Physical training, inflammation and bone integrity in elite female rowers

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Abstract

This study examined whether fluctuations in training load during an Olympic year lead to changes in mineral properties and factors that regulate bone (sclerostin (SOST), osteoprotegerin (OPG)), and receptor activator of nuclear factor kappa-B ligand (RANKL)) and energy metabolism (insulin-like growth factor-1 (IGF-1) and leptin), and inflammation (tumor necrosis factor-α (TNF-α)) in elite heavyweight female rowers. Blood samples were drawn from female heavy-weight rowers (n=15) (27.0±0.8y, 80.9±1.3 kg, 179.4±1.4 cm) at baseline (T1 – 45 weeks pre-Olympic Games) and following 7, 9, 20, 25 and 42 weeks (T1-6, respectively). Serum was analyzed by Multiplex assays (EMD Millipore, Toronto, CAN). Total weekly training load was recorded over the weeks prior to each time point. Bone mineral density (BMD) was measured by dual energy X-ray absorptiometry at T1 and T6. Total BMD increased significantly pre- to post-training (+1.6%). OPG, IGF-1, and leptin were not different across all time points. OPG/RANKL was significantly higher at both T4 and 5 compared to T1 and 2. High training load (T5) was associated with the highest TNF-α levels (2.1 pg/ml), and a parallel increase in SOST (993.1 pg/ml), while low training load (T6 - recovery) was associated with significantly lower TNF-α (1.5 pg/ml) and a parallel decrease in SOST (741.0 pg/ml). Leptin was a significant determinant of bone-mineral properties in these athletes. These results suggest exercise training can lead to an increase in OPG/RANKL, and training load periodization can control the inflammatory response associated with intense training, and combined with adequate caloric intake can preserve bone mineral integrity in elite female athletes.

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Table of Contents

List of Figures v
List of Tables vi
List of Abbreviations vii
CHAPTER 1: LITERATURE REVIEW 1
1.1 Bone 1
1.2 Bone Turnover 1
1.2.1 Cells involved in bone remodeling 2
1.2.2 Osteoclasts 2
1.2.3 Osteoblasts 4
1.2.4 Osteocytes 5
1.3 Serum markers of bone remodelling 6
1.3.1 Formation Markers 6
1.4 Tumor Necrosis Factor-Alpha effect on bone remodelling 8
1.5 Insulin Like Growth Factor-1 10
1.6 Leptin 11
1.6.1 Leptin Signaling 11
1.6.2 Leptin's effects on cell signalling in bone 13
1.7 Female rowers and bone 15
1.8 Monitoring serum markers of bone turnover during training 17
1.9 Introduction to Wnt Proteins 19
1.10 Canonical Wnt/β-Catenin Signalling 20
1.10.1 Interaction of Wnt signalling with the OPG/RANKL axis 22
1.10.2 Wnt signalling controls osteoblastogenesis 25
1.11 SOST – a Wnt antagonist 26
1.12 TNF-α response to various modes of exercise training 33
1.13 IGF-1 response to various modes of exercise training 35
1.14 Leptin response to various modes of exercise training 39
1.14.1 Short term training effects on Leptin (<12 weeks) 42
1.14.2 Long term training effects on Leptin (>12 weeks) 43
1.15 OPG response to exercise training 45

1.16 SOST response to various modes of exercise training 48
CHAPTER 2: INTRODUCTION TO RESEARCH PAPER 52
CHAPTER 3: METHODS 55
3.1 Participants 55
3.2 Study Design and Procedures 55
3.3 Calculation of Training Volume, Intensity, and Load 56
3.4 Biochemical Analysis 57
3.5 Bone Measurements and Dietary Intake 57
3.6 Statistical analysis 58
CHAPTER 4: RESULTS 59
CHAPTER 5: DISCUSSION 64
5.1 Bone mineral properties and anthropometric outcomes pre- and post-training 65
5.2 SOST response to fluctuations in training load 66
5.3 OPG, RANKL and OPG/RANKL responses to fluctuations in training load 68
5.4 Leptin's association with bone mineral properties 69
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS 70
6.1 Conclusion 70
6.2 Limitations 70
6.3 Future Directions 71
REFERENCES 74
APPENDIX 100

List of Figures

Figure 1. Regulation of osteoclastogenesis 3
Figure 2. Regulation of osteoblastogenesis 4
Figure 3. Leptin signalling's effect on bone osteoclastogenesis 12
Figure 4. Factors associated with Wnt/β-catenin signalling 22
Figure 5. Weekly training volume from the 3 weeks preceeding each blood draw and mean ± SEM of resting a) SOST, b) OPG, c) RANKL, and d) OPG/RANKL serum concentrations in elite heavyweight female rowers 61
Figure 6. Weekly training volume from the 3 weeks preceeding each blood draw and mean ± SEM of resting a) TNF-α, b) leptin, c) IGF-1, and d) estrogen serum concentrations in elite heavyweight female rowers (n=15). A RM ANOVA was used to ascertain any significant changes across time in biomarkers. a = $p<0.05$, significantly different from week 1; significantly different from week 25; f = $p<0.05$, significantly different from week 42. 62
Figure 7. The effect of increased training volume on Wnt/β-catenin and RANK:RANKL:OPG signalling cascades; (Adapted from Servier Medical Art by Servier©). 65

List of Tables

Table 1. Studies investigating the response of TNF-α to exercise training. 33
Table 2. Studies investigating the response of IGF-1 to exercise training. 38
Table 3. Studies examining leptin's response to exercise training. 40
Table 4. Studies assessing OPG's response to exercise training. 47
Table 5. Studies assessing SOST's response to exercise training 49
Table 6. Bone outcomes of interest at pre- compared to post-season in all rowers 59
Table 7. Percent coefficient of variation (%CV) of all biomarkers for individual participants across time 60
Table 8. Regression models predicting total BMC and BMD using TNF-α and leptin. 63

List of Abbreviations

BMD – bone mineral density

M-CSF – macrophage-colony stimulating factor

RANKL – receptor activator of NF-kβ ligand

TRAP– tartrate resistant acid phosphatase-positive

C-fms – colony stimulating factor receptor

Osx – osterix

RUNX2 – runt related

BAP – bone alkaline phosphatase

OC – osteocalcin

SOST - sclerostin

DKK-1 – dickkopf-related protein 1

TNF-α – tumor necrosis factor α

IGF-1 – insulin like growth factor 1

PI3K – phosphatidylinositol 3-kinase

IGFBP-3 – IGF binding protein 3

ALS – acid labile unit

GH – growth hormone

GSK – glycogen synthase kinase

CART – cocaine-amphetamine-related transcript

JAK2 – janus kinase 2

STAT3 – signal transducer and activator of transcription 3

MAPK – mitogen activated protein kinase

mTOR – mammalian target of rapamycin

PTH – parathyroid hormone

Wnt – wingless and INT-1

APC – adenomatous polyposis coli

FZD – frizzled

LRP 5/6 – Lipoprotein-related protein 5 and 6

OPG – osteoprotegerin

OVX – ovariectomized

TSH – thyroid stimulating hormone

DHEA – dehydroepiandrosterone

CHAPTER 1: LITERATURE REVIEW

1.1 Bone

Bone is a dynamic tissue that provides structural support to the body, and allows for locomotion and protection. It also supports the body's homeostasis, acting as a reservoir for minerals, and of course supporting hematopoiesis within the supplying mineral marrow space [1]. There are 4 general categories of bone; long, short, flat and irregular bones. Within each category of bone there are anatomical differences. These differences involve changes in the amount of cortical and trabecular bone.

Cortical bone is dense, solid and surrounds the marrow space. In contrast, trabecular bone is a meshwork of bone interspersed in the bone marrow compartment and is mainly present in the vertebrae, pelvis and metaphysis of long bone. Trabecular bone makes up ~20% of the adult skeleton and tends to be more metabolically active than cortical bone, which makes up ~80% of the adult skeleton [1]. However, there are specific bones that have varying make-ups of bone types. For example, vertebrae (irregular bone), femoral head (long bone) and radial diaphysis are composed of 25:75, 50:50, and 95:5 cortical to trabecular bone respectively [1].

1.2 Bone Turnover

Bone turnover is a continual process of bone formation and resorption that leads to healthy and stable bone [2]. Metabolic disorders like osteoporosis and Paget's disease are characterized by a low bone mineral density (BMD) and changes in the microarchitecture of bone, and high fragility and risk of stress fractures because of a misbalance of whole body bone turnover (negative turnover balance). A negative turnover balance is

characterized by a push towards an increase in bone resorption and/or a decrease in bone formation, which ultimately leads to alterations in bone remodelling and thus a lower BMD [3].

Bone turnover determines the quality and quantity of bone [2]. This is the process of resorbing old damaged bone and forming new bone. This continual process maintains bone strength and mineral homeostasis, and is accomplished by removal of discrete packets of old bone (i.e., bone resorption), and replacement of these packets with newly synthesized proteinaceous matrix, and subsequent mineralization of the matrix to form new bone (i.e., bone formation).

1.2.1 Cells involved in bone remodeling

The bone remodeling process can be simplified to the relationship/balance of the cells that form bone and cells that resorb bone. Osteoblasts are derived from pluripotential mesenchymal stem cells from bone marrow and form bone. Osteoclasts are derived from circulating hematopoietic monocyte precursors and resorb bone [4]. These cells communicate through various pathways and their activity is tightly regulated to maintain adequate bone integrity and mass. Bone remodeling is conducted by osteoclasts and osteoblasts that are tightly coupled and are involved in a sequential cycle involving: 1) Activation. 2) Resorption, 3) Reversal, and 4) Formation [1].

1.2.2 Osteoclasts

Osteoclasts are multinucleated cells that require two factors for activation (i.e., osteoclastogenesis) that are depicted in Figure 1. These two factors include macrophage-colony stimulating factor (M-CSF), which signals through its receptor Fms [5], and receptor activator of NF-kβ ligand (RANKL), which signals through its receptor (RANK)

[6]. Furthermore, the downstream intracellular signalling effectors of M-CSF and RANKL; TNF receptor associated factor 6 (TRAF6) [7], activator protein 1 (AP-1) [8], nuclear factor-kβ (NF-kβ) [9], and nuclear factor of activated t-cells (NFAT) [10] have also been shown to be essential for osteoclastogenesis to occur. Specifically, knockout models, or *in vitro* inhibition of these effectors result in the inhibition of progression of pre-osteoclasts through the stages of differentiation. For example, the absence of these effector cells halts the progression to tartrate resistant acid phosphatase-positive (TRAP) multinucleated cells that express the receptor for M-CSF, which takes approximately 3 days *in vitro*. Furthermore, factors like tumor necrosis factor α (TNF-α) can induce an increase in osteoclastogenesis by increasing the expression of RANKL or M-CSF.

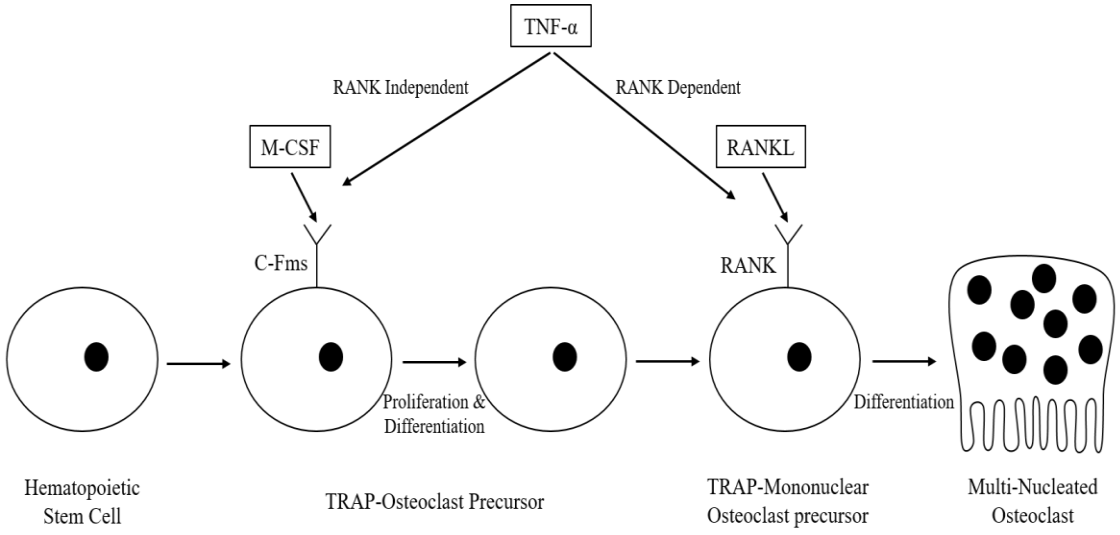

Figure 1. Regulation of osteoclastogenesis. M-CSF and RANKL signalling are critical for the progression from a hematopoietic stem cell to a mature multi-nucleated osteoclast. As osteoclast matures it becomes able to produce and secrete enzymes (TRAP) and factors that break down bone. M-CSF=macrophage colony-stimulating factor, C-Fms=colony stimulating factor receptor

Activation of resorption involves: recruitment and activation of mononuclear monocyte-macrophage osteoclast precursors, lifting of the endosteum that contains bone lining cells, and attachment of mononucleated osteoclast precursors to bone matrix, which subsequently become multinucleated preosteoclasts [1]. Resorption takes ~2-4wks during each cycle while the process of bone formation proceeds resorption and takes approximately 4-6 months [11]. Reversal involves the transition from bone resorption to bone formation.

1.2.3 Osteoblasts

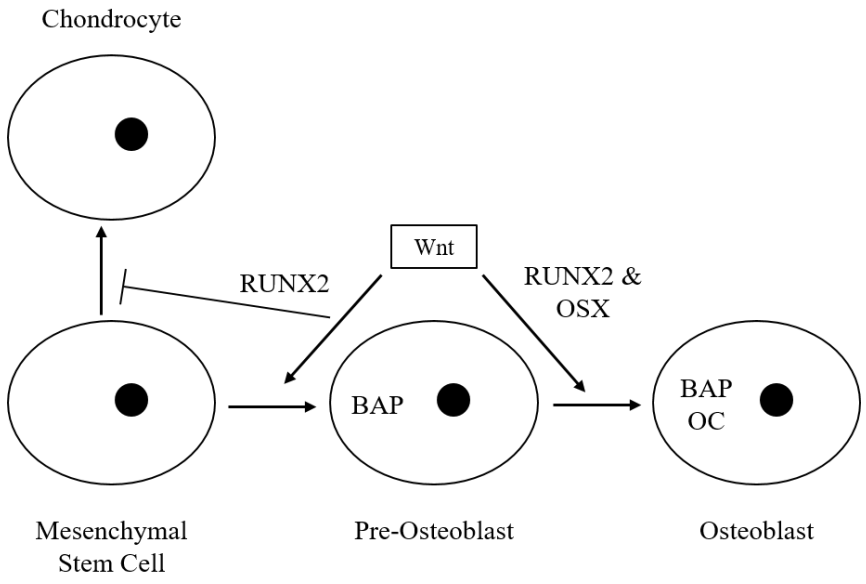

Figure 2. Regulation of osteoblastogenesis. Wnt signalling regulates the increase in osteoblastogensis through the increase in production of RUNX2 and OSX transcription factors. Upon progression from a mesenchymal stem cell, the osteoblast matures and becomes able to produce factors (BAP and OC) that lead to increase bone formation. OSX=osterix, BAP=bone alkaline phosphatase, OC=osteocalcin.

Bone formation involves synthesis of collagenous matrix by osteoblasts. Matrix is regulated by osteoblasts, which release membrane bound vesicles that concentrate calcium and phosphate and enzymatically destroy inhibitors of mineralization, where the matrix that has filled the resorbing pits will go on to mineralize and form new bone [12]. Mesenchymal stem cells are precursors of osteoblasts and are located within the bone marrow. In vitro, these cells can be differentiated into osteogenic, chondrogenic and adipogenic lineages [13]. The Wnt/β-catenin signalling has been shown to regulate osteoblastogenesis (Figure 2) and will be discussed further below. Osteoblasts that are in the newly formed matrix become osteocytes that have an extensive canalicular network connecting them to the bone surface osteoblasts. However, approximately 50-70% of osteoblasts that form new bone undergo apoptosis, while the remaining 50-30% turn into osteocytes or into bone-lining cells [14].

1.2.4 Osteocytes

Osteocytes account for 90-95% of all adult bone cells and can live up to a decade within mineralized bone matrix [15]. Their location within bone matrix is likely critical for their development into osteocytes from osteoblasts [16], and once within mineralizing bone they act as sensors to changes in mechanical load/stimuli (i.e., exercise) [17]. These mechanosensory cells can modulate osteoclast activity as well by expressing and secreting RANKL on their dendritic processes [18]. Osteocytes can also increase osteoblast differentiation, as seen with the treatment of mesenchymal stem cells with medium from osteocytes *in vitro* (MLO-Y4) [19]. Evidence of osteocytes regulation of both osteoclastogenesis and osteoblastoglenesis suggests that they are important in the regulation of bone remodeling in general. Also, more recently there is evidence of

osteocytes mineralizing bone within their canicular network [20]. Osteocytes also highly express Wnt negative regulators sclerostin (SOST) and dickkopf-related protein 1 (DKK-1), which supplies further evidence of their role in regulating bone remodeling [15].

