session was set for 20 min for session 1 and incremented by 0.5 min each session. The slope of the treadmill was held constant at 15°. The control group only performed maximal running distance tests for exercise capacity and were placed on a static treadmill for 5 minutes 3 days per week to simulate the environmental change of handling.

After the intervention, rats from control and trained groups were again tested for maximal treadmill running distance as an indicator of exercise capacity. Rats were sacrificed two days after their last exercise session to avoid acute metabolic effects; tibia bones were exposed, removed, and immediately frozen in liquid nitrogen and stored at −80°C.

5.2.1.3. Deuterium enrichment

Rats received a gavage of 7.2 ml·kg BW^{-1} 70% D_2O for 3 days at the end of the of the 16-week intervention period. A blood sample was taken ~2 hours after the D_2O bolus to calculate peak enrichment. The final blood was collected three days later at necropsy and plasma was separated by centrifugation and stored frozen at −20°C until analysis. Baseline blood (non-enriched) was collected from different group of rats that did not receive D_2O.

5.2.2. Bone samples collection and isolation and derivatisation of bone collagen proteins

Tibia bones were rapidly exposed after necropsy, removed, and immediately frozen by complete immersion in liquid nitrogen and were kept frozen at −80°C until analysis. Bone samples from three different sites of the tibia were obtained using an electric hand saw (Dremel 3000 Rotary Tool, USA) and standing clamp (**Figure 13**, page 99). Like in the *Young* cohort (Chapter 4), samples from the tibial bones of the *Old cohort* were obtained from the tibial proximal epiphysis-metaphysis (T-PRO), the tibial mid-shaft diaphysis (T-MID), and the tibial distal epiphysis-metaphysis (T-DIS), each sample was ~20% of the tibial length (**Figure 14**, page 100). The weights for T-PRO, T-MID and T-DIS were 0.21 ± 0.03 g, 0.12 ± 0.11 g, and 0.10 ± 0.09 g.

The demineralisation and collagen extraction of bone samples was completed using the optimised method described in Chapter 4. Briefly, bone samples were transferred into 0.5 M HCl solution for ~13 days or until samples were completely decalcified and appeared translucent and flexible. The HCl solution was changed for a fresh solution every 2-3 days with bouts of vortexing before and after each change, removing the mineral component dissolved in the solution. Any visually remaining connective tissue was manually removed with a sharp scalpel before or during the demineralisation process. Following demineralisation, bone samples were transferred to 0.3 M NaOH to dissolve and remove the remaining bone marrow and soluble proteins, leaving the bone collagen proteins. The NaOH solution was changed >3 times over ~5 days, or until it visually appeared that the bone marrow was completely removed, with bouts of vortexing and centrifuging before and after each change.

Bone collagen proteins were hydrolysed to free amino acids by incubating in 0.1 M HCl in Dowex H^+ resin slurry overnight at 110ºC before being eluted from the resin with 2 M NH_4OH and evaporated to dryness. Amino acids were then derivatised as their N-methoxycarbonyl methyl esters. Tibia dried samples were suspended in 120 µl of distilled water and 64 µl of methanol, and following vortex, 20 µl of pyridine and 16 µl of methyl chloroformate were added. Samples were vortexed for 30 s and left to react at room temperature for 5 min. The newly formed N-methoxycarbonyl methyl ester amino acids were then extracted into 200 µl of chloroform. A molecular sieve was added to each sample for ~20 s before being transferred to a clean glass gas chromatography insert, removing any remaining water by size exclusion adsorption.

5.2.3. GC-MS/MS body water enrichment analyses

The deuterium enrichment was measured in plasma collected at necropsy, by incubating 100 µl of each sample with 2 µl of 10 M NaOH and 1 µl of acetone for 24 h at room temperature. Following incubation, the acetone was extracted into 200 µl of n-heptane, and 0.5 µl of the heptane phase was injected into a TRACE 1310 Gas Chromatograph connected to TSQ 8000 triple quadrupole GC-MS/MS (Thermo Scientific) for analysis. A standard curve of known D_2O enrichment was run alongside the samples for calculation of enrichment.

5.2.4. GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates

Protein-bound alanine enrichment was determined by pyrolysis-gas chromatography with isotope-ratio mass spectrometry (Delta V Advantage; Thermo Scientific). Bone collagen fractional synthetic rate (FSR) was calculated from the incorporation of deuterium-labelled alanine into protein using the enrichment of body water [corrected for the mean number of deuterium moieties incorporated per alanine (3.7) and the dilution from the total number of hydrogens in the derivative (*i.e.*, 11)] as the surrogate precursor labelling over the 3-day period of D_2O labelling. The equation used was:

$$\text{FSR} = -\ln\left[\frac{1-\left(\frac{APEala}{APEp}\right)}{t}\right]$$

where APEala equals deuterium enrichment of protein-bound alanine, APEp indicates mean precursor enrichment over the time period, and *t* represents time (3 days).

5.2.5. Statistical analyses

Data from all rats in the *Old cohort* (independently of the sex, phenotype, and training groups) were pooled and analysed together. Some data points showed negative FSR values (**Figure 19**), which are not physiologically feasible and reflect the insufficient ^{2}H incorporation into the bone samples (*i.e.*, protein-bound alanine enrichment). Initially, data were analysed in two different ways, *i.e.*, with and without removing negative FSR data points, which did not significantly affect the results (data not shown). It was decided, however, to keep the negative data points for the analyses because they represented noise around 0 and to avoid skewing the data by shifting the mean FSR values higher.

Descriptive statistics were performed to check for normal distribution (accepted if $p > 0.05$) using the Shapiro-Wilk test, homogeneity of variances by the Levene test (accepted if $p > 0.050$) and outliers by the Rosner's test. Data were transformed (squared root) to comply with ANOVA assumptions. A one-way ANOVA was used to compare T-PRO, T-MID and T-DIS bone sites. *Post-hoc* analysis was performed using Turkey's range test to determine the differences between two bone sites. The effect size Cohen's *d* was estimated for differences between two bone sites, defined as small ($d = 0.20$), moderate ($d = 0.50$) and large ($d = 0.80$). All analyses were performed on RStudio (version 1.4.1717) with packages *tidyverse*, *ggpubr*, *EnvStats*, and *rstatix*. The level of significance was set at $p \leq 0.050$. All data are presented as mean ± 1SD and 95% confidence intervals [95% CI].

5.3. Results

The average body water enrichment was 0.672 ± 0.115 APE in this cohort of old rats. The average ^{2}H incorporation in tibia samples was ~10x fold lower in the old rats (T-PRO, 0.007 ± 0.005 APE; T-MID 0.002 ± 0.003 APE; T-DIS 0.002 ± 0.002 APE), which had a short labelling period of 3 days compared to young rats (T-PRO, 0.099 ± 0.062 APE; T-MID 0.027 ± 0.024 APE; T-DIS 0.013 ± 0.008 APE), which had a longer labelling period of 3 weeks as described in Chapter 4 (section 4.2.1., page 95) (**Figure 18**). Bone collagen FSRs were 0.097 ± 0.068 %·d^{-1} (95% CI [0.084-0.111] %·d^{-1}) at the T-PRO site, 0.033 ± 0.039 %·d^{-1} (95% CI [0.020-0.047]) at the T-MID site, and 0.024 ± 0.033 %·d^{-1} (95% CI [0.011-0.037] %·d^{-1}) at the T-DIS site (**Figure 19**). The three tibial sites had significantly different FSRs ($p < 0.001$, **Figure 20**). Bone collagen FSRs were significantly greater at the T-PRO site compared to T-MID ($p < 0.001$; $d = 1.01$) and T-DIS ($p < 0.001$; $d = 1.13$) sites, but there were no significant differences between T-MID and T-DIS ($p = 0.880$; $d = 0.12$).

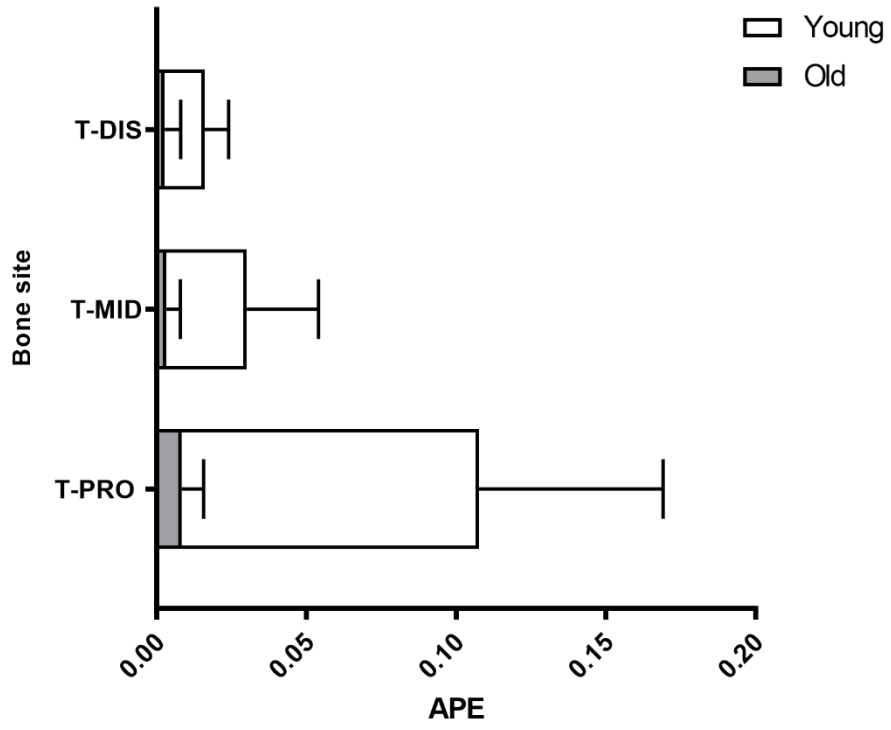


Figure 18. Comparison between protein-bound alanine deuterium enrichment, showed as atom percent excess (APE) after 3 weeks of D_2O labelling in young rats (white) and 3 days of D_2O labelling in old rats (grey), and across three bone sites (tibia proximal, T-PRO; tibia mid-shaft, T-MID; tibia distal, T-DIS).

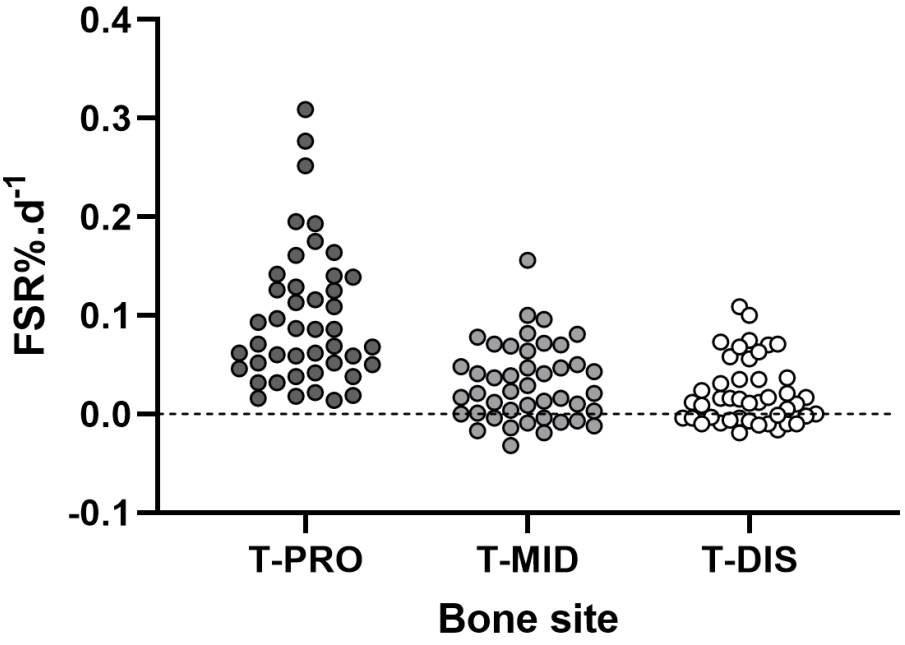


Figure 19. Bone collagen fractional synthetic rates (FSR%·d^{-1}) of old rats across the proximal (T-PRO), mid-shaft (T-MID) and distal (T-DIS) sites of the tibia, individual data including negative values (below dashed line).

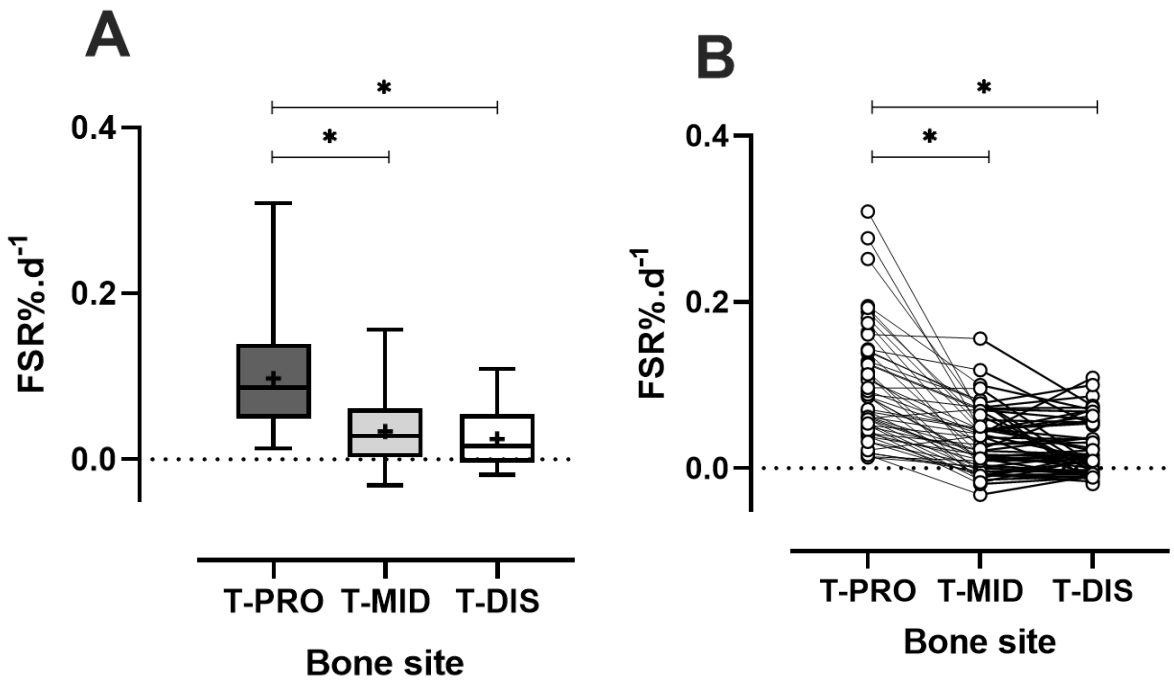


Figure 20. Bone collagen fractional synthetic rates (FSR%·d^{-1}) of old rats across the proximal (T-PRO), mid-shaft (T-MID) and distal (T-DIS) sites of the tibia. A) Data represented as box plots, + represents the mean. B) Individual values. * Post hoc Turkey's test $p < 0.001$.

5.4. Discussion

This Chapter comprises the continuation of the development and optimisation of the method described in Chapter 4, by determining whether low levels of deuterium incorporation are able to quantify bone collagen synthesis at the proximal, mid-shaft, and distal tibial sites in old rats (22 months old). The results showed that in old rats (used herein), which had a 3-day D_2O labelling period, and in young rats (used in Chapter 4), which had a 3-week D_2O labelling period, deuterium body water enrichment was maintained at similar levels (~0.7%). The protein-bound alanine enrichment levels, measured in the proximal, mid-shaft and distal tibia, were, however, ~10x fold lower in the old rats compared to the young rats (**Figure 18**). The very low levels of δ^2H at the different tibial sites of the old rats were not sufficient to adequately quantify bone collagen synthesis at the mid-shaft and distal tibia sites in some animals, indicating that, even when using sensitive GC-*pyrolysis*-IRMS techniques, the resultant data lied close to the limits of detection at the sites with slower bone collagen synthesis rates.

D_2O labelling can safely maintain ^2H body water enrichment for prolonged periods of time (days, weeks, months). In Chapter 4 it was shown that a 3-week labelling period using low levels (<1%) of ^2H body water enrichment was suitable for the quantification of bone collagen synthesis in non-growing young adult rats using sensitive GC-*pyrolysis*-IRMS techniques. Herein, similar levels of ^2H body water enrichment were maintained using a 3-day labelling period in older rats, providing a shorter period to allow ^2H to be incorporated into bone collagen proteins. Results demonstrate that, while GC-*pyrolysis*-IRMS is highly sensitive and was able to detect protein-bound enrichments as little as 0.001 APE (**Figure 18**), the levels of bone δ^2H were insufficient to quantify bone collagen synthesis rates in some animals, which resulted in the estimation of negative bone collagen FSRs values reported at the tibia mid-shaft (in 21% of rats) and distal tibia (in 33% of rats) sites (**Figure 19**). Although a previous study using D_2O tracer techniques combined with LC-MS/MS was able to quantify skull bone synthesis rates in rodents with a 24 h labelling period, they used higher levels (~5 %) of body water enrichment (Cross *et al*., 2020). The lower (<1%) body water enrichment levels used herein are more suitable for human investigations because they reduce the side effects (*e.g*., dizziness, nausea) associated with the

consumption of large doses of D_2O required to achieve higher levels of enrichment. Human studies using D_2O methods with low levels (<1%) of ^2H body water enrichment have measured muscle protein synthesis over 48-72 h periods (Damas *et al*., 2016; Wilkinson *et al*., 2014), but, because bone has a slower turnover than muscle (Smeets *et al.*, 2019), the labelling periods of studies that are interested in measuring human bone collagen synthesis would likely require longer periods of D_2O labelling, and even longer periods (*e.g*., weeks) if using less sensitive mass spectrometry equipment (Wilkinson, 2016).

Despite this short labelling period, most animals did have sufficient, albeit low, δ^2H levels to confidently estimate bone collagen FSRs. For this reason, the subsequent analysis that compared the synthesis rates across tibial sites was carried out as part of this method development study but, because some negative FSRs values were included in this analysis, the results need to be interpreted with caution and taking this factor into consideration. As per Chapter 4, different bone collagen FSRs were quantified at three different sites across tibial bone: proximal tibia, tibial mid-shaft, and distal tibia. Using the same method, differences in bone collagen FSRs between tibial bone sites were detected herein in old rats. The mean synthesis rate was faster at the proximal tibial site compared to the mid-shaft and distal sites (**Figure 20**), confirming the results reported in Chapter 4 in young rats (**Figure 17**, page 106), where the same differences were shown. The different composition of trabecular and cortical bone at the three tibial sites may explain the faster bone collagen synthesis at the proximal tibia, composed of more trabecular bone and with higher bone (re)modelling rates in comparison to the mid-shaft and distal sites of the rat tibia, highly composed of cortical bone (Robling, Castillo, & Turner, 2006; Seeman, 2013). Interestingly, the results reported herein suggest that these differences in bone collagen synthesis rates across tibial sites persist across age and were not influenced by the presence of open growth plates (Wilsman *et al*., 2008), at least in this cohort of old rats.

In conclusion, the results from this study indicate that, the short labelling period (3 days), rather than the low levels of body water enrichment, are the reason why there was insufficient δ^2H at the tibial mid-shaft and distal tibia sites. These results highlight the slow bone turnover rates, particularly at the highly

cortical sites (*e.g.*, mid-shaft and distal tibia) in rodents and suggest that longer D_2O labelling periods than 3 days would be required to allow sufficient time for ^2H to be incorporated into bone proteins and effectively quantify bone collagen synthesis at slow turnover bone sites in old rodents and, potentially, in human populations. This information is relevant for the future applicability of this method and has important implications for the design of animal and human studies that use it.

CHAPTER 6:

THE EFFECTS OF PHENOTYPE, SEX, AND RUNNING TRAINING ON BONE COLLAGEN SYNTHESIS IN YOUNG RATS

6.1. Introduction

Following the development and optimisation of the D_2O method for quantifying bone collagen synthesis (Chapter 4), it was important to determine whether any of the physiological characteristics and interventions measured in these rodent models influenced bone collagen synthesis. One of the main intended uses of this novel method in the future is to be better able to determine the effects of interventions and physiological characteristics on bone collagen synthesis, both in animal and in human models. For example, the LRT and HRT rat model offered the opportunity to investigate if bone collagen synthesis was affected by the acquired phenotype. The development of the LRT and HRT rat model was driven by the hypothesis that aerobic (*i.e.*, endurance) exercise is an effective prescription for enhancing various clinical conditions (Koch & Britton, 2008; Koch, Pollott, & Britton, 2013). This model allows us to study the adaptational components of exercise capacity, rather than the intrinsic components, and their connection to health. This model has already shown that a higher level of aerobic (oxidative) exercise capacity acquired by a higher response phenotype is associated with improved health outcomes (Koch, Pollott, & Britton, 2013).

The bone samples analysed herein are a secondary analysis performed on samples obtained from a prior study primarily designed to investigate training responses following high intensity training across sex and phenotypes, using the LRT and HRT rat model as specified in Chapter 4, section 4.2.1. (page 95). This study was not designed to directly investigate the effects of phenotype, sex, and training on bone; as such, the running training intervention was not designed to maximise osteogenic responses. These three variables, however, have important implications for bone health. The potential effects of exercise on bone remodelling are described in Chapter 2 section 2.7. (page 40) and 2.8. (page 43), and the importance of sex and genetics (which determines the phenotype) for bone health is detailed in Chapter 2 section 2.2. (page 9).

As such, the aim of this study was to conduct a secondary analysis investigating the differences in bone collagen synthesis between (i) low and high responders to endurance running training, (ii) males and

females, and (iii) trained and control, in young rats. Given the findings regarding the differences across bone sites (section 4.3., page 104), these three differences were investigated and analysed separately in the femur diaphysis, tibial proximal metaphysis-epiphysis, tibial mid-shaft diaphysis, and tibial distal metaphysis-epiphysis.

6.2. Methods

As described in Chapter 4, section 4.2.1. (page 95), femur and tibia bones were derived from young, male and female rats artificially selected for yielding low (LRT) or high (HRT) responses to endurance running training (Koch, Pollott, & Britton, 2013). Analyses of the *Young cohort* of rats are described in this chapter.

6.2.1. Animals and intervention

Forty-six ~9 month old rats were included in the *Young cohort* (**Table 11**, page 97). During a 7-week intervention period, rats of both phenotypes were divided into trained and control groups, where rats in the trained groups underwent interval running training on a treadmill as detailed in section 4.2.1. (page 95). Briefly, interval running training consisted of a warm-up for 5 min at 50 – 60% of maximum speed (individual speed for each rat). This was followed by a 15 min session that comprised 3 x 3 min bouts of running at 85–90% maximum speed, which were interspersed by 2 min recovery periods at 50% maximum speed, with 15° uphill inclination. Training was completed three times per week with a 1-day rest period between each session. Rats in the control groups were kept in the same conditions as the exercising rats but did not complete any training during the 7-week intervention period. During the last 3 weeks of the 7-week intervention period rats received a gavage of 7.2 ml·kg BW^{-1} 70% D_2O and drinking water was enriched to 2% D_2O to maintain body-water enrichment. Blood samples (~5 ml) were collected at necropsy and plasma was separated by centrifugation and stored frozen at –20ºC until analysis. Baseline blood (non-enriched) was collected from a different group of rats that did not receive D_2O.

6.2.2. Bone sample collection and isolation and derivatisation of bone collagen protein

Left femur and tibia bones were rapidly exposed after necropsy, removed, and immediately frozen by complete immersion in liquid nitrogen and were kept frozen until analysis. Bone samples were demineralised, and collagen proteins were isolated and then hydrolysed to free amino acids using the processes listed in section 4.2.2. (page 99). Amino acids were then derivatised as their N-methoxycarbonyl methyl esters as described in section 4.2.3. (page 102).

6.2.3. GC-MS/MS body water enrichment analyses and GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates

Body water enrichment was measured in plasma by GC-MS/MS as described in section 4.2.4. (page 102). Protein-bound alanine enrichment was determined by GC-*pyrolysis*-IRMS, and bone collagen fractional synthetic rates (FSRs) were calculated as described in section 4.2.5. (page 103).

6.2.4. Statistical analyses

Each bone site was considered as an independent data set with potential independent effects. Descriptive statistics were performed for data sets to check for normal distribution (accepted if $p > 0.05$) using the Shapiro-Wilk test and homogeneity of variances by the Levene test (accepted if $p > 0.050$). Data sets for the T-MID and the T-DIS were non-normally distributed, so both were log base (log10) transformed to achieve a normal distribution prior to analysis. The data set for the FEM had unequal variances and was therefore log base (log10) transformed to achieve homogeneity of variances prior to analysis. Rosner's test was used to identify extreme outliers. One outlier was identified at the T-DIS site, although a decision was made not to remove it from analysis because it was considered within the possible physiological range. Instead, a Robust three-way ANOVA test was used to analyse the data from this site to take account of the potential outlier.

Differences in bone collagen FSRs at the FEM, T-PRO, and T-MID sites were determined using three-way (phenotype x sex x training effect) ANOVA tests. The same analysis was conducted at the T-DIS

site using a Robust three-way ANOVA test due to the presence of one outlier. All data are presented as mean ± 1SD and 95% confidence intervals [95% CI]. The accepted level of significance was set at $p \leq 0.050$. The effect size generalised eta squared (η^2_g), defined as small ($\eta^2_g = 0.01$), medium ($\eta^2_g = 0.06$), and large ($\eta^2_g = 0.14$) effects was estimated for ANOVA analyses. All analyses were performed on RStudio (version 1.4.1717) with packages *tidyverse*, *ggpubr*, *EnvStats*, *rstatix* and *WRS2*.