1.3 Serum markers of bone remodelling

Recently there has been extensive assessment and characterization of cellular and extracellular factors of the skeletal matrix associated with bone formation and resorption [21]. These factors are a practical therapeutic tool for assessing bone metabolism. Serum bone formation markers include bone alkaline phosphatase (BAP), osteocalcin (OC) and P1NP, bone resorption markers include products of type 1 collagen degradation (PYD, DPD, CTX, and NTX) and an enzyme secreted by osteoclasts (TRAP) have been used. These markers of bone turnover are more sensitive to changes in bone metabolism and can be used to monitor/diagnose metabolic bone diseases more effectively when compared to measurements of BMD (i.e., dual energy x-ray absorptiometry (DXA)), as BMD takes months to years to have measurable changes to occur [21]. However, it is important to understand that changes in these formation or resorption markers explain the activity of either osteoblasts or osteoclasts in a specific moment in time, and do not explain what is leading to the change in activity or bone remodelling.

1.3.1 Formation Markers

Tissue non-specific alkaline phosphatase (AP) is a membrane bound isoenzyme secreted by liver, kidney and bone. Bone specific AP (BAP) is secreted by osteoblasts and makes up ~50% of total serum AP [22]. BAP's mechanism of action in bone remains unclear, however there is strong evidence that BAP hydrolyzes the mineralization inhibitor PP_i to

allow for mineralization and growth, as well as provides inorganic phosphate to promote mineralization [23, 24]. Specifically, in homozygous BAP knockout *in vitro* and *in vivo* models there is no initialization of mineralization by osteoblasts and an increase in mineralization inhibitor, suggesting BAP is required for mineralization and the prevention of inhibition of mineralization [25, 26]. BAP has been extensively studied as a serum marker of bone formation with the use of bone specific immunoassays. It appears that there is little effect of food, stability, half-life and intra-individual variability on BAP levels. However, the limitation of this marker is that there is cross-reactivity with liver isoforms [27].

Osteocalcin (OC) is a noncollagenous protein secreted by differentiated osteoblasts and is the most abundant protein of the bone matrix [28, 29]. OC contains 3 residues of gamma-carboxyglutamic acid, which can be post-translationally modified by being carboxylated [28]. Carboxylation is vitamin K dependent and determines calcium binding properties of OC. OC has been established as both a marker of formation and resorption and is released during both processes. Early research suggests a role in osteoid mineralization, because OC is mainly expressed during bone formation. However, analysis of OC knockout murine models shows increased cortical and trabecular thickness and increased bone rigidity [30]. These results are conflicting and suggest a potential negative feedback mechanism of OC. OC also appears to be regulated by 25(OH)D, renal function, menstrual phase and circadian variability [27]. Procollagen type 1 carboxy and amino terminal propeptide (P1CP and P1NP) are derived from extracellular cleavage of type-1 collagen by proteases during formation [31]. P1NP is the most accepted bone formation marker and marker of proliferating osteoblasts, however

type 1 collagen is not specific to bone, and therefore peripheral levels may not reflect dynamic changes in bone formation [27].

1.3.2 Resorption Markers

Carboxy and amino terminal cross-linked telopeptides of type 1 collagen (CTX and NTX) are derived from extracellular degradation by proteases (cathepsin K) during bone resorption [31]. These markers have variation in levels following food intake and NTX is the most reliable bone resorption marker. Deoxypyridinoline and pyridinoline (DPD and PYD) are products of type I collagen breakdown, which are released into circulation in a free or bound state. These pyridinium crosslinks therefore represent bone resorption [32]. These markers are assessed in urine and need to be corrected to creatinine levels, and they have high circadian variation and depend on liver function as well [27].

Tartrate resistant acid phosphatase-isoform 5b (TRAP) is an enzyme that cleaves type I collagen into fragments and is secreted by osteoclasts into circulation during bone resorption and reflects osteoclast differentiation [33]. Serum levels appear to be affected by acute exercise, circadian rhythm and are unstable at room temperature. Cathepsin K is a cysteine protease that is present in the outer membrane of actively resorbing osteoclasts and cleaves telopeptides and helical regions of type I collagen [33].

1.4 Tumor Necrosis Factor-Alpha effect on bone remodelling

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is involved in both systemic and local inflammation. TNF-α is ubiquitously expressed in most cell types, including lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal cells, however it is mainly produced by macrophages [34]. TNF-receptor1 is

constitutively expressed in most tissues, and TNF-α can effect intracellular signalling in the majority of cell types [34].

TNF-α is associated with chronic inflammatory bone diseases, such as rheumatoid arthritis, post-menopausal osteoporosis, periodontal disease and aseptic periprosthetic bone resorption [35]. TNF-α has been shown to induce apoptosis of osteoblasts *in vitro*, and increase RANKL expression in osteoblasts and stromal cells [36, 37], ultimately leading to inhibited bone formation and increased osteoclastogenesis [38]. *In vitro* studies have shown that TNF-α can induce osteoclastogenesis, however when pre-osteoclasts were cultured with OPG and TNF-α, there was an increase in osteoclastogenesis independent of RANKL/RANK by increasing M-CSF [39]. Thus, TNF-α increases bone resorption through the increase in osteoclast activity, and decreases formation through osteoblast apoptosis. Furthermore, Kim et al. 2012 highlighted a relationship between TNF-α and bone when they found an increase in SOST expression, a negative regulator on Wnt/β-catenin signalling, in mice with low estrogen while SOST expression was inhibited when a TNF-α blocker was administered [40]. Also, when TNF-α is administered to cultured fibroblast-like synoviocytes from synovial tissue of rheumatoid arthritis patients there is a distinct increase in expression of SOST. Furthermore, when mice over-express recombinant human TNF-α there is SOST expression present in fibroblast-like synoviocytes, whereas WT mice fibroblast-like synoviocytes do not express SOST [41], which suggests an indirect role of TNF-α can induce SOST expression *in vivo* and *in vitro.*

1.5 Insulin Like Growth Factor-1

Insulin like growth factor 1 (IGF-1) operates through a homologous receptor tyrosine kinase that regulates cell metabolism, differentiation, proliferation, and protection against apoptosis [42, 43]. Cell signalling networks associated with the receptor tyrosine kinase include phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/extracellular signal-regulated kinase (ERK), and together these cascades mediate the actions of IGF-1 [44]. The majority (70-80%) of IGF-1 circulates in a 150-kDa complex composed of IGF-1, IGF-binding protein 3 (IGFBP-3), and the acid labile subunit (ALS) [45]. This complex preserves the half-life of IGF-1 and facilitates their endocrine action by inhibiting secretion into extravascular compartments. Hepatocytes produce the majority of IGF-1, however all tissues express IGF-1 [46].

Serum levels of IGF-1 are mostly the product of the liver. In murine models, when hepatic IGF-1 production is ablated through out the lifespan there is a 70% decrease in circulating IGF-1, increased growth hormone (GH) levels, and increased liver GH signalling (STAT5B phosphorylation) [47]. These changes are associated with increased liver inflammation, oxidative damage, and despite the increase in GH, the decrease in IGF-1 is thought to lead to compromised skeletal integrity, and accelerated bone loss [47].

Studies analyzing murine models of IGF-1R/IGF-1 gene knock outs have shown that IGF-1 receptor haploinsufficiency in a female mouse model (IGF-1R(+/-)) leads to inhibited osteoblast differentiation and decreased femoral and calvariae BMD. Reduced IGF-1 also resulted in significant reduction in Osx and Runx2 when compared to wild types [48], which suggests an implication of altered IGF-1 signalling in osteopenia. Furthermore, IGF-1 has been shown to inhibit glycogen synthase kinase-3 (GSK3) by

increased PI3K and Ras/ERK intracellular signalling [49], which suggests cross talk between IGF-1 signalling and Wnt/β-catenin signalling. Wnt/β-catenin signalling will be discussed in detail below.

1.6 Leptin

Leptin is a 167-amino acid protein product of the *ob* gene [50] expressed primarily in adipose tissue, however has been recently shown to be produced in placenta, ovaries, skeletal muscle and the stomach [50]. Leptin receptor is located ubiquitously and is a member of the class I cytokine receptor family [51]. Circulating leptin levels have been shown to represent the relative number of adipocytes in normal weight and obese individuals, and therefore, body fat [52] and body mass index [53]. Leptin acts through receptors located centrally, specifically in the hypothalamus and hindbrain, and peripherally in skeletal muscle, bone, and cartilage [54]. There is indirect evidence of leptin's role in bone metabolism. Leptin deficient female athletes were found to have an increased risk of osteopenia and stress fractures [55]. In addition, long-term administration of metreleptin treatments to lean hypoleptinemic women resulted in an increased BMD and BMC at the lumbar spine [56].

1.6.1 Leptin Signaling

Leptin receptor activation leads to the activation of a receptor-associated janus kinase JAK. Leptin acts on the hypothalamus, which leads to the inhibition of transcription factors neuropeptide Y and cocaine- amphetamine-related transcript (CART), and subsequently inhibits food intake [57]. However, leptin also acts on several peripheral tissues, such as bone. Leptin's actions are mediated through its receptor, and upon

binding it dimerizes and can activate several pathways associated with growth and survival. These pathways include the janus kinase 2(JAK2)/signal transducer and activator of transcription 3 (STAT3), the mitogen-activated protein kinase (MAPK), and the PI3K/Akt/mammalian target of rapamycin (mTOR) to name a few [58]. Inactivation of these pathways can result in aberrant osteoblast/osteoclast activity and lead to bone loss [59, 60]. More recently, leptin has been shown to activate the nuclear kappa-light-chain-enhancer of activated B cells (NF-kβ) pathway as well [61], which highlights differential ways leptin can exert actions on bone metabolism.

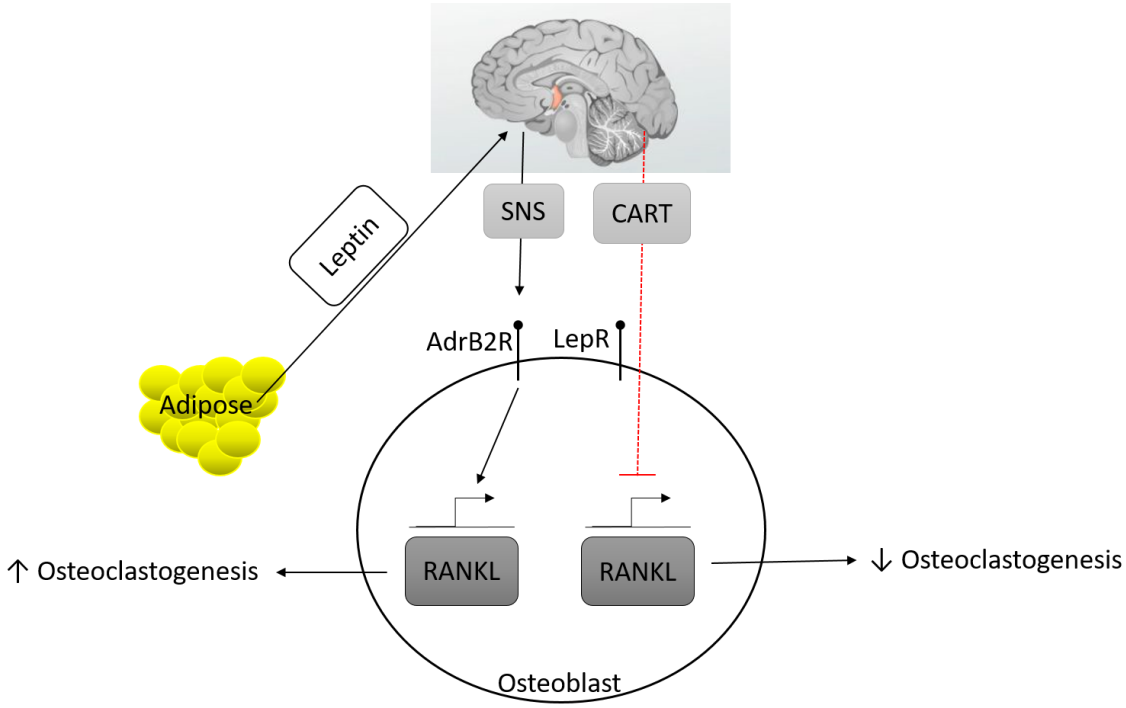

Figure 3. Leptin signalling's effect on bone osteoclastogenesis. Leptin acts either peripherally or centrally, which have opposing effects on osteoclastogensis. AdrB2R= adrenergic beta 2 receptor; LepR = leptin receptor; SNS = sympathetic nervous system; CART = cocaine amphetamine regulated transcript.

1.6.2 Leptin's effects on cell signalling in bone

Leptin replacement therapy in women with leptin deficiency induced by amenorrhea has been shown to increase free triiodothyronine, free thyroxine, IGF-1, IGFBP-3, BAP, and OC [62], and decrease parathyroid hormone (PTH) and RANKL/OPG after 36 weeks of treatment [63] and improved reproductive outcomes, recovery of menstruation, GH, and adrenal axes [64]. These changes translate into improved BMC and BMD across 24 months of leptin replacement in centrally hypothalamic amenorrhea as well [56]. Figure 3 compared how the central and peripheral pathways of leptin effect bone. Findings that support leptin's differential effects on bone are highlighted below.

Mice with mutations in the *ob* gene (inhibited leptin production) tend to be obese, diabetic, have reduced activity, metabolism and body temperature, and upon administration of leptin there is reduced food intake, weight, fat mass and improvement in metabolic rate, activity and body temperature [65, 66]. This data suggests an important role of leptin in regulating body weight and fat deposition through effects on metabolism and appetite. Furthermore, mice deficient in leptin show infertility and inhibited hypothalamic-pituitary function [67]. This model also appears to have increased mesenchymal precursor migration to adipose tissue from distant organs in order to increase adipogenesis, which can be explained by the increased production of TNF-α within adipose tissue [68]. When leptin is administered either peripherally or centrally there is a significant decrease in bone marrow adipocyte number due to an increase in apoptosis in WT or leptin deficient mice and rats compared to vehicle treated controls [69-72]. More recently when leptin was administered both peripherally and centrally there was no difference in bone growth between the two modes of administration [73]. However, these results appear to be inconsistent with recent

findings. Specifically, investigation of the bone microarchitecture of leptin deficient mice found these mice having significantly shorter femora, lower femora BMC, BMD, cortical thickness, trabecular bone volume, and higher bone marrow adipocyte number when compared to lean WT mice [70]. However, this same study showed that leptin deficient mice have significantly increased vertebral length, lumbar BMC, BMD, and trabecular bone volume compared to lean WT [70]. These results indicate that the axial and appendicular skeleton may respond differently to leptin and that there is likely more than one pathway regulating leptin's actions on bone and that central and peripheral leptin may have inhibitory or stimulatory effects on bone growth.

Previous research promotes the hypothesis that leptin inhibits bone formation centrally, thus increasing bone loss by the regulation of osteoblast activity through the sympathetic nervous system [74]. Specifically, when leptin is administered by intracerebroventricular infusion (administered centrally) there is an increased bone loss in leptin-deficient and WT mice when compared to controls [74]. Furthermore, when leptin is administered centrally into WT mice that have been treated with propranolol, a β-blocker, for 5 weeks there is no change in bone mass, suggesting a role of the sympathetic nervous system in the anti-osteogenic effects observed with central leptin administration [75]. Assessment of leptin deficient mice, who have had leptin repletion through recombinant adeno-associated virus leptin gene therapy in the hypothalamus with no change in peripheral leptin levels, has shown to lead to normalization of the skeletal phenotype, as seen with increased femoral length, total bone volume and decreased femoral and vertebral cancellous bone volume compared to pre-treatment, vehicle control and WT mice [76].

In contrast to centrally administered leptin, leptin administered intraperitoneally has been shown to significantly increase cortical and trabecular bone mass, as well as total bone mass in leptin deficient mice when compared to vehicle treated controls [77]. Leptin also regulates OC production, which suggests a direct mechanism of leptin on bone metabolism [78]. Furthermore, the identification of adipocytes in bone marrow that secrete leptin highlights a route to lead to increased leptin action in the bone micro-environment [79]. These results indicate the peripheral leptin pathway increases bone mass, while the central leptin pathway leads to lower bone mass.

1.7 Female rowers and bone

Healthy female rowers tend to have significantly higher lumbar spine BMD compared to a control population [80]. This high BMD in the lumbar spine of competitive rowers has been seen to occur in non-elite rowers, where novice male rowers performed ~10hrs of total training volume/week for 7-months and had a 2.9% increase in lumbar spine BMD [81]. This higher BMD is most likely a result to the mechanical loading of 4.6 times body mass at the lumbar spine, which occurs during a rowing stroke in elite female rowers [82].

However, there is evidence of no effect on BMD in general and at the lumbar spine in particular after 9-months of training in female college-level rowers [83]. The training consisted of 2 d/wk resistance training (~60% 1 rep maximum) and 5 d/wk on water moderate intensity training. Despite no change in BMD, however, there was a significant decrease in 2000m erg times (-18s in post compared to baseline), as well as increased lean mass and decreased body fat. The improved performance and body composition (baseline body fat of 31% to 27% post season) could be a result of being at a

relatively lower level of performance compared to elite athletes at baseline. In another study, control (non-rowing college students), novice (3-months experiences) and experienced collegiate female rowers (2.2y) were compared at baseline and after a 6-month competitive season [84]. All rowers trained together, and performed similar number of strokes. However, the experienced rowers had significantly faster 2000m, and 6000m ergometer times, indicating more force production. The experienced group had a 2.5% increase in spine BMD from pre- to post-competition period when compared to novice rowers. Interestingly, novice rowers had a slight decrease in spine BMD, although they started off with a 3% higher BMD than experienced rowers. The BMD changes in both rowing groups was not different than controls [84]. The similar BMD changes in female rowers compared to age matched controls after a training season creates more questions concerning the effect high level competitive rowing has on BMD.

A systematic review [85] published in 2011 in the Journal of Sports Medicine outlined the epidemiology, mechanisms, risk factors and effectiveness of prevention strategies for rib stress fractures in rowers. Assessment of 140 journal articles resulted in the identification of an 8-16% occurrence of rib stress fractures in elite rowers, and the incidence was the same in scullers and sweepers, as well as in males and females. They hypothesized based on their findings that the increased risk of stress fractures may be a result of low calcium and vitamin D intake, eating disorders in general, or low testosterone [85]. They discussed that a higher rate of bone resorption and a lower rate of bone formation may be resulting in the increase in fracture, however they did not review any studies that measured these factors specifically in rowers. Furthermore, disordered eating, menstrual dysfunction, or energy availability may be the reason some researchers

observed decreases in BMD or alterations in markers of osteoblast and osteoclast activity, however they don't explain what precedes these changes in bone remodelling.

1.8 Monitoring serum markers of bone turnover during training

Understanding the response of BMD across a training season in athletes can provide a general overview of the influence raining has on bone. However, detectable changes occur only after relatively long term periods. Monitoring factors that are released during bone formation or resorption provides a snap shot picture of ongoing formation and resorption processes. Furthermore, changes in formation and resorption markers occur following relatively shorter periods. Thus, some studies have attempted to illustrate how changes in training volume can affect various markers of bone turnover. Lombardi et al. [86] assessed serum bone and energy metabolism markers at rest in the unfed state in 9 professional male cyclists competing in the *Giro d'Italia* stage race. Blood was drawn at baseline, day 12 (154 km/d, net energy expenditure = 3402kcal), and day 22 (154km/d, net energy expenditure = 3756 kcal). 12 days into the 3wk stage race resulted in a significant decrease in total OC and leptin, which remained lower 22 days into the race when compared to baseline. Furthermore, 22 days into the race resulted in a significant increase in adiponectin and TRAP activity when compared to baseline. Despite the changes in bone resorption markers across the stage race, serum BAP levels were unchanged. O'Kane et al. [87] highlighted differences in urine bone resorption (NTX) and collagen breakdown (CTX) markers between college level rowers, runners, swimmers and age matched controls. Rowers had the highest levels of resting urine NTX compared to all groups, and had lower levels of CTX compared to runners. The authors suggest rowers have more of an osteo-stimulatory effect (higher bone turnover).

However, they did not measure any markers of bone formation in this study nor did they describe what period of training these athletes were in.

Sansoni et al. [88] assessed serum samples taken from 17 male marathoners pre- and post- a 65-km mountain ultra marathon as well as from 12-age matched controls. Pre-race levels showed significantly higher P1NP and lower decarboxylated OC compared to age matched controls. Post-race levels showed a decrease in decarboxylated OC and P1NP compared to pre-race levels. These results suggest that ultramarathoners have higher osteoblastic activity than age matched controls, and that an extended period of high volume exercise can lead to a decrease in bone formation, which is interesting, because an increase in resorption found in elite cyclists during a 3-week stage race was also not matched by an increase in bone formation [86]. Zanker et al. [89] evaluated the effect of energy balance on markers of bone turnover in 8 elite male distance runners, who averaged 50 km/wk of running at a high intensity. Energy balance was manipulated by diet, while the exercise protocol during each week of balance or energy deficit would remain the same and lasted 3 consecutive days. Athletes who were in an energy balance had no change in P1NP, OC, Dpd, NTX, or IGF-1 from pre- to post- 3d of training. In contrast, the same athletes performing the same 3d exercise protocol but consuming 50% of their estimated energy requirement had significant decreases in P1NP and IGF-1 from pre- to post-training, which were also positively correlated. These results suggest that the bone formation response to repetitive endurance training is highly dependent on energy availability.

De Souza et al. [90] assessed young (24 years old) recreationally active females across the menstrual cycle and separated them into either (1) energy and estrogen replete,

(2) energy and estrogen deficient, (3) energy replete and estrogen deficient, or (4) energy deficient and estrogen replete. Energy and estrogen deficient group had significantly lower P1NP and significantly higher urinary CTX levels compared to groups that were either energy replete or estrogen replete. Furthermore, CTX was shown to be a predictor of lumbar spine BMD and leptin was correlated with bone formation markers [90].

In summary, there appears to be a push towards an increase in bone resorption while some studies have suggested a decrease in formation following strenuous periods of training or competition. However, monitoring direct markers of bone formation/resorption only paints a picture of the current state of bone turnover. Therefore, monitoring both BMD and markers that precede bone turnover may be a better way to assess how training is impacting bone during periods of intense training in athletes. One pathway that may be useful for monitoring changes in bone metabolism during exercise training is the Wnt/β-catenin pathway.

1.9 Introduction to Wnt Proteins

Wingless and INT-1 (Wnt) is a family of 20 identified signalling glycoproteins that activate various signal transduction pathways [91]. These pathways include the canonical Wnt/β-catenin cascade, the non-canonical planar cell polarity pathway, and the Wnt/Ca^{2+} pathway. Of these pathways the canonical pathway is the most understood, and is involved in development, cancer, and tissue self renewal [92]. This section will focus on the canonical pathway and the proteins/mediators involved in tissue self renewal, specifically its role in bone formation and resorption.

Wnt undergo extensive intracellular processing before being secreted from their producing cells into the extracellular matrix, where they will act on their effector cell.

Post-translational modifications that have been shown to be essential for Wnt signalling to occur are glycosylation and acetylation. This is highlighted by experiments where site specific mutagenesis was induced at sites of either glycosylation [93] or acetylation [94], which resulted in impaired secretion or inhibition of signalling, respectively. Following post-translational modifications, Wnt proteins are recruited to the endoplasmic reticulum, where chaperone proteins guide Wnt to the extracellular space. However, the ER protein Oto appears to anchor Wnt1 and 3a with the addition of glycophosphatidylinositol to the endoplasmic reticulum, which results in the accumulation of Wnt and decreased signalling. Knockdown models of Oto *in vivo*, as well as over-expression of phospholipases to colorectal cancer cells leads to increased Wnt signalling [95, 96]. Following post-translational modifications and retention in the endoplasmic reticulum, Wnt protein is targeted for secretion and is removed from the cell by an endosome as a lipid modified protein [97]. The combination of post-translational modifications, as well the retention of Wnt to the endoplasmic reticulum can contribute to the activity of Wnt signalling before Wnt has even left the cell.

1.10 Canonical Wnt/β-Catenin Signalling

Figure 4 summarizes the Wnt/β-Catenin signal transduction pathway within osteoblasts. Briefly, following secretion of Wnt into the extracellular matrix, it can act on its effector cell through cell surface receptors. However, prior to Wnt signalling, β-catenin, an essential second messenger for Wnt signalling, is phosphorylated by GSK3 with facilitation of scaffolding proteins Axin and adenomatous polyposis coli (APC) [98]. This phosphorylation marks β-catenin for degradation by β-Transducin repeat containing protein mediated ubiquitin/proteasome pathway. Activation of Wnt/β-catenin cascade

occurs upon the binding of Wnt to a 7-transmembrane domain-spanning G-protein coupled receptor frizzled (FZD) as well as low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) co-receptors [99]. This transmembrane domain also contains membrane proteins such as disheveled, axin, and Frat-1, which in conjunction lead to disruption of the protein complex APC, Axin and GSK3. This disruption in the protein complex leads to inhibition of GSK-3, and thus β-catenin phosphorylation by GSK-3. Therefore, Wnt signalling results in β-catenin stabilization and accumulation in the cytoplasm. Now stable, β-catenin can translocate into the nucleus. Translocation of β-catenin into the nucleus leads to formation of complexes with members of LEF/TCF family of transcription factors, and mediates transcription of Wnt-responsive genes. Translocation of β-catenin is also dose dependent, where an increase in Wnt signalling leads to increased translocation [100]. Extracellular regulators of Wnt signalling include, but are not limited to DKK-1 and SOST, which act on LRP5/6 to inhibit signal transduction.

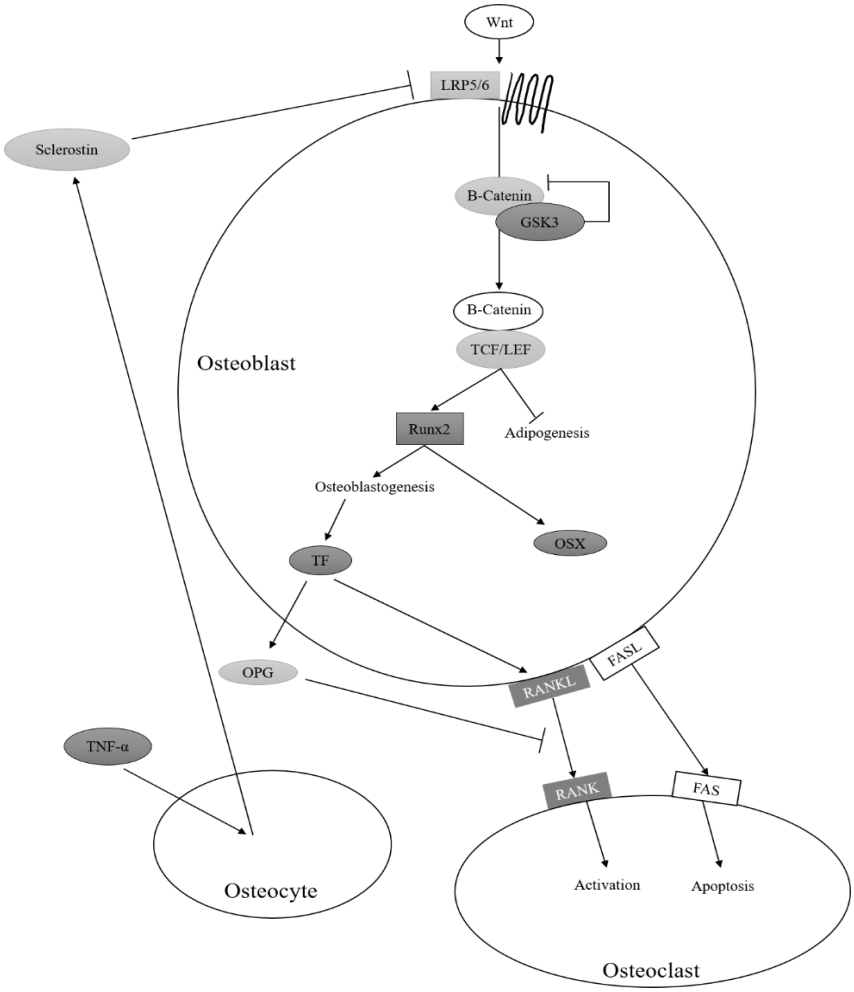

Figure 4. Factors associated with Wnt/β-catenin signalling. Wnt signalling is tightly regulated to have sufficient osteoblastogenesis as well as osteoclastogenesis, which result in adequate bone growth. LRP5/6 = Low-Density Lipoprotein Receptor-Related Protein 5/6; GSK3 = Glycogen Synthase Kinase 3; TCF/LEF = T-Cell Factor/Lymphoid Enhancer Factor; RUNX2 = Runt Relate Transcription Factor 2; TF = Transcription Factor; OSX = Osterix; FASL = Fas Ligand; RANK = Receptor Activator of Nuclear Kappa-β; RANKL = RANK Ligand.

1.10.1 Interaction of Wnt signalling with the OPG/RANKL axis

Figure 4 illustrates the relationship between Wnt and OPG/RANKL signalling. Briefly, murine knock out models of β-catenin and APC in mature osteoblasts have been shown to reduce trabecular and cortical bone volume [101]. Furthermore, bone loss phenotype of osteoblast β-catenin knockout mice appears to be associated with a reduced osteoblast

differentiation and matrix mineralization, and increased osteoclast activity, which is attributed to the decreased OPG and increased RANKL expression. Furthermore, mouse models with constitutive activation of β-catenin have high bone mass phenotype that results from reduced osteoclast activity and increased osteoprotegerin (OPG) production which inhibits RANKL expression [101].

Evidence of Wnt signalling inhibiting osteoclastogenesis through activation of osteoblasts comes from assessment of co-cultures of mouse osteoblasts and mononuclear spleen cells [102]. Cells stimulation with LiCl (mimicking Wnt signalling by inhibiting GSK3) resulted in complete inhibition of RANKL mRNA expression and inhibition of osteoclast formation when compared to vehicle control. Furthermore, β-catenin's role in RANKL inhibition was assessed through transfecting ST2 stromal cells with full length β-catenin or β-catenin lacking the C-terminal domain needed for TCF/LEF dependent gene transcription. Overexpression of full length β-catenin reduced endogenous RANKL promotor activity, and in contrast, β-catenin lacking its C-terminal domain had no effect on RANKL expression when compared to control. These results indicate canonical Wnt signalling in osteoblasts can lead to decreased RANKL expression through increased Wnt signalling, which ultimately inhibits osteoclastogenesis [102].

Further evidence of the importance of Wnt signalling in osteoclastogenesis comes from transgenic 3-month old mice, where LRP6 in osteoblasts is knocked out [103]. KO mice had significantly reduced femoral trabecular bone volume, and increased bone separation in the secondary spongiosa area when compared to WT mice. In addition, the number of osteoblasts, but not the number of osteoclasts, were significantly reduced in KO mice when compared to WT mice, and interestingly osteoclast activity was

unchanged between KO and WT mice. This decrease in osteoblast number was associated with increased apoptosis, where proliferation was unchanged in KO mice compared to WT. Importantly, β-catenin target gene expression of Axin2, Naked2, BMP4 and OPG were significantly down-regulated by 30-40% in KO mice when compared to WT. However, RANKL/OPG ratio remained unchanged between the KO and WT mice [103]. These results indicate LRP6 knockout in osteoblasts results in bone loss due to decreased bone formation with no change in osteoclastic bone resorption despite a decrease in OPG expression in adult mice.

β-catenin's function was further assessed *in vivo*, where point mutations were introduced to generate osteoblast specific loss of function or gain of function mouse models [104]. In gain of function mouse models there was a significant reduction in osteoclast number and Dpd (collagen breakdown), and increased bone mass. However, osteoblast number was unchanged when compared to WT mice [104]. Micro-array analysis highlighted a significant increase in OPG expression (3.2-fold), which encodes a soluble TNF-α receptor that acts to inhibit RANKL and therefore osteoclastogenesis, when compared to WT mice. Significant reduction in bone-mass and an increase in osteoclast number, and bone resorption rate were observed in mice lacking β-catenin compared to WT mice [104]. Interestingly, the number of osteoblasts and bone formation rate were unchanged between the mutant animal models [104]. LEF/TCF proteins appear to be critical in the expression of OPG, as seen with TCF null mouse models and osteoblast cell lines. These results suggest Wnt signal transduction in osteoblasts is responsible for the regulation of osteoclast differentiation, whereas mutations in Wnt LRP5 receptor may not be critical in the activation of β-catenin.

1.10.2 Wnt signalling controls osteoblastogenesis

Commitment of mesenchymal stem cells to the osteoblast lineage requires activation of the canonical Wnt/β-catenin pathway [105]. Therefore, Wnt signalling increases bone mass by an increase in osteoblast number. In vitro assessment of human mesenchymal stem cells suggests that increased Wnt signalling leads to inhibition of adipogenic differentiation and an increase in alkaline phosphatase, indicating increased osteogenesis. Specifically, Wnt treatment resulted in no change in the levels of differentiation, but it rather led to an increase in proliferation of progenitor cells in a dose dependent manner. Also, over-expression of stabilized β-catenin lead to BAP induction, suggesting stimulation of osteochondral differentiation [106]. Furthermore, stimulation of mesenchymal precursor cells with LiCl resulted in osteoblast differentiation through the induction of Runx2 and OSX transcription factors [107, 108]. Additionally, when mice overexpress Wnt proteins there is a significant increase in bone volume and strength, as well as stimulated osteoblastogenesis by the induction of Runx2 and OSX [107]. *In vivo* evidence of Wnt signalling leading to increased osteblastogenesis comes from analysis of the inhibition of GSK3β, and therefore constitutive activation of β-catenin [109]. Four weeks of LiCl administration seems to lead to a significant increase in bone formation rate and number of osteoblasts when compared to mice being treated with vehicle control [109].

The importance of Runx2 is highlighted *in vivo* with deficient murine models, which results in the absence of differentiated osteoblasts and mineralization compared to WT mice [110, 111]. However, in mice overexpressing Runx2 there is an increase in bone formation and resorption, and osteoclast number, which further highlights the need

for osteoblast differentiation to drive osteoclastogenesis [112]. Osteoblast driven osteoclastogenesis is further underscored by assessment of increased expression of Runx2 in co-cultures of primary murine pre-osteoblasts, which resulted in an increased differentiation of splenocytes into osteoclasts [113], which was associated with Runx2 mediated increased RANKL secretion and association with the osteoblast membrane [114]. Also, increased β-catenin levels leads to an increase in OPG levels [104]. The above results suggest that Wnt signalling mediates both osteoblastogenesis, and the regulation of osteoclastogenesis through the increase in Runx2, further illustrating a tight relationship between bone formation and resorption.