6.3. Results

There was a significant large main effect of phenotype at the femur site (**Figure 21**; $F_{[1, 32]} = 28.73$; $p < 0.001$; $\eta^2_g = 0.473$), where HRT rats showed greater bone collagen FSRs than LRT rats (HRT 0.178 ± 0.080 [95% CI 0.152-0.205] %·d^{-1}; LRT 0.079 ± 0.026 [95% CI 0.051-0.107] %·d^{-1}). There were no other significant interactions or main effects at the femur site.

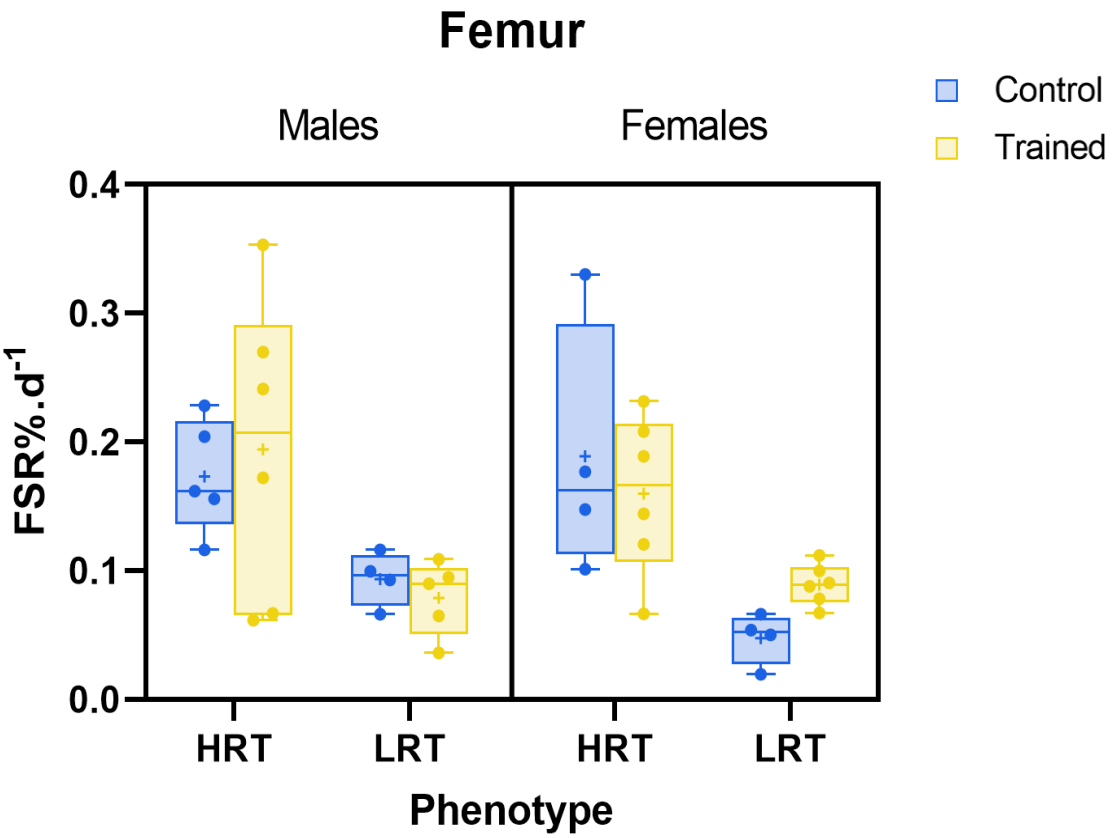


Figure 19. Comparison of femur diaphysis bone collagen synthesis rates (FSR%·d^{-1}) between phenotypes of high (HRT) and low (LRT) responders to endurance running training ($p < 0.001$), male and female ($p = 0.420$), and trained and control ($p = 0.627$) in young rats. Data shown as raw values (*i.e.*, prior to any transformation). *p* value represents main effects.

There was a significant large main effect of phenotype ($F_{[1, 36]} = 7.77$; $p = 0.008$; ($\eta^2_g = 0.178$) and a significant large main effect of sex ($F_{[1, 36]} = 8.75$; $p = 0.005$; $\eta^2_g = 0.196$) at the proximal site of the tibia **(Figure 22**). Rats from the HRT phenotype showed greater bone collagen FSRs than rats from the LRT phenotype (HRT 0.248 ± 0.136 [95% CI 0.198-0.298] %·d^{-1}; LRT 0.159 ± 0.091[95% CI 0.109-0.209] %·d^{-1}), and male rats also showed greater bone collagen FSRs compared to female rats (males 0.250 ± 0.131[95% CI 0.199-0.301] %·d^{-1}; females 0.161 ± 0.101[95% CI 0.112-0.210] %·d^{-1}). There were no other significant interactions or main effects at the proximal tibia site.

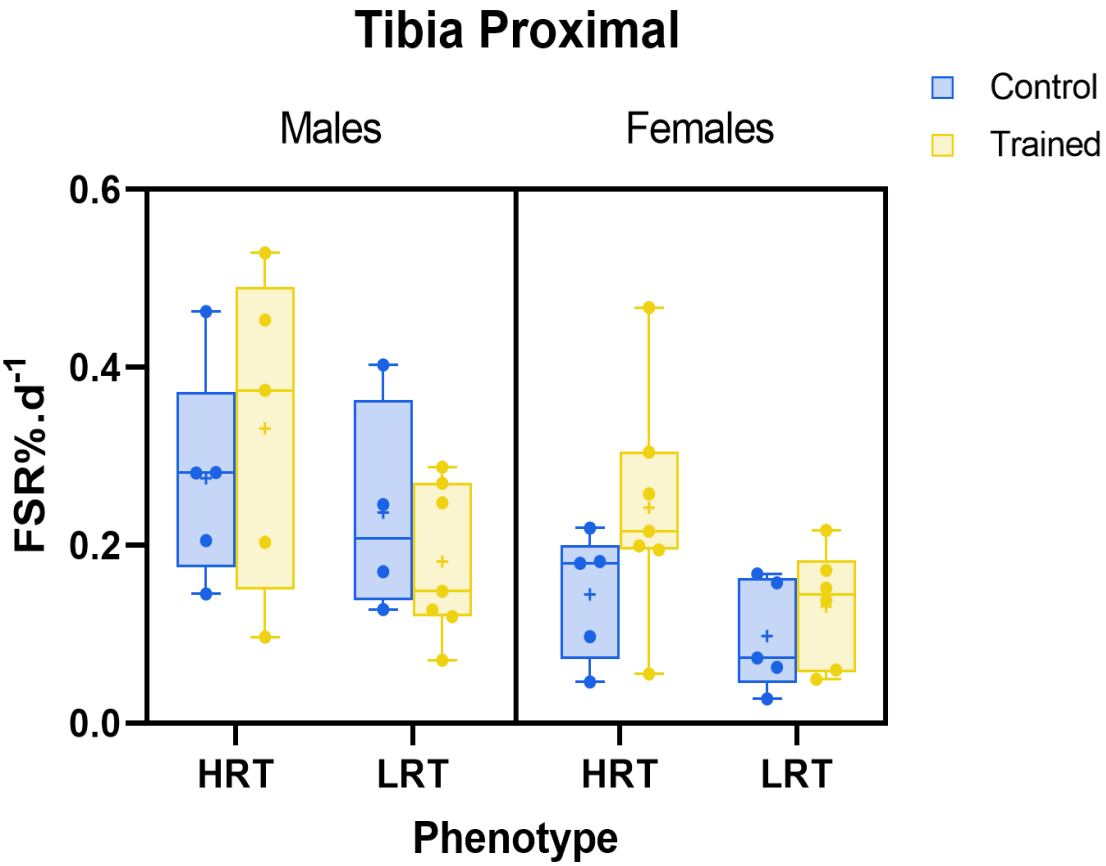


Figure 20. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) on the proximal tibia (T-PRO) between phenotypes of high (HRT) and low (LRT) responders to endurance running training ($p = 0.008$), male and female ($p = 0.005$), and trained and control ($p = 0.296$) in young rats. p value represents main effects.

There was a significant large main effect of training at the mid-shaft site of the tibia (**Figure 23**; $F_{[1, 37]}$ = 7.01; p = 0.012; η^2_g = 0.159), with rats that underwent running training having greater bone collagen FSRs than control rats (trained 0.066 ± 0.055 [95% CI 0.047-0.085] %·d^{-1}; control 0.039 ± 0.036 [95% CI 0.017-0.062] %·d^{-1}). There were no other significant interactions or main effects at the mid-shaft tibial site.

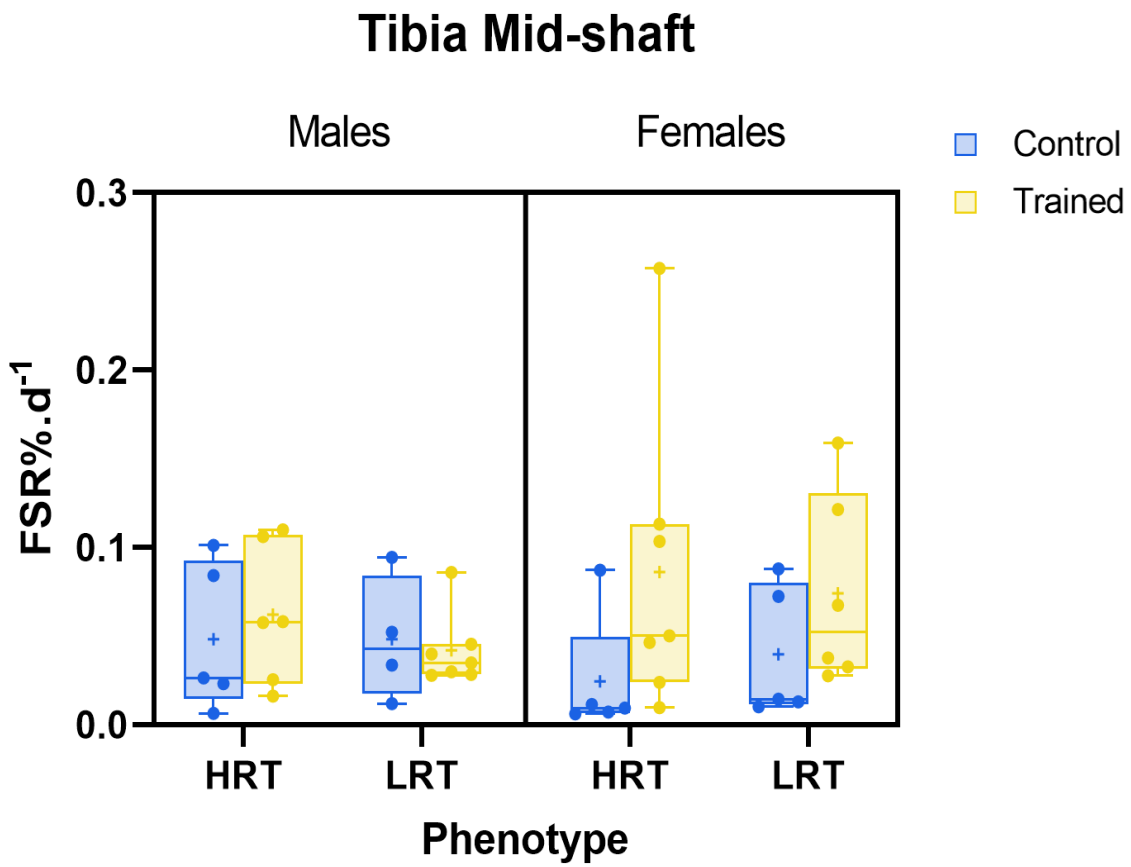


Figure 21. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the tibial mid-shaft (T-MID) between phenotypes of high (HRT) and low (LRT) responders to endurance running training (p = 0.617), male and female (p = 0.670), and trained and control (p = 0.012) in young rats. Data shown as raw values (*i.e.*, prior to any transformation). p value represents main effects.

Similarly, there was a significant large main effect of training at the distal site of the tibia (**Figure 24**; $F_{[1, 36]}$ = 6.94; p = 0.050; η^2_g = 0.156), with rats in the running training group having greater bone collagen FSRs compared to rats in the control group (trained 0.031 ± 0.017 [95% CI 0.026-0.037] %·d^{-1}; control 0.020 ± 0.010 [95% CI 0.014-0.027] %·d^{-1}). There were no other significant interactions or main effects at the distal tibia site.

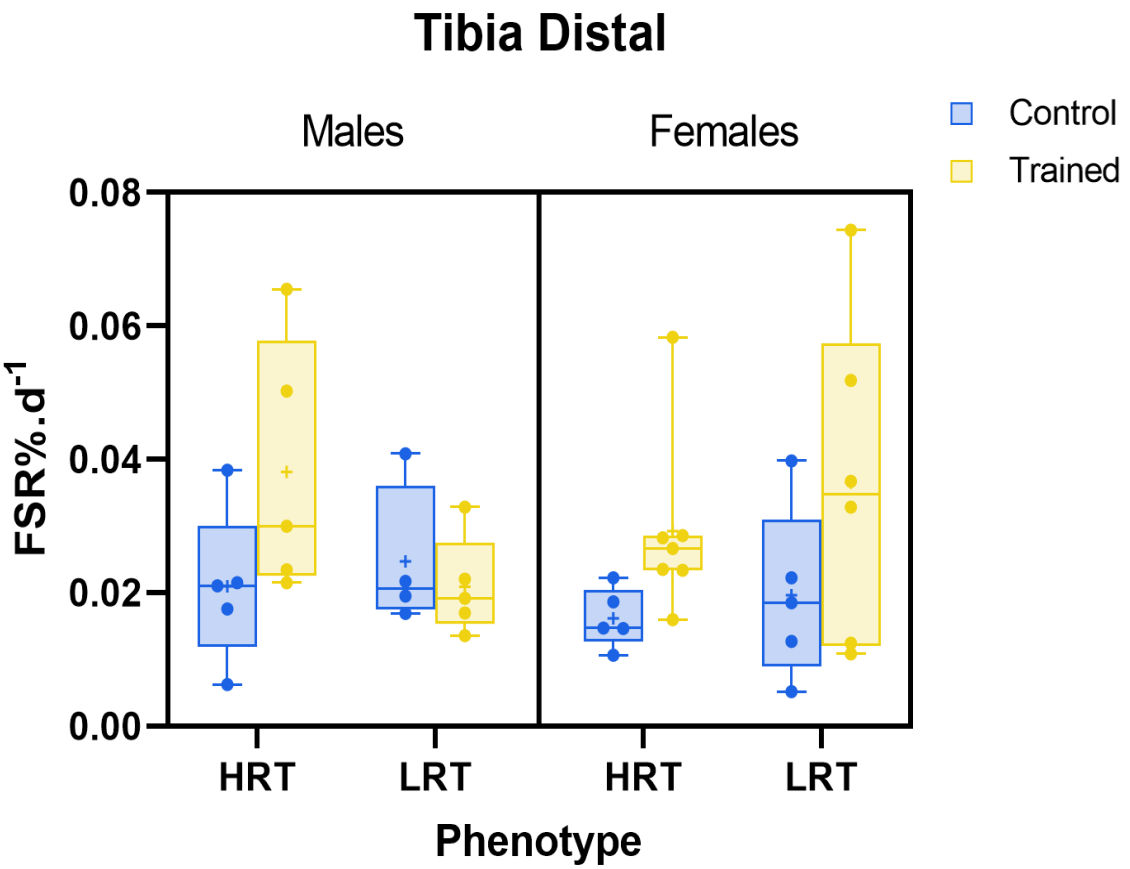


Figure 22. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the distal tibia (T-DIS) between phenotypes of high (HRT) and low (LRT) responders to endurance running training (p = 0.930), male and female (p = 0.503), and trained and control (p = 0.050) in young rats. Data shown as raw values (*i.e.*, prior to any transformation). p value represents main effects.

6.4. Discussion

In this study the effects of phenotype, sex, and running training on bone collagen synthesis in 9-month-old (36 weeks) rats were investigated by using the deuterium oxide stable isotope tracer method developed in Chapter 4. This is the first study to report the effects of these physiological differences on bone collagen synthesis using the direct incorporation of stable isotopes in rat femoral and tibial bones. The effects of these three variables (phenotype, sex, and running training) on bone collagen synthesis differed depending upon the site of measurement (femur diaphysis, proximal tibia, tibia mid-shaft, and distal tibia). Compared to LRT rats, rats from the HRT phenotype showed greater bone collagen synthesis rates at the femur diaphysis and proximal tibia. Likewise, running training increased bone

collagen synthesis rates at the tibial mid-shaft and distal tibia. Male rats had greater bone collagen synthesis rates than female rats, but only at the proximal tibia.

Herein, main effects of phenotype on bone collagen synthesis were reported at the femur diaphysis and proximal tibia; with high responders to endurance running training (HRT) rats showing greater bone collagen FSRs compared to low responders to endurance running training (LRT) rats. This evidence indicates that having a heritability trait of high trainability to endurance training may induce greater bone formation and/or greater overall bone remodelling independently of sex and training on these two bone sites. In contrast, there were no significant phenotype effects on the mid-shaft and distal tibial sites, which agrees somewhat with a recent study that also measured bone synthesis rates in growing mice (12 weeks old) using a similar D_2O method and reported similar values between inherently high and low active inbred mice (both groups showing bone FSRs of ~0.035 $\%\cdot d^{-1}$), albeit in the skull rather than in the limbs (Cross *et al*., 2020). That said, the skull and the tibial diaphysis are comprised of predominantly cortical bone and, taken together, the results of these two studies suggest that genetic or hereditary factors might influence this type of bone to a lesser extent than trabecular bone (*e.g*., the predominant type of bone found in the proximal tibia). In support of this assertion, Paternoster *et al*. (2013) showed that cortical and trabecular human bone are affected differently and in a site-specific manner by genetic determinants, and Judex *et al*. (2004) reported more evident differences between genetic variations in trabecular regions than in cortical regions in mice.

The effects of HRT and LRT phenotypes on musculoskeletal tissues are not fully understood, with previous studies using the same rat model to investigate skeletal muscle morphology and physiology showing mixed results. Ahtiainen *et al*. (2018) failed to show differences on skeletal muscle morphological characteristics between HRT and LRT rats in control and resistance trained (ladder-climbing) conditions. Two subsequent studies, however, reported impaired skeletal muscle hypertrophy in response to functional overload (West *et al*., 2021) and exacerbated atrophy after immobilisation (Thompson *et al*., 2022) in LRT compared to HRT rats. The present study is the first to report HRT and LRT phenotype differences in bone tissue. Further research exploring the phenotype effects on bone

and other musculoskeletal tissues with this rat model may help the understanding of musculoskeletal adaptations of highly trained individuals, such as endurance athletes.

Animal and human skeletal sexual dimorphism (*e.g.*, greater bone size and bone mass in males) becomes more prominent with growth and sexual maturation (Hendrickx, Boudin, & Van Hul, 2015). Herein sex differences on bone collagen synthesis were only evident at the tibial proximal site and not at the distal tibia or the tibial mid-shaft. In addition, there were no sex differences on bone collagen synthesis at the femur. Even though this study was conducted in adult (non-growing) rats, the potential presence of open growth plates in the proximal tibial epiphyses (Wilsman *et al*., 2008) of these rats may explain the greater collagen FSRs in males compared to females at this bone site. The collagen synthesis reported on the proximal tibia may reflect the synthesis of collagen of cartilage from growth plates, indicating greater bone growth in male rats due to the evident sex differences during maturation.

Conversely, research has suggested that animal and human sex-related differences in bone structural adaptations (measured by DXA and pQCT) to exercise/loading exist due to differences in the hormonal environment (*e.g.*, oestrogen) (Callewaert *et al*., 2010). Whilst some studies have shown blunted responses to running activity at the femur diaphysis in adult female rats compared to adult males (Järvinen *et al*., 2003) and at the tibial diaphysis in mice (Wallace *et al*., 2007), these outcomes are not supported by the data presented herein, where, although running training positively affected bone collagen synthesis, there was no effect of sex on bone collagen synthesis at the tibial mid-shaft and distal tibia. This fact indicates that sex differences in bone mineral adaptations may not be due to variances in bone formation (by the newly synthesised collagen) across sexes but may be influenced by other factors such as overall bone remodelling balance. Unfortunately, human research directly comparing dynamic measures of bone metabolism (*e.g.*, using bone (re)modelling markers) in males and females is lacking and should be urgently addressed in future investigations.

Bone adaptations to loading are a local phenomenon, as shown in human (Bass *et al*., 2002; Herbert *et al*., 2021) and animal studies (Miller *et al*., 2021; Sugiyama, Price, & Lanyon, 2010); these adaptations

generally occur on the shaft of long bones, with increases in cortical periosteal apposition (Miller *et al*., 2021; Wang *et al*., 2009). Somewhat in agreement, herein, rats completing a 7-week interval running training programme reported greater bone collagen synthesis rates than control rats at the mid-shaft and distal sites of the tibia, which suggests that the training intervention had an osteogenic effect by increasing formation of newly synthesised bone matrix at these two sites. Despite high variability in bone collagen FSRs, especially in the trained rats (**Figures 23** and **24**), these effects were statistically large for this sample. Evidence from the mineral adaptations of bone have shown similar outcomes. A study in young growing rats (3 weeks old) showed increased BMC (measured by DXA) at the middle and distal tibial sites, but not at the proximal tibia after a 7-week exercise intervention, where the rats ran on a treadmill (Iwamoto *et al*., 2004). These site-specific osteogenic effects from running may be due to differences in cortical and trabecular bone adaptations to loading (Hagino *et al*., 2001). Indeed, a recent study in mice (14 weeks old), which applied controlled mechanical loading for 4 weeks, reported small or no effects on trabecular bone (at the proximal tibia site) and greater effects on cortical bone (at the tibia diaphysis), particularly at the periosteal surface (Robinson, Shyu, & Guo, 2021). Differences in loading magnitude may also be reflected in bone adaptations; although these are usually studied using artificial loading interventions, which have reported higher loading magnitudes in the proximal tibia and lower magnitudes in the more distal tibial sites (Miller *et al*., 2021; Sugiyama *et al*., 2012). Due to the lack of methods for measuring loads across bone sites during live exercise (Meakin, Price, & Lanyon, 2014), the site-specific loading characteristics of running and if/how this factor may have influenced the results reported herein remain unknown. In addition, the exercise intervention (*i.e*., 7 weeks of interval running training) used herein was not designed to optimise bone osteogenic adaptations because this study is part of a secondary analysis of a previous study with a different purpose. Other exercise-based interventions may produce different/greater responses on bone collagen synthesis that can be studied using the D_2O method developed herein.

A limitation of this study was that histological analyses were not performed on the bone samples. As such, it is not possible to determine exactly whether the significant effects reported herein denote differences between cortical and trabecular bone. Future studies should consider including these

analyses and determining the effects of non-modifiable factors, such as sex and genetic profile, and interventions, such as exercise and drugs, on separate components of trabecular and cortical bone given their distinct material and biological properties. Bone samples for all sites could have included both trabecular and cortical bone, which could potentially have influenced some effects; particularly in the femur diaphysis and the proximal tibial sites, which would have a greater mixture of trabecular and cortical bone (Gao *et al*., 2019). Moreover, small sample sizes and high variability in the data were present in this study; further research confirming the outcomes reported here would provide more confidence in the results.

6.4.1. Conclusions

Bone collagen synthesis reflects the formation of newly synthesised bone matrix during the bone (re)modelling cycle. The different physiological effects (phenotype, sex and running training) on bone collagen synthesis shown at four bone sites (femur diaphysis, proximal tibia, tibial mid-shaft and distal tibia) in this study indicate that these variables affect bone formation in a site-specific manner. This evidence further emphasises, as discussed in Chapter 4, the importance of investigating bone (re)modelling on a site-specific basis and the limitations of using whole-body indicators (*e.g*., whole-body BMD, bone markers). Whilst non-modifiable factors had effects on bone collagen synthesis at the femur diaphysis (phenotype) and proximal tibia (phenotype and sex), interval running training had a positive effect on bone collagen synthesis at the tibial mid-shaft and distal sites.

Further animal and human research combining stable isotope techniques and histology, using larger sample sizes, could provide further insight into physiological effects on bone collagen synthesis on trabecular and cortical bone sites. Studies focusing on the bone adaptations to exercise should explore how other types of exercise regimens (*e.g*., shorter/longer interventions, short bouts of running or jumping) affect bone formation and collagen synthesis at different sites. It remains unknown whether site-specific bone collagen synthesis changes are linked to changes on the bone mineral structure at the same bone sites.

CHAPTER 7:

THE EFFECTS OF AGE, PHENOTYPE, SEX, AND RUNNING TRAINING ON BONE COLLAGEN SYNTHESIS IN OLD RATS

7.1. Introduction

Age is a significant mediating factor in bone health and bone (re)modelling, as described in Chapter 2 section 2.2. (page 9). With ageing, there is a decrease in bone mass causing weakening of the bone, which can lead to reduced bone strength and disease (*e.g.*, osteoporosis, fragility fractures) (Hendrickx, Boudin, & Van Hul, 2015). Animal and human studies have usually determined ageing-associated changes in bone remodelling by focusing upon the mineral compartment of bone tissue using imaging (Duan *et al*., 2001; Riggs *et al*., 2004; Willinghamm *et al*., 2010) and histological (Ferguson *et al*., 2003; Glatt *et al*., 2007; Parfitt *et al*., 1995) techniques. Increased bone resorption (Seeman, 2002), diminished bone formation (Parfitt *et al*., 1995), and altered collagen crosslinking (Saito & Marumo, 2010) have been reported in older populations.

No *in vivo* studies have examined the differences between bone collagen synthesis in young and old populations using the direct incorporation of stable isotopes. Bone collagen synthesis rates of young rats were quantified using the D_2O tracer developed in the study described in Chapter 4. This method was subsequently used in older rats from the same rat model in the study described in Chapter 5. Therefore, differences in bone formation across age could be determined by comparing bone collagen synthesis rates between these two cohorts (*i.e.*, young vs old rats), where older rats were expected to have slower bone formation rates. Furthermore, and like in Chapter 6, the rat model and training intervention of the *Old cohort* allows an investigation of whether the differences between LRT and HRT phenotypes, male and female, and running training influence bone collagen synthesis in these rats.

The first aim of this study was to conduct a secondary analysis comparing the bone collagen synthesis rates at three tibial sites between a young and an old cohort of rats. The following aims of this study were to conduct a secondary analysis investigating the differences on bone collagen synthesis between (i) low and high responders to endurance running training, (ii) males and females, and (iii) trained and control old rats. Given the results regarding the differences across bone sites in the old cohort of rats

(Chapter 5), these three effects were independently investigated in the proximal metaphysis-epiphysis of the tibia, the tibial mid-shaft diaphysis, and the tibial distal metaphysis-epiphysis.

7.2. Methods

As described in Chapter 4 (section 4.2.1., page 95) and Chapter 5 (section 5.2.1., page 111), tibial bones were derived from young and old rats and from male and female rats artificially selected for yielding low (LRT) or high (HRT) responses to endurance running training (Koch, Pollott, & Britton, 2013) and analysed as a secondary analysis in this study.

7.2.1. Animals

7.2.1.1. Young cohort

Forty-six ~9-month-old rats were included in the *Young cohort* (**Table 11**, page 97), as detailed in section 4.2.1. (page 95). During a 7-week intervention period, rats of both phenotypes were divided into trained and control groups, only the control groups were included in this study for the comparison between young and old rats because the running interventions differed between cohorts. Rats received a gavage of 7.2 ml·kg BW^{-1} 70% D_2O during the last 3 weeks of the 7-week intervention period and drinking water was enriched to 2% D_2O to maintain body-water enrichment. Blood samples were collected at necropsy (~5 ml) and plasma was separated by centrifugation and stored frozen at –20ºC until analysis. A baseline blood sample (non-enriched) was collected from a different group of rats that did not receive D_2O.

7.2.1.2. Old cohort

Fifty-five, male and female, ~22 months old rats were included in the *Old cohort* (**Table 12**, page 112) Rats from LRT and HRT phenotypes were divided into trained and control groups, where rats in the trained groups underwent interval running training on a treadmill for 16 weeks as detailed in section 5.2.1. (page 111). Briefly, rats were first familiarised with treadmill running for one week before they were tested for maximal treadmill running capacity as previously described (Koch, Pollott, & Britton,

2013). All rats (control and trained) ran on a motor driven treadmill set at a constant grade of 15° and an initial speed of 10 $m \cdot min^{-1}$. Speed was progressively increased by 1 $m \cdot min^{-1}$ every 2 min until exhaustion (*i.e.*, third time a rat remained on the shock grid for 2 s). After the familiarisation period, rats in the trained groups underwent moderate running training on a treadmill 2-3 times per week at 60% of their maximum tested running speed during a 16-week intervention period (37 trials in total). The treadmill speed started at 10 $m \cdot min^{-1}$ in session one and increased by 1 $m \cdot min^{-1}$ every other session up to a maximum speed of 21 $m \cdot min^{-1}$. The duration for each training session was set for 20 min in the first trial and incremented by 0.5 min in each subsequent session. The slope of the treadmill was held constant at 15°. Rats in the control groups were placed on a static treadmill for 5 minutes 3 days per week to simulate the environmental change of handling. Post-intervention, rats from control and trained groups were tested for maximal treadmill running distance as an indicator of exercise capacity. Rats were sacrificed two days after their last exercise session. Rats received a gavage of 7.2 $ml \cdot kg\ BW^{-1}$ 70% D_2O for 3 days at the end of the of the 16-week intervention period. A blood sample was taken ~2 hours after the D_2O bolus to calculate peak enrichment. A final blood sample was collected at necropsy three days later at necropsy and plasma was separated by centrifugation and stored frozen at –20ºC until analysis. A baseline blood sample (non-enriched) was collected from a different group of rats that did not receive D_2O.

7.2.2. Bone samples collection and isolation and derivatisation of bone collagen proteins

Tibial bones of the *Young* (Chapter 4) and *Old* (Chapter 5) rats were rapidly removed after necropsy and immediately frozen by complete immersion in liquid nitrogen and kept frozen at −80°C until analysis. Bone samples were demineralised, and collagen proteins were isolated and then hydrolysed to free amino acids using the processes listed in Chapter 4 section 4.2.2. (page 99) and Chapter 5 section 5.2.2. (page 113). Amino acids were then derivatised as their N-methoxycarbonyl methyl esters as described in section 4.2.3. (page 102) and 5.2.2. (page 113).

6.2.3. GC-MS/MS body water enrichment analyses and GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates

Body water enrichment was measured in plasma by GC-MS/MS as described in section 4.2.4. (page 102). Protein-bound alanine enrichment was determined by GC-*pyrolysis*-IRMS, and bone collagen fractional synthetic rates (FSRs) were calculated as described in section 4.2.5. (page 103).

7.2.3. GC-MS/MS body water enrichment analyses and GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates

Body water enrichment was measured in plasma by GC-MS/MS as described in sections 4.2.4 (page 102) and 5.2.3. (page 114). Protein-bound alanine enrichment was determined by GC-*pyrolysis*-IRMS and bone collagen fractional synthetic rates (FSRs) were calculated as described in sections 4.2.5. (page 103) and 5.2.4. (page 114).

7.2.4. Statistical analyses

Each bone site was considered as an independent data set with potential independent effects. As detailed in Chapter 5 (section 5.2.5., page 115), some data points from this study showed negative FSR values (**Figure 19**, page 117), but it was decided to include these data points (reflecting data points outside the limit of detection for protein-bound enrichment due to insufficient ^{2}H incorporation into the bone samples). Descriptive statistics were performed to check for normal distribution (accepted if $p > 0.05$) using the Shapiro-Wilk test, homogeneity of variances by the Levene test (accepted if $p > 0.050$), and outliers by the Rosner's test.

Comparisons between bone collagen FSRs in young and old rats were investigated using unpaired samples non-parametric *t*-test (Wilcoxon test), because data sets included outliers (T-PRO and T-MID) and/or were not normally distributed (T-MID and T-DIS). For these age comparisons, only samples from the control groups ($n = 44$) were included; due to differences in the running interventions between the *Young* and *Old* cohorts it was not appropriate to include trained rats in this analysis. Differences in

bone collagen FSRs at the T-PRO, T-MID, and T-DIS sites in old rats were determined using three-way (phenotype x sex x training effect) ANOVA tests. *Post-hoc* analysis was performed using Bonferroni multiple pairwise comparisons. The data sets for T-PRO, T-MID, and T-DIS did not include outliers, but T-PRO and T-MID were not normally distributed and were therefore transformed (log10 and squared root) to achieve normal distribution prior to ANOVA analyses.

All data are presented as mean ± 1SD and 95% confidence intervals [95% CI]. The accepted level of significance was set at $p \leq 0.050$. The effect size generalised eta squared (η^2_g), defined as small (η^2_g = 0.01), medium (η^2_g = 0.06), and large (η^2_g = 0.14) effects, was estimated for ANOVA analyses. The effect size Cohen's *d* was estimated for differences between two variables, defined as small (*d* = 0.20), moderate (*d* = 0.50) and large (*d* = 0.80). All analyses were performed on RStudio (version 1.4.1717) with packages *tidyverse*, *ggpubr*, *EnvStats*, *rstatix* and *WRS2*.

7.3. Results

There was a large significant difference between young and old rats (control groups) at the proximal tibia (p = 0.006; d = 0.95; **Figure 25**), where old rats showed lower bone collagen FSRs (0.101 ± 0.066 %·d^{-1}; 95% CI [0.065-0.137] %·d^{-1}) compared to young rats (0.186 ± 0.114 %·d^{-1}; 95% CI [0.145-0.228] %·d^{-1}). There were no significant differences in bone collagen FSRs between young and old rats at the tibial mid-shaft (p = 0.835; d = 0.08; **Figure 25**) or at the distal tibia (p = 0.670; d = 0.39).

There were no significant effects of phenotype, sex, or running training at the proximal tibia (**Figure 26**). There was a significant main effect of sex ($F_{[1, 34]}$ = 5.40; p = 0.026; η^2_g = 0.137) at the mid-shaft tibial site (**Figure 27**), with male rats showing greater bone collagen FSRs compared to female rats (males 0.220 ± 0.092 [95% CI 0.184-0.256] %/d^{-1}; females 0.167 ± 0.074 [95% CI 0.129-0.205] %/d^{-1}). In addition, there was a significant two-way interaction of phenotype x training ($F_{[1, 34]}$ = 4.62; p = 0.039; η^2_g = 0.120) at the tibial mid-shaft, although *post-hoc* analysis showed no further significant differences (p = 0.075; η^2_g = 0.081). There were no other significant interactions or main effects at the

mid-shaft tibial site. There was a significant two-way interaction of sex x phenotype ($F_{[1, 46]} = 5.33$; $p = 0.026$; $\eta^2_g = 0.104$) and a significant two-way interaction of phenotype x training ($F_{[1, 46]} = 31.61$; $p < 0.001$; $\eta^2_g = 0.407$) at the distal tibia (**Figure 28**). *Post-hoc* analysis showed a significant difference ($p = 0.048$; $d = 0.81$) between male (0.040 ± 0.039 [95% CI 0.019-0.060] %·d^{-1}) and female (0.013 ± 0.028 [95% CI -0.004-0.030] %·d^{-1}) rats of the LRT phenotype group, but not the HRT group ($p = 0.282$; $d = 0.41$); and significant differences between control and trained rats for both HRT ($p < 0.001$; $d = 2.48$) and LRT ($p = 0.004$; $d = 0.99$) phenotypes. Within the HRT group ($p < 0.001$; $d = 2.48$) trained rats (0.001 ± 0.011 [95% CI -0.013-0.014] %·d^{-1}) had lower bone collagen FSRs at the distal tibia than control rats (0.051 ± 0.027 [95% CI 0.037-0.065] %·d^{-1}); and within the LRT group ($p = 0.004$; $d = 0.99$) trained rats (0.037 ± 0.034 [95% CI 0.023-0.052] %·d^{-1}) had higher bone collagen FSRs than the LRT control rats (0.006 ± 0.029 [95% CI -0.001-0.022] %·d^{-1}). There were no other significant interactions or main effects at the distal tibial site.

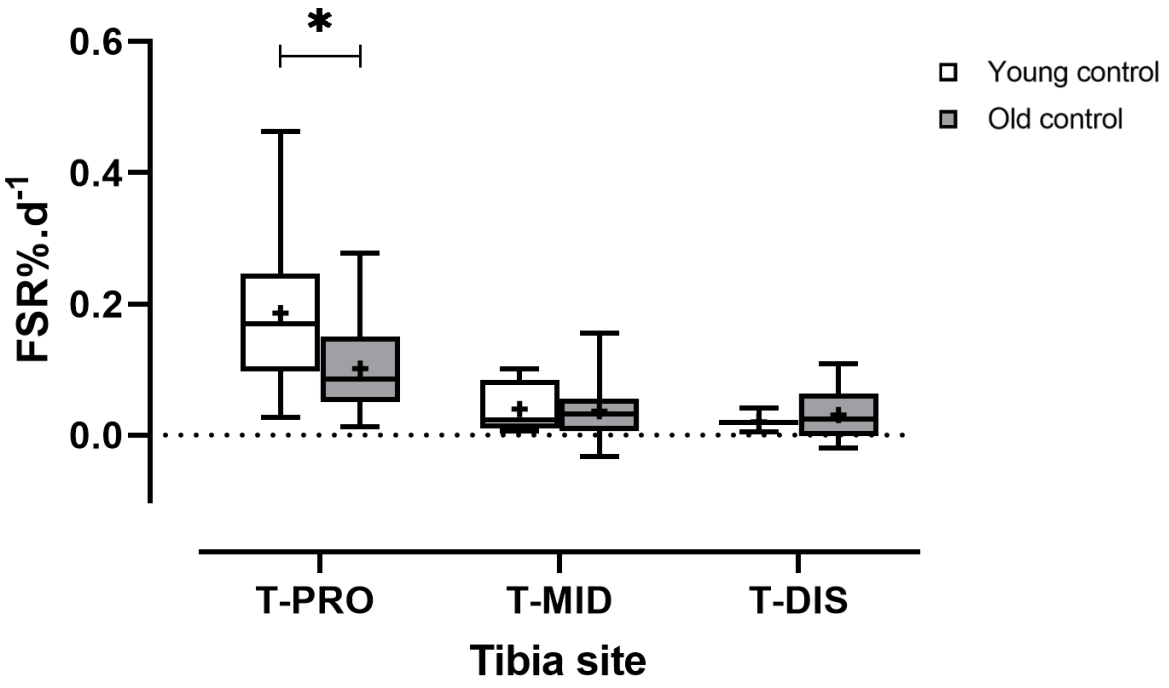


Figure 23. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) on the tibia proximal (T-PRO), mid-shaft (T-MID), distal (T-DIS) tibia sites between young (white) and old (grey) rats. Data represented as box plots, + represents mean. * Wilcoxon unpaired samples t-test $p = 0.006$.

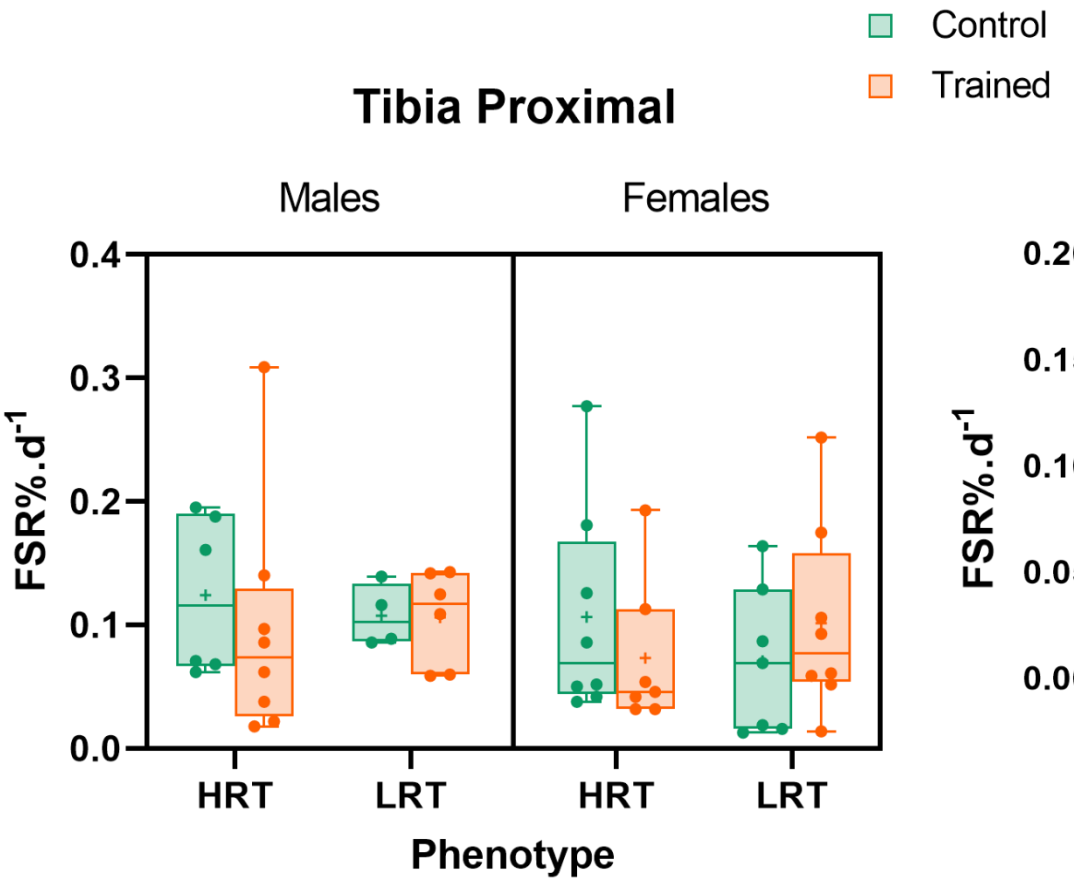


Figure 24. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the proximal tibia (T-PRO) between phenotypes of high (HRT) and low (LRT) responders to endurance running training (p = 0.943), male and female (p = 0.127), and trained and control (p = 0.619) in young rats. Data shown as raw values (*i.e.*, prior to any transformation). p value represents main effects.

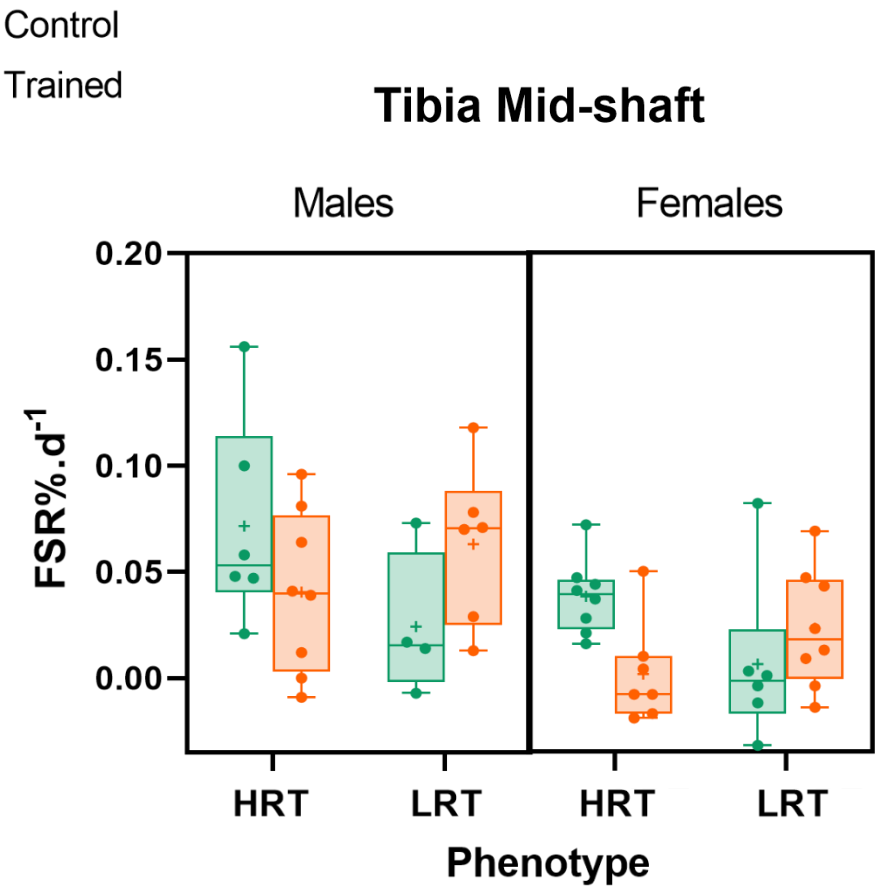


Figure 25. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the mid-shaft of the tibia (T-MID) between phenotypes of high (HRT) and low (LRT) responders to endurance running training (p = 0.786), male and female (p = 0.026), and trained and control (p = 0.702) in young rats. Data shown as raw values (*i.e.*, prior to any transformation). p value represents main effects.

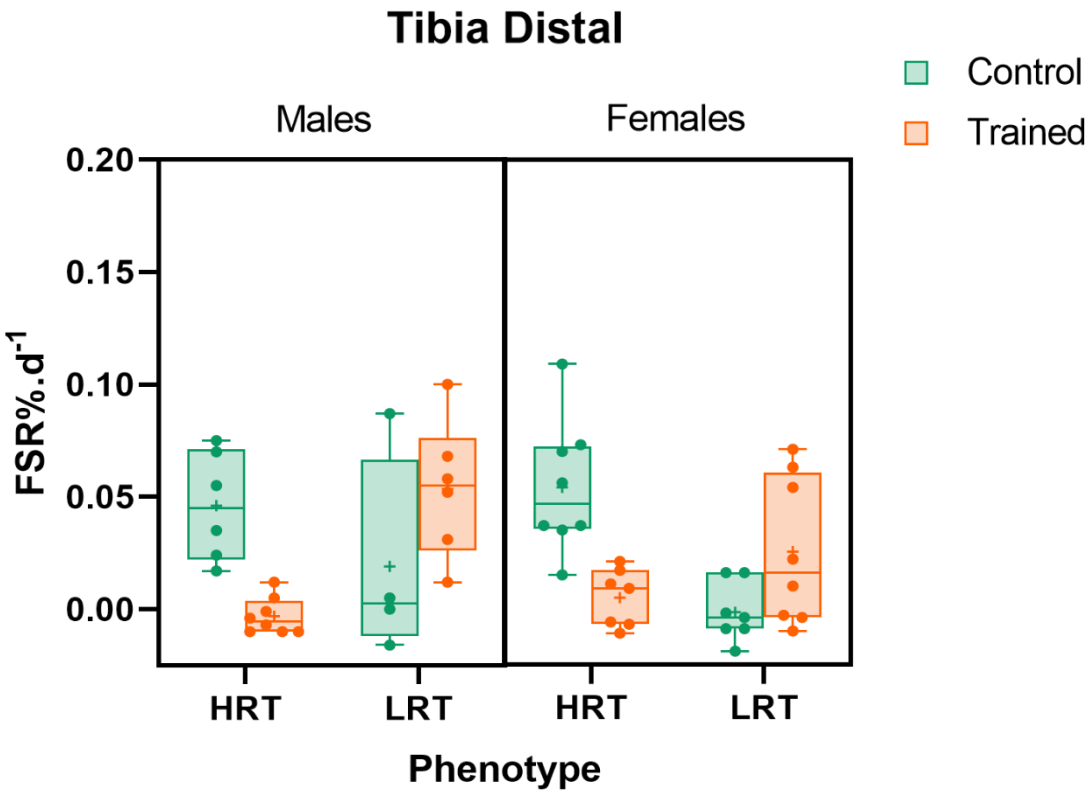


Figure 26. Comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the distal tibia (T-DIS) between phenotypes of high (HRT) and low (LRT) responders to endurance running training (p = 0.987), male and female (p = 0.328), and trained and control (p = 0.077) in young rats. Data shown as raw values (*i.e.*, prior to any transformation). p value represents main effects.

7.4. Discussion

This is the first study to compare bone collagen synthesis rates between young and old rats using the direct incorporation of stable isotopes in rat tibial bone, with lower rates of bone collagen synthesis at the proximal tibia reported in old rats compared to young rats. The effects of phenotype, sex, and running training on bone collagen synthesis in old rats differed depending upon the site of measurement, but these results need to be interpreted with caution due to some data points being outside the limit of detection for protein-bound enrichment (*i.e.*, negative FSR values). Independently of phenotype, male rats showed greater bone collagen FSRs compared to female rats at the tibial mid-shaft, and only male rats in the LRT phenotype reported greater bone collagen FSRs than LRT female rats at the distal tibia.

Opposite training effects were reported in HRT phenotyped rats, with lower bone collagen FSRs at the distal tibia reported in the trained compared to the control rats; and LRT phenotyped rats, with greater bone collagen FSRs at the distal tibia in the trained compared to the control rats.

Age-related bone loss occurs due to the imbalance between bone resorption and formation, where bone resorption exceeds bone formation leading to a net loss of bone mass (Seeman, 2002). Decreases in bone formation in older humans and animals have been shown in studies using imaging and histology/ histomorphometry techniques (Duan *et al*., 2001; Ferguson *et al*., 2003; Parfitt *et al*., 1995; Willinghamm *et al*., 2010). Herein, bone collagen FSRs at the proximal tibia were lower in old rats (22 months old) compared to young rats (9 months old) of the same rat model (Koch, Pollott, & Britton, 2013). This difference agrees with previous studies that have shown reduced bone formation in long bones of old mice (20-26 months old) using histomorphometry (Ferguson *et al.*, 2003) and μCT scans (Willinghamm *et al*., 2010). Interestingly, these two studies reported most prominent changes in mineralisation at the femoral proximal epiphysis (Ferguson *et al,*. 2003) and the tibial proximal metaphysis (Willinghamm *et al*., 2010), which are highly trabecular bone sites. Herein, age differences in bone collagen synthesis were only present in the proximal tibia, and no age differences were reported at the tibial mid-shaft and distal tibia, which are predominantly made up of cortical bone. In fact, periosteal and endocortical bone formation continue with ageing, whilst age-associated loss of trabecular bone (*e.g*., thinning and perforation) is more evident (Parfitt, 1984), especially during the early stages of ageing (Riggs *et al.*, 2004). The measurements of bone collagen FSRs at the tibial mid-shaft and distal tibia might, however, have been influenced by the insufficient protein-bound deuterium enrichment at these two sites (showed in Chapter 5). Future studies investigating age differences in bone collagen synthesis at the mid-shaft and distal tibia are warranted, given that the thinning of the cortical cortex is considered a crucial aspect of age-associated bone loss (Seeman, 2002) and is associated with fragility fractures (Johnston *et al*., 1985; Power *et al*., 2003). Nonetheless, the lack of age differences at the tibial mid-shaft and distal tibia could indicate that age-related cortical bone thinning is not primarily caused by reduced bone formation, but rather increased resorption.