1.11 SOST – a Wnt antagonist

One ligand for LRP5 is the *Sost* gene product SOST. SOST is mainly found in the osteocytes and has extensively been shown to inhibit Wnt/β-catenin in *vitro* and in *vivo*. Mutations in the Sost gene in humans leads to lack of SOST production, leading to sclerosteosis or van Buchem disease, which are forms of sclerosing bone dysplasias (bone overgrowth) [115, 116]. Processes associated with SOST includes cellular response to PTH, negative regulation of BMP signalling pathway, negative regulation of Wnt signalling pathway involved in dorsal/ventral axis specification, negative regulation of the canonical Wnt pathway, negative regulation of ossificiation, negative regulation of protein complex assembly, and response to mechanical stimulus.

Co-immunoprecipitation of SOST in *vitro* found that SOST directly antagonizes LRP5/6 co-receptors of the canonical Wnt pathway [117]. Also, activation of LRP6/FZD induced by Wnt1 cell treatment was abolished by ectopic SOST treatment [117]. Moreover, production of *Xenopus* embryos with LRP6ΔC, which has a mutation in the

cytoplasmic domain, resulted in no interaction with frizzled upon SOST administration and therefore inhibition of Wnt signalling (i.e., axis formation) [118]. These data suggest a critical relationship between SOST levels and activation of LRP5 in the canonical Wnt pathway.

Further assessment of SOST's function was done in *Xenopus* embryos, which highlighted that SOST inhibits the canonical Wnt pathway resulting in inhibited bone formation [117]. Also, assessment of human osteoblastic (HEK293T) cells transfected with Wnt-1 expressing plasmid and co-expression with a *sost* expression plasmid illustrated that SOST inhibited Wnt signalling in a dose-dependent manner [117].

The in *vitro* investigation of murine iliac bone cells found a high proportion of osteocytes positive for SOST while osteoblasts bone lining cells and periosteal osteoblasts had no SOST, suggesting osteocytes were the cells that were solely expressing SOST [119]. Furthermore, osteocytes that were positive for SOST were further away from bone surfaces than osteocytes negative for SOST [119]. This suggests that osteoblasts within forming osteons have protection from SOST inhibition by a layer of SOST negative osteocytes. Osteons in the process of bone formation (BAP positive cells) contained significantly greater SOST negative osteocytes than SOST positive osteocytes, and osteons not forming bone (BAP negative) had osteocytes that were nearly all positive for SOST [119]. Also, analysis of murine bone biopsies has found that newly embedded osteocytes are negative for SOST, and only after the onset of mineralization are these cells able to produce SOST mRNA. Also, ~2/3 of non-remodelling cortical osteons contained SOST positive osteocytes exclusively, indicating that after mineralization there is an increase in this inhibitory signal [119]. Additionally, there was

no alterations in the recruitment of osteocytes in the osteons of mice with a loss of function mutation in *Sost* or controls, which suggests that SOST affects the later stages of bone formation [119]. These results indicate that SOST is critical in the maintenance of bone microarchitecture, which is based on the evidence of the tight regulation of SOST's production and location and coincides with the positive association of increased resting serum SOST levels and BMC [120].

In *vitro* analysis of a human osteoblastic cell line that expresses SOST has illustrated that transcription factors activated upon Wnt signalling (i.e, Runx2 and OSX), bind to specific regions at the human *sost* promotor, and together activate SOST expression in a dose dependent manner [121, 122]. Also, Runx2 and OSX levels have been shown to be positively correlated with SOST levels [121]. These studies provide evidence for a potential feedback control mechanism involved in bone formation.

Identification of additional potential pathways targeted by SOST, comes from in *vivo* studies assessing DNA electroporation of gastrocnemius of mice with expression plasmids for BMP and SOST, and in *vitro* in osteoblastic cell lines with exogenous BMP and/or SOST treatment [123]. BMP ectopic bone formation appears to be prevented by co-expression of SOST in *vivo*, and there was no evidence of SOST acting as a direct BMP antagonist. Various osteoblast cell lines have been assessed for the effect of endogenous SOST treatment. This analysis found that SOST affected both the Wnt and TGF-β/BMP signalling pathways, however there was only evidence of direct association with Wnt (Wnt1, Wnt3, and Wnt3a) and indirect association with BMP (down regulation of BMP target genes) [123]. These results suggest SOST is a direct canonical Wnt inhibitor and an indirect BMP antagonist.

To assess if SOST production or inhibition was mediated autonomously or through a hormonal response (i.e., alterations in PTH), cultured osteocytes were analyzed to determine the cellular mechanisms underlying the response to mechanical loading and unloading [124]. In *vitro,* 3-dimensional cultures underwent loading and unloading, which was accomplished by non-rotating (static) or rotating (simulated microgravity) cultures for 3 days. Unloading increased SOST expression and RANKL/OPG ratio in unloaded cells when compared to loaded cells, which illustrated osteocytes ability to independently respond to mechanical loading. Also, there were no alterations in osteocalcin, BAP, and OSX mRNA levels as well as pathways associated with increased SOST expression [124], demonstrating SOST response is not a consequence of altered transcriptional activity in general. Finally, when endogenous PTH and PGE_2 were added to unloaded cultures the SOST response was attenuated, which illustrates that SOST's response is not only affected by autocrine action, but paracrine and endocrine as well.

The effects of SOST antibody administration on temporal changes in systemic and local expression of bone turnover marker and its long-term effect on osteoblast, osteoclasts, and osteocytes has been assessed in ovariectomized rats [125, 126]. Six weeks post anti-SOST administration there were significant increases in P1NP and OC, and after 26 weeks' elevation these markers were normalized back to levels comparable to animals being treated with vehicle control. In addition, levels of TRAP were decreased at 6 weeks and were back to levels comparable to control after 26 weeks of anti-SOST treatment. Increased bone formation at 6 weeks was confirmed with histomorphometric analysis, which showed an increase in bone volume fraction and L3 trabecular bone in ovary ectomized(OVX)-anti-SOST compared to OVX-vehicle controls. Ex *vivo*

osteoclastogenesis of OVX animals being treated with vehicle control had significantly higher TRAP-positive osteoclast-like cells compared to cultures from animals being administered anti-SOST. Surprisingly, there were no changes in the osteoclast regulatory proteins OPG or RANKL. Finally, anti-SOST administration has led to a significant increase in DKK1 and SOST mRNA expression suggesting a mechanism of preventing excessive bone accrual in response to anabolism/bone formation [125]. These data show the impact that SOST has on bone formation and markers of bone turnover.

In contrast to the previously discussed studies [125, 126], SOST antibody treatment has also been shown to increase lumbar vertebrae and femur-tibia BMD increased progressively through 26 weeks [127]. Increases in BMD at week 6 was attributed to decreased osteoclast activity and increased trabecular and cortical bone formation, and increases in BMD at week 26 were attributed to residual endocortical and trabecular osteoblast stimulation and decreased osteoclast activity [127]. These findings support decreased osteoclast activity at 26 weeks of SOST antibody administration, where other authors have reported no change in *in vivo* and *ex vivo* osteoclast activity when compared to vehicle control [125, 126]. These studies suggest that there is a complex relationship between SOST and bone formation, as *in vitro* studies clearly show mechanistically that this factor inhibits bone formation.

1.11.1 Factors that mediate SOST production

Seasonal variation of serum SOST levels has been assessed in healthy men and women 65 years of age [128]. SOST appeared to increase in wintertime by 20% and declined through spring and summer seasons. In contrast, in the fall SOST was 20% higher compared to mean levels. Interestingly, OC, parathyroid hormone (PTH) and physical

activity levels were unchanged at all time points. These results suggest a potential seasonal variation in SOST levels in healthy men and women over the age of 65. No such studies exist in younger adults.

Comparison of serum SOST, PTH and free estrogen levels in pre- (27 years of age) and postmenopausal women (57 years of age) has been done [129]. SOST levels were significantly higher in the postmenopausal women when compared to premenopausal women, whereas PTH and 25(OH)D levels were not different between groups. Multiple regression analysis in the post-menopausal women showed that PTH and free estrogen index could be predictors of SOST. Further analysis in pre- and postmenopausal women has found that SOST increases progressively up until age 45 and remains elevated post-menopause [130]. Estradiol, FSH, PTH, and age for postmenopausal women, and serum OC, FSH and estradiol for pre- and post-menopausal women were shown to be determinants of serum SOST levels [130]. This data suggest that estrogen deficiency can lead to increased SOST levels, and that PTH, estrogen, FSH, and age can regulate SOST expression.

PTH is a known inhibitor of SOST production [131-133]. Intermittent increases in PTH lead to increased osteoblast number by attenuating apoptosis. Specifically, infusion or intermittent administration of PTH into WT rats or mice has shown up to a ~90% decrease in SOST mRNA and SOST levels as well as an increase in markers of bone remodelling in vertebral bone, secondary metaphyseal trabeculae, diaphyseal bone and in epiphyseal trabeculae [132, 133]. However, a single injection of PTH resulted in a 50% reduction in SOST mRNA at 2 hours while 4 daily injections had no effect on SOST

mRNA or SOST [132]. These results indicate a tight regulation of osteoblastogenesis mediated by osteocytes, and illustrates a negative role of SOST in bone formation.

Elevated osteoclasts and intracortical/calvarial porosity is exacerbated by overexpressing SOST and is reversed by blocking resorption. Rhee et al. (2013) used intermittent PTH injections into WT rats and found no direct relationship between SOST expression and levels of OPG and RANKL [134]. Also, OPG/RANKL ratio had an inverse response to PTH treatment in primary (rise) and secondary (falling) metaphyseal bone [134]. These results illustrate different metabolic needs of various compartments of bone, where primary metaphyseal bone undergoes modeling and secondary bone undergoes remodeling. In post-menopausal women, serum SOST levels were found to be positively correlated with both lumbar spine BMD and T-score and negatively correlated with PTH [135].

OVX mice are often used as a model to assess estrogen-deficiency. This model appears to lead to an increase in SOST expression, and when TNF-α blocker or β-estradiol were administered 3 times per week OVX SOST expression was reversed back to WT levels [40]. These results indicate that estrogen may regulate SOST levels through TNF-α. This hypothesis is supported by previous studies that have shown that TNF-α null mice do not lose bone mass following ovariectomy like WT mice do. Decreased estrogen levels in this model have been shown to increase T-cell production of TNF-α, which in turn augments RANKL-induced osteoclastogensis [136-139]. Constitutively, estrogen supplementation has been shown to prevent OVX bone loss through a TGF-β dependent mechanism, which inhibits T-cell activation [139]. Kim et al. (2015) have also shown that postmenopausal women taking aromatase inhibitors, which block the conversion of

steroids to estrogen, have significantly higher serum SOST compared to control post-menopausal women [140], suggesting a protective effect of estrogen on SOST levels.

Interestingly, examination of the effect of the transcription factors OSX and RUNX2 on SOST expression in human bone cell lines has found that each of these two transcription factors alone led to increased SOST expression, and together they acted synergistically [121]. This is surprising, because RUNX2 has been shown to be responsible for pre-osteoblasts' differentiation into mature osteoblasts, following which levels of SOST decline [141]. These findings suggest a negative feedback loop, as increased OSX and RUNX2 are also required for osteoblastogenesis.

1.12 TNF-α response to various modes of exercise training

Table 1. Studies investigating the response of TNF-α to exercise training.

Training	Effect on TNF-α	Other findings	Conclusions	Reference
8 M competitive cyclists trained for 6 wk (wk 1-2 low training, 7 h/wk; wk 3-4 high intensity, 14 h/wk; wk 5-6 taper, 3.5 h/wk)	↔	↓ Performance and mood state	TNF-α is not a useful measure of measuring changes in training stress in cyclists	[142]
8 M competitive rowers trained for (wk 1 no training, 10 h/wk; wk 2-3 high volume, 18 h/wk; wk 4 taper, 10 h/wk)	↑	↑ TNF-α following endurance exercise only after wk 3 ↔ Resting Leptin ↓ Leptin following endurance exercise only after wk 3	TNF-α increase and leptin decrease post-exercise suggests higher stress to lipid metabolism in higher energy deficit conditions	[143]
4 M and 4 F competitive rowers trained for 8 wk (wk 1-6 high volume, 24.8 h/wk; wk 7, low volume, 2.4 h/d; wk 8 taper, 1.8 h/d)	↑	↑ IL-6 ↔ Training intensity TNF-α was associated with perceived stress scale, training duration and distance rowed	Monitoring resting levels of TNF-α may indicate levels of training stress in elite rowers	[144]

F – Female, M – Male

Smith et al. [145] proposed the cytokine hypothesis of overtraining, which suggests that repetitive trauma to the skeletal system, due to high intensity/volume training, with a lack of appropriate recovery time, can lead to overtraining. It is suggested that resting levels of TNF-α, a secreted myokine and pro-inflammatory cytokine, may be

elevated due to repetitive increases in acute inflammation as a response to adaptive microtrauma, which subsequently results in systemic chronic inflammation [146]. If the training plan was successful, and the athlete adapted to the stress of training, then hypothetically elevated basal TNF-α levels during high training intensity/volume would normalize during tapering. There is extensive evidence of IL-6 increasing following acute strenuous exercise, however TNF-α's response appears to be variable (reviewed in [147]). Furthermore, some studies have measured resting TNF-α levels in athletes and have found contrasting results (Table 1). Ramson et al. [143] attempted to assess if 2 weeks of high volume (18h/wk) training could alter resting serum TNF-α levels in competitive rowers. They found no differences in resting levels. However, when they assessed the immediate post-exercise response of TNF-α they found a significant increase post-exercise during the 2 weeks of high volume training, whereas levels were unchanged post-exercise during a tapering week. They found similar results with leptin, however instead of increasing, leptin decreased. The authors suggested that since high volume training is highly dependent on lipid metabolism, the marked increase in TNF-α and decrease in leptin are suggestive of an energy deficit. Despite these findings, resting levels of TNF-α were not sensitive to changes in training volume. However, in this study training intensity was not taken into account, and the training duration was only 2 weeks long, which might be too short to see changes.

Another study assessing competitive male cyclists performing 2 weeks of low volume training, 2 weeks of high volume and intensity training followed by 2 weeks of tapering showed that TNF-α was unresponsive to changes in training volume, and were not associated with declines in performance and mood state [142]. However, the cytokine

hypothesis to overtraining requires excessive microtrauma to occur, and since cyclists perform mainly concentric contractions with limited loading there may not have been enough stress to elicit a change in cytokines. Furthermore, these athletes had very high levels of TNF-α initially (6.3 pg/ml), which could have masked any changes seen following 2 weeks of high volume training (8.3 pg/ml). Assessment of recreationally active adolescent boys has supported the cytokine hypothesis, which showed that resting serum levels of TNF-α is significantly increased during basketball [148] and wrestling [149] competitive periods compared to pre-training. Lastly, Main et al. [144] showed that resting TNF-α levels are sensitive to changes in training volume and distance rowed in competitive male and female rowers, suggesting TNF-α is a useful marker for assessing training volume in elite rowers. Despite these findings, there is limited evidence in elite level athletes. Thus the utility of TNF-α as a marker of training stress and its association with bone metabolism remains speculative.

1.13 IGF-1 response to various modes of exercise training

Monitoring IGF-1 levels in athletes has been proposed as a useful tool for assessing training stress/overtraining [150]. It is suggested that there is an increase in central catabolism and local anabolism early in the adaptation to increased exercise volume. This is likely a way of conserving energy while increasing local tissue growth [151]. However, there have been disparate results in studies that have assessed resting serum IGF-1 levels in athletes and non-athletes. Sartorio et al. [152] highlighted that resting IGF-1 levels in elite male and female sprinters, triathletes, runners, walkers, cyclists, rowers, skiers, hockey players, and swimmers were all within normal range for the age of these athletes. Furthermore, despite a higher GH level, IGF-1 serum levels were not different when

compared to non-elite athletes and sedentary controls [153], which the authors attributed to a high paracrine action of IGF-1 in active muscle. In contrast, Antonelli et al. [154] found that salivary IGF-1 levels were lower in well-trained female volleyball players compared to sedentary females. These findings suggest that athletes have variable levels of IGF-1 compared to controls, however IGF-1 response to training has found contrasting results as well.

Table 2 presents the studies on the IGF-1 responses to exercise training. Hecksteden et al. [155] assessed IGF-1 response to a 6-day training camp and following 2 days of subsequent recovery in professional cyclists, team sport athletes, and strength trained athletes in their respective preparatory periods. Similar results have been found in elite adolescent athletes as well [156, 157]. The 6-day training camp elicited a significant decrease in performance, which was restored following 2 days of recovery. Furthermore, cyclists and sports trained athletes showed a significant decrease in IGF-1 from pre-training camp to post-training camp, and levels in cyclists and strength trained athletes increased following recovery compared to pre-training camp levels. The author suggests that IGF-1 may be a good peripheral marker for monitoring endurance training, however due to high inter-individual responses following an acute change in training volume this conclusion may be too generalized. Measurement of IGF-1 levels in elite handball players was assessed over a slightly longer period of time and found that serum IGF-1 levels declines significantly following 2 weeks of intense training and returns to baseline following 2 weeks of tapering [158]. These results suggest a catabolic state with intense training and are in agreement with previous findings in adolescent gymnasts, where IGF-1 decreased in a state of negative energy balance [159]. Nemet et al. [149] tested this

hypothesis that decreased IGF-1 or increased IGF-1 would be associated with energy deficit or excess following a 7-day excessive exercise program. Following the program men who were in an energy deficit had a decline in IGF-1 following training, and over fed participants had no change in IGF-1, which suggests that energy balance during exercise training influences IGF-1 levels and that adequate diet may occlude changes in IGF-1 following fluctuations in training volume. Lastly, elite level rowers training for 5 weeks showed that as volume and intensity of training tapered, IGF-1 levels increased, which suggests that tapering period is critical for an anabolic response to training [160]. Furthermore, this ties back to the cytokine hypothesis [150], which suggests that if the training plan was successful and resulted in a stress (inflammation), and the athlete was able to recover than there should be an anabolic increase, and a catabolic decrease, which would suggest an increase in IGF-1 and decrease in TNF-α during tapering. This is supported by *in vitro* assessment of myotubes that shows higher levels of IGF-1 leads to inhibited expression of TNF-α.

Table 2. Studies investigating the response of IGF-1 to exercise training.

Training	Effect on IGF-1	Other Findings	Conclusions	Reference
3 days of intensive exercise in 11-year-old trained F gymnasts	↓	↑ DHEA, testosterone ↓ T3, cortisol, body mass	The authors suggest that depressed T3 and IGF-1 leads to growth depression, and retardation of bone age	[156]
Adolescent wrestlers compared to active controls had blood draws pre- and post 4 months of training	↓	↑ GH ↓ Testosterone, body mass	Nutrition may lead to alteration in IGF-1 levels	[157]
12 elite M rowers had fasted blood draws at: 1. relatively low volume of 11.6±0.4 h/wk. 2. 6-month extended preparatory period of high training volume 16.8±0.6 h/wk	↑	Increased by 20.2% at high vs low training volume period IGF-1 correlated with OC	Bone formation marker OC is related to IGF-1 levels, indicating a possible metabolic implication of IGF-1 in bone cell activity	[161]
Young, healthy females did either 8wk of no exercise (control), resistance, aerobic or combined exercise training	↔	No time, group or interaction effects with immunoreactive and bioactive IGF-1 levels and all IGFBPs. ↑ Training specific neuromuscular outcomes	Endocrine derived IGF-1 does not reflect positive anabolic neuromuscular outcomes	[162]
73 competitive cyclists and strength trained athletes did 8d of an intense training camp (fatigue) then 2d of recovery	↓	↑ CK	Periods of fatigue induces a decrease in IGF-1, which can be returned to baseline following 2d of recovery	[155]
Elite handball player who trained for 4 wks; 2 wks of intense training followed by 2 wks of tapering	↓	Parallel changes in subjective physical conditioning	Periods of intense exercise can induce a decrease in anabolism (IGF-1 levels) and can lead to a decrease in subjective physical conditioning	[158]
7 internationally ranked artistic gymnasts training for 16 wks	↓	↓ 31% energy intake compared to recommendation, IGFBP3\-3, IGF-1:C ↑ Cortisol	Energy deficit may induce a catabolic state, as seen with a decrease in IGF-1:cortisol.	[159]
10 male rowers trained for 18d straight for 3.2 h/d	↓	↓ Performance, mood state Normalized following tapering	Overreaching is needed for competition preparation and can be monitored with IGF-1	[160]

F – Female, M – Male

1.14 Leptin response to various modes of exercise training

Leptin follows a diurnal pattern of nadir (0900h) and peak (0100h). [163]. Endurance athletes appear to have lower levels of resting leptin when compared to age matched-controls [88]. Leptin responds to increased and decreased energy availability by decreasing and increasing, respectively. Decreased leptin results in energy conservation and thermogenesis, and leptin increases to inhibit food intake [164]. Furthermore, leptin has been negatively correlated with measures of performance, and appears to have a dose response relationship with training volume [165]. Thus, leptin's role as a potential tool to monitor changes in energy availability or training volume in athletes has been explored but with conflicting results. Specifically, leptin has been shown to be sensitive [143, 165-171] and not sensitive to changes in training volume [161, 172-176].

Table 3. Studies examining leptin's response to exercise training.

Training	Effect on Leptin	Other findings	Conclusions	Reference
6 well-trained rowers had blood taken after 1wk of normal intensity training (BL), 3 wk of 9.1h RT/wk and 5.5h ET/wk (RT), 1 wk of recovery, 3 wk of 14.3h ET/wk (HVLIT), and a second week of recovery (RE2)	↓	RT compared to BL ↓ cortisol RE1 compared to BL ↓ Performance ET compared to BL ↑ Performance Correlates with training intensity and Pmax but negatively with VO_{2max}	Decreases leptin with increased intensity may have been caused by increased flux of energy, and/or hypocortisolism from overtraining. Leptin is a potential marker for monitoring training and metabolic energy expenditure	[166]
12 highly trained M rowers had fasted blood taken at 9:00 during training 9.3 hrs/wk (BL), after 3 weeks of training at 17.5 hrs/wk (HVLIT), and after 2wk of training at 8.9hrs/wk (RE)	↓	HVLIT compared to BL: ↑ CK ↓ Insulin ↔ Cortisol, glucose, PB% RE compared to BL: ↔ Cortisol, glucose, insulin, CK, PBF RE compared to HVLIT: ↔ Cortisol, glucose, insulin, BF%	An increase in training volume of 100% lead to a 40% decrease in leptin levels. There appears to be a dose response relationship between training stress and leptin, suggesting leptin can be used as a signal for human metabolic adaptation to heavy training stress in highly trained male rowers.	[165]
17 F rowers and recreationally active controls had resting serum taken throughout a 20 wk training block at pre-, 5 wk (high intensity), 10 wk (moderate intensity) and 20 wk (moderate intensity)	↓ Leptin when fT3 ↓ as well at 5 and 10wk ↔ Leptin when fT3 ↔		TSH and fT3 were decreased with decreased leptin suggesting a lower hypothalamic-pituitary signaling action and a means of energy conservation	[167]
11 M rowers participated in 36wk of intense endurance training and one session of acute exercise pre- and post-training	↔		Resting leptin levels were unchanged. However, the ability to recover basal leptin levels 24h following an acute bout of exercise was improved after 36wk of training, which suggests an improved energy and metabolism regulation.	[172, 173]
13 M collegiate distance runners participated in 8 days of a strenuous training camp (~284.1 km ran)	↔	24 wks compared to 0 wks: ↑ Cortisol 20wks compared to 0wks: ↓ BF%, testosterone	Increase in cortisol and decrease in testosterone indicates a state of overtraining. Leptin was unchanged, and was not related to cortisol or %BF. However, leptin was correlated with testosterone, indicating a role in modulation of overtraining.	[174]
11 elite M rowers preparing for 2004 Olympics; 6 rowers were selected and 5 were not. Testing was done at beginning of preparatory period and 24 wk later	Selected Rowers: ↔ Non-selected rowers: ↓	Competition period compared to preparatory period: ↑ Training volume (23.4%), VO_{2max} (3%), Pa_{max} (5%) ↓ BF% (0.9%), insulin ↔ Weight, 2000m sculling time	Decreased adipokines post exercise in lower performing athletes may be indicative of the inadequate recovery of these athletes.	[168]

during the competition period. Blood was taken pre- and immediately post 2000m single sculling, and 30 min post exercise.		Non-selected compared to selected rowers at competition period: ↓ testosterone		
12 nationally and internationally ranked M rowers. Fasted blood draws at: 1. Relatively low training volume of 11.6±0.4 h/wk. 2. 6-month extended preparatory period of high training volume 16.8±0.6 h/wk	↔	2 compared to 1: ↑ OC (17%) and IGF-1 (20%) ↔ Testosterone, cortisol, insulin, leptin, adiponectin	Leptin is not a sensitive marker for training volume	[161]
12 M internationally and nationally ranked rowers; 24 wk training with resting blood draws at 0, 4, 8, 12, 16, 20 and 24 weeks.	↓	24 wk compared to 0 wk: ↑ Performance, training volume (41%) 20wks compared to 0wks: ↓ Leptin ↔ Resting adiponectin at all times	Adiponectin levels were negatively correlated with leptin levels and body fat mass, which indicates its importance and potential use for assessing energy expenditure/body mass control	[169]
8 trained M rowers had fasted resting blood draws after 1wk of 6-8h (BL), 2 wk of 10-16h (HVT), and 1 wk of 8h of training (RE)	↓	HVT compared BL: ↑ Training volume, energy expenditure, social stress, fatigue ↓ Relative caloric intake (-455kcal/day), RESTQ-index, leptin (29%) ↔ Insulin, ghrelin, TNF-α and glucose RE compared to BL: ↓ Ghrelin ↑ Success ↔ Training volume, insulin, TNF-α and glucose RE compared to HVT: ↑ RESTQ-Index ↓ Training volume, physical complaints ↔ Leptin, insulin, ghrelin, TNF-α and glucose	HVT at low intensity for 2-weeks resulted in a significant decrease in leptin when compared to baseline, the athletes were also in an energy deficit at this time, which confirms previous findings that leptin can monitor training stress and energy expenditure	[143]
13 M competitive heavy weight rowers performed an acute bout of exercise at the 3rd and 36th week of the training season and serum was taken pre-, and immediately, 2h and 24h post-exercise	. ↔	Post- compared to pre-training ↑ Post-exercise recovery of leptin levels	Resting leptin levels were unchanged. However, the ability to recover leptin levels following an acute bout of exercise to resting levels 24h post-exercise was achieved only in the 36th week of training,. This could be attributed to an alteration in energy balance.	[175]
12 M national and international level rowers had blood draws following a reference week (R) (10h), high volume week (T1) (~19h) and after a recovery week (T2) (10h)	↓	T1 compared to R ↔ Insulin, NPY, ghrelin T1 compared to T2 ↔ Insulin, NPY, ghrelin T2 compared to R ↔ Insulin, NPY, ghrelin	Fasting leptin levels is a sensitive measure for assessing changes in volume of training	[170]

8 M trained cyclists had blood draws throughout a 6-month heavy cycling training plan	↓	Post 6-months training compared to pre: ↑ Aerobic capacity ↔ Resting adiponectin	This data suggests 6-months of heavy chronic endurance exercise does not affect adiponectin levels, however decreases leptin synthesis. Also, adiponectin is not associated with aerobic capacity or insulin resistance.	[171]
80 F elite rhythmic gymnasts were divided into intensely and very intensely trained (mean training volume = 40.8 h/wk) and were monitored during a 20-week training period	↔	Very intensely trained compared to intensely trained: ↑ Adiponectin 20 wk compared to 0 wk: ↓ BF%, testosterone	Adiponectin levels was associated with training intensity, and may reflect deterioration of energy balance rather than training stress and leptin is not a sensitive marker to changes in training load	[176]

F – Female, M – Male, EU – Eumenorrheic, AU – Amenorrheic, BL – Baseline, RT – Resistance Training, HVLIT – High Volume Low Intensity Training, ET – Endurance Training, RE – Recovery, BF% - Body Fat Percentage, HVT – High Volume Training, LIT – Low Intensity Training.

1.14.1 Short term training effects on Leptin (<12 weeks)

Table 3 presents the studies on the leptin responses to exercise training. Simsch et al. [166] assessed resting serum leptin levels in well-trained male rowers before and after a 3 week high intensity resistance training block, one week of recovery, 3 weeks of endurance training, and 1 week of recovery. Leptin levels decreased following 3 weeks of high intensity resistance training and remained lower than resting levels the second week of recovery during a moderate intensity training block and prior to the high intensity training block (1.3, 1.1, and 0.83ng/ml respectively). Leptin levels returned to pre-training levels following the endurance training block and remained constant at the end of the 1 week of recovery. Leptin levels were correlated with thyroid stimulating hormone (TSH) levels following resistance training, suggesting that high intensity training rather than endurance training can lead to suppression in the hypothalamic-thyroid-axis and leptin. Furthermore, Jurimae et al. [165] compared 3 weeks of high volume training (17.5 h/wk) and 2 weeks of tapering (8.9 h/wk) in highly trained male rowers. Resting leptin decreased significantly following the 3-week high volume training when compared to

pre-training (1.5 compared to 2.5 ng/ml). Furthermore, resting leptin increased significantly following 2 weeks of tapering compared to post- 3 weeks of high volume training (2.0 compared to 1.5 ng/ml) yet it was still significantly lower than pre-training levels. The authors suggest leptin is a sensitive marker for training volume and could be used to monitor training status [165] and is supported by more recent studies in elite male rowers [170]. These results contrast findings from swimmers, who train at high intensity, but when training volume increased during the training season there was no change in resting leptin compared to pre-season levels, despite a decline in fat mass. Ramson et al. [143] also attempted to better understand resting leptin levels following 1 week of low intensity training (6-8h), 2 weeks of high volume training (10-16h) and a subsequent week of recovery (8h) in competitive male rowers. There was no change in resting serum leptin levels before the 1 week of low intensity, 2 weeks of high volume, or after a recovery week (1.1, 1.1 and 1.0 ng/ml respectively. Conflicting evidence from short term training may be a result of training status prior to assessing leptin levels, thus leptin's utility as a marker of energy homeostasis remains speculative, especially when monitoring athletes in the short term.

1.14.2 Long term training effects on Leptin (>12 weeks)

Baylor and colleagues [167] assessed leptin, TSH, T3, and T4 responses to a 20 wk training block in female crew level rowers. The first 1-9 weeks were high volume training, while weeks 10-20 were moderate volume training with week 5 being a recovery week. Athletes were grouped into ether responders (decreased T3) or non-responders (no change in T3). Responders had significant decreased T3 and TSH at week 5 and at 10 compared to baseline with levels returning to baseline after 20 weeks. Resting leptin was

not different in responders compared to sedentary age matched controls and non-responders (12.0 compared to 12.9 and 12.6ng/ml respectively). Leptin decreased from pre-training to week 5 (-26.1%) and week 10 (-28.9%) and returned to pre-training levels following the moderate training phase at week 20. In contrast, as non-responders had no change in any hormones at any time point [167]. These results indicate decreased hypothalamic-pituitary signalling in these athletes may be a way of conserving energy, and decreases in leptin may be a response to changes in energy status and not changes in training volume, as low intensity high volume training relies on lipid metabolism.

Desgorces et al. [177, 178] examined the effect of 36 weeks of intense endurance training in highly trained male rowers on leptin and free fatty acid serum levels. They found that 36 weeks of intense endurance training did not elicit a change in resting leptin (1.75 and 1.69ng/ml for pre- and post-36wk of training respectively), however leptin was reduced immediately post-exercise while energy intake increased as training progressed. These results suggest that repeated hypoleptinemia following acute exercise leads to an increase in energy intake, which normalizes resting leptin. Furthermore, Jurimae et al. [169] assessed the effect of 24 weeks of volume extended training on resting levels of leptin in elite male rowers at week 0, 4, 8, 12, 16, 20, and 24. Training volume was initially 99 min/day, and there was a steady increase from week 8 to week 20 (127-168 min/d) while at week 24 volume decreased to 114min/d. Leptin only decreased at week 20 compared to week 0 (0.97 compared to 1.02ng/ml) suggesting that these athletes may be in an energy deficit at this time, or may be due to a decline in fat mass. The effect of a 6-month volume extended training plan on leptin and bone remodelling has been assessed in elite male rowers [161]. Average weekly training volume was higher (16.8h/wk)

compared to relative rest week prior to training (11.6h/wk), while intensity remained the same. Arm BMD was the only site that was affected and increased compared to pre-training BMD levels. Resting leptin was unchanged (1.02 compared to 0.99ng/ml) and IGF-1 levels increased and were correlated with OC, which also increased. These results suggest an important role of IGF-1 with bone formation and that BMD remains stable during the preparatory period in elite rowers [161]. The recent association of leptin with BMD [179, 180], as seen with the relationship between serum leptin concentrations and fluctuations in markers of bone remodelling, warrants further investigation.

Thus, leptin appears to be a sensitive marker to changes in energy expenditure, and plays a role in fat and glucose metabolism and energy homeostasis. In addition, various modes of training can impact the resting levels of leptin, albeit results are inconsistent. The lack of longitudinal studies on changes in training modes across a training season (9-months) has not been completed, therefore conclusive evidence of the fluctuations of leptin to various training loads across a training year can not be confirmed. Also, the limited literature on the association of leptin with bone metabolism warrants further investigation.

1.15 OPG response to exercise training

OPG increases with age and is higher in women with osteoporosis than in age-matched controls. Normal, resting OPG serum levels for pre-menopausal women are around 200 pg/ml. Also, women with higher OPG levels have higher rates of bone turnover, suggesting a compensatory mechanism to enhanced osteoclastic bone resorption rather

than a cause of osteoporosis [181]. Herrmann et al. [182] highlighted a potential protective effect of oral contraceptive use in female endurance athletes, as seen with increase markers of bone formation. However, they saw no group difference in OPG levels in endurance female athletes compared to age matched controls. In contrast, West et al. [183] emphasized that eumenorrheic endurance athletes have higher OPG levels compared to sedentary controls. However, Scott et al. [184] found no difference in trained and untrained male athletes, suggesting a potential sex difference or that the participants in this study were not trained enough.

Several studies (Table 4) have shown that OPG responds to various modes and volumes of exercise training. Bergstrom et al. [186] assessed the effect of moderate training 3 d/wk in post-menopausal women and found OPG increased by 7.6 pg/ml and BMD increased by 0.008 g/cm^2 at the hip when compared to non-exercising controls. This increased BMD was only associated with an increase in OPG, and was independent of changes in SOST or RANKL. In contrast, obese and overweight patients performing aerobic exercise 4 h/wk for 6 months found no change in OPG levels despite positive body composition changes following exercise training. Also, when post-menopausal women trained on a cycle-ergometer at 70-80% of workload 40 min 3d/wk for 8wk there was no change in OPG levels, however there was a decrease in OC following training (Table 4).

Table 4. Studies assessing OPG's response to exercise training.