Sex differences in bone (re)modelling and bone mass are expected in older populations, both in animals and humans, due to changes in sex hormone concentrations in older females (*i.e*., low oestrogen levels) that increase bone resorption in relation to bone formation, leading to subsequent bone loss (Krum & Brown, 2008; Nakamura *et al.*, 2007). Male rats had greater bone collagen synthesis compared to female rats at the tibial mid-shaft (independently of phenotype) and distal tibia (only in LRT phenotyped rats), and there were no sex differences at the proximal tibia. In older humans, it has been shown that males have greater periosteal bone formation (*i.e*., outer cortical bone at the diaphysis of long bones) than females (Duan *et al*., 2001). These differences in bone formation at cortical bone sites, agree with the results reported in the present study at the tibial mid-shaft and distal tibia. Whilst there is also evidence of sexual dimorphism in age-related bone changes in older rodents, these seem to be around the increased resorption of trabecular bone in females compared to males (Glatt *et al*., 2007). Furthermore, the fact that there were no differences in bone collagen synthesis between HRT male and female rats at the distal tibia, suggests that old female rats that are high responders to endurance running training (*i.e*., HRT phenotyped) have similar bone formation than males of the same phenotype. This outcome indicates that the HRT phenotype could have a protective effect on bone in older females and might have important implications for highly trained endurance individuals.

Interestingly, a 16-week endurance running training intervention had opposite effects depending upon the rats' phenotype at the distal tibia. Whilst, within HRT phenotyped rats, the trained rats had lower bone collagen FSRs at the distal tibia compared to the control rats; in the LRT phenotyped rats, the trained rats had higher bone collagen FSRs at the distal tibia compared to the control rats. The effects reported in the LRT rats agree with the results shown in Chapter 6, where a 7-week interval running training intervention increased bone collagen synthesis rates at the tibial mid-shaft and distal tibia of young rats (9 months old). There is, in fact, evidence that mechanical loading can yield positive (*i.e*., osteogenic) effects and increase strength of the long bones in old rodents (>19 months old) (Birkhold *et al*., 2014a; Brodt & Silva, 2010; Buhl *et al*., 2001; de Farias Junior *et al*., 2020), including with treadmill running interventions (Leppänen *et al*., 2008; Raab *et al*., 1990). Some rodent studies have determined, however, that these responses are blunted when compared to their younger counterparts

(Birkhold *et al.*, 2014b; McDonald, Hegenauer, & Saltman, 1986; Razi *et al.*, 2015; Turner, Takano, & Owan, 1995). The differences shown in the HRT rats, however, would suggest the contrary, indicating that running training could have negative effects on bone collagen synthesis in older rats with the HRT phenotype. Nonetheless, it is important to emphasise that the results reported at the mid-shaft and distal tibia need to be interpreted with extra caution given that the protein-bound deuterium enrichment was too low in some animals, resulting in negative FSR values. Therefore, further research evaluating the effects of phenotype and running training at different bone-loaded sites is needed.

7.4.1. Conclusions

Age is a crucial factor affecting the remodelling of bone tissue. Herein, lower rates of bone collagen synthesis at the proximal tibia were reported in old rats in comparison to young rats using a stable isotope tracer method. These results demonstrate the age-related decline in bone formation. The D_2O tracer method was sensitive enough to detect age differences in bone collagen synthesis at the proximal tibia despite the low synthesis rates, although the lack of significant differences at the tibial mid-shaft and distal tibia needs further investigation, since these results may have been affected by the insufficient deuterium incorporation into bone proteins at these two tibial sites. The effects of phenotype, sex, and running training in old rats differed depending upon the site of measurement (proximal tibia, tibial mid-shaft, and distal tibia). Sex and training effects were shown at the tibial mid-shaft and distal tibia, which were largely influenced by phenotype. Future animal and human research should continue to investigate the effects of age, phenotype, sex, and exercise training on bone remodelling in a site-specific manner, specially at highly cortical sites (*e.g.*, mid-shaft of long bones).

CHAPTER 8:

EXPRESSION OF BONE REMODELLING AND COLLAGEN GENES

8.1. Introduction

There are many proteins (*e.g.*, growth factors, receptors, ligands) involved in the bone (re)modelling process, as detailed in Chapter 2 section 2.5. (page 23). The cellular synthesis of these proteins is encoded by genetic information via messenger RNA (mRNA) in the gene expression process. Gene expression dictates how cells respond to changes in their environment (*e.g.*, exercise training) and ultimately affect the phenotype. By obtaining RNA from the tibial diaphyses of rats (from tibial bones used previously in Chapters 4 and 6) and measuring mRNA expression, information about the activation of regulatory mechanisms and pathways underlying the phenotype, sex, and training effects on bone collagen synthesis can be obtained. In Chapter 6, significant main effects of phenotype (femur diaphysis and proximal tibia), sex (proximal tibia), and running training (mid-shaft and distal tibia) on bone collagen synthesis rates were reported. The examination of mRNA expression of genes involved in bone formation and resorption and collagen formation can indicate a pathway or gene-set activation that influenced these effects on bone collagen synthesis in the tibial mid-shaft diaphysis, providing a mechanistic insight in the results produced in this previous chapter.

Hence, the aim of this study was to investigate the expression of genes that underpin bone remodelling and bone collagen formation in tibial diaphyses of young rats. To measure gene expression, the extraction and isolation of good quality RNA from bone is required, which is potentially trickier than from other tissues due to the hard nature of the tissue. As such, this chapter also describes some method development for these processes.

8.1.1. Selection of genes of interest

The genes of interest included in this study were: *Bglap* (osteocalcin), *COL1A1*, *COL1A2*, *COL5A1*, integrin-binding sialoprotein (*IBSP*), *TNFRSF11B* (osteoprotegerin), *RANK*, *RANKL*, *Runx2*, *SOST* (sclerostin), *TGF-β* and *Wnt16*. The associations and key functions of these genes in relation to bone formation, bone resorption, and collagen formation are described below.

Several genes are associated with bone formation, such as *Runx2*, *Wnt16*, *TNFRSF11B* (gene encoding OPG), and *IBSP*. Whilst Runx2 is a transcription factor required for osteoblast differentiation (Bruderer *et al*., 2014), *Wnt16* (Movérare-Skrtic *et al*., 2014) and *OPG* (Lacey *et al*., 1998) inhibit osteoclastogenesis. Furthermore, the *Wnt16* gene (or its polymorphisms) are associated with greater BMD, bone strength, cortical bone thickness and lower incidence/risk of fragility fractures (Estrada *et al*., 2012; García-Ibarbia *et al*., 2013; Hendrickx *et al*., 2014; Koller *et al*., 2013; Medina-Gomez *et al*., 2012; Movérare-Skrtic *et al*., 2014; Zheng *et al*., 2012). Sialoprotein, encoded by the *IBSP* gene, positively regulates osteoblastic differentiation and bone matrix mineralisation (Bouet *et al*., 2015; Gordon *et al*., 2007) by interacting with collagen and stimulating hydroxyapatite nucleation (Baht, Hunter, & Goldberg, 2008; Fujisawa, Nodasaka, & Kuboki, 1995). Collagen genes, such as *COL1A1*, *COL1A2*, and *COL5A1*, provide the instructions to fabricate collagen types 1 and 5 present in bone and mutations on these genes are linked to musculoskeletal diseases, such as carpal tunnel syndrome (Burger, de Wet, & Collins, 2015) and osteogenesis imperfecta (Etich *et al*., 2020).

Other factors and proteins, such as TGF-β and osteocalcin, have been associated with both bone formation and resorption. While *TGF-β* promotes both osteoblast and osteoclast formation (Quinn *et al*., 2001; Tang *et al*., 2009), the role of osteocalcin – also known as bone γ-carboxyglutamic acid protein (*Bglap*) – is not well understood and conflicting results have been obtained from studies worldwide. Although osteocalcin has been considered a marker of bone formation (Li *et al*., 2016), genetic evidence has shown osteocalcin-deficient murine have increased bone mass and bone strength (Ducy *et al*., 1996; Lambert *et al*., 2016). These animal studies indicate that osteocalcin is a negative regulator of bone remodelling, and are in line with human data suggesting that genetic variation of the *Bglap* gene might predispose postmenopausal women to osteoporosis (Raymond *et al*., 1999).

Bone resorption can be linked to RANK, its ligand RANKL and sclerostin (Kenkre & Bassett, 2018). The binding of RANKL to RANK is essential for the activation, function, and survival of osteoclasts (Boyle, Simonet, & Lacey, 2003; Teitelbaum, 2007), which enable bone resorption. Sclerostin, encoded by the *SOST* gene, prevents bone formation by antagonizing BMPs and the Wnt/β-catenin signalling

pathway (Delgado-Calle, Sato, & Bellido, 2017; Lin *et al*., 2009; Poole *et al*., 2005), and osteocyte-downregulation of sclerostin is required for facilitating the osteogenic response to mechanical loading (Delgado-Calle, Sato, & Bellido, 2017; Robling *et al*., 2008; Tu *et al*., 2013). Moreover, mutations in the *SOST* gene are associated with bone disorders, such as sclerosteosis (Moester *et al*., 2010).

8.2. Methods

8.2.1. Design of primers

Primers for five housekeeping (reference) genes and the twelve genes of interest (listed above) were designed using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and validated for efficiency (**Table 13**). Validation of primers was completed using a small set of samples to test the RNA extraction method (not included in the main analysis, see section 8.2.3., page 150). For each gene, a standard curve was used to evaluate primer efficiency following the methodology described in the sections below and by using a serial cDNA dilution (1:10, 1:20, 1:40, 1:80). Primer efficiencies were calculated using the cycle threshold (Ct) standard curve, calculating the slope of the trend line, and using the equation: $efficiency = -1+10^{(-1/slope)}$. Efficiencies between 90-110% were accepted; otherwise, primers were redesigned and validated again.

8.2.2. Bone samples

The bone samples used for this study were from the remaining tibial diaphyses of the *Young* rats (**Figure 29**) used in the prior studies (as described in Chapters 4 and 6) that were stored at –80°C. From the complete cohort of young rats (n = 46), it was not possible to include data from seven rats due to (a) not enough bone tissue available or (b) the low quality of the extracted RNA. Therefore, samples from thirty-nine rats were included in the main analysis. Small chunks (~23 g) of bone were obtained using pestle and mortar and cleaned with 70% ethanol to remove residual bone marrow. To avoid RNA degradation, samples were kept at a cold temperature during this process by keeping the pestle and mortar in liquid nitrogen while the bone was chipped into small pieces. Chipped bone samples were placed in nuclease-free tubes and stored at −80°C until further analysis.

Table 13. Efficiency of successfully validated primers of reference genes and genes of interest.

Gene name	Category	Primer sequences	Efficiency
18S (ribosomal RNA)	reference gene	Fwd: GATGGTAGTCGCCGTGCC Rev: GCCTGCTGCCTTCCTTGG	110.33%
ACTB (Actin-Beta)	reference gene	Fwd: CCCGCGAGTACAACCTTCTT Rev: CGCAGCGATATCGTCATCCA	100.58%
B2M (Beta-2-Microglobulin*)*	reference gene	Fwd: CGGGGTGGTGATGAGAAGTT Rev: AAGGCTCCTTGTCCCTTGAC	96.68%
EEFG1 (Elongation factor 1-gamma)	reference gene	Fwd: ACTGGCTGACATCACGGTTG Rev: ACCATCGGTTGGTATTGGGGA	95.00%
RPL13 (ribosomal protein L13)	reference gene	Fwd: CTGCCGAAGATGGCGGAG Rev: AGCGTACAACCACCACCTTT	95.00%
Bglap (osteocalcin)	gene of interest	Fwd: GTTTGAGGGGCCTGGGATTG Rev: ACACAACTGCAGGTCGAGTTT	105.26%
COL1A1	gene of interest	Fwd: GTACATCAGCCCAAACCCCA Rev: CAGGATCGGAACCTTCGCTT	107.38%
COL1A2	gene of interest	Fwd: GGGGTTGATGCAGACAGTCA Rev: CCCACTCACTGCACATCACT	97.31%
COL5A1	gene of interest	Fwd: CCCAAAGAAAACCCAGGTTCC Rev: CACAGGGTTGCCTTCAGCAT	106.47%
IBSP (integrin-binding sialoprotein)	gene of interest	Fwd: GCCACACTCTCAGGGGTAAC Rev: TGCATCTCCAGCCTTCTTGG	91.00%
TNFRSF11B/OPG (osteoprotegerin)	gene of interest	Fwd: TGCTCCTGGCACCTACCTAA Rev: GCACTCCTGTTTCACGGTCT	101.04%
RANK	gene of interest	Fwd: GCTACCACTGGAACGCAGACT Rev: CGTTGAGCTGCAAGGGATGTT	99.96%
RANKL	gene of interest	Fwd: GTCCAGGTGTCCAACCCTTC Rev: CCATGCTAAGGCTCCACAAA	106.20%
Runx2 (runt-related transcription factor 2)	gene of interest	Fwd: CGCCTCACAAACAACCACAG Rev: AATGACTCGGTTGGTCTCGG	98.00%
SOST (sclerostin)	gene of interest	Fwd: CAACCAGACCATGAACCGGG Rev: TGTACTCGGACACGTCTTTGG	102.26%
TGF-β (transforming growth factor beta)	gene of interest	Fwd: CAGTGCTGAGGAGAAACCGT Rev: GCTCTCCATTGTCCCAGGTC	99.00%
Wnt16	gene of interest	Fwd: AGCATGACCGATGTCCACAC Rev: AACACTCTTACAGGCAGCGA	100.00%

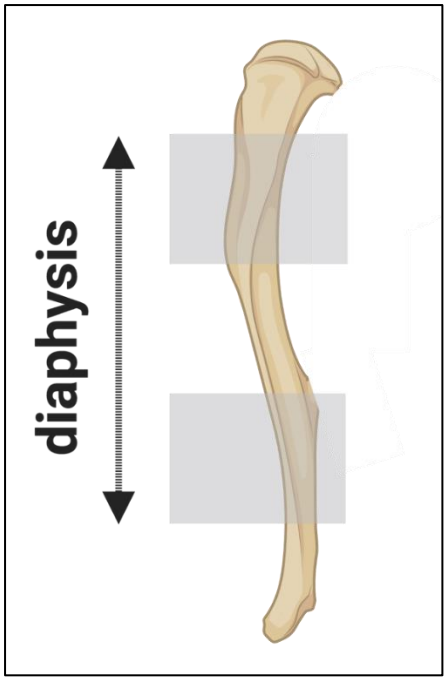


Figure 27. Sampling areas of tibia diaphysis highlighted in grey. Created with BioRender.com

8.2.3. RNA isolation

The isolation of good quality RNA from frozen bone tissue presented some challenges, since RNA is rapidly degraded when it becomes warm and contamination from bone marrow, which affects gene expression measurements (Kelly *et al*., 2014), is possible. For this reason, the methodology of the processes of RNA extraction and isolation was first tested and optimised in a small group of bone samples (not included in the main analysis).

Frozen chipped bone samples in nuclease-free tubes were placed on ice and 400 µl of room-temperature Tri-Reagent were added. One stainless steel bead was added into each tube in order to break down bone tissue and allow homogenisation. After 5 minutes on ice, samples were placed in a cold (kept in the fridge) TissueLyser tube-adapter and tissue was homogenised using TissueLyser II (Qiagen) shaker. Two shaking bouts of 30 seconds alternating with ~2 minutes with the tubes on ice between bouts were performed. Then a third, longer bout of 1 minute in the TissueLyser II shaker was completed. Placing the tubes containing the bone samples on ice between bouts of shaking made sure that the tissue was

maintained at a cold temperature to avoid degradation. If tissue homogenisation seemed incomplete (little or no cloudiness in the appearance of the Tri-Reagent solution) after the three bouts, and extra bout (~1 minute) was added.

Next, 80 µl of chloroform was added into each tube, tubes were vortexed for 30 seconds and left to incubate for 5 minutes. Samples were then centrifuged at 17000 x g for 15 minutes at 4°C. The samples were then separated into three layers; with the RNA being in the upper aqueous phase, which was carefully measured, removed, and placed into a fresh nuclease-free tube. An equal volume of 2-propanol was added into to new tube with the RNA. Before leaving the tube on ice for 30 minutes, 1µl of GlycoBlue ™ (Invitrogen™) coprecipitant was added and the tube was inverted a few times to ensure thorough mixing. After 30 minutes, samples were centrifuged at 17000 x g for 10 minutes at 4°C. A pellet was formed at the base of the tube, if needed (no clear pellet appeared) samples were centrifuged for 5-10 more minutes. RNA pellets were washed with 70% ethanol and centrifuged at 17000 x g for 1 minute at 4°C; a process that was repeated three times. After removal of the last 70% ethanol solution, the pellet was air dried by keeping the lid of the tube open for ~5 minutes and was then resuspended in 20µl of DEPC-treated water. RNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific). For samples with low RNA concentration (<50 $ng \cdot \mu l^{-1}$) or low quality (260/280 ratio <1.70 and/or 260/230 ratio <0.80), the RNA extraction process was repeated to obtain a suitable sample, with a maximum of three total attempts made due to limited tissue availability.

The overall quality of RNA was tested via agarose gel electrophoresis in the set of samples used to test the method. A mix of 4 µl of RNA sample and 2 µl of gel-loading buffer (Sigma-Aldrich) was electrophoresed on 1% (g·100 ml) agarose gel for 30 minutes at 100 V. This analysis indicated that good quality RNA was isolated by showing two distinct bands for 28S and 18S rRNA, where 28S band was more prominent (Imbeaud *et al*., 2005; Schroeder *et al*., 2006) (**Figure 30**).

Another agarose gel was used to test a group of samples included in the main analysis, however only faint bands were visible (not shown), potentially due to smaller RNA concentrations and/or RNA

degradation. To further check for degradation in the RNA of the samples used in the main analysis, a ribosomal RNA gene (*i.e.*, 18S) was included as a reference gene.

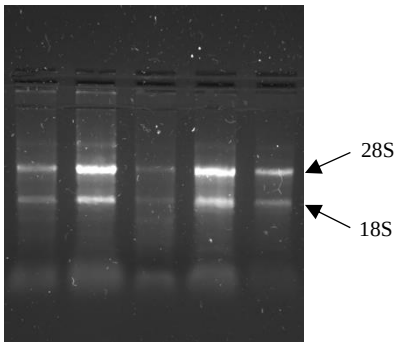


Figure 28. Imaged agarose gel electrophoresis showing intact RNA.

8.2.4. Gene expression

Following the extraction and quantification of RNA, equal quantities of 500 ng RNA were separated into a new RNase-free tube by adding the required quantity of RNA for each sample and DEPC-treated water to form a total volume of 10 µl. All components of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) were combined into a master mix and RNA was reverse transcribed to cDNA following manufacturer instructions (Applied Biosystems) using a thermal cycler (Bio-Rad iCycler IQ). cDNA samples were diluted (1:5 dilution) by adding DEPC-treated water, and a final volume of 100 µl was used for the analysis of mRNA expression. A master mix was prepared for each gene by mixing Power Up ™ SYBR Green Master Mix (Applied Biosystems), DEPC-treated water, and appropriate primers (**Table 14**). cDNA samples (1 µl) and master mix (6 µl) were pipetted into a 384-well PCR plate, which was then centrifuged at 25000 x g (Thermo Scientific Heraeus Megafuge 40R) for 1 minute, two times. The 384-well plate was placed into a Viia 7 real-time PCR machine (Applied Biosystems) for amplification using the following cycling conditions: an initial hold stage at 95 °C for 20 s, then 40 cycles of 95 °C for 1 s and 60 °C for 20 s (1.6 °C/s ramp rate), with a final melt curve stage of 95 °C for 15 s, 60 °C for 60 s; finishing at 95 °C for 15 s (dissociation).

Table 14. Master mix volumes for RT-qPCR analysis.

Reagent	Volume for x1 µl
SYBR Green	3.50
Forward primer	0.02
Reverse primer	0.02
Water	2.46

8.2.5. Data and statistical analyses

Cycle threshold (Ct) values were used to calculate the fold difference in the expression of genes of interest. Using the *BestKeeper* excel-based tool (Pfaffl *et al*., 2004), B2M was determined as the most stable (*i.e*., lowest SD) reference gene and, therefore, it was selected for further calculations. Fold difference was calculated using the delta-delta Ct method (Livak & Schmittgen, 2001) against the B2M reference gene, and relative to the control groups for the LRT and HRT groups.

Descriptive statistics were performed for data sets to check for homogeneity of variances by the Levene test, and normality by the Shapiro-Wilk test (accepted if $p > 0.050$). Data for the *SOST* gene were log base (log10) transformed to achieve a normal distribution prior to analysis. Rosner's test was used to identify extreme outliers. For genes *COL1A1* (four outliers), *COL1A2* (five outliers), *COL5A1* (five outliers), *IBSP* (four outliers) *RANK* (two outliers) and *TGF-β* (two outliers) extreme outliers were identified, but were not removed, and robust statistic approaches (see below) were used to analyse these data (*i.e.*, analyses that takes into consideration outliers in the data).

Datasets for genes *Bglap, OPG, RANKL, Runx2, SOST* and *Wnt16* were analysed for interactions of phenotype, sex, and training effects on gene expression using a three-way ANOVA. The same analysis was completed for genes *COL1A1*, *COL1A2*, *COL5A1*, *IBSP*, *RANK*, *TGF-β* using a Robust three-way ANOVA test due to the presence of extreme outliers. Pearson correlations or Robust correlations (R)

were performed to investigate associations between gene expression and bone collagen synthesis rates (FSR%·d^{-1}) at the tibia mid-shaft (Chapters 4 and 6). Cook's distance was evaluated for all significant correlations to identify influential data points.

The level of significance was set at $p \leq 0.050$. All data are presented as mean ± 1SD and 95% confidence intervals [95% CI]. The effect size generalised eta squared (η^2_g), defined as small ($\eta^2_g = 0.01$), medium ($\eta^2_g = 0.06$), and large ($\eta^2_g = 0.14$) effects, was estimated for all ANOVA analyses. All analyses were performed on RStudio (version 1.4.1717) with packages *tidyverse*, *ggpubr*, *rstatix*, and *WRS2*.

8.3. Results

All reference genes (**Figure 31**) and genes of interest (**Figure 32**) were expressed in the bone samples. All samples showed low Ct values for the 18S rRNA gene, confirming adequate integrity of RNA.

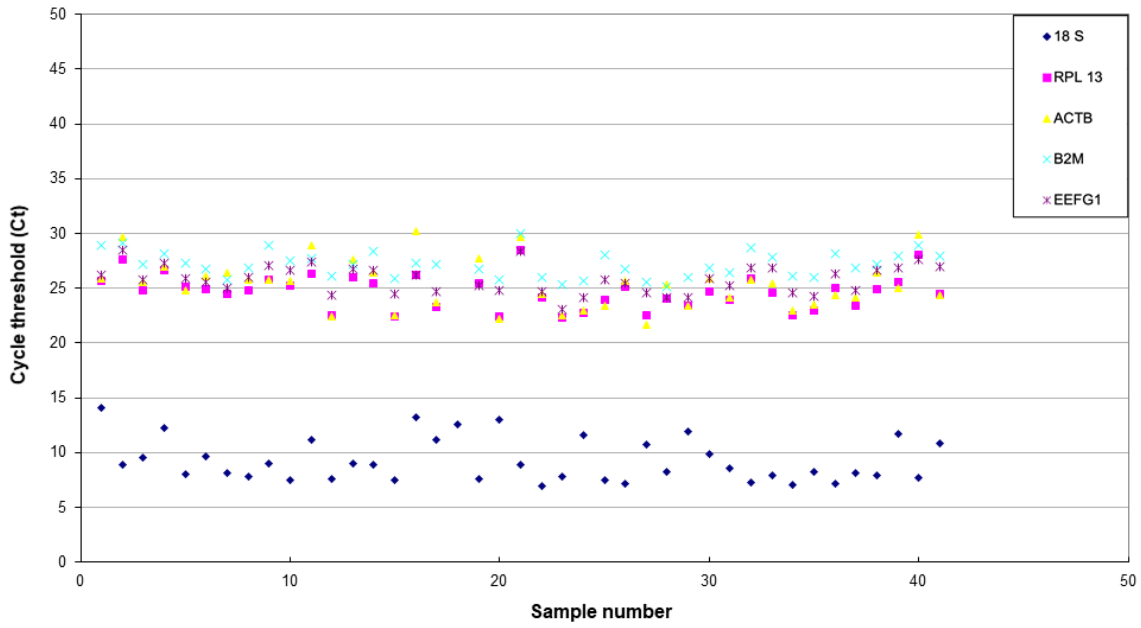


Figure 29. Cycle threshold (Ct) individual values of reference genes.