Training	Effect on OPG	Other findings	Conclusions	Reference
Comparison of F endurance athletes (training 20 h/wk) and matched controls	↔	Grouped: ↔ sRANKL, BAP ↑ CTx Athletes using Oral Contraceptive vs. Not: ↑ CTx, BAP, OPG	Athletes have increased bone turnover than controls, and athletes taking oral contraceptives have lower bone turnover and lower bone resorption than non-users, suggesting a protective effect on bone	[182]
Comparison of EU sedentary (1), exercising women (2) and exercising AU young women (3)	2 compared to 1 & 3: ↑	3 had ↑ CTX and CTX/OPG than 1 & 2. 3 had ↓ lumbar spine BMD than 2.	OPG responds to exercise training and that ↓ OPG may be involved in the etiology of increased bone resorption and decreased BMD.	[183]
27 postmenopausal F performed cycle-ergometer at 70-80% of workload for 40 min, 3 d/wk for 8 wk	↔	↔ CTx, insulin ↓ OC, waist:hip OC and OPG correlated only at pre-training	Regular exercise resulted in a decrease in OC and had no impact on OPG levels	[185]
112 post-menopausal F walked for 30 min 3 d/wk and 1h of aerobic and strength training 1-2 d/wk for 1 year compared to sedentary controls	↑	↔ SOST, RANKL, CTX and BAP ↑ BMD	Exercise training balances bone turnover and increases OPG to counter balance RANKL signaling and increase BMD	[186]
21 overweight and obese patients (M and F) performed 6 months of aerobic training 4 h/wk	↔	↔ CRP, RANKL ↓ Weight, waist circumference, BP	Exercise resulted in positive changes in body composition, however training resulted in no changes in OPG or RANKL	[187]
9 M experienced Crossfit athletes had blood draws before, immediately and 24h following a workout consisting of resistance and anaerobic exercises for 2 days in a row	↓ pre- to 48h post	Immediately post-exercise: ↑ IL-6, IL-10 (only on day 1) ↓ IL-10/IL-6	Consecutive days of CrossFit training results in suppression of the immune-system, as well as a decline in OPG levels	[188]

F – Female, M – Male, EU – Eumenorrheic, AU – Amenorrheic

Furthermore, despite *in vitro* evidence of mechanical load increasing OPG levels, Maimoun et al. [189] illustrated that OPG levels are not different in pre- or post-monarchial girls who were either not active or participating in high impact, low impact, or non-impact sport disciplines, despite a higher BMD associated with the high impact group. Marques et al. [190] supports these findings in their study on older adults performing 32 weeks of loaded exercise (resistance exercise) training 3 d/wk, which

found no change in OPG levels from pre- to post-resistance training, suggesting training with load does not have an impact on OPG levels. Furthermore, Marques et al. [191] showed that 8 months of either resistance or aerobic training in post-menopausal women results in no change in serum levels of OPG or RANKL, however BMD is increased compared to sedentary controls. The hypothesis that volume and not intensity mediates OPG response is contradicted in a study that assessed 10 weeks of moderate intensity walking 50 mins/d, 5 d/wk for 10 wks in middle aged men, which found no change in OPG and a decrease in RANKL serum levels [192]. The results of these studies suggest individual differences in the OPG response to exercise training (Table 4).

1.16 SOST response to various modes of exercise training

In the past 2 decades, bone metabolism in endurance athletes has gained a great deal of attention as highlighted in previous sections. However, less is known about the female endurance athlete. In particular, serum markers associated with the Wnt/β-catenin pathway during extensive periods of training have been rarely assessed. Current research in this field provides some evidence from healthy non-athletes as well as elite level athletes that suggests SOST is affected by various modes of exercise training (Table 5).

Table 5. Studies assessing SOST's response to exercise training

Training	Effect on SOST	Other findings	Conclusions	Reference
112 post-menopausal F walked for 30 mins 3 d/wk and 1h of aerobic training 1-2 d/wk for 1 year compared to sedentary controls	↔	↔ RANKL, CTX and BAP ↑ BMD and OPG	Exercise training balances bone turnover by increasing OPG to counter balance RANKL signaling and increase BMD	[186]
58 healthy F trained for 8 wks, 4 d/wk and compared to 62 controls	↓	↑ IGF-1 (107%), PINP, BAP, CTX and OC	Exercise training decreases SOST expression in non-athletes	[193]
Compared athletes in various sport disciplines with sedentary controls	Weight Bearing sports: ↑ Non-weight bearing sports: ↔ F compared to M: ↑	SOST and BAP were inversely correlated in sedentary individuals, but not athletes	Increased bone anabolism by mechanical loading leads to a negative feedback loop, which increases inhibitors of anabolism (i.e., SOST)	[194]
50 female EU or AU athletes and sedentary controls ~19.8 years of age	EU and AU athletes compared to controls: ↑	EU athletes SOST levels were positively correlated with lumbar spine BMD, and control SOST levels were inversely associated with lumbar spine BMD	SOST asserts differential effects on bone in athletes and non-athletes	[195]
9 M cyclists competing in the Giro D'Italia stage race: Comparing baseline, mid and final stage	Continual ↑	↑ CK, uCa Positive Correlations: SOST and estradiol, PO/m, NEE, uCa, and CK - Correlations: SOST and DHEA	SOST could be a marker for both muscle and bone metabolic activity and damage	[196]
43 professional M soccer players ~26.5 years of age compared to 16 M healthy controls	↑	↑ 25(OH)D3, P1NP, sCa ↓ PTH ↔ Energy, Ca or vitamin D intake - Correlations: SOST and sCa	Training for multiple years may be associated with increased serum SOST	[197]

F – Female, M – Male, EU – Eumenorrheic, AU – Amenorrheic, CK – Creatine Kinase, u/sCa – Urinary/Serum Calcium, PO/m – Power Output/meter, NEE – Net Energy Expenditure

Pre-menopausal women participating in resistance and aerobic training for 90 min for 4d/wk for a year have shown no changes in serum SOST, but had increased serum OPG levels and BMD when compared to healthy sedentary controls [186]. In contrast, a similar population following an intervention of 120 min/wk for 8wks was shown to have significantly lower (37%) serum SOST levels and higher IGF-1 (107%) levels when

compares to sedentary controls [193]. These conflicting results in healthy non-athletes suggest that serum SOST levels may be affected by different training durations and that the response to training may be variable depending not only on volume, but performance level as well. This is highlighted by the comparison of SOST levels in elite athletes of various sports and healthy non-athlete controls, which has shown that SOST is higher in females compared to males in general, and in athletes competing in weight bearing sports when compared to non-weight bearing sports and non-athletes [194]. Furthermore, these athletes in general had an inverse age-related correlation with SOST and that individuals with higher levels of physical activity (i.e., endurance athletes) have higher SOST levels (20% higher) compared to individuals with lower levels of physical activity. These findings are in contrast to findings in healthy individuals aged 44±10 years, which found that men had higher SOST than women, and that age in general was positively correlated with serum SOST levels [120]. Furthermore, SOST is significantly higher in athletes when compared to healthy non-athlete controls [195]. Surprisingly, SOST was positively associated with lumbar spine BMD and Z-score, while in non-athletes, SOST was inversely associated with lumbar spine BMD. In addition, athletes had significantly higher CTX than non-athletes, however P1NP was not different, yet it was positively associated with SOST. These results indicate that despite SOST being a known bone formation inhibitor, it can be positively (eumenorrheic) or negatively (non-athletes) associated with BMD.

Continued assessment of SOST's response to exercise training in athletes was done during the *Giro d'Italia* 2012 stage race, where 9 professional male cyclists had 3 fasted blood draws prior, 12d and 23d after the start of competition [196]. SOST was

significantly increased at 12d and 23d of competition compared to the beginning of competition, and it was directly related to estradiol and inversely related to dehydroepiandrosterone (DHEA) levels. Estradiol was also correlated with urinary calcium and phosphorous levels. This data suggests that increased muscular effort in 12d intervals, as seen with increased CK levels in the absence of loading, stimulates osteocytes to increase SOST expression, which increases bone resorption. Ultramarathoners are a great model of assessing changes of bone metabolism after extreme energy expenditure. Spartathlon race is a 246-km ultradistance race, and assessment of serum levels of SOST has shown no change from pre- to post-race [198]. Despite the absence of an immediate response, sera taken 3 days following the race showed a significant decrease in SOST when compared to pre- and post-race levels. This is in agreement of previous work that has shown that athletes have lower resting SOST levels than non-athletes [120], and suggests a long term positive effect of endurance training on bone health. Assessment of SOST levels in professional male soccer players has shown to be significantly higher when compared to age matched, healthy non-athletes [197], which further suggests a complex relationship between peripheral levels of SOST and physical activity. These data suggest that SOST levels may be affected not only by training level, but also by the type of training being performed, and can be associated with either higher or lower BMD. However, this suggestion is limited by the lack of longitudinal studies.

CHAPTER 2: INTRODUCTION TO RESEARCH PAPER

Exercise training has generally been shown to be beneficial to bone health in young athletes compared to non-athletic controls [199, 200]. However, the osteogenic benefits of exercise training may have a threshold, as periods of high volume/load training have been shown to lead to greater bone resorption over formation [89, 196, 201], and consequently, may increase the risk of stress fractures in elite female rowers [202].

Several cross-sectional studies suggest that elite female rowers, and endurance athletes in general, are susceptible to low bone mineral density (BMD), due to consistent high volume training/load (endurance runners ~ 26 miles/week; elite rowers ~1080 min/week) [203, 204], accompanied by inadequate energy intake, resulting in low energy availability [205]. Competitive female rowers have also been found having higher resting serum levels of type 1 collagen breakdown compared to non-athletes and competitive runners and swimmers [87], suggesting higher rate of bone turnover. In elite, heavyweight male rowers, Jurimae et al. (2006) [161] demonstrated that 6 months of training can elicit an osteogenic response, as seen with a 6% increase in BMD and 17% increase in resting osteocalcin serum levels, suggesting that extended periods of high volume training can lead to a beneficial osteogenic response. However, these studies report bone marker levels only pre- and post-training, without taking into account fluctuations in training volume/load during a training period. More importantly, previously examined bone markers reflect bone turnover activity, but do not address the mechanisms responsible for changes in formation or resorption.

The anabolic Wnt/β-catenin signal transduction pathway regulates osteoblast and osteoclast activation and differentiation [99]. Greater Wnt binding to its receptor increases bone formation and inhibits bone resorption [206]. In addition, Wnt signalling increases expression of osteoprotegerin (OPG), which down-regulates osteoclastogenesis by inhibiting the catabolic receptor activator of nuclear factor kappa-B (RANK):RANK ligand (RANKL) signalling cascade, a pathway critical for osteoclastogenesis[207]. Sclerostin (SOST), an osteocyte-derived Wnt signalling antagonist [119], has been implemented in assessing stress fracture risk [208] and has been proposed to unbalance bone turnover toward bone resorption following a 3-week stage race in elite male cyclists [196]. Thus, the Wnt signalling pathway may be partially mediating changes seen in the bone response to high volume training. However, no study has assessed how SOST or the downstream products, OPG and RANKL, are affected by fluctuations in training load, which takes into account both training volume and intensity.

Furthermore, SOST's, OPG's, and RANKL's relationship with markers of energy homeostasis and stress has yet to be explored. Three markers that are used to assess variations in energy homeostasis or stress in elite athletes include tumor necrosis factor alpha (TNF-α), leptin, and insulin like growth factor 1 (IGF-1) [164]. Resting TNF-α levels have been suggested to increase due to excessive training stress and lack of recovery in elite male rowers [143-145]. In addition, TNF-α has been shown to increase SOST expression [40, 209][210], as well as RANKL expression [211], warranting investigation into its role in Wnt/β-catenin and RANK:RANKL signalling during fluctuations in training volume/load. Leptin is related to mean daily energy intake and expenditure and decreases following periods of high volume training as a result of

increased energy expenditure (reviewed in [164]). Low levels of leptin have been associated with a lower BMD in female athletes [56, 179, 180], as well as with lower OPG and higher RANKL levels [56, 63]. IGF-1 seems to reflect energy status, and bone formation, as a combination of high volume training and low energy intake reduces IGF-1 and type 1 collagen production[89], and has been shown to be positively associated with BMD and BMC of the femoral neck and spine in female athletes [212].These results suggest a negative impact of TNF-α on bone, but a protective effect of leptin and IGF-1 in elite athletes.

The objective of this study was to examine whether fluctuations in training load during an Olympic year lead to changes in bone mineral properties and serum biomarkers of Wnt and RANK:RANKL signalling (SOST, OPG, and RANKL), and how these bone-specific changes relate to markers of training stress (TNF-α, leptin, and IGF-1). It is noteworthy that since heavyweight rowers are not required to make a certain weight category, their examination allows for the assessment of the bone and cytokine response independent of low energy balance. We hypothesize that SOST and TNF-α will increase while leptin and IGF-1 will decrease following periods of high load training, and that subsequently will all return to baseline following periods of low training load. Lastly, since these athletes will be in relative energy balance throughout the year, it is expected that there will have no changes in their BMD from pre- to post training.

CHAPTER 3: METHODS

3.1 Participants

Fifteen elite, heavyweight, female rowers, who were training to represent Canada at the 2016 Olympiad in Rio de Janeiro were studied. Athletes were 27.0 ± 0.8 years of age, 179.4 ± 1.4 cm tall, 80.9 ± 1.3 kg, 20.8 ± 0.6% body fat, and had 9.3 ± 1.1 years of experience in competitive rowing. All participants gave written informed consent and the study was approved by the Research Ethics Boards of Brock University and the Canadian Sport Institute Ontario.

3.2 Study Design and Procedures

Over the course of the 42-week study, participants arrived at their daily training environment (Rowing Canada's National Training Centre, London, ON, CAN) at the same time (08:00-09:00) and had blood draws on six occasions: prior to the beginning of the training season in September 2015 (T1), 7 weeks (T2), 9 weeks (T3), 20 weeks (T4), 25 weeks (T5), and 42 weeks (T6) into the training season. Each testing day occurred on a Monday prior to their training for the week in a rested state and following the consumption of a consistent breakfast. Total energy intake at T1, T2 and T4-6 were: 4246±401, 3740±181, 3883±242, 3445±213, 3563±157 kcal, respectively, and there was no difference across time (p=0.22). Total protein intake at T1, T2 and T4-6 were: 158±8, 144±7, 147±9, 130±10, and 154±12g, respectively, and there was no difference across time (p=0.22). Total carbohydrate intake at T1, T2 and T4-6 were: 611±91, 521±34, 504±29, 426±30, and 410±26g, respectively, and there was no difference across time (p=0.09). Total fat intake at T1, T2 and T4-6 were: 136±8, 123±7, 135±8, 123±7,

135±10, 121±9, and 146±6g, respectively, and there was no difference across time (p=0.10). Menstrual status was not controlled for, as blood draws needed to be done on the same day for all athletes. The last blood draw took place 4 weeks before competition.

Blood was drawn from an antecubital vein into Vacutainers. Following blood draws, the samples sat at room temperature for 25 min, then were transported at 4^{o}C for 1.7h from the training center to the University laboratory, where blood was centrifuged at 1,000xg, serum isolated and aliquoted, and stored at -80^{o}C for future analysis.

3.3 Calculation of Training Volume, Intensity, and Load

Training volume (min/wk) was prescribed by the National Team coaches, and both volume and intensity were the same for all athletes throughout the year training plan. A modified Banister's training impulse (TRIMP) [213] was used to quantify weekly training intensity based on heart rate response coupled with the duration of training for each training session. Each athlete's heart rate (HR) intensity zones were determined via ergometer step tests, which were done throughout the season, and adjustments to intensity zones were made as fitness progressed. There were no drastic differences between TRIMP at T1-6. Training volumes for T1-6 were: 1020, 942, 972, 955, 1097, and 880 min/wk, respectively. T5 had the highest training volume, and T6 had the lowest. Training load (intensity × time × min^{-1}) was calculated as the product of the average weekly intensity and training volume from the previous 3 weeks of training. Specifically, training load for T1-6 were: 5319, 3808, 4875, 4221, 5660, and 4808 intensity*h*min^{-1}, respectively. T5 had the highest training load, and T2 had the lowest.

3.4 Biochemical Analysis

Serum SOST, OPG, TNF-α, leptin, RANKL, and IGF-1 were analyzed in triplicate using Milliplex MAGPIX kits (EMD Millipore Corporation, Bellerica, MA, USA). SOST, OPG (OPG levels include bound (to RANKL) and the unbound forms), TNF-α, and leptin were measured on a Human Bone Panel (4 panels were used to analyze all samples). The average intra-assay coefficient of variation for SOST was 5.3% and the inter-assay coefficient of variation was 7.4%. The average intra-assay coefficient of variation for OPG was 7.2%, and the inter-assay coefficient of variation was 8.2%. The average intra-assay coefficient of variation for TNF-α was 6.1%, and the inter-assay coefficient of variation was 5.7%. The average intra-assay coefficient of variation for leptin was 4.3%, and the inter-assay coefficient of variation was 5.1%. Human RANKL (RANKL levels only include the unbound form) was measured using a single human RANKL Panel (2 panels were used to analyze all samples). The average intra-assay coefficient of variation for RANKL was 3.4%, and the inter-assay coefficient of variation was 4.1%. Human IGF-1 was measured using a single Human IGF-1 Panel (4 panels were used to analyze all samples). The average intra-assay coefficient of variation for IGF-1 was 7.5%, and the inter-assay coefficient of variation was 5.4%. Human estradiol was measured in duplicate using 2 enzyme linked immunosorbent assays (ELISA) (R&D, MN, USA). The average intra-assay coefficient of variation for estradiol was 4.8%, and the inter-assay coefficient of variation was 5.9%.