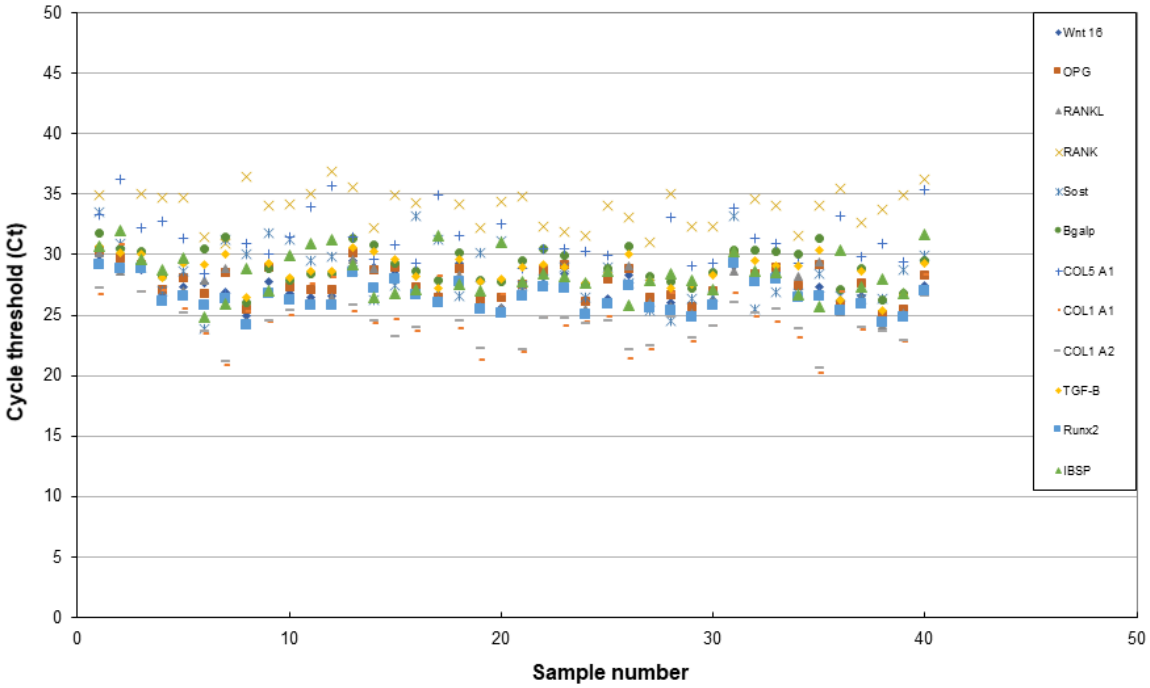


Figure 30. Cycle threshold (Ct) individual values of genes of interest.

8.3.1. Three-way ANOVA

There were no significant phenotype, sex, or running training effects on any genes (**Figures 33 and 34**). The sex main effects on *COL1A2* (F [1, 30] = 2.01; $p = 0.131$; $\eta^2_g = 0.064$), *COL5A1* (F [1, 31] = 3.10; $p = 0.129$; $\eta^2_g = 0.092$), and *IBSP* (F [1, 31] = 4.28; $p = 0.100$; $\eta^2_g = 0.124$) expression were not significant (**Figure 33**) but had medium effect sizes, indicating that 6-12% of the variance of gene expression was explained by training. Notably, there was large variability on these datasets.

The training main effects on *Bglap* (F [1, 31] = 2.38; $p = 0.133$; $\eta^2_g = 0.071$; **Figure 33**), *OPG* (F [1, 31] = 3.32; $p = 0.078$; $\eta^2_g = 0.097$; **Figure 33**), and *TGF-β* (F [1, 31] = 3.98; $p = 0.067$; $\eta^2_g = 0.110$; **Figure 34**) expression were not significant but had medium effect sizes, where 7-11% of the variance of gene expression was explained by training. *Bglap* gene expression was 1.88 ± 1.12 [95% CI 1.40 – 2.34] fold difference in rats that underwent running training, and 1.36 ± 1.00 [95% CI 0.85 – 1.87] fold difference in control rats. Expression of *OPG* was 1.66 ± 0.87 [95% CI 1.32 – 2.00] fold difference in

trained rats compared to 1.18 ± 0.65 [95% CI 0.815 – 1.55] fold difference in control rats. *TGF-β* expression was 1.81 ± 1.19 [95% CI 1.39 – 2.23] fold difference in trained rats, and 1.15 ± 0.57 [95% CI 0.69 – 1.61] fold difference in control rats.

8.3.1. Correlations

Pearson correlations showed significant positive associations between *Bglap* (R = 0.437; $p = 0.006$), *OPG* (R = 0.538; $p < 0.001$), *RANKL* (R = 0.340; $p = 0.034$), and *Wnt16* (R = 0.349; $p = 0.030$) gene expression and bone collagen FSRs at the tibia mid-shaft (**Figure 35**). Robust correlations showed significant positive associations between *TGF-β* expression (R = 0.582; $p < 0.001$) and bone collagen FSRs at the tibia mid-shaft, whilst *RANK* expression showed a significant negative correlation with bone collagen FSRs at the mid-shaft tibia (R = -0.459; $p = 0.005$) (**Figure 35**). For the correlation between *TGF-β* and bone collagen FSRs at the tibia mid-shaft one influential data point (>1.0 Cook's distance) was detected, however, the correlation was still significant after removing this point (R = 0.554; $p < 0.001$).

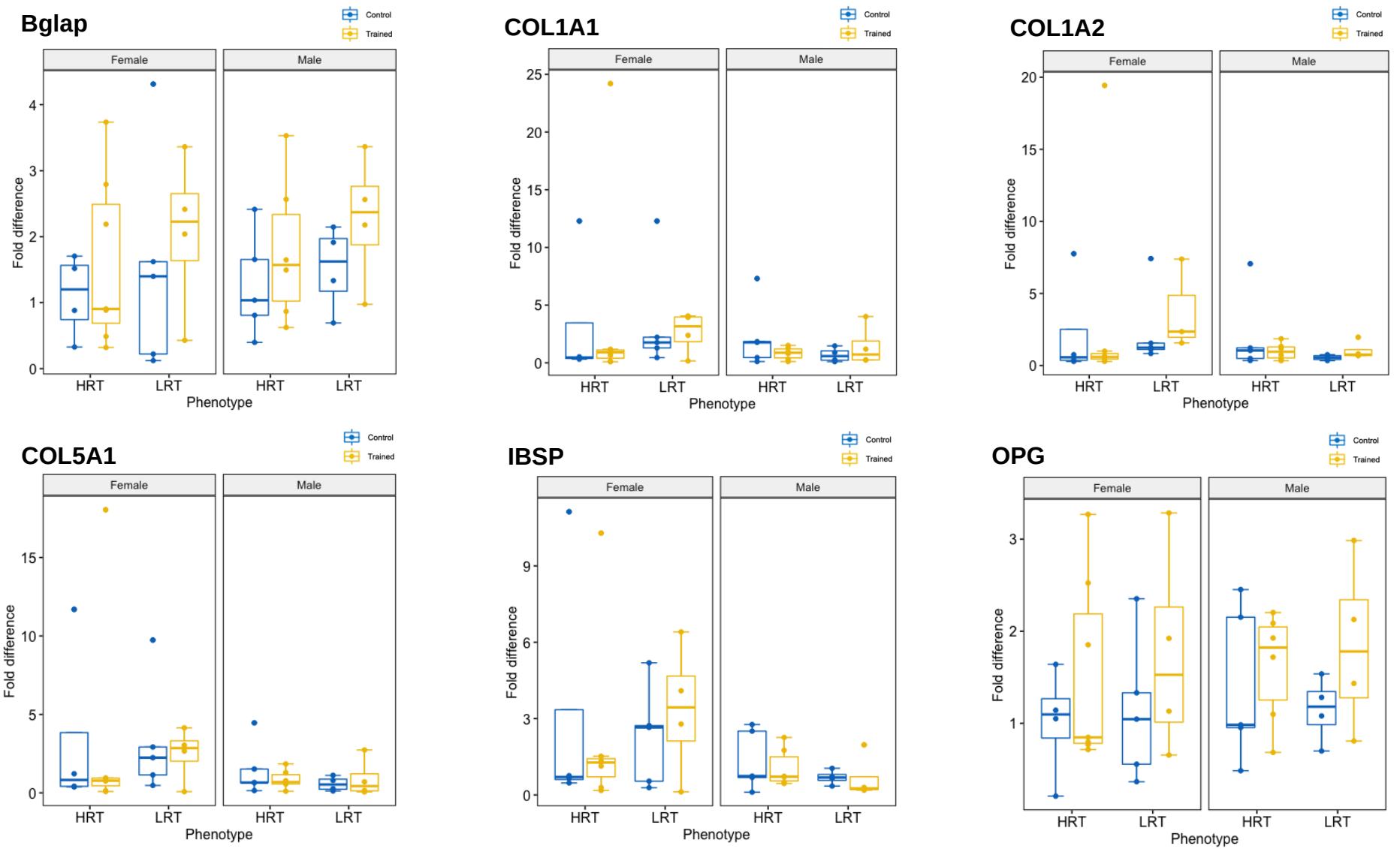


Figure 31. Comparisons of *Bglap*, *COL1A1*, *COL1A2*, *COL5A1*, *IBSP*, and *OPG* gene expression (fold difference) between high (HRT) and low (LRT) responders to endurance running training, female and male, and trained (yellow) and control (blue) young rats.

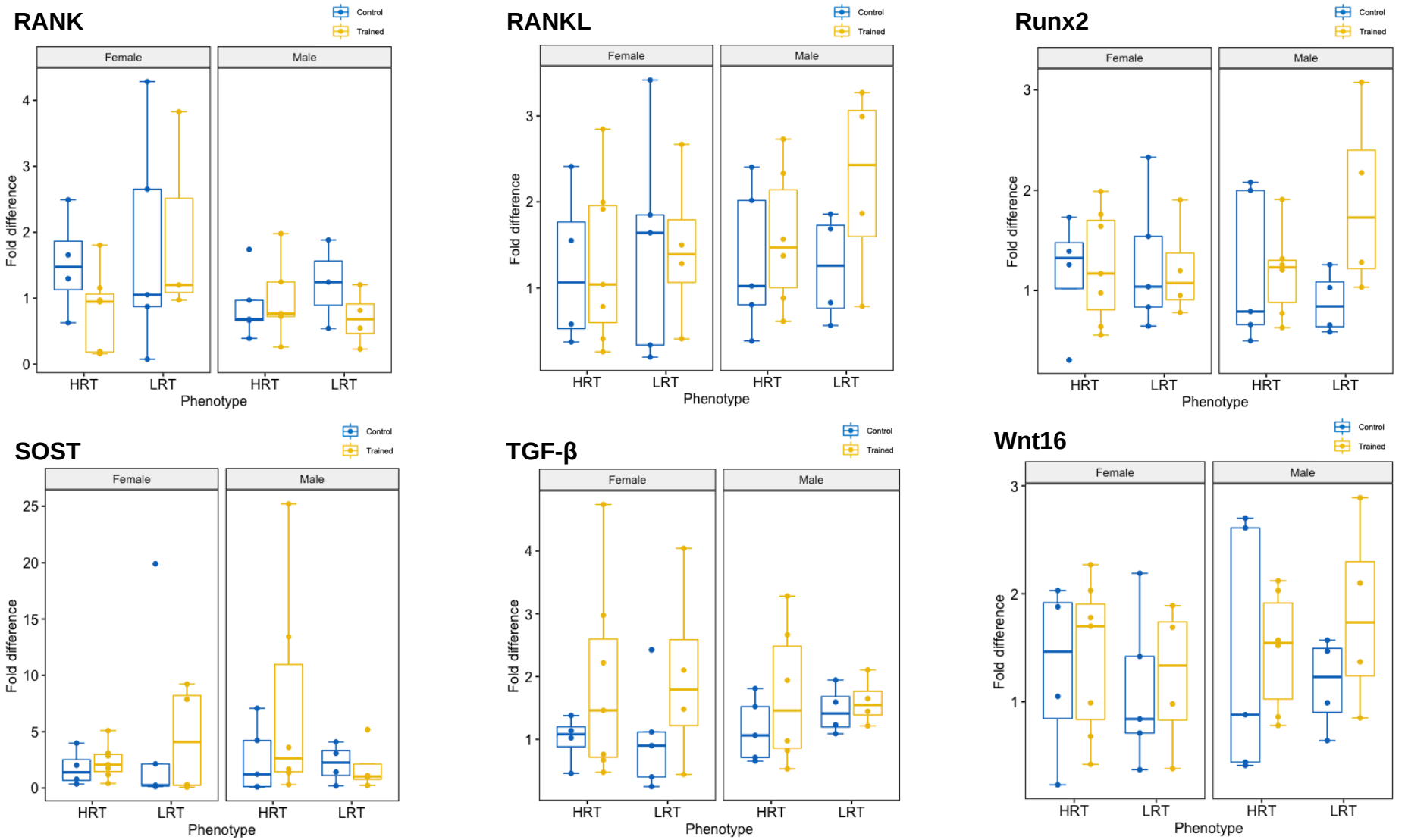


Figure 32. Comparisons of *RANK*, *RANKL*, *Runx2*, *SOST*, *TGF-β*, and *Wnt16* gene expression (fold difference) between high (HRT) and low (LRT) responders to endurance running training, female and male, and trained (yellow) and control (blue) young rats.

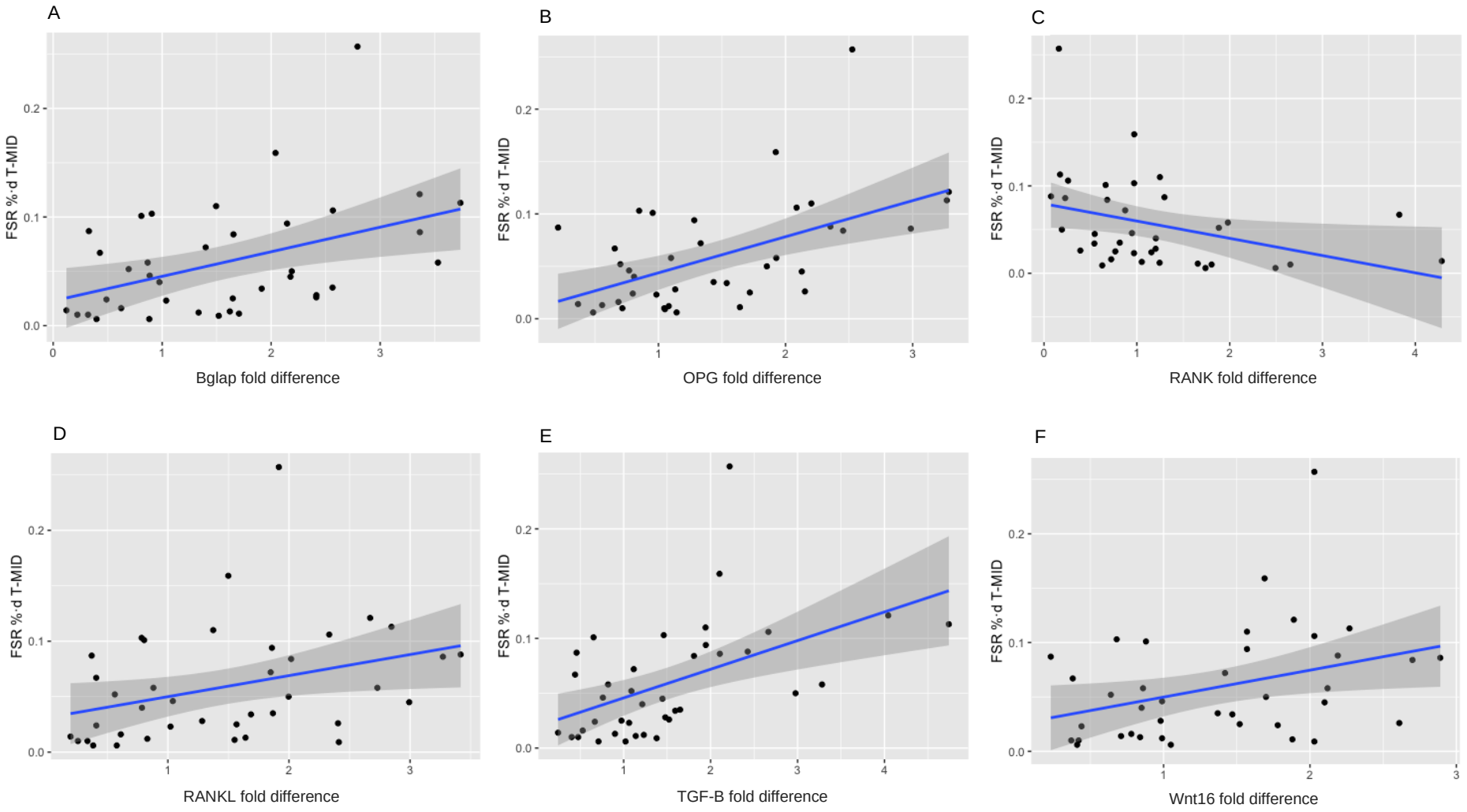


Figure 33. Pearson (*Bglap*, *OPG*, *RANKL* and *Wnt16*) and Robust (*TGF-β* and *RANK*) correlations between (A) *Bglap* (R = 0.437; $p = 0.006$), (B) *OPG* (R = 0.538; $p < 0.001$), (C) *RANK* (R = -0.459; $p = 0.005$), (D) *RANKL* (R = 0.340; $p = 0.034$), (E) *TGF-β* (R = 0.582; $p < 0.001$), and (F) *Wnt16* (R = 0.349; $p = 0.030$) gene expression (fold difference) and bone collagen synthesis rates (FSR%·d^{-1}) at the tibia mid-shaft (T-MID).

8.4. Discussion

RNA was extracted from bone samples from the tibial diaphysis of young rats. The effects of phenotype, sex, and running training on the expression of genes involved in bone remodelling and collagen formation and were examined, showing no significant effects on any of the genes. Positive correlations between gene expression and bone collagen synthesis (tibial mid-shaft diaphysis) were reported for *Bglap* (osteocalcin), *OPG* (osteoprotegerin), *RANKL*, *TGF-β*, and *Wnt16*; and a negative correlation was reported for *RANK*.

Although no significant effects were reported herein, running training had a medium effect (explaining ~10% of the variance) on osteocalcin (*Bglap*), *TGF-β*, and *OPG* encoding gene expression. The small sample sizes of the present study might not have been enough to generate a significant effect of training on these genes, and these outcomes warrant further investigation because a significant main effect of running training on bone collagen synthesis was shown at the tibial mid-shaft (Chapter 6), where running training increased bone collagen synthesis rates in the same rats. Furthermore, osteocalcin, *TGF-β*, and *OPG* were positively correlated with tibial mid-shaft bone collagen synthesis. Taken together, this evidence suggests that the 7-week interval running training intervention may have positively affected expression of osteocalcin, *TGF-β*, and *OPG* encoding gene and these three genes might underpin bone adaptations from exercise. Future studies with greater sample sizes should test this hypothesis to better understand the mechanisms and pathways that underpin osteogenic adaptations to exercise.

In fact, osteocalcin (*Bglap*) (Bouchard *et al*., 2022; Silva *et al*., 2012) and *TGF-β* (Raab-Cullen *et al*., 1994) expression in rodent tibias increases when mechanical loading is applied. Whilst there is controversy surrounding osteocalcin and its functions (Manolagas, 2020), it appears to be indispensable for the alignment of biological apatite crystallites parallel to collagen fibres, which is a determinant for optimal bone strength (Moriishi *et al*., 2020). This evidence supports the association between the osteocalcin gene expression and bone collagen synthesis reported in the present study, indicating the

potential coupling link between mineralisation and newly synthesised collagen. Growth factors, such as TGF-β, are influenced by bone loading and can stimulate cortical periosteal bone formation (Deng *et al*., 2021; Raab-Cullen *et al.*, 1994; Westerlind & Turner, 1995), which could explain the positive association between *TGF-β* expression and bone collagen synthesis at the mid-shaft tibia. OPG is secreted by osteoblasts and it is associated with bone formation because it inhibits osteoclastogenesis (Lacey *et al*., 1998). A recent study reported higher expression of *TNFRSF11B* (the gene encoding OPG) after a single artificial loading session in murine tibia (Bouchard *et al*., 2022), and similar results have been reported previously *in vitro* (Kim *et al*., 2006; Tang, Lin, & Li, 2006). In addition, human studies have shown increased circulating levels of OPG after acute (Ziegler *et al*., 2005) and chronic (Bergström *et al*., 2012; West, Scheid, & De Souza, 2009) exercise.

Additionally, expression of *RANKL* genes were positively associated with bone collagen FSRs at the tibial mid-shaft. RANKL, however, stimulates osteoclast differentiation and activation after binding with RANK (Boyce & Xing, 2007). Both OPG and RANKL are secreted by osteoblasts, and the ratio of RANKL to OPG is crucial for the regulation of bone resorption (Boyce *et al*., 2012). The positive association of *OPG* and *RANKL* with bone collagen synthesis may be an indicator of the overall remodelling of bone at the tibial diaphysis, where bone resorption is followed by bone formation. *Wnt16* gene expression, which has been associated with bone formation and beneficial bone health outcomes in humans (Estrada *et al.*, 2012; García-Ibarbia *et al*., 2013; Koller *et al*., 2013), was also positively associated with bone collagen FSRs at the tibial mid-shaft. In agreement with this outcome, *Wnt16* expression has been positively linked to tibial cortical thickness in animals (Movérare-Skrtic *et al*., 2014; Ohlsson *et al*., 2018; Wergedal *et al*., 2015; Zheng *et al*., 2012). This evidence supports the positive relationship between *Wnt16* expression and bone collagen synthesis at the tibial diaphysis (a highly cortical bone site) reported herein. Interestingly, expression of *RANK*, which promotes osteoclast differentiation and thereby bone resorption (Boyle, Simonet, & Lacey, 2003), was negatively correlated with bone collagen FSRs at the tibial mid-shaft. Recently, research has shown that *RANK* silencing promotes osteoblast differentiation and bone formation *in vitro* (Cao, 2018; Chen *et al*., 2018), indicating that downregulation of RANK may be required for increased bone formation. This evidence

supports the negative association of *RANK* expression and rates of newly synthesised bone collagen (bone formation) reported in this study.

There were no significant associations between collagen (*COL1A1*, *COL1A2*, *COL5A1*), *Runx2*, and sialoprotein genes and bone collagen synthesis measured at the tibial mid-shaft, despite their previous association with bone formation (Bouet *et al*., 2015; Bruderer *et al*., 2014; Burger, de Wet, & Collins, 2015; Gordon *et al*., 2007; Etich *et al*., 2020). Furthermore, no associations were shown between sclerostin (*SOST*) gene, which prevents bone formation by antagonising osteogenic pathways (Delgado-Calle, Sato, & Bellido, 2017; Lin *et al*., 2009; Poole *et al*., 2005), and bone collagen synthesis. The reason why some of the genes included in this study failed to show associations with bone collagen synthesis (bone formation), may be due to the presence of extreme outliers in the data, but also due to potential site-specific differences on gene expression. In particular, the lack of correlations between collagen gene expression and bone collagen FSRs at the tibial mid-shaft (Chapter 6) is incongruous, and this question should be addressed by future research.

The current study was not without its challenges and limitations. Whilst integrity and purity of RNA are key for successfully and reliably quantifying gene expression, isolating RNA with suitable quality from bone tissue is difficult because of its toughness, which required longer and a greater number of shaking cycles to ensure enough RNA was extracted from the tissue. This, however, can result in a significant increase in the temperature of the tissue, which, in turn, increases the risk of degradation (*i.e*., lower integrity). It has also been suggested that the critical phase for avoiding RNA degradation occurs before homogenisation (Pedersen *et al*., 2019), and failing to keep samples frozen and ice-cold can result in degraded RNA that cannot be reliably quantified (Pedersen *et al*., 2019; Streicher *et al*., 2017). Herein, strategies to minimise degradation before (keeping mortar in liquid nitrogen while crushing bone samples) and during (adding cooling periods between shaking bouts) homogenisation were applied, and RNA quality for this methodology was assessed by analysing 18S and 28S rRNA bands using agarose gel electrophoresis (not included in the analysis). In addition, all samples included in analyses expressed the 18S rRNA gene, confirming the integrity of the RNA. The application of the

RNA Integrity Number, which is considered the most robust and reliable method for the assessment of the degradation and integrity of RNA (Imbeaud *et al*., 2005; Schroeder *et al*., 2006), nonetheless, was not evaluated because the resources to measure it were not available at the time of the study. Regarding RNA purity, ratios 260/280 and 260/230 were used to estimate samples purity. While the 260/280 ratio (~1.7-2.0) indicated limited protein contaminants overall, some of the 260/230 ratio values were <1.8, indicating the potential presence of organic compounds such as phenol, sugars, or alcohol; and thereby compromised RNA purity (Imbeaud *et al*., 2005). Although efforts were made to ensure and evaluate the quality of RNA, contaminants may have lowered the quality of bone RNA and affected the analyses.

Outlier data points were identified in many genes' datasets (*i.e*., *COL1A1*, *COL1A2*, *COL5A1*, *IBSP*, *RANK*, and *TGF-β*). These outliers in the data may be due to methodological issues such as the presence of phenol, sugars or alcohol contaminants or the potential presence of bone marrow in some samples, but this cannot be confirmed and, therefore, it was decided to include the outliers in the analyses. Although care was taken to remove any visible bone marrow by cleaning samples with ethanol, other methods, such as centrifuging, have been proposed to effectively remove bone marrow from bone samples for gene expression analysis (Kelly *et al*., 2014). Because bone samples in this study were used retrospectively and were already frozen, using the centrifugation method would have required bone samples to thaw, which would have compromised bone integrity (Pedersen *et al*., 2019). Another factor that could have influenced analyses is the difference in gene expression between cortical and trabecular bone (Kelly *et al*., 2014). Even though the bone samples used in this study were likely from sites of predominantly cortical bone, this was not confirmed by histological analyses.

8.4.1. Conclusions

While results from this study should be interpreted cautiously because of the methodological challenges around the isolation of high-quality RNA from rat's bone samples, there is evidence that osteocalcin, osteoprotegerin, and *TGF-β* gene expression was linked to bone collagen synthesis, and thus, bone formation; and may be influenced by running training. Therefore, the activation of these three genes may be a key regulatory factor for osteogenic bone adaptations. Future animal and human research

should further investigate the associations between bone collagen synthesis and bone formation and *RANK*, *RANKL*, and *Wnt16* gene expression since the expression of these genes was also associated with bone collagen synthesis in this study.

CHAPTER 9:

GENERAL DISCUSSION

The aims of this research programme were to (i) investigate the responses and individual variability of reference bone (re)modelling markers to an acute running-based exercise intervention in a human population of healthy adult males, (ii) develop a novel deuterium oxide tracer method to quantify bone collagen synthesis *in vivo*, (iii) examine site-specific and physiological differences in bone collagen synthesis in young and old rats using the new method, and (iv) explore the expression of genes that regulate pathways involved in bone remodelling and bone collagen formation. This thesis adds new contributions to the understanding of bone (re)modelling markers in response to a bout of running exercise and to the feasibility of novel approaches using direct incorporation tracer techniques to quantify bone collagen synthesis, which are intended to have eventual applicability to human research. To support this, examinations of the ability of this method to distinguish differences in bone collagen synthesis across sites of loaded long-bones and to determine the effects of age, sex, phenotype and running training on bone collagen synthesis were also undertaken in a rodent model, in addition to the examination of how key genes are linked to bone collagen formation.

It was important to establish whether short-term (*i.e*., hours/days) bone responses to a single bout of exercise could be determined by bone (re)modelling markers, as it is one of the commonly used approaches to measure dynamic changes in bone formation and resorption. Only a few individual studies have tried to answer this question directly using reference bone (re)modelling markers P1NP and/or β-CTX-1 and their results are somewhat inconclusive, which might be due to the varied study designs and different exercise interventions (Evans *et al*., 2020; Gombos *et al*., 2016; Guerriere *et al*., 2018; Prawiradilaga *et al*., 2020; Rogers *et al*., 2011; Scott *et al*., 2010). Meta-analytical approaches of aggregate data and individual participant data can provide better estimates of mean responses and effects at the participant level by pooling data from selected studies (Riley, Lambert, & Abo-Zaid, 2010). These approaches are essential to understand the magnitude and context of short-term exercise-induced changes in bone metabolism and systematically assess the quality of available studies. The systematic review and individual participant meta-analysis reported in Chapter 3 included data from 8 studies (and a total of 139 participants) that measured changes in bone (re)modelling reference markers P1NP and β-CTX-1 during and after (*i.e*., hours and days) a continuous, prolonged running bout and/or

at rest (control) relative to baseline. This meta-analysis determined that prolonged bouts of treadmill running did not result in bone responses, as determined by P1NP and β-CTX-1, in young adult healthy males. Although transient increases were reported in P1NP, these were likely caused by biological artefacts (*e.g.*, shifts in plasma volume, leakage from other connective tissues) rather than being reflective of bone formation. Small decreases in β-CTX-1 were shown during and shortly after running, as well as in resting conditions, suggesting that these changes were more likely due to the marker's circadian rhythm rather than being caused by the running intervention.

From this study, two possible conclusions could be drawn: (a) a single running bout does not stimulate bone (re)modelling or (b) that it does yield bone responses but P1NP and β-CTX-1 markers failed to capture them. Conclusion (a) might suggest that the exercise intervention was insufficient or inadequate to stimulate a bone adaptation and, therefore, further single or meta-analytic studies should evaluate if other exercise interventions (*e.g.*, impact level, duration, and intensity, intermittent/continuous) are able to elicit a response that is captured by bone (re)modelling markers. Indeed, a different systematic review and aggregate data meta-analysis of a broader scope conducted on this topic determined that these markers, particularly β-CTX-1, seem to be more responsive to cycling interventions compared to exercise interventions with higher impact such as jumping or resistance exercise (Dolan *et al*., 2022). Further high-quality research directly comparing bone (re)modelling responses to high-impact and low impact acute exercise interventions is warranted. An important caveat of this broad meta-analysis (Dolan *et al*., 2022), however, was the lack of control (non-exercise) data against which to compare the exercise responses. This limitation was also highlighted in the individual participant meta-analysis reported in Chapter 3, where most of the included studies did not have a control group and individual participant control data had to be obtained from other similar studies.

The second conclusion (b) is more complex to confirm. First, it could be argued that more sampling at smaller intervals during the days after an acute intervention might be needed to capture changes in bone (re)modelling markers. While this seems unlikely, it is also not very feasible in terms of study designs and associated costs. It is also possible that these markers better reflect longer-term adaptations to

exercise. For example, some cross-sectional studies have determined that athletic populations of certain groups (*e.g.*, team-sports, horse-riding, swimming, decathlon) have altered baseline levels of bone (re)modelling markers compared to control groups (Creighton *et al.*, 2001; Dolan *et al.*, 2012; Maïmoun *et al.*, 2004; Maïmoun *et al.*, 2008; Mcveigh *et al.*, 2015). Nonetheless, there is little understanding of what changes in these markers mean and how they reflect bone adaptations at the tissue level. Bone (re)modelling markers are systemic and it is not clear whether they can predict changes detected by imaging techniques, such as DXA or pQCT (Vasikaran *et al.*, 2011). These markers represent whole-body bone (re)modelling and cannot be used to directly determine local bone adaptations, which is a major limitation in the context of exercise research, given that bone responses to mechanical loads are largely site-specific (Bass *et al.*, 2002; Judex, Gross, & Zernicke, 1997; Kannus *et al.*, 1994). It stands to reason that changes in bone markers following an exercise intervention would likely represent changes in bone (re)modelling in the exercising limbs, but bone marker data do not always support chronic adaptations in the mineral compartment of bone and it is important to remember that these markers have yet to be validated against direct and local measures of bone collagen formation or breakdown. Furthermore, given the lack of tissue specificity of most of these markers, it could be suggested that *in vitro* studies are needed to help understand which biochemical markers are secreted by different collagenous tissues, in order to reconcile what each of these markers indicates within the activation and progression of the bone (re)modelling process. Although current bone (re)modelling markers are convenient and accessible tools to measure changes in bone (re)modelling, their utility is undetermined and the interpretations that can be made from them are limited. This information emphasises the need for alternative methods that can measure short-term bone (re)modelling and the effects of exercise on bone turnover.

In response to this need, a novel deuterium oxide tracer method able to quantify bone collagen synthesis was developed in the studies described in Chapters 4 and 5. In contrast to the use of bone (re)modelling markers, stable isotopically labelled tracers, such as deuterium oxide (D_2O), can be used to provide direct and site-specific measurements of bone metabolism. These methods allow quantification of bone collagen synthesis, which reflects the formation of newly synthesised bone matrix during the bone

(re)modelling cycle. The main downside to the use of tracer methods for this purpose, however, is that they require access to bone tissue samples, which is more invasive than a blood sample. For this reason, the choice was made to develop the method using available femoral and tibial bones from a rat model, as part of a secondary analysis from other studies (Ahtiainen *et al*., 2018; Brook *et al*., 2017; Nokia *et al*., 2016; Thompson *et al*., 2022; West *et al*., 2021). Whilst prior studies have used D_2O to measure bone proteins synthesis in rodents (Busch *et al*., 2006; Cross *et al*., 2020; Do *et al*., 2006; Jeong *et al*., 2005), these were done in young growing animals (≤ 3 months old) and used high ^2H body enrichment levels (2.5-5%). In contrast, the D_2O method developed as part of this programme of work used lower levels (<1%) of ^2H body water enrichment in adult, non-growing animals. This lower level of body water enrichment is more suitable to future human investigations and non-growing animals better represent the low bone turnover expected in adult humans. Therefore, the intention was to have a method that could be more easily applied to human investigations.

D_2O tracer methods have not yet been employed to measure bone protein synthesis rates in humans and only three studies have used stable isotope tracer techniques to determine human bone collagen synthesis (Babraj *et al*., 2005a; Scrimgeour *et al*., 1993; Smeets *et al*., 2019), although they used amino acid tracers, which are infused intravenously rather than being orally ingested as with a D_2O tracer. Because the D_2O tracer is ingested, rather than being intravenously infused, the labelling period for D_2O studies aiming to quantify bone protein synthesis is not yet known. Several studies have been using D_2O tracers to measure human muscle protein synthesis (Bell *et al*., 2015; Brook *et al*., 2015; Franchi *et al*., 2015; Gharahdaghi *et al*., 2019; Groennebaek *et al*., 2018; Murphy *et al*., 2016; Scalzo *et al*., 2014; Wilkinson *et al*., 2014), which usually use labelling periods between 2 days and 6 weeks. Indeed, human muscle protein synthesis can be determined in only a few days (2-3 days) after ingesting D_2O, while maintaining low levels of ^2H body water enrichment (Murphy *et al*., 2016; Wilkinson *et al*., 2014). Human bone, however, appears to have slower synthesis rates compared to other musculoskeletal tissues (Smeets *et al*., 2019), and the bone collagen synthesis rates measured herein, in rats, were ~30 fold lower than mean muscle protein synthesis rates measured in the same group of rats (data not shown). For this reason, studies interested in measuring bone protein synthesis using D_2O tracer

methods will likely require longer periods of D_2O labelling to achieve adequate levels of protein-bound ^2H enrichment in the bone tissue and, subsequently, quantify fractional synthetic rates.

The results from the first method development study (described in Chapter 4) showed that the D_2O tracer method was capable of quantifying bone collagen synthesis *in vivo*, using low levels (<1%) of ^2H body water enrichment over a 3-week period in non-growing young rats. It was also important to investigate whether bone collagen synthesis could be quantified with lower levels of ^2H incorporation into the tissue. This question was addressed in the next study (described in Chapter 5), where bone collagen synthesis was quantified using similar ^2H body water enrichment levels, but over a shorter D_2O labelling period of 3 days in older rats. This short labelling period and the older age of the animals resulted in very low ^2H protein-bound alanine enrichments in the bone samples. Despite using highly sensitive mass spectrometry equipment (GC-*pyrolysis*-IRMS), which was able to detect protein-bound enrichments as little as 0.001 APE, the resultant data were close to the limits of detection at the tibial mid-shaft and distal tibia sites, which have the slower bone collagen synthesis rates. Where the levels of bone ^2H protein-bound enrichments were too low (*i.e.*, too similar to baseline), bone collagen synthesis rates could not be adequately quantified, resulting in negative values. Hence, it was determined that longer labelling periods (>3 days) are required to allow sufficient time for ^2H to be incorporated into bone proteins and to effectively quantify bone collagen synthesis at bone sites with slow turnover in old rodents. This would also most likely be the case for human participants, who might even require exposure to longer labelling periods because of the expected slower protein turnover compared to small animals. This information is crucial for the design of future animal and human investigations using this D_2O method in combination of GC-*pyrolysis*-IRMS analyses and for D_2O studies using mass spectrometry equipment with lower limits of detection, which will require even longer labelling periods and/or higher ^2H body water enrichment levels for the measurement of bone synthesis.

Other important parts of the method development were to determine an effectual way to acquire bone samples in a precise manner from the whole femur and tibial bones, and to optimise a method to extract

and isolate collagen proteins from the bone samples. Given that bone adaptations occur at local and site-specific locations across bones, it was important to obtain samples from specific bone sites. This was deemed not possible by using a pestle and mortar technique (used on the femur samples), but samples from three specific sites of the tibia were obtained by using an electric hand saw and a standing clamp. Previous rodent studies that used D_2O methods to study bone did not extract and isolate bone collagen proteins, meaning that they measured mixed bone protein synthesis (Busch *et al*., 2006; Cross *et al*., 2020; Do *et al.*, 2006; Jeong *et al*., 2005). Herein, the development of the new method included the exaction and isolation of collagen proteins from bone samples to allow for the quantification of collagen-specific fractional synthetic rates. It was determined that bone samples required ~13 days in 0.5 M HCl solution, followed by ~5 days in 0.3 M NaOH solution (with each solution changed after a few days and with bouts of vortexing before and after each change of solution) for effective demineralisation and removal of bone marrow and alkali soluble proteins. A limitation of this process was that histological analyses were not performed to confirm the purity of the collagen and the characterisation of trabecular and cortical bone.

Acquiring samples from different anatomical sites of the bones enabled the comparison of bone collagen synthesis rates between them. It was established that the new D_2O method was sensitive enough to detect differences in bone collagen synthesis across bone sites in young rats, where the synthesis rates were greater at the femur diaphysis compared to the tibial mid-shaft diaphysis and greater at the tibial proximal epiphysis-metaphysis compared to the tibial mid-shaft and tibial distal epiphysis-metaphysis. Interestingly, the same outcomes across tibial sites were reported in old rats in the subsequent study. It was speculated that these differences could be due to variations in the mechanical stresses placed upon these different sites (Sugiyama, Price, & Lanyon, 2010) and/or due to differences in trabecular and cortical compositions (Robling, Castillo, & Turner, 2006; Seeman, 2013). In fact, overall, the bone sites with a potentially greater mixture of trabecular and cortical bone (*i.e*., femur diaphysis and proximal tibia) had faster synthesis rates than the bone sites that would be predominately made up of cortical bone (*i.e*., mid-shaft and distal tibia). These results have implications for future studies, which should control and report the bone sites used in their analyses and should continue investigating how the

heterogeneity of bone tissue and its complex structure affects its turnover rates. For example, studies combining histological analysis and tracer methods could establish if there is indeed a difference in the rates at which new collagen is formed between trabecular and cortical bone.

Bone and its (re)modelling are influenced by many factors, some are non-modifiable (*e.g.*, genetics, sex, age) and some are modifiable (*e.g.*, diet, physical activity, or exercise) (Weaver *et al*., 2016). Because the rat model used for the development and optimisation of the D_2O method comprised various groups and cohorts, it allowed the examination of the effects of some of these factors on bone collagen synthesis. The studies described in Chapters 6 and 7 are the first studies to report the effects of age, sex, phenotype, and running training (**Figure 36**) using the direct incorporation of stable isotopes in rat femoral and tibial bone sites. Interestingly, and highlighting that bone adaptations are site-specific, these physiological effects differed depending upon the bone site of measurement (femur diaphysis, proximal tibia, mid-shaft tibia and distal tibia). In young rats, overall, non-modifiable factors (*i.e.*, sex, phenotype) had greater effects at the femur diaphysis and proximal tibia and modifiable factors (*i.e.*, running training) had greater effects at the mid-shaft and distal tibial sites. In old rats, however, significant effects of sex and running training were only shown at the tibial mid-shaft and distal tibia, which were largely influenced by phenotype.

Ageing is associated with low bone mass and poor bone health, which can lead to serious conditions, such as osteoporosis and fragility fractures (Hendrickx, Boudin, & Van Hul, 2015). Having measured bone collagen synthesis in young (9 months old) and old (22 months old) rats from the same rat model and using the same method, it was possible to perform an age comparison between these two cohorts (only including the control groups that did not undergo running training). The results showed that old rats had slower bone collagen formation at the proximal tibia compared to the young rats, which agrees with previous studies in ageing mice showing reduced bone formation (*i.e.*, mineralisation) in highly trabecular bone sites of long bones measured by histomorphometric analysis (Ferguson *et al*., 2003) and micro-CT scans (Willinghamm *et al*., 2010). Although the lack of age differences at the tibial mid-shaft and distal tibia might suggest that age-related cortical bone thinning is caused by increased

resorption rather than reduced bone formation, these outcomes warrant further investigation as results might have been influenced by the low/insufficient protein-bound deuterium enrichment at these two sites.

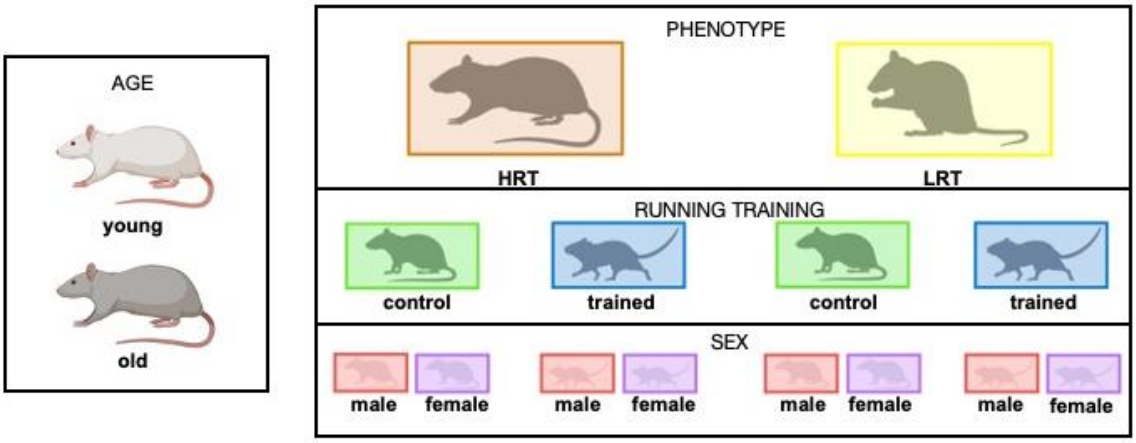


Figure 34. Diagram representing the different effects investigated in the studies reported in Chapters 6 and 7. Age effect, young and old rats (left side); phenotype effect, high responders to endurance running training (HRT, orange) and low responders to endurance running training (LRT, yellow) rats; running training effect, control (green) and trained (blue) rats; sex effect, male (red) and female (purple).

Bone responds to mechanical loading (Frost, 1987), and weight-bearing exercise is generally considered to be beneficial for bone health and is associated with increased bone mass, particularly at load bearing sites (Bass *et al*., 2002). But the short- and long-term bone responses to endurance running training are not clear (Scofield & Hecht, 2012). The results from the rodent studies reported herein (Chapters 6 and 7) demonstrate that running training can increase bone collagen formation at highly cortical loaded sites in young and old rats. In young rats, a 7-week interval running training intervention increased the rates of bone collagen synthesis at the tibial mid-shaft and distal tibia. In old LRT rats, a 16-week endurance running training intervention increased bone collagen synthesis at the distal tibia. In addition, the faster bone collagen synthesis rates reported in young HRT phenotyped rats compared to LRT phenotyped rats, might be indicating that HRT rats have chronic upregulation of bone formation processes, as has been suggested by some authors in the context of human exposure to exercise training (for a review, please see Dolan *et al*., 2020a).

The unclear effects of endurance running on bone turnover and health have been discussed throughout this thesis. This type of exercise is not considered to be optimal for producing an osteogenic response for various reasons, including its loading pattern causing microdamage accumulation (Hoenig *et al*., 2022). Nonetheless, the results from the animal studies described in Chapters 6 and 7 using stable isotope direct incorporation techniques would suggest that, in the short-term, endurance running is osteogenic by enhancing bone formation and that, in the long-term, the phenotype associated with this type of exercise includes an upregulation of bone formation. Something else important to consider, however, is that only bone collagen synthesis, and thus bone formation, was measured with the D_2O method in these studies and the effects of mechanical loading or exercise on bone resorption are equally important to appreciate the net change in bone mass. While rates of whole-body and muscle protein breakdown can be measured using stable isotopic tracer techniques (Biolo *et al*., 1995; Tipton, Hamilton & Gallagher, 2018), these methods have not been applied to measure bone collagen breakdown. Future research could help develop similar approaches to provide more direct measurements of bone resorption.

In the study reported in Chapter 8, the expression of bone remodelling and collagen genes was examined to provide some mechanistic insight to the results produced in this previous chapters. The extraction and isolation of high-quality RNA from bone is challenging and, although several studies have proposed various "improved" methods for this purpose (Carter *et al*., 2012; Kelly *et al*., 2014; Pedersen *et al*., 2019), there is currently no agreement on the best method to use and nor has there been a development of a standardised protocol. Despite the methodological challenges, this study provided evidence that the expression of osteocalcin, osteoprotegerin, and *TGF-β* genes were linked to bone formation at the tibial mid-shaft, indicating that the activation of these three genes may be a key regulatory factor for osteogenic responses. Other genes, including *RANK*, *RANKL* and *Wnt16* were also associated with bone collagen synthesis. Future research should confirm the associations between the expression of these genes and bone collagen synthesis and bone formation and examine their role in mechanistic pathways leading to bone adaptations from loading/exercise.

9.1. General conclusions and research impact

The key outcomes of this programme of work corresponding to the aims stated above, the impact of the research conducted herein, and the novel aspects of the work are summarised below:

Table 15. Key outcomes and impact of the research

Aim	Key outcomes	Impact
(i)	The individual participant data meta-analysis determined that: • A prolonged, continuous treadmill running bout does not elicit short-term bone responses in healthy adult males, measured by reference bone (re)modelling markers (P1NP and β-CTX-1). • Inter-individual variability was mostly similar between running and control conditions.	A novel use of meta-analytic analyses of individual participant data was able to establish responses and individual variability of reference bone (re)modelling markers to acute exercise in very specific conditions. It is unclear whether a single running bout does yield bone adaptations or not, but these potential responses were not captured by P1NP and β-CTX-1 markers. There is a need for specific study designs that investigate the acute responses of bone (re)modelling markers to different types of exercises across different populations.
(ii)	A collagen extraction and deuterium oxide tracer method, in combination with highly sensitive GC-*pyrolysis*-IRMS analysis, was developed for the quantification of bone collagen synthesis *in vivo*. Using this D_2O tracer method, the fractional synthetic rates of femoral and tibial bone collagen were quantified and differences across bone sites were determined.	These differences across bone sites emphasise the complex and heterogeneous structure of bone and how it should be carefully considered when studying bone turnover, especially in response to loading. This new method, developed in rodents, uses D_2O loading protocols applicable to human research.
(iii)	The new D_2O method was able to detect significant effects of age, phenotype, sex, and running training on bone collagen synthesis, providing evidence that: • Old rats synthesise bone collagen at a slower rate than young rats at specific bone sites. • Running training can produce osteogenic bone adaptations at highly cortical loaded bone sites in young and old animals.	These studies are the first to establish age, phenotype, sex, and running training physiological differences in rodent loaded long-bones using gold standard direct incorporation techniques.

(iv)	The effects of phenotype, sex, and running training on the expression of genes involved in bone remodelling and collagen formation were examined, showing no significant effects on any of the genes. Positive correlations between gene expression and bone formation at the tibial mid-shaft were reported for osteocalcin, osteoprotegerin, *RANKL*, *TGF-β*, and *Wnt16*; and a negative correlation was reported for *RANK*.	Particularly, osteocalcin, osteoprotegerin, and *TGF-β* genes appear to be key regulatory factors for osteogenic responses and require further investigation. Extracting quality RNA from bone tissue was proven to be challenging and more robust approaches and standardised methods are warranted. Mechanistic insights underpinning osteogenic adaptations to exercise can be studied in combination with tracer techniques.

9.2. Future directions

Further research needs to establish if bone (re)modelling markers respond to other acute exercise interventions within the hours and –days after the intervention and, if they do, by what magnitude and in which direction. Given the inter-individual variation and circadian rhythms of these markers, individual studies using exercise interventions should include a control (non-exercise) group and should be designed following the current recommendations to minimise pre-analytical and analytical sources of variability of bone (re)modelling markers (Bauer *et al*., 2012; Morris *et al*., 2017; Szulc *et al*., 2017; Vasikaran *et al*., 2011). Furthermore, approaches using direct incorporation of stable isotopes into bone, such as the D_2O method used herein, could be used to investigate if/how changes in bone (re)modelling markers are associated with changes in bone collagen synthesis. There was an intention to investigate this association within this programme of work. Unfortunately, the analysis of bone (re)modelling markers on plasma samples from the rats included in the studies herein was not successful, primarily due to problems with the assay kits to perform the analyses and the limited amount of plasma available, which also had to be used to perform body water enrichment analyses.

The development and optimisation of a new D_2O method capable of quantifying of rodent bone collagen synthesis *in vivo* was achieved in this programme of work and, therefore, future studies need to continue this work by using this method to determine human bone collagen synthesis. Although this method

comes with the challenge of obtaining bone tissue samples, there are ways to achieve this in human populations (*e.g.*, bone removed in surgery, bone biopsies). It is unknown whether the differences in bone synthesis across different long-bone sites reported herein in rodents (quadrupedal) would also be reported in humans (bipedal), and this also requires further investigation. Similarly, these differences across bone sites suggested that trabecular bone might have faster turnover rates compared to cortical bone, but these results need confirmation from future studies using histological analyses in combination with direct tracer incorporation techniques.

Lastly, this D_2O tracer method could be used to further understand age and sex differences in bone formation in human populations, and to study differences in bone collagen synthesis in health and disease, for example investigating how bone diseases (*e.g.*, osteoporosis, osteogenesis imperfecta, fractures) affect bone collagen formation. Given that this new D_2O method in combination with GC-*pyrolysis*-IRMS analyses has proved to be highly sensitive, the method has great future applicability for interventional studies investigating the efficacy and short-term (days-weeks) effects of strategies that can promote bone health such as exercise, diet, and drug interventions. One important consideration for future human bone research using this D_2O method with low ^2H body water enrichment levels is that it would require longer labelling periods compared to studies measuring protein synthesis in human muscle (Wilkinson *et al*., 2014).