3.5 Bone Measurements and Dietary Intake

Total-body BMD and BMC were measured by dual-energy x-ray absorptiometry (DXA) (Lunar iDXA ME 2000087, GE Healthcare, Burlington, ON, CAN). Lumbar spine, ribs,

pelvis, arms, and leg BMD values were also ascertained from region-of-interest values reported with the whole-body scan. Dietary variables were assessed from three-day dietary records for each athlete prior to each blood draw. Athlete food diaries for the 3 days leading into each blood draw were analyzed for total energy, and macro- and micronutrient intake using Food Processor Nutrition Analysis Software (ESHA, Salem, OR, SUA).

3.6 Statistical analysis

All data were screened for normality using the Kolmogorov-Smirnov test. Descriptive statistics on anthropometric characteristics were calculated for the total group. A paired *t*-test was used to compare the pre- and post-training DXA scans. A one-way analysis of variance for repeated measures (RM ANOVA) was used to assess changes over time in biochemical markers. In the event of a significant time effect, further pairwise comparisons were made using LSD correction to determine significant differences between time points. In addition, percent coefficient of variation (%CV) for each biochemical marker was determined for each participant to ascertain fluctuations in marker concentrations throughout the season and to assess how marker variability affected bone mineral properties. Finally, data from T1 and T6 were combined into continuous data sets and linear regression was used to determine predictors of BMD, BMC, and percent change (%change) in BMD and BMC. Significance was accepted at an alpha level of <0.05 for all analyses, and means±SEM are reported in all Figures and Tables. Statistical Analysis was performed using SPSS version 22 for Windows.

CHAPTER 4: RESULTS

From pre-season (T1) to post-season (T6) there was no change in body mass. However, there was a significant decrease in adiposity (20.7 ± 0.6 to 19.9 ± 0.8%, p = 0.02). Table 6 presents changes in bone mineral properties from pre- to post-season. Total BMD increased significantly (+1.6%, p = 0.05) from pre- to post-season (42 weeks). However, total BMC remained stable (+1.9%, p = 0.34).

Table 6. Bone outcomes of interest at pre- compared to post-season in all rowers (values are Mean±SEM).

Measurements	Pre-Season (T1)	Post-Season (T6)	% Δ	*P*-value
Total BMC (g)	3115 ± 95	3176 ± 64	+1.9	0.34
Total BMD (g/cm^2)	1.25 ± 0.02	1.27 ± 0.02	+1.6	0.05*
Trunk BMD (g/cm^2)	1.10 ± 0.03	1.13 ± 0.02	+2.7	0.09
Pelvis BMD (g/cm^2)	1.22 ± 0.03	1.26 ± 0.03	+3.2	0.06
Ribs BMD (g/cm^2)	0.89 ± 0.02	0.91 ± 0.02	+2.2	0.34
Spine BMD (g/cm^2)	1.20 ± 0.03	1.23 ± 0.03	+2.6	0.19

*= $P<0.05$; %Δ = percent change calculated as (post-season subtract pre-season)/pre-season X 100

The mean percent coefficient of variation (%CV) of all biochemical markers over the training season were estimated using the individual %CV across time points, and are presented in Table 7. These values represent the variability of each marker during the season within each individual. Estradiol had the highest CV (37.8%) and OPG had the lowest CV (19.9%) during the season.

Table 7. Percent coefficient of variation (%CV) of all biomarkers for individual participants across time (values are Mean±SEM).

Biomarker	%CV
Estradiol	37.8±4.7
IGF-1	20.4±3.0
Leptin	32.0±2.5
TNF-α	21.7±2.9
SOST	25.7±3.3
OPG	19.9±2.1
RANKL	33.0±6.0
OPG/RANKL	36.9±22.3

IGF-1=insulin growth factor-1; TNF-α=tumor necrosis factor-α; SOST=sclerostin; OPG=osteoprotegerin; RANKL=receptor activator of nuclear factor kappa-B ligand;

Figure 5 shows the changes of SOST (a), OPG (b), RANKL (c), and OPG/RANKL ratio (d), along with the training load fluctuations, across the 42-week study period. SOST appeared sensitive to changes in training load, as levels increased at T5, compared to T6, T4, T3 and T2 ($p = 0.006$) and not different from T1. That is, SOST followed the fluctuations in training volume with its levels being highest at T5, when training volume was also at its highest, compared to the tapering week (T6) with the lowest training volume (993.1 vs. 741.0 pg/mL, respectively, $p = 0.006$). OPG was unchanged over the season. RANKL decreased significantly at T4 and remained reduced at T5 and T6 compared to T1 ($p = 0.002$). The OPG/RANKL ratio was significantly increased at T4 and T6 compared to T1 and T2 ($p = 0.02$).

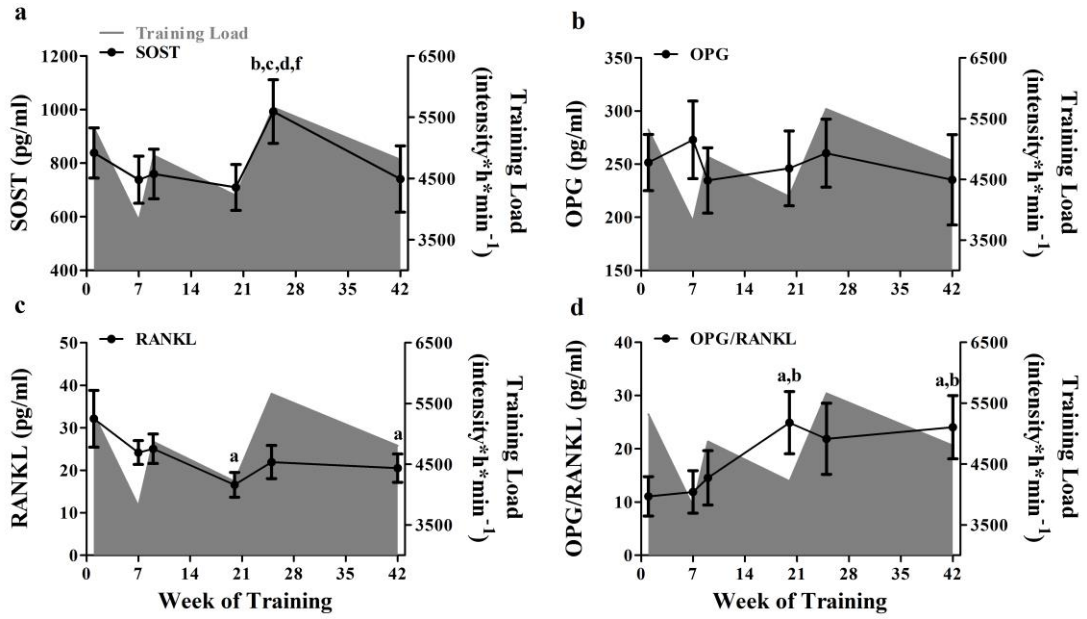

Figure 5. Weekly training volume from the 3 weeks preceding each blood draw and mean ± SEM of resting a) SOST, b) OPG, c) RANKL, and d) OPG/RANKL serum concentrations in elite heavyweight female rowers (n=15). A RM ANOVA was used to ascertain any significant changes across time in osteokines. a = $p<0.05$, significantly different from week 0; b = $p<0.05$, significantly different from week 7; c = $p<0.05$, significantly different from week 9; d = $p<0.05$, significantly different from week 20; f = $p<0.05$, significantly different from week 42.

Figure 6 shows the changes in the inflammatory and metabolic markers, along with training load fluctuations, across time. TNF-α (a) showed a similar pattern to (SOST), and was significantly higher at T1 and T5 compared to T6 ($p = 0.002$). Leptin (b) did not change consistently with fluctuations in training load or volume, and was lowest at T2 (1224.5 pg/ml). However, there were no significant differences between any time points. IGF-1 (c) remained unchanged throughout the training season.

Estrogen was measured to control for menstrual status, because all athletes had their blood drawn on the same day, thus at different stages of their menstrual cycles. Estrogen was not significantly different at any time point. Figure 6d shows the mean ±

SEM concentration change of estrogen across the 42-week long study period. Also, estrogen had no significant relationships with any other serum biomarkers or bone outcomes at any time point or overall.

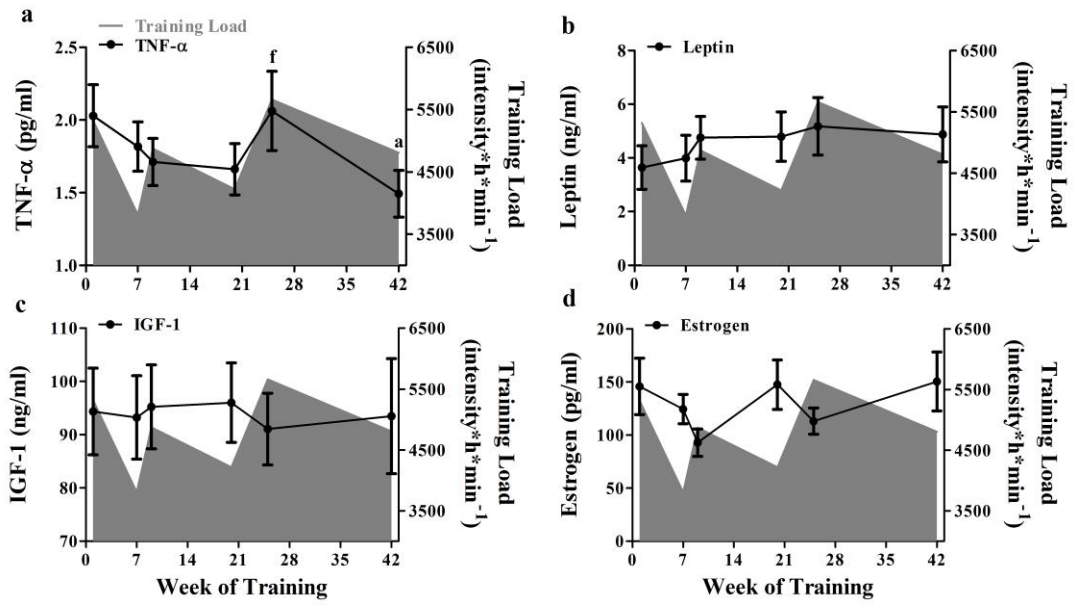

Figure 6. Weekly training volume from the 3 weeks preceding each blood draw and mean ± SEM of resting a) TNF-α, b) leptin, c) IGF-1, and d) estrogen serum concentrations in elite heavyweight female rowers (n=15). A RM ANOVA was used to ascertain any significant changes across time in biomarkers. a = $p<0.05$, significantly different from week 1; significantly different from week 25; f = $p<0.05$, significantly different from week 42.

Table 8 shows the regression results for total BMC and BMD. Model 1 for total BMC included TNF-α as the strongest negative predictor, which together with leptin (model 2) could explain up to 31% of the variance in total BMC. For total BMD, leptin was a positive predictor and could explain 14% of the variance in total BMD (Table 3). Stepwise regressions were also performed for %change of BMC and %change of BMD.

The model for %change of BMC included OPG/RANKL %CV as a negative predictor and could explain 30% of the variance in %change of BMC. No other variables assessed in this study were accepted into the stepwise regressions and no variables explained the variance in %change of BMD (entry at $F<0.05$). The relationships between total BMD and BMC and biomarkers were independent of estrogen, body composition, menstrual status and oral contraceptive use, as none of these variables entered in the predictive models.

Table 8. Regression models predicting total BMC and BMD using TNF-α and leptin.

Variables	Model 1	Model 2
Total BMC		
TNF-α	-158.3(-0.42)	-182.1(-0.49)
Leptin		0.03(0.42)
Adjusted R^2	0.15	0.31
p-value	0.02*	0.003*
Total BMD		
Leptin	$9.3E^{-6}$(0.37)	
Adjusted R^2	0.14	
p-value	0.04*	

Unstandardized β-coefficients are reported with Beta in parentheses

CHAPTER 5: DISCUSSION

This is the first study to examine the effect of training load fluctuations on bone markers (SOST, OPG and RANKL), and their relationship with changes in inflammatory and metabolic markers across an Olympic year in elite athletes in general, and in female, heavyweight rowers in particular. Our findings demonstrate that TNF-α, an inflammatory marker, and SOST, a bone formation inhibitor, were sensitive to changes in training volume and training load. Additionally, SOST appears to increase in parallel with TNF-α, suggesting that high volume/load training can induce systemic inflammation, and inhibit osteoblastogenesis. Thus, this study presents new evidence in support of the mechanism, illustrated in Figure 7, of how high training load leads to a transient decrease in bone formation through an upregulation of SOST via TNF-α. In contrast, the intermittent periods of lower volume/load training were accompanied by decreases in both TNF-α and SOST, further supporting the proposed mechanism. Over the course of the season, a decrease in RANKL, along with the relatively stable OPG, led to an overall increase in OPG/RANKL, which is an indicator of an overall decrease in osteoclastogenesis. Lastly, leptin, appears to be a positive predictor of BMD and BMC. In contrast, TNF-α, which was sensitive to training load fluctuations, appears to be a negative predictor of BMC. These catabolic responses, however, can be reversed with intermittent periods of reduced training load, which combined with a healthy metabolic profile (i.e., stable leptin), seem to protect the bone integrity in elite female athletes.

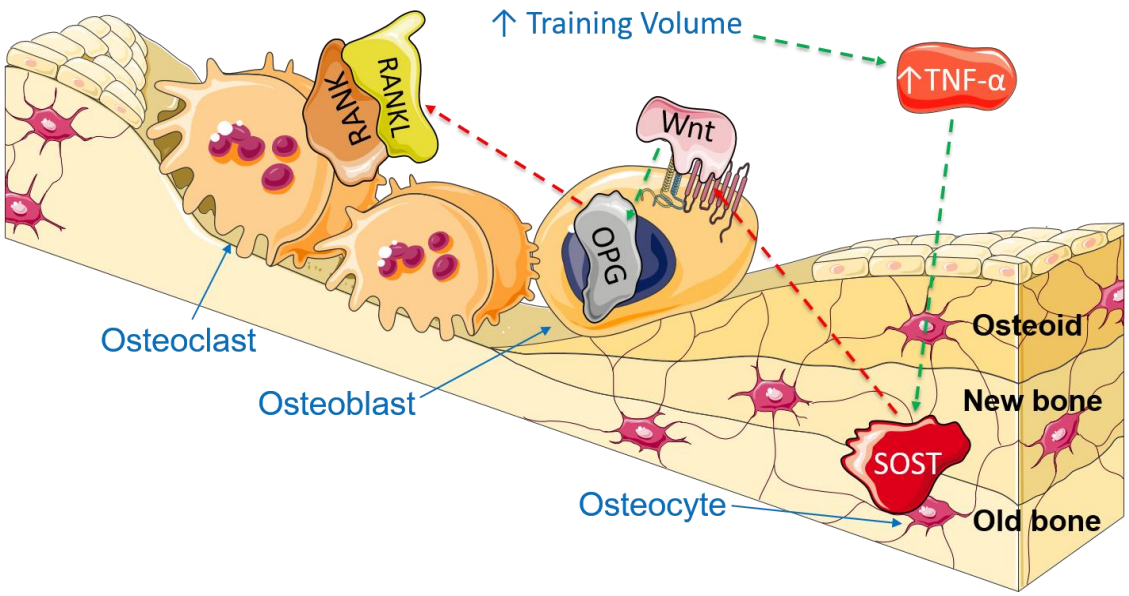

Figure 7. The effect of increased training volume on Wnt/β-catenin and RANK:RANKL:OPG signalling cascades; (Adapted from Servier Medical Art by Servier©).

5.1 Bone mineral properties and anthropometric outcomes pre- and post-training

There was a significant increase in BMD over the training season. This finding is in line with previous studies that have reported site specific (i.e., lumbar spine) [81, 82] increases in BMD in elite male and female rowers. However, this the first study to assess changes in BMD in elite heavyweight female rowers across a full competitive season, and highlight an increase. Body mass did not change, suggesting that rowers were in energy balance. This is supported by the observations that IGF-1 and leptin remained relatively constant, and the fact the rowers received frequent nutritional counseling. Therefore, our results suggest that the increase in BMD is due to the high quantity of intense muscle contractions [214]. This provides further support for the mechanostat theory [214], according to which the increase in BMD is due to the high quantity of intense muscle contractions.

The increase in total BMC, although of similar magnitude (1.9%), was statistically not significant. This is puzzling and is probably due to the higher variability in individual values. On the other hand, although there was no change in stature, it is possible that the participants were still accruing bone mass. Peak bone mass, defined as the highest level of bone mass achieved as a result of normal growth, is largely achieved by age 18 to early 20s depending on the bone [215]. In girls, approximately 50% of peak bone mass is accrued around the time of peak height velocity [216], with 90% of total body BMC accrued by the end of the second decade [217], and the remaining 5-10% achieved by the third decade [216]. Overall, the significantly higher BMD and stable BMC clearly indicate that the bone mineral properties of these elite, heavyweight rowers were at least preserved during the season.