9.2.1. Planned human study

The next step in the development and application of the new deuterium oxide tracer method is to test this approach in human populations. There was, in fact, a human surgical study planned for this programme of work, which was postponed due to major delays arising from the COVID-19 pandemic, which resulted in many of the elective surgeries that this trial would count upon being stopped. Although this study is, therefore, not part of the thesis, the design and planning were carried out during the development of this programme of work. In summary, this study was a prospective observational cohort study on osteoarthritic patients undergoing total knee replacement or arthroplasty elective surgery in

collaboration with an orthopaedic surgeon from the Nottingham University Hospitals NHS Trust. Because the D_2O method requires access to tissue samples to determine protein synthesis rates and obtaining human bone tissue *in vivo* from healthy individuals can be challenging, this approach would allow access to human bone samples from patients with osteoarthritis that are routinely removed during these types of surgeries. In addition to bone samples, the collection of tendon and muscle samples via biopsies was also part of the proposed study, with the purpose being to investigate how osteoarthritis may affect protein synthesis of different musculoskeletal tissues. The patients would have ingested the D_2O tracer during the weeks before the surgery.

There were different intended measures and analyses for this study. Firstly, because not all parts of subchondral bone that are removed during surgery are osteoarthritic and these patients have different grades of osteoarthritis, it was planned to perform histological analyses on the collected samples of bone to determine the presence of osteoarthritis and its severity grade. These analyses would have allowed a comparison of the bone collagen synthesis between osteoarthritic and healthier bone samples. Secondly, by taking a muscle biopsy on each leg at the start of the surgery, comparisons between the muscle protein synthesis of the thigh muscle of the leg affected by osteoarthritis and the same muscle of the healthier leg would have been performed. In addition, by taking a tendon biopsy during the surgery, protein synthesis of the patella tendon of the osteoarthritic knee would have been quantified. Thirdly, and given that most patients undergoing knee replacement surgeries are obese/overweight, a sub-analysis comparing protein synthesis in these musculoskeletal tissues between obese/overweight and normal-weight patients was planned if enough participants from each group were recruited. As exploratory measures, gene expression analyses in these musculoskeletal tissues would have been conducted.

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APPENDICES

Appendix A: Spreadsheet data extraction (https://drive.google.com/drive/folders/1wEmyIm18VDbMqUVjnUy8tKj5uK1Y20aq?usp=sharing)

Study number	Author	Year	Journal	Title	Aim	Design	N	Inclusion criteria	Participant Overview (health status, gender)	VO2max	Age (mean)	Age (SD)	BMI (mean)	BMI (SD)	Nutritional Intervention	Nutritional Intervention	Standarisation	Time of exercise	Fasting before baseline	Fasting status during trials	Gorup included	Exercise Stimulus	Intensity	Duration (min)	Total Work (intensity*min)	Timing of samples	Biomarker	Biomarker (code)	Bone Process	Sample Type	Sample type code (1=serum; 2=plasma)	Method	Intra-assay variability	Inter-assay variability	Unit	Baseline (time of day)	CONTROL DATA
1	Lehrskov et al.	2020	Journal of the Endocrine Society	Interleukin-6 may not affect bone resorption marker CTX or bone formation marker P1NP in humans	To assess the tole of IL-6 in regulating CTX and P1NP in a crossover design during an acute exercise bout (Study 1)	Placebo-controlled single blinded cross-over study whereby participants were infused with either saline or the IL-6 receptor antibody (to block IL-6 signaling) prior to an exercise bout. PLACEBO ONLY	5		healthy males	VO2max 56±4	21	2	22.3	0.9	Yes	mixed-meal tolerance test	Avoid vigorous physical activity and alcohol for 48 h preceding study days	morning	Fasted >10h, food consumed after exercise	Fasted baseline	saline, placebo	treadmill run	75% VO2max	60	4500	Baseline, 1h post-baseline, 20min during ex, 40min during ex, 60min post-ex	P1NP	1	Formation	EDTA plasma	2	IDS-iSYS (CrossLaps, Immunodiagnostic Systems) chemiluminescence	5.4% (18.96 µg/L), 6.5% (48.48 µg/L), and 6.1% (122.10 µg/L)	5.4% (18.96 µg/L), 6.5% (48.48 µg/L), and 6.1% (122.10 µg/L)				
																										CTX	2	Resorption	EDTA plasma	2				ug/L	morning	YES
2	Sale et al.	2015	Journal of Applied Physiology	Effect of carbohydrate feeding on the bone metabolic response to running	Investigate the immediate and short-term bone metabolic response to carbohydrate feeding during treadmill running	Experimental randomized, repeated measures, cross-over design, whereby men took part in 2 identical exercise trials, one where they were fed CHO immediately before, during and after exercise, and the other placebo. USE PLACEBO ONLY	10	nonsmokers, had not suffered a bone fracture or injury of any type in the previous 12 mo, were free from musculoskeletal injury, were not taking any medication, and were not suffering from any condition known to affect bone metabolism	Healthy, physically active men	VO2max 53±6	24	3			Yes	CHO (8% glucose immediately before, every 20 minutes during and immediately after exercise or placebo.	normal diet and refrained from exercise or strenuous physical exertion. Participants recorded their dietary intake and were asked about lifestyle activity (e.g., feelings of fatigue, sleep patterns), with diet and sleep patterns being replicated between trials.	morning, ~0900	Fasted 12h	Fasted until end of exercise	placebo drink	treadmill run, 0% gradient	70% VO2 max	120	8400	baseline, immediately post-ex, 1h post-ex, 2h post-ex, 24h post-ex, 48h post-ex, 72h post-ex	P1NP	1	Formation	EDTA plasma	2	ECLIA (Roche)		<3% (0.2-1.5ug/L)	ng/ml	0830	NO
																										CTX	2	Resorption	EDTA plasma	2	ECLIA (Roche)		<3% (20-600ug/L)	ng/ml	0830	NO
3	Scott et al.	2011	Journal of Applied Physiology	The role of exercise intensity in the bone metabolic response to an acute bout of weight-bearing exercise	To compare the effects of three different cardiovascular exercise intensities on changes in bone turnover markers during, and for 4 days following, acute endurance running under highly standardized conditions.	Repeated measures, counterbalanced, experimental design, whereby participants took part in 3 experiments, each one involving an exercise test conducted at a different intensity.	10	nonsmokers, had not suffered a bone fracture of any type in the previous 12 mo, were free from musculoskeletal injury, and did not suffer from any condition or take any medication known to affect bone metabolism. Additional inclusion criteria were a fasting vitamin D concentration of >12 ng/ml or, if vitamin D was <12 ng/ml, a fasting PTH concentration of <6.9 pmol/l.	Healthy males (team sports players, recreational runners, club runner)	VO2max 56±8	28	4	23.2	2.1	No		standarised diet after the trial for 3 days	morning,~ 0815	Fasted 11h	Fasted until 1200, water consumed	65% intensity	treadmill running	65% VO2max	60	3900	baseline, 20min during ex, 40min during ex, immediately post-ex, 30min post-ex, 1h post-ex, 2h post-ex, 3h post-ex, 24h post-ex, 48h post-ex, 72h post-ex	P1NP	1	Formation		2	RIA (Orion Diagnostica)	22% duplicates	3.5-5.4%	ng/ml	0800	NO
																										CTX	2	Resorption		2	ECLIA (Roche)		<8% (0.2-1.5ug/L)	ng/ml	0800	NO
																					75% intensity	treadmill running	75% VO2max	60	4500		P1NP	1	Formation		2	RIA (Orion Diagnostica)	22% duplicates	3.5-5.4%	ng/ml	0800	NO
																											CTX	2	Resorption		2	ECLIA (Roche)		<8% (0.2-1.5ug/L)	ng/ml	0800	NO
4	Scott et al.	2012	Bone	Effect of fasting versus feeding on the bone metabolic response to running	To investigate the effect of an overnight fast, versus feeding, on the bone metabolic response to an acute bout of treadmill exercise.	Repeated measures, experimental design, whereby participants took part in two, counterbalanced, experiments comprising exercise conducted in either a fasted, or fed, state. ONLY FASTED	10	good physical condition, with a history of weightbearing exercise and average to above average levels of fitness	Physically-active males	VO2max 52±6	28	4			Yes	fasted vs fed	standarised died after the trial for 3 days?	morning, ~1030	overnight fast	Fasted until 3h post exercise	Fasted	treadmill run	65% VO2max	60	3900	baseline, 1h post-baseline, 1.5h post-baseline, 2.25h post-baseline, 30min during ex, immediately post-ex, 1h post-ex, 2h post-ex, 3h post-ex, 24h post-ex, 48h post-ex, 72h post-ex	P1NP	1	Formation	EDTA plasma	2	RIA (Orion Diagnostica)				0800	YES
																										CTX	2	Resorption	EDTA plasma	2	ECLIA (Roche)		<8% (0.2-1.5ug/L)	ng/ml	0800	YES
5	Townsend et al.	2017	Medicine and Science in Science and Sports and Exercise	The effect of postexercise carbohydrate and protein ingestion on bone metabolism	To investigate whether feeding CHO and protein immediatley, or 2 hours after a prolonged intense running bout will impact the bone biomarker respoonse to that exercise bout .	Randomized, counterbalanced, placebo controlled and single blinded crossover study, whereby participants took part in 3 experimental trials, i.e., placebo control trial, immediate feeding (CHO/PRO) ingested straight after exercise and delayed feeding (CHO and PRO) ingested 2 hours post exercise.	10	Participants were non-smokers, had not suffered a fracture in the last 12 months, were free from musculoskeletal injury and did not suffer from any condition known to affect bone metabolism.	male endurance runners who had been competing and training consistently >2 years in 10 km, half marathon, marathon or ultra-distance races, without a significant break.	VO2max 63±5	28	6			Yes	CHO feeding, CHO and protein immediatley, or 2 hours after a prolonged intense running	On days 1 and 2, participants refrained from all exercise and followed a prescribed diet. A diet consisting of 55% CHO, 30% fat and 15% PRO, and isocaloric with habitual diets was designed using dietary analysis softwar for each participant to consume on days 1 and 2 of each trial.	morning, ~0850	overnight fast from 2000 (>12h)	Fasted	Placebo	treadmill run to volitional exhaustion	75% VO2max	75 (average time till exhaustion)	5625	baseline, immediately post-ex, 1h post-ex, 2h post-ex, 3h post-ex, 4h post-ex, 24h post-ex	P1NP	1	Formation	EDTA plasma	2	ECLIA (Roche)		<3% (20-600 ug/L)		0840	NO
																					Placebo					CTX	2	Resorption	EDTA plasma	2	ECLIA (Roche)		<3% (0.2-1.5 ug/L)		0840	NO
																					Delayed feeding		75% VO2max	75	5625		P1NP	1	Formation	EDTA plasma	2	ECLIA (Roche)		<3% (20-600 ug/L)		0840	NO
																					Delayed feeding						CTX	2	Resorption	EDTA plasma	2	ECLIA (Roche)		<3% (0.2-1.5 ug/L)		0840	NO
																					Immediate feeding		75% VO2max	75	5625		P1NP	1	Formation	EDTA plasma	2	ECLIA (Roche)		<3% (20-600 ug/L)		0840	NO
																					Immediate feeding						CTX	2	Resorption	EDTA plasma	2	ECLIA (Roche)		<3% (0.2-1.5 ug/L)		0840	NO
6	Varley	PhD thesis		A Preliminary Investigation into Genetic Associations with Bone Resorption Following Treadmill Running (Thesis Chapter)	To determine whether specific SNPs are associated with bone resorption (β-CTX) prior to, and following, 120 min of treadmill running and attempt to offer a mechanistic explanation to the findings in studies 2 and 3.		42	Healthy males. At least three exercise sessions per w, were non-smokers, aged 18-35y and not taking medication that influenced bone metabolism.	Healthy males	VO2max 52±6	23	4			No		Throughout the experimental trial participants recorded and maintained their dietary intake, physical activity, lifestyle activity and refrained from alcohol and caffeine consumption (D4 only) and prolonged (>15 min) or intense physical activity.	morning, ~0900	overnight fast	Fasted	ALL	treadmill running	70% VO2max	120	8400	baseline, immediatey post-ex, 24h post-ex, 48h post-ex, 72h post-ex	CTX	2	Resorption	EDTA plasma	2	ELISA (IDS)	1.80%	2.50%	ng/ml	~0830	NO
7	Scott et al.	2010	Journal of Clinical Endocrinology and Metabolism	The effect of training status on the metabolic response of bone to an acute bout of exhaustive treadmill running		To investigate if training status influences the bone biomarker response to an acute bout of strenuous running exercise.	10	Subjects were included if they were nonsmokers, had not suffered a bone fracture in the previous 12 months, were free from musculoskeletal injury, and were not taking any medication or suffering from any condition	Recreationally active males	VO2max 54±3	26	3	24	2.2			All subjects refrained from physical activity, followed a prescribed diet, and attended the laboratory for follow-up analyses		overnight fast	Fasted	CONTROL	REST/CONTROL				24h post-baseline, 48h post-baseline, 72h post-baseline, 96h post-baseline	P1NP	1	Formation	EDTA plasma	2	RIA (Orion Diagnostica)	22% duplicates	3.5-5.4%	ng/ml	0800-0830	YES
																						REST/CONTROL					CTX	2	Resorption	EDTA plasma	2	ECLIA (Roche)		<8% (0.2-1.5ug/L)	ng/ml	0800-0830	YES
8	Evans et al.	2020	European Journal of Sport Science	The effect of intermittent running on biomarkers of bone turnover		To exmaine the effect of intermittent running on bone turnover markers using altered exercise to rest intervals.	12	Healthy men participating in >3 impact exercise sessions per week	Healthy males	VO2max 53±7	23	4			No		12 h fasted state prior, no exercise >48 h prior, limit physical activity the morning of testing, euhydrated state (drinking to thirst), no alcohol and psychoactive substances >24 h prior		12h fasting	Fasted	CONTROL	REST/CONTROL				1h post-baseline, 2h post-baseline, 24h post-baseline	P1NP	1	Formation	plasma (adjusted)	2	IDS-iSYS	3.4-5.3%	3.9-5.5%	ng/ml	0700-0800	YES
																						REST/CONTROL					CTX	2	Resorption	plasma (adjusted)	2	IDS-iSYS	3.2-3.5%	4.4-5.3%	ng/ml	0700-0800	YES

Appendix B: Codebook examples (https://drive.google.com/drive/folders/1wEmyIm18VDbMqUVjnUy8tKj5uK1Y20aq?usp=sharing)

Study	Author (date)	Biomarker	Biomarker code	Unit	Baseline	During20	During40	Immediate	Post0.5	Post1	Post2	Post3	Post4
1	Lehrskov et al. 2020	P1NP	1	ng/ml									
1	Lehrskov et al. 2020	P1NP	1	ng/ml									
1	Lehrskov et al. 2020	P1NP	1	ng/ml									
1	Lehrskov et al. 2020	P1NP	1	ng/ml									
1	Lehrskov et al. 2020	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
2	Sale et al 2015	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
3	Scott et al 2011	P1NP	1	ng/ml									
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4	Scott et al 2012	P1NP	1	ng/ml									
4	Scott et al 2012	P1NP	1	ng/ml									
4	Scott et al 2012	P1NP	1	ng/ml									
5	Townsend et al 2017	P1NP	1	ng/ml									
5	Townsend et al 2017	P1NP	1	ng/ml									
5	Townsend et al 2017	P1NP	1	ng/ml									
5	Townsend et al 2017	P1NP	1	ng/ml									
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5	Townsend et al 2017	P1NP	1	ng/ml									
5	Townsend et al 2017	P1NP	1	ng/ml									
5	Townsend et al 2017	P1NP	1	ng/ml									

ID	Study	Author (date)	Biomarker	Biomarker code	Unit	Baseline	Remeasure	Difference
1	1	Lehrskov et al. 2020	CTX	2	ng/ml			
2	1	Lehrskov et al. 2020	CTX	2	ng/ml			
3	1	Lehrskov et al. 2020	CTX	2	ng/ml			
4	1	Lehrskov et al. 2020	CTX	2	ng/ml			
5	1	Lehrskov et al. 2020	CTX	2	ng/ml			
6	4	Scott et al 2012	CTX	2	ng/ml			
7	4	Scott et al 2012	CTX	2	ng/ml			
8	4	Scott et al 2012	CTX	2	ng/ml			
9	4	Scott et al 2012	CTX	2	ng/ml			
10	4	Scott et al 2012	CTX	2	ng/ml			
11	4	Scott et al 2012	CTX	2	ng/ml			
12	4	Scott et al 2012	CTX	2	ng/ml			
13	4	Scott et al 2012	CTX	2	ng/ml			
14	4	Scott et al 2012	CTX	2	ng/ml			
15	4	Scott et al 2012	CTX	2	ng/ml			
6	4	Scott et al 2012	CTX	2	ng/ml			
7	4	Scott et al 2012	CTX	2	ng/ml			
8	4	Scott et al 2012	CTX	2	ng/ml			
9	4	Scott et al 2012	CTX	2	ng/ml			
10	4	Scott et al 2012	CTX	2	ng/ml			
11	4	Scott et al 2012	CTX	2	ng/ml			
12	4	Scott et al 2012	CTX	2	ng/ml			
13	4	Scott et al 2012	CTX	2	ng/ml			
14	4	Scott et al 2012	CTX	2	ng/ml			
15	4	Scott et al 2012	CTX	2	ng/ml			
16	8	Evans et al 2020	CTX	2	ng/ml			
18	8	Evans et al 2020	CTX	2	ng/ml			
19	8	Evans et al 2020	CTX	2	ng/ml			
20	8	Evans et al 2020	CTX	2	ng/ml			
21	8	Evans et al 2020	CTX	2	ng/ml			
22	8	Evans et al 2020	CTX	2	ng/ml			
23	8	Evans et al 2020	CTX	2	ng/ml			
24	8	Evans et al 2020	CTX	2	ng/ml			
25	8	Evans et al 2020	CTX	2	ng/ml			
26	8	Evans et al 2020	CTX	2	ng/ml			
27	8	Evans et al 2020	CTX	2	ng/ml			
16	8	Evans et al 2020	CTX	2	ng/ml			
17	8	Evans et al 2020	CTX	2	ng/ml			
18	8	Evans et al 2020	CTX	2	ng/ml			
19	8	Evans et al 2020	CTX	2	ng/ml			
20	8	Evans et al 2020	CTX	2	ng/ml			
21	8	Evans et al 2020	CTX	2	ng/ml			
22	8	Evans et al 2020	CTX	2	ng/ml			
23	8	Evans et al 2020	CTX	2	ng/ml			
24	8	Evans et al 2020	CTX	2	ng/ml			
25	8	Evans et al 2020	CTX	2	ng/ml			
26	8	Evans et al 2020	CTX	2	ng/ml			
27	8	Evans et al 2020	CTX	2	ng/ml			

Appendix C. Modified Downs & Black checklist

P1NP and β-CTX-1 responses to a prolonged continuous running bout in adult healthy males: a systematic review and individual participant data meta-analysis

Supplementary File

Modified Downs & Black checklist

Q.1. *Is the hypothesis/aim/objective of the study clearly described*? Yes = 1; No = 0

Q.2. *Are the main outcomes to be measured clearly described in the introduction or methods section?* If the main outcomes are first mentioned in the results section, answer no. Yes = 1; No = 0.

Q.3. *Are the characteristics (e.g., age, height, weight, training and health status) and the inclusion/exclusion criteria (e.g., non-smokers, injury free for the previous 12 months, not taking any medication, or suffering from any condition known to affect bone metabolism) of the participants included in the study clearly described?* Yes = 2 (characteristics AND inclusion/exclusion criteria); Yes = 1 (characteristics OR inclusion/exclusion criteria); No = 0.

Q.4. *Are the characteristics of the exercise bout clearly described?* Including, type, intensity and duration should be described. For control studies, resting should be confirmed. If they provide a nutritional supplement the exact type and dose should be provided. Yes = 1, No = 0.

Q.5. *Does the study provide estimates of the random variability in the data for the main outcomes?* In non-normal data, inter-quartile range should be reported. In normal data, standard deviation, standard error or confidence intervals should be reported. Yes = 1; No = 0.

Q.6. *Are the main results of the study clearly described?* Simple outcome data should be reported (including tables and figures) for all major results so the reader can check the major analyses and conclusions. This does not cover statistical tests. Yes = 1; No = 0.

Q.7. *Were the interventions approved by an ethics committee?* Answer yes if they confirm they have ethical approval Yes = 1; No = 0.

Q.8. *If any of the results of the study were based on 'data dredging' was this made clear?* Any analyses that had not been planned at the outset should be clearly indicated. If no retrospective subgroup analyses were reported, then answer yes. Yes = 1; No = 0; Unable to determine = 0.

Q.9. *Was the timing of blood sampling clearly described?* Answer yes if the precise time-points were provided. Answer no if it is not clear exactly when the blood samples were drawn during the trial. Yes = 1; No = 0; Unable to determine = 0.

Q.10. *Were the laboratory methodologies (e.g., blood samples storage and handling, CV of assays used) appropriately described?* Yes = 2 (information about sample storage and handling, AND assays CV is provided); Yes = 1 (information about sample storage and handling, OR assays' CV is provided); No = 0; Unable to determine = 0.

Q.11. Were study participants randomised to intervention groups or was the order of trials randomised if using *a crossover design?* Answer yes if the order of trials, treatment, or allocation to groups, was randomly assigned. If it was not possible for the study to be

randomised (*e.g.*, single-trial studies) or if the intervention of interest was not exercise answer yes. Yes = 1; No = 0; Unable to determine = 0.

Q.12. *Were multiple trials separated by a clear washout period?* Answer yes if there was a washout period between multiple-trials in crossover/counterbalanced designs. For studies with different designs (*e.g.*, single-trial, randomised groups) answer yes. Yes = 1; No = 0; Unable to determine = 0.

Q.13. *Was at least one familiarization session conducted prior to exercise testing?* Answer yes if they conducted a familiarization exercise trial, **or if familiarization was not necessary** (*e.g.*, if the study uses a single or **non-performance-based** exercise bout, and control/resting interventions). Yes = 1; No = 0; Unable to determine = 0.

Q.14. *Were the exercise test conditions adequately standardised and described?* Factors to consider include confirmation of the time of day that testing was conducted (score yes if the exact time of day that tests were conducted was reported, and this was the same for all participants), and control for unusual activity (score yes if they requested that participants avoid unusual or very strenuous activity for at least 24 hours prior to the test) or nutritional factors in the days prior to the exercise test (score yes if they request participants to maintain usual feeding habits the day before). Yes (all 3 factors considered) = 3; Yes (most factors (2 of the 3) considered) = 2; Yes (some (1 of the 3) factors considered) = 1; No = 0; Unable to determine = 0.

Q.15. *Was fasting status for baseline blood sampling confirmed*? Answer yes if the strategy for standardization is indicated as an overnight fast. Yes = 1, No = 0, Unable to determine = 0.

Q.16. Were samples corrected for plasma volume changes *or appropriately accounted for with fluid intake*? Answer yes for resting/control studies. Yes = 1; No = 0; Unable to determine = 0

Max attainable score = 20. The combined score will be used to categorise each study according to 4 categories, i.e., High (18 - 20), Moderate (15 - 17), Low (11 - 14) or Very Low (≤ 10)

Note: For any question where the response cannot be ascertained based on the information presented in the article, score 0 (unable to tell).

Original Reference:

Downs, S.H., & Black, N., 1998. The feasibility of creating a checklist for the assessment of the methodological quality both of randomised and non-randomised studies of health care interventions. *Journal of Epidemiology and Community Health*, 52(6), pp.377–384. https://doi.org/10.1136/jech.52.6.377

Appendix D. Publication of the study reported in Chapter 4.

A collagen extraction and deuterium oxide stable isotope tracer method for the quantification of bone collagen synthesis rates *in vivo*

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Running head

Bone collagen synthesis measurement with a D_2O method

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Abstract

The development of safe and practical strategies to prevent weakening of bone tissue is vital, yet attempts to achieve this have been hindered by a lack of understanding of the short-term (days-weeks) physiology of bone collagen turnover. To address this, we have developed a method to quantify bone collagen turnover *in vivo,* using deuterium oxide (D_2O) tracer incorporation techniques combined with gas chromatography pyrolysis isotope-ratio mass spectrometry (GC-*pyrolysis*-IRMS). Forty-six male and female rats from a selectively bred model, ingested D_2O for 3 weeks. Femur diaphyses (FEM), tibia proximal (T-PRO), and distal (T-DIS) epiphyses-metaphyses and tibia mid-shaft diaphyses (T-MID) were obtained from all rats after necropsy. After demineralisation, collagen proteins were isolated, hydrolysed and collagen fractional synthetic rates (FSR) determined by incorporation of deuterium into protein-bound alanine via GC-*pyrolysis*-IRMS. The collagen FSR for the FEM (0.131 ± 0.078 %/day; 95% CI [0.106-0.156]) was greater than the FSR at T-MID (0.055 ± 0.049 %/day; 95% CI [0.040-0.070]; $P < 0.001$). The T-PRO site had the highest FSR (0.203 ± 0.123 %/day; 95% CI [0.166-0.241]) and T-DIS the lowest (0.027 ± 0.015 %/day; 95% CI [0.022-0.031]). The three tibial sites exhibited different FSRs ($P < 0.001$). Herein we have developed a sensitive method to quantify *in vivo* bone collagen turnover and identified site-specific rates of turnover, which could be applicable to studies of human bone collagen synthesis.

Key words

Bone turnover, collagen synthesis, deuterium oxide, GC-*pyrolysis*-IRMS, stable isotopes

Abbreviations

D_2O	Deuterium oxide
GC-*pyrolysis*-IRMS	Gas chromatography-pyrolysis-mass spectrometry
LRT	Low adaptive response to exercise training
HRT	High adaptive response to exercise training
FEM	Femur diaphysis site
T-PRO	Tibia proximal epiphysis and metaphysis site
T-MID	Tibia mid-shaft site
T-DIS	Tibia distal epiphysis and metaphysis site
FSR	Fractional synthetic rate
GC-MS/MS	Gas chromatography tandem mass spectrometry
DXA	Dual-energy X-ray absorptiometry
BMD	Bone mineral density
LOD	Limit of detection
μCT	X-ray micro-computed tomography

New and noteworthy

We have developed a method to quantify bone collagen turnover *in vivo*, using D_2O in adult rodents combined with highly sensitive GC-*pyrolysis*-IRMS techniques. This method is ideally suited to the measurement of slow turnover proteins, such as collagen, and its high sensitivity will permit the application of D_2O loading protocols that are well tolerated in humans (*i.e.*, 150 ml + 50 ml/week^{-1}). Therefore, our D_2O GC-*pyrolysis*-IRMS approach has great potential applicability to study human bone collagen turnover.

Introduction

Understanding bone remodelling in ageing and disease (*e.g.*, osteoporosis), and developing strategies to maintain bone tissue are vital. Imaging techniques, such as dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography, enable the measurement of the mineral compartment of bone. Changes in mineralised bone, however, can only be determined over a long period (*e.g.*, months/years), and DXA-derived bone mineral density only relates to about two-thirds of the bone's strength (1). Other factors in the non-mineral compartments of bone, are equally important (4). The extracellular matrix of bone, largely made up of collagen proteins, is vital in providing underlying strength to the bone (4).

Collagen is the most abundant protein in the human body, comprising ~30% of total body protein in humans (25). In bone, 90% of the organic matrix is made up by type I collagen; and collagen (types I, II, III and V) is also an important component of tendon, skin, ligaments and muscle (25). Human muscle collagen synthesis is slower than tendon collagen synthesis at rest (20)(24), although the question as to whether or not bone has a slower turnover than other musculoskeletal tissues remains controversial (25)(24). The rate of bone collagen turnover is important in determining bone strength because it influences the pattern of mature/immature collagen crosslinking in bone, which is important for bone quality and strength (4)(27)(3). The mechanisms by which collagen turnover is altered in bone-affecting diseases or in response to potentially favourable interventions (*e.g.*, drugs, exercise, diet) to improve bone strength, is, however, poorly defined due to the lack of robust analytical approaches to its measurement.

Indirect measures of bone formation and resorption, known as bone (re)modelling markers, can be measured in the blood. Although these biomarkers are widely used for assessing short-term changes in bone collagen turnover, they have yet to be validated against direct measures of bone collagen synthesis or breakdown (2). Bone (re)modelling markers have some key limitations, including pre-analytical (*e.g.*, biological causes; sample collection, handling and storage requirements) and analytical (*e.g.*, within and inter laboratory variation, assay reproducibility) variability (11)(17), as well as a lack of tissue and site specificity (7)(25). As such, studies that have used bone biomarkers to measure collagen changes need to be interpreted carefully.

The direct incorporation of isotopically labelled tracers is generally regarded as the gold standard in determining fractional synthetic rates and can be performed on bone if a tissue sample can be collected (29). Traditionally, amino acid isotope tracers, such as carbon (^{13}C), deuterium (^{2}H) or nitrogen (^{15}N), have been used to measure *in vivo* synthesis of human musculoskeletal tissues, including bone (2)(22)(24). These amino acid tracers are, however, hindered by the heterogeneity of amino acid body pools (21)(28), and require preparation of high-cost infusions and venous/arterial cannulation (30).

These studies are also restricted by time (generally <24h), which makes it challenging to accurately measure very low rates of bone collagen synthesis. Furthermore, the use of variable stable isotope tracers and different bone protein fractions makes comparisons between studies difficult (2)(22)(24).

Using deuterium oxide (D_2O or "heavy water") as a stable isotope tracer can overcome some of these limitations. For instance, D_2O can be easily ingested orally, with the deuterium becoming rapidly equilibrated within the body water and intracellular amino acid pools (30). The potential to use protein-bound alanine to quantify collagen synthesis offers a major advantage to detect low rates of tissue turnover such as in bone. First, up to four hydrogens are replaced by deuterium before free alanine is incorporated into newly made protein. This acts to amplify the amount of deuterium incorporated into the bound end product. Further, alanine has been robustly validated in the application of D_2O methodologies (30), with rapid transamination reactions meaning alanine enrichment is not easily perturbed overtime (8). This allows D_2O to be administered with minimal interference to an individual's normal daily activities, with enrichment in the precursor pool easily maintained over weeks and months (29), making this tracer more suited to the measurement of slow turnover proteins, such as collagen.

Previous assessments of bone collagen synthesis rates using D_2O have been made in rodents, however these required high levels of ^2H body water enrichment (~3%) and were performed in growing rats, where collagen synthesis rates are considerably higher (12)(5)(6). As such, methods using D_2O to measure collagen synthesis rates in adult animals' scenarios and potentially in humans, where collagen synthesis rates are considerably lower, are lacking. To address this, we have developed a method to quantify low levels of bone collagen turnover *in vivo*, using lower levels (<1%) of ^2H body water enrichment in adult rodents. Combining sensitive GC-*pyrolysis*-IRMS techniques, this method enables the measurement of slow turnover proteins such as collagen, with the potential to determine short term changes in bone collagen synthesis.

Methods

Animals. All experiments were approved by the Animal Care and Use Committee of Southern Finland, license number ESAVI-2010-07989/Ym-23, STH 534A (21.9.2010) and complements ESAVI/1968/04.10.03/2011, PH308A (30.3.2011) and ESAVI/722/04.10.07/2013, PH275A (1.3. 2013); and were conducted in accordance with the Guidelines of the European Community Council Directive 86/609/EEC. Bones were derived from 46 adult male (n = 22) and female (n = 24) rats (9 ± 3 months), which were selectively bred for yielding low or high aerobic responses to exercise training (15). The background of the experimental animals is not relevant to the present method development

and the bone was opportunistically harvested for this purpose as an addition to other independent investigations already being conducted.

Rats were single-housed in air-conditioned rooms at an ambient temperature of 21 ± 2°C and relative humidity at 50 ± 10%. Artificial lighting provided light cycles of 12:12-h light-total darkness. Commercially available pelleted rodent diet (R36; Labfor; Lantmän nen, Malmö, Sweden) and tap water (from the municipal water system of Jyväskylä, Finland) was available *ad libitum* throughout the study. The energy content of the feed was 1,260 kJ/100 g (300.93 kcal/100 g). The feed contained 18.5% raw protein, 4.0% raw fat, 55.7% nitrogen-free extracts, 3.5% fibre, 6.3% ash, and 12% water. Rats were divided into two groups of control or exercise trained, with samples collected from both groups for method development.

Deuterium enrichment. Rats received a gavage of 7.2 ml/kg 70% D_2O, thereafter, animals were provided with free access to drinking water enriched with 2% (v/v) of D_2O. Body water enrichment was determined from plasma and was used to calculate the average precursor enrichment. Blood samples were collected at necropsy (~5 ml) and plasma was separated by centrifugation and stored frozen until analysis. Body water enrichment was measured in plasma by incubating 100 µl of each sample with 2 µl of 10 M NaOH and 1 µl of acetone for 24 h at room temperature. Following incubation, the acetone was extracted into 200 µl of n-heptane and 0.5 µl of the heptane phase was injected into the GC-MS/MS for analysis. A standard curve of known D_2O enrichment was run alongside the samples for calculation of enrichment.

Bone sample collection. Forty-eight hours after the last training bout, animals were anesthetised with carbon dioxide and killed by cardiac puncture and thereafter immediately necropsied. Left femur and tibia bones were rapidly exposed, removed, and immediately frozen by complete immersion in liquid nitrogen and were kept at -80^{o}C until analysis. We speculated that different anatomical bone sites might have different turnover rates. As such, we obtained bone samples from the femur diaphysis (not site controlled) with pestle and mortar (FEM, 0.10 ± 0.03 g), and three different sites of the tibia using an electric hand saw (Dremel 3000 Rotary Tool, USA): tibial proximal epiphysis-metaphysis (T-PRO, 30 ± 0.08 g), the tibial mid-shaft diaphysis (T-MID, 9 ± 0.04 g), and the tibial distal epiphysis-metaphysis (T-DIS, 11 ± 0.02 g); each sample was ~20% of the total tibia length.

Isolation and derivatisation of bone collagen protein. Bone samples were transferred into 0.3-0.5 M HCl until samples were completely decalcified and appeared translucent and flexible. This process typically took 10-15 days with the HCl solution being changed every 1-4 days. Following demineralisation, bone samples were transferred to 0.3 M NaOH in order to dissolve and remove the remaining bone marrow and soluble proteins, leaving the bone collagen proteins. The NaOH solution

was changed ~3 times over 2-5 days with bouts of vortexing and centrifuging to help remove bone marrow particles. The remaining bone collagen proteins were hydrolysed to free amino acids by incubating in 0.1 M HCl in Dowex H^+ resin slurry overnight at 110°C before being eluted from the resin with 2 M NH_4OH and evaporated to dryness. Amino acids were then derivatised as their N-methoxycarbonyl methyl esters. Dried samples were suspended in 60 µl of distilled water and 32 µl of methanol, and following vortex, 10 µl of pyridine and 8 µl of methyl chloroformate were added. Samples were vortexed for 30 s and left to react at room temperature for 5 min. The newly formed N-methoxycarbonyl methyl ester amino acids were then extracted into 100 µl of chloroform. A molecular sieve was added to each sample for ~20 s before being transferred to a clean glass gas chromatography insert, removing any remaining water by size exclusion adsorption.

GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates. Protein-bound alanine enrichment was determined by gas chromatography pyrolysis isotope-ratio mass spectrometry (GC-*pyrolysis*-IRMS) and body water enrichment by gas chromatography tandem mass spectrometry (GC-MS/MS). Bone collagen fractional synthetic rates (FSR) were calculated from the incorporation of deuterium-labelled alanine [corrected for the mean number of deuterium moieties incorporated per alanine (3.7) and the dilution from the total number of hydrogens in the derivative (*i.e.*, 11)] into protein using the enrichment of body water as the surrogate precursor labelling over the 3-week time period of D_2O labelling. The equation used was:

$$\mathrm{FSR} = -\ln\left[\frac{1-\left(\frac{APEala}{APEp}\right)}{t}\right]$$

where APEala equals deuterium enrichment of protein-bound alanine, APEp indicates mean precursor enrichment over the time period, and *t* represents time (3 weeks or 21 days)(30).

Statistical analysis. Data from all rats were pooled and analysed together independently of the sex, phenotype and exercise for this study. Descriptive statistics were performed for all data sets to check for normal distribution (accepted if $P > 0.05$) using the Shapiro-Wilk test. All data are presented as means ± 1SD. Differences between collagen FSR of the FEM and T-MID samples were analysed by Wilcoxon matched pairs test. The Kruskal-Wallis test was used to compare T-PRO, T-MID and T-DIS samples. *Post hoc* analysis was performed using Dunn's multiple comparisons test to determine the differences between each of the tibial sites. All analyses were performed on GraphPad Prism 8 (La Jolla, CA, USA). The level of significance was set at $P < 0.05$.

Results

The difference in rats' body weight over a 7-week period was ~6 %. The average body water enrichment in rats was 0.685 ± 0.089 APE, whilst the average change in the deuterium labelling, expressed as delta per mil deuterium (δ^2H) was FEM 352 ± 38 δ^2H, T-PRO 548 ± 45 δ^2H, T-MID 170 ± 21 δ^2H and T-DIS 83 ± 10 δ^2H (**Figure 1**) the higher the value reflecting the greater incorporation of labelled alanine. The calculated average collagen FSR for FEM (0.131 ± 0.078 %/day; 95% CI [0.106-0.156]) were significantly greater than the FSR at T-MID (0.055 ± 0.049 %/day; 95% CI [0.040-0.070]; P < 0.001, **Figure 2**). The highest FSR was at the T-PRO site (0.203 ± 0.123 %/day; 95% CI [0.166-0.241]) and the lowest at the T-DIS (0.027 ± 0.015 %/day; 95% CI [0.022-0.031]). The three tibial sites had significantly different FSRs (P < 0.001, **Figure 3**). T-PRO was significantly different from T-MID (P <0.001) and T-DIS (P <0.001), but the difference between T-MID and T-DIS was only approaching significance (P = 0.057).

Figure 1

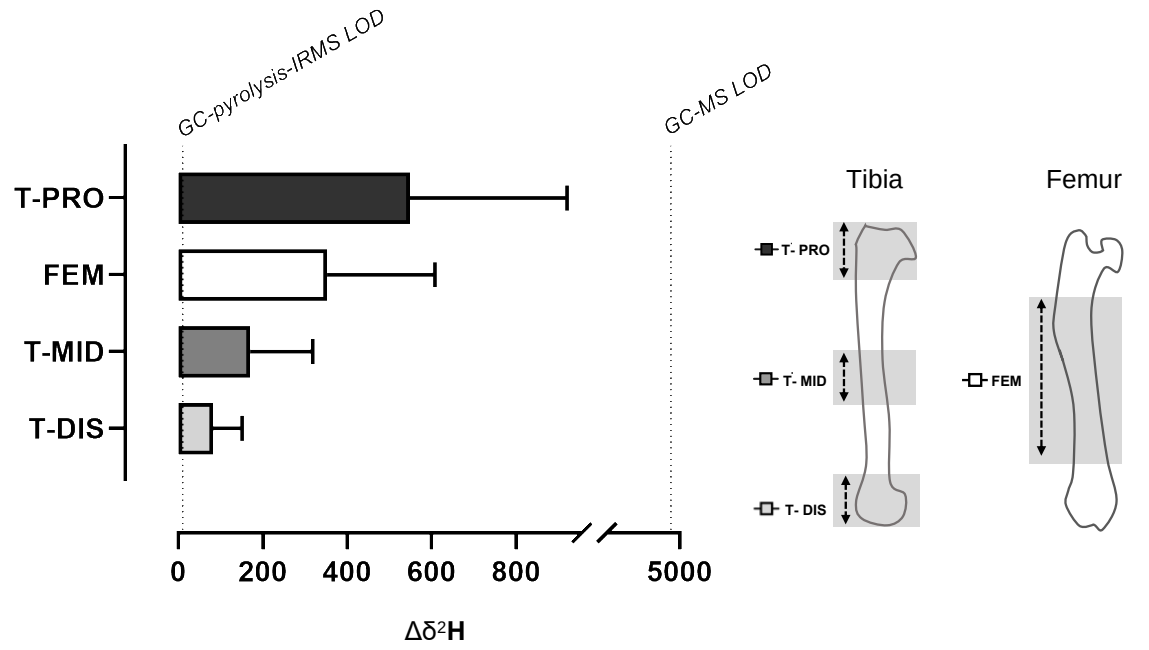

Figure 1. Change in bound deuterium enrichment ($\Delta\delta^2$H) across the tibia proximal (T-PRO), mid-shaft (T-MID), distal (T-DIS) and femur (FEM). GC-*pyrolysis*-MS limit of detection (LOD) shown as 10 δ^2H and GC-MS LOD shown as 4700 δ^2H. Sampling areas of tibia and femur shown in highlighted in grey.

Figure 2

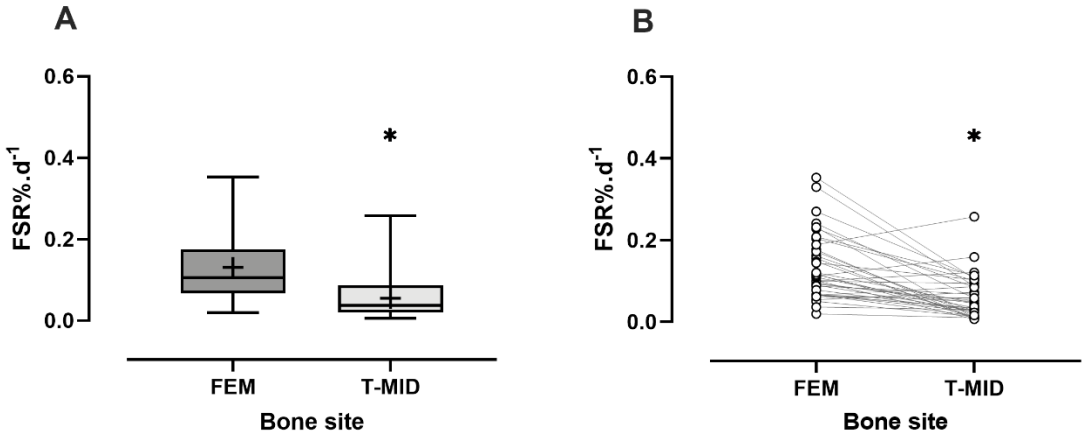

Figure 2. Collagen fractional synthetic rate (FSR) for the femur (FEM) and the mid -shaft of the tibia (T-MID). **A)** Data represented as box plots, + represents mean. **B)** Individual values. * Wilcoxon matched pairs test $P < 0.001$.

Figure 3

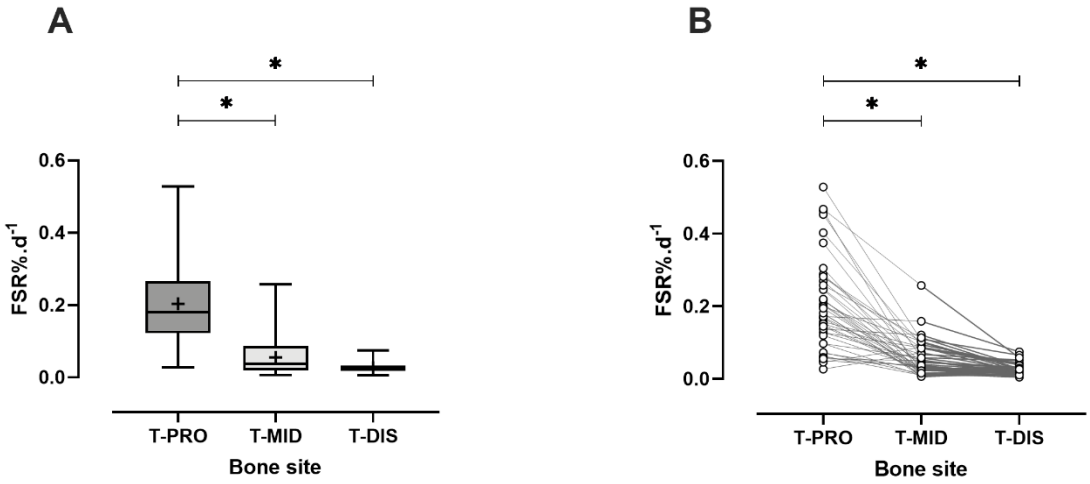

Figure 3. Collagen fractional synthetic rate (FSR) across the proximal (T-PRO), mid-shaft (T-MID) and distal (T-DIS) sites of the tibia. **A)** Data represented as box plots, + represents the mean. **B)** Individual values. * *Post hoc* Dunn's multiple comparisons test $P < 0.001$.

Discussion

We have developed a novel D_2O stable isotope tracer method capable of detecting low levels of ^2H incorporation and have tested this method for its ability to quantify a range of bone collagen synthesis rates *in vivo* over a 3-week period in rodents. This method was able to detect differences in bone collagen synthesis between the femur and the tibia and differences in collagen FSR at different sites along the length of the same bone (tibia). Our measures of bone collagen FSR ranged between 0.005-0.529 %/day, being ~30 fold lower than mean muscle protein synthesis rates measured in this cohort. Nonetheless, our data and others (2)(24) suggest bone collagen has a greater turnover rate than previously suggested by semi-quantitative estimates (3-25%/year) or calcium turnover (8-15%/year) (24). Previous measures of murine bone collagen synthesis using D_2O have showed active synthesis (6)(12) with one study reporting rates of ~17 %/week in young growing mice (5).

Tibial samples were obtained from the mid-shaft diaphysis site (corrected by the length of each rat's bone). In addition, samples from proximal and distal epiphyses-metaphyses were obtained, in order to investigate collagen FSRs across bone regions that have different compositions of trabecular and cortical bone. Diaphysis synthesis rates in the femur (not site-controlled) and tibia (mid-shaft) were significantly different. The synthesis rate was faster at the proximal tibial site than at both the mid-shaft and distal sites. The differences between collagen FSR across bone sites reported herein highlight the potential limitations in the utility of bone turnover biomarkers that estimate whole body bone turnover. In fact, previous work in humans has pointed to the incongruities between the PINP biomarker and changes in bone (2) and tendon (19) turnover. Further, our results highlight the importance of controlling and reporting the bone site used for analysis in future studies.

Such differences between synthesis rates among bone sites may be due to variability of strain distribution and magnitude across bone surfaces when physical loading is applied, producing an osteogenic effect (*i.e.*, stimulation of bone formation). For example, similar bone-site differences in 19-week-old mice were shown using μCT and histomorphometry analyses (26). After receiving *in vivo* artificial loading for 2 weeks, murine tibia showed greater changes and new bone formation in the proximal and mid-shaft sites compared to the distal site (26). The heterogeneity of bone may well be important when considering how mechanical loading affects trabecular and cortical bone, since they appear to respond different to loading (33). Trabecular bone, compared to cortical bone, has shown a higher response to changes in the loading environment in mice vertebrae (16) and tibia (9). A different study showed that cortical and trabecular bone expressed different genes at baseline and in response to *in vivo* mechanical loading (13), suggesting that the cellular mechanisms of the mechanical loading responses in trabecular and cortical bone are different. This could explain the higher collagen FSRs at the proximal site of the rat tibia (composed of more trabecular bone), compared to the mid-shaft and distal tibia (composed of more cortical bone) shown herein.

Another important factor influencing an osteogenic response is the muscle contractile forces exerted upon the skeleton during movement (10). The direct insertion of healthy and active muscle tissue onto the bone periosteum promotes localised bone formation without mechanical stimulation (10). Herein, we showed greater collagen synthesis at the proximal site of the tibia, with major muscles being adjacent to this region of the knee. We are confident that the differences in collagen FSRs shown across different bone sites were not due to contamination with protein or amino acids from bone marrow or connective tissue, since care was taken during sample preparation to ensure bone samples were clean. Bone marrow and alkali soluble protein was thoroughly removed with 0.3 M NaOH and the remaining connective tissue was manually removed with a sharp scalpel during the demineralisation process.

Additionally, the potential presence of periosteum and growth plate in the tibial epiphyses (proximal and distal) may also have affected our measurements of bone collagen synthesis. Wilsman *et al.* suggested that the tibial proximal growth plate has a greater growth rate compared the tibial distal growth plate in 2-4-week-old rats (32)(31). We showed a higher collagen synthesis rate in the proximal tibia than in the distal tibia and mid-shaft, although the collagen synthesis rate in the distal tibia had a slower synthesis rate than the mid-shaft site (without a growth plate). Whilst rats used in our study were 9 months old at the start of the study, histological evidence suggests that tibial proximal growth plates are still active (areas of resting cells, cell proliferation, cell maturation and lacunar hypertrophy) and cartilage is still present in up to 25 month old rats, despite bony bridging being complete and without longitudinal bone growth (18). As such, it is possible that there were some elements of the growth plates present in the proximal and distal tibial sites measures, although we cannot determine the exact extent to which this might have affected our interpretation of their collagen synthesis rates. However, this issue will likely be minimised in human studies, where the control for the bone site during sampling can be made more easily in larger bones and the growth plates close in late puberty (14)(23). Future application of this method in interventional studies will provide further validity of the method and its sensitivity.

To date, no studies have used D_2O as a direct incorporation tracer technique to determine human bone collagen synthesis. The use of D_2O has many advantages for determining bone turnover compared to traditional amino acid tracer approaches, where changes in bone synthesis can only be captured in a short timeframe (*i.e.*, over hours). D_2O labelling with continued oral ingestion of heavy water can safely maintain body water enrichment for days, weeks, or months (29). This is especially important for slow turnover proteins, such as collagen, which may need longer periods of labelling for longer term interventional studies looking at changes in bone synthesis. Our bone collagen extraction and D_2O GC-*pyrolysis*-IRMS method offers a highly sensitive technique for quantifying small changes in δ^2H and therefore bone collagen synthesis *in vivo*. Despite very low collagen synthesis rates, this will ultimately permit measures of bone collagen synthesis in humans using well tolerated D_2O loading protocols (*i.e.*,

150 ml + 50 ml/week^{-1}). As such, there is great future applicability to human investigations, which are crucial in determining differences in bone turnover between age, sex, health and disease and responses to interventions, such as exercise, diet and drugs.

Author contribution statement

Conceptualisation: RC, MSB, KJE, LS, IV, DJW, KS, CS, and PJA. Methodology: RC, MSB, HK, SL, LGK, SLB, DJW, KS, CS, and PJA. Formal analysis: RC and MSB. Investigation: RC, MSB, HK, SL, LGK, and SLB. Data curation: RC, MSB, CS and PJA. Resources: MSB, HK, SL, LGK, SLB, DJW, KS, CS, and PJA. Writing original draft: RC. Writing review and editing: RC, MSB, KJE, LS, IV, HK, SL, LGK, SLB, DJW, KS, CS, and PJA. Visualisation: RC, MSB, CS and PJA. Supervision: MSB, KJE, LS, IV, DJW, KS, CS and PJA. Funding acquisition: CS and PJA.

Acknowledgements

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