5.2 SOST response to fluctuations in training load

SOST fluctuated parallel to training load, which is in contradiction to studies that have shown extensively that increased mechanical loading decreases the expression and protein levels of SOST in *vivo* [218]. On the other hand, elite cyclists who were competing in a 3-week stage race showed a continual increase in SOST from pre- to 1.5 and 3 weeks into the race [196]. Another study found a significant decrease in SOST from pre- and post-3 days of recovery following a race in spartathlon participants [198]. These studies suggest that SOST may increase with increased training volume/load and can decrease with recovery, despite mechanical unloading increasing SOST expression *in vivo* [218]. Our findings further support these findings found in elite athletes, as SOST increased during the weeks of higher training load (T5) and decreased during periods of lower training load. It is also interesting that SOST fluctuated in parallel to training

volume; at T6, during the tapering period of the lowest training volume, SOST reached its lowest levels while it reached its highest levels during the week of the highest training load (T5). This suggests that simple changes in training volume (as in hours/week) can lead to alterations in bone metabolism.

Our results also support the previous suggestions that extended periods of high volume/load training can lead to a transient suppression of bone formation due to inhibition of Wnt signalling by SOST [196]. *In vitro* assessment of SOST found that its expression is only seen in osteons following mineralization, and SOST is highest in osteons that are undergoing bone resorption [119]. The relationship between SOST expression and bone resorption suggests that either the number of osteons undergoing bone resorption increased, or the osteons undergoing bone resorption increased SOST expression as training volume/load increased. SOST fluctuated in parallel, and was positively related to TNF-α levels, a proposed inflammatory marker of muscular stress [145], which suggests that excessively high training load can lead to elevated resting TNF-α levels and subsequently, increased SOST expression.

Lastly, estrogen was not correlated to either TNF-α or SOST at each time point or overall, despite previous evidence of estrogen being a significant predictor of SOST in pre- and post-menopausal women [129], and estrogen deficient mice appear to increase SOST expression in a TNF-α dependent mechanism [40]. In contrast, our results suggest that TNF-α may upregulate SOST expression independent of estrogen as a response to increased training load.

5.3 OPG, RANKL and OPG/RANKL responses to fluctuations in training load

OPG did not respond to fluctuations in training load, and remained fairly stable. However, despite no change in OPG, RANKL decreased significantly at midseason compared to pre-season and remained suppressed to post-season, leading to an increase in OPG/RANKL ratio from T1 to T4 and T6. Taken together, these results suggest there may be a decrease in osteoclast number due to a decrease in available RANKL, and thus a decrease in bone resorption over the training period, possibly explaining the increase in BMD. This suggestion is supported by the regression analysis in Table 3, which shows that the variability of OPG/RANKL throughout the season, as determined by %CV across all time points, accounted for 30% of the variance in the %change of BMC.

OPG has been hypothesized to increase with exercise training, subsequently decreasing unbound RANKL levels, thus protecting the skeleton from bone loss [219]. In this study, OPG levels remained unchanged across the training year, however unbound RANKL levels significantly decreased, suggesting a decrease in osteoclastogenesis either by a decrease in RANKL levels, or due to a higher proportion of OPG being bound to RANKL. Also, the OPG/RANKL ratio was not associated with BMC or BMD in this study despite being elevated at week T4 and T6 compared to pre-training levels, suggesting a protective effect on bone and subsequently contributing to the increase in BMD from pre- to post-training. In elite female gymnasts and endurance athletes, BMD is significantly higher than age-matched controls, although OPG/RANKL ratio was similar [220],[182]. There are limited studies that assessed the OPG/RANKL ratio in athletes and this is the first to assess the training response. Thus, our finding that OPG/RANKL ratio is not a predictive marker for BMD, is in agreement with previous

findings [220]. However, monitoring the OPG/RANKL ratio may suggest directional changes in BMD, as we have shown that this ratio increases throughout a training season and remains elevated. The elevated ratio may also translate to the improved BMD from pre- to post-season.

5.4 Leptin's association with bone mineral properties

Leptin was not sensitive to changes in training load but was found to be a significant predictor of total BMD and BMC. This finding agrees with one previous study also reporting leptin to be a significant predictor of total BMC and BMD in active females [221]. Leptin replacement therapy has also been shown to increase bone formation and improve BMD in female athletes with low BMD and serum leptin levels [56, 222, 223]. Interestingly, leptin's influence on bone mineral properties seems to be independent of training since leptin did not directly respond to the fluctuations in training load, and showed moderate variability (32 %CV) throughout the training season. These findings, and supporting literature, suggest that adequately stable levels of leptin may be critical for BMD preservation in elite female athletes. Despite these findings, the exact mechanism of how leptin preserves BMD remains unclear, as leptin acts on many tissues, which increase the secretion of anabolic endocrine effectors, such as IGF-1 and estrogen [55], and inhibit the production of RANKL [224], which together, improves BMD. Our findings only support leptin may be important for preservation or improvement in BMD in elite female athletes.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Fluctuations in training load were accompanied by parallel fluctuations in SOST and TNF-α, suggesting that increasing training load may inhibit bone formation. This catabolic response can be counterbalanced by periods of lower training load, which lower TNF-α and SOST. Furthermore, leptin levels, which appear insensitive to training load fluctuations. Also, leptin levels and the low variability in the OPG/RANKL ratio during the training season seem to protect the bone mineral properties in these elite, heavyweight rowers. This is new evidence that training load periodization can control the inflammatory response associated with intense training, and coupled with adequate nutrient intake can preserve bone mineral integrity in elite female athletes.

6.2 Limitations

This is the first study to assess the effect of training load fluctuations on bone markers (SOST, OPG and RANKL), and their relationship with changes in inflammatory and metabolic markers during an Olympic year in elite athletes in general, and in female, heavyweight rowers in particular. Despite the attempt to limit the factors that may confound our results, this study did have some flaws.

First, we have not measured bone events directly, and our interpretation of the results are based on inferences from indirect bone markers measured in blood. Also, the design of this study did not allow for controlling for menstrual status or food intake. In order not to interfere with training schedule, all blood draws were performed in all athletes on the same morning, right before workouts. This meant athletes were in

different phases of their menstrual cycles, and thus had different levels of hormones. Additionally, since practice immediately followed the blood draw, all athletes were in a fed state during blood draws. Also, multiplex assays are an effective way at assessing multiple analytes simultaneously with a small amount of sample. However, this method has a higher lower limit of detection for most analytes, and has higher variability than ELISA.

6.3 Future Directions

Future studies are needed to assess elite lightweight female athletes, compared with heavyweight rowers and with non-exercising controls. This will help understand the response of SOST, as well as other Wnt related markers, and inflammatory cytokines to fluctuations in training load with or without energy expenditure/intake balance in elite athletes. Also, taking multiple draws during recovery periods following periods of high load training will help elucidate how long a recovery period is required to have levels of SOST and TNF-α to return to baseline. Furthermore, following resting levels of SOST, and other Wnt related markers, in non-athletic and athletic populations at multiple time points from pre- to post- an exercise training program with various training modalities will give an insight into how initialization vs. long term training of various modes of exercise (i.e., low impact vs. high impact, high intensity vs. high volume) impact these markers.

Lastly, there is still much to be learned about what role osteocytes have in bone mineralization and maintenance, and the mechanism of why there is this paradoxical increase in SOST with increased training load in elite athletes while there is a decrease with excessive loading in murine models. Since we are unable to take bone biopsies,

future studies should first utilize animal models to assess osteocyte, osteoblast, and osteoclast cell number, expression and localization of SOST and other Wnt related signalling molecules within bone and peripheral tissues, as well as bone microarchitecture following exercise training rather than utilizing loading murine models. Making assertions from loading experiments and not exercise specific studies may lead to a misinterpretation as to what may be occurring with exercise, as exercise stimulates the utilization of all organ systems and secretion of signalling molecules, while the loading experiments only focuses on sedentary behaviour coupled with excessive mechanical loading. Assessing bone cell ratios will give an insight into how the bone multicellular unit is responding to exercise training and give an insight into why we are seeing the changes in SOST (i.e., if there is a change in osteocyte number over osteoblast number). Evaluating expression and localization of SOST will answer the question of whether there is a change in expression or the response to exercise is based on the quantity of SOST within the canaliculi lacunae system. Also, assessing localization of SOST, and other Wnt associated signalling molecules, with peripheral tissues has never been done, since we know SOST is in high concentration within serum and that LRP5/6 receptors are a ubiquitous signalling system we would expect that SOST may inhibit Wnt signalling in peripheral tissues, such as muscle. Also, assessing Wnt expression, protein levels, and Wnt-receptor association following acute exercise, as well as training, in bone and muscle will help elucidate the mechanisms that lead to the anabolic responses observed. Measuring bone microarchitecture changes will give an improved understanding of how bone is responding to exercise training along with changes in SOST expression. These studies will provide insight into what impact the SOST response to exercise training has

on bone microarchitecture. Furthermore, assessing how Wnt signalling is modulated following exercise (acute and training) can help elucidate why we see the response observed in serum and what they mean.

REFERENCES

1. Clarke, B., *Normal Bone Anatomy and Physiology.* Clinical Journal of the American Society of Nephrology : CJASN, 2008. **3**(Suppl 3): p. S131-S139.
2. Sims, N.A. and J.H. Gooi, *Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption.* Seminars in Cell & Developmental Biology, 2008. **19**(5): p. 444-451.
3. Zhang, Q., R.C. Riddle, and T.L. Clemens, *Bone and the regulation of global energy balance.* Journal of internal medicine, 2015. **277**(6): p. 681-689.
4. Martin, T.J. and K.W. Ng, *Mechanisms by which cells of the osteoblast lineage control osteoclast formation and activity.* J Cell Biochem, 1994. **56**(3): p. 357-66.
5. Tsurukai, T., et al., *Roles of macrophage-colony stimulating factor and osteoclast differentiation factor in osteoclastogenesis.* J Bone Miner Metab, 2000. **18**(4): p. 177-84.
6. Lacey, D.L., et al., *Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.* Cell, 1998. **93**.
7. Kobayashi, N., et al., *Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis.* Embo j, 2001. **20**(6): p. 1271-80.
8. Johnson, R.S., B.M. Spiegelman, and V. Papaioannou, *Pleiotropic effects of a null mutation in the c-fos proto-oncogene.* Cell, 1992. **71**(4): p. 577-86.
9. Franzoso, G., et al., *Requirement for NF-kappaB in osteoclast and B-cell development.* Genes Dev, 1997. **11**(24): p. 3482-96.

10. Ishida, N., et al., *Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator.* J Biol Chem, 2002. **277**(43): p. 41147-56.

11. Boyle, W.J., W.S. Simonet, and D.L. Lacey, *Osteoclast differentiation and activation.* Nature, 2003. **423**(6937): p. 337-42.

12. Crockett, J.C., et al., *Bone remodelling at a glance.* Journal of Cell Science, 2011. **124**(7): p. 991-998.

13. Ullah, M., et al., *Transdifferentiation of mesenchymal stem cells-derived adipogenic-differentiated cells into osteogenic- or chondrogenic-differentiated cells proceeds via dedifferentiation and have a correlation with cell cycle arresting and driving genes.* Differentiation, 2013. **85**(3): p. 78-90.

14. Franz-Odendaal, T.A., B.K. Hall, and P.E. Witten, *Buried alive: How osteoblasts become osteocytes.* Developmental Dynamics, 2006. **235**(1): p. 176-190.

15. Bonewald, L.F., *The amazing osteocyte.* J Bone Miner Res, 2011. **26**(2): p. 229-38.

16. Franz-Odendaal, T.A., B.K. Hall, and P.E. Witten, *Buried alive: how osteoblasts become osteocytes.* Dev Dyn, 2006. **235**(1): p. 176-90.

17. Pead, M.J., et al., *Increased 3H-uridine levels in osteocytes following a single short period of dynamic bone loading in vivo.* Calcif Tissue Int, 1988. **43**(2): p. 92-6.

18. Xiong, J., et al., *Osteocyte-derived RANKL is a critical mediator of the increased bone resorption caused by dietary calcium deficiency.* Bone, 2014. **66**: p. 146-54.

19. Heino, T.J., T.A. Hentunen, and H.K. Vaananen, *Conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation into osteoblasts.* Exp Cell Res, 2004. **294**(2): p. 458-68.

20. Ren, Y. and J.Q. Feng, *Osteocytes Play a Key Role in the Formation and Maintenance of Mineralized Bone.* The FASEB Journal, 2017. **31**(1 Supplement): p. 7.1.

21. Cavalier, E., et al., *The role of biochemical of bone turnover markers in osteoporosis and metabolic bone disease: a consensus paper of the Belgian Bone Club.* Osteoporosis International, 2016. **27**(7): p. 2181-2195.

22. Green, S., C.L. Anstiss, and W.H. Fishman, *Automated differential isoenzyme analysis. II. The fractionation of serum alkaline phosphatases into "liver", "intestinal" and "other" components.* Enzymologia, 1971. **41**(1): p. 9-26.

23. Orimo, H., *The mechanism of mineralization and the role of alkaline phosphatase in health and disease.* J Nippon Med Sch, 2010. **77**(1): p. 4-12.

24. Millán, J.L., *Alkaline Phosphatases: Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes.* Purinergic Signalling, 2006. **2**(2): p. 335-341.

25. Wennberg, C., et al., *Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice.* J Bone Miner Res, 2000. **15**(10): p. 1879-88.

26. Johnson, K.A., et al., *Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(4): p. R1365-77.

27. Wheater G., et al., *The clinical utility of bone marker measurements in osteoporosis.* Journal of Translational Medicine, 2013. **11**(201).

28. Hauschka, P.V., *Osteocalcin: the vitamin K-dependent Ca2+-binding protein of bone matrix.* Haemostasis, 1986. **16**(3-4): p. 258-72.

29. Hauschka, P.V., et al., *Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone.* Physiological Reviews, 1989. **69**(3): p. 990-1047.

30. Ducy, P., et al., *Increased bone formation in osteocalcin-deficient mice.* Nature, 1996. **382**(6590): p. 448-52.

31. Hlaing, T.T. and J.E. Compston, *Biochemical markers of bone turnover - uses and limitations.* Ann Clin Biochem, 2014. **51**(Pt 2): p. 189-202.

32. Costa, L., et al., *Prospective evaluation of the peptide-bound collagen type I cross-links N-telopeptide and C-telopeptide in predicting bone metastases status.* J Clin Oncol, 2002. **20**(3): p. 850-6.

33. Henriksen, K., et al., *Assessment of osteoclast number and function: application in the development of new and improved treatment modalities for bone diseases.* Osteoporosis International, 2007. **18**(5): p. 681-685.

34. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Tumor necrosis factor signaling.* Cell Death Differ, 2002. **10**(1): p. 45-65.

35. Popa, C., et al., *The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk.* J Lipid Res, 2007. **48**(4): p. 751-62.

36. Weitzmann, M.N. and R. Pacifici, *Estrogen deficiency and bone loss: an inflammatory tale.* The Journal of Clinical Investigation, 2006. **116**(5): p. 1186-1194.

37. Weitzmann, M.N. and R. Pacifici, *The role of T lymphocytes in bone metabolism.* Immunological Reviews, 2005. **208**(1): p. 154-168.

38. Pascher, E., et al., *Effect of 1alpha,25(OH)2-vitamin D3 on TNF alpha-mediated apoptosis of human primary osteoblast-like cells in vitro.* Horm Metab Res, 1999. **31**(12): p. 653-6.

39. Kobayashi, K., et al., *Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction.* J Exp Med, 2000. **191**(2): p. 275-86.

40. Kim, B.J., et al., *TNF-alpha mediates the stimulation of sclerostin expression in an estrogen-deficient condition.* Biochem Biophys Res Commun, 2012. **424**(1): p. 170-5.

41. Wehmeyer, C., et al., *Sclerostin inhibition promotes TNF-dependent inflammatory joint destruction.* Science Translational Medicine, 2016. **8**(330): p. 330ra35-330ra35.

42. Kavran, J.M., et al., *How IGF-1 activates its receptor.* eLife, 2014. **3**: p. e03772.

43. Luigi, L., N. Annalisa, and G. Francesco, *The IGF-I Signaling Pathway.* Current Pharmaceutical Design, 2007. **13**(7): p. 663-669.

44. Taniguchi, C.M., B. Emanuelli, and C.R. Kahn, *Critical nodes in signalling pathways: insights into insulin action.* Nat Rev Mol Cell Biol, 2006. **7**(2): p. 85-96.

45. Yakar, S., et al., *Circulating levels of IGF-1 directly regulate bone growth and density.* J Clin Invest, 2002. **110**(6): p. 771-81.

46. Le Roith, D., et al., *The somatomedin hypothesis: 2001.* Endocr Rev, 2001. **22**(1): p. 53-74.

47. Gong, Z., et al., *Reductions in serum IGF-1 during aging impair health span.* Aging Cell, 2014. **13**(3): p. 408-18.

48. Yeh, L.C., et al., *IGF-1 Receptor Insufficiency Leads to Age-Dependent Attenuation of Osteoblast Differentiation.* Endocrinology, 2015. **156**(8): p. 2872-9.

49. Cross, D.A., et al., *The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf.* Biochem J, 1994. **303 (Pt 1)**: p. 21-6.

50. Margetic, S., et al., *Leptin: a review of its peripheral actions and interactions.* Int J Obes Relat Metab Disord, 2002. **26**(11): p. 1407-33.

51. Chua, S.C., Jr., et al., *Fine structure of the murine leptin receptor gene: splice site suppression is required to form two alternatively spliced transcripts.* Genomics, 1997. **45**(2): p. 264-70.

52. Maffei, M., et al., *Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects.* Nat Med, 1995. **1**(11): p. 1155-61.

53. Farooqi, I.S., et al., *Partial leptin deficiency and human adiposity.* Nature, 2001. **414**(6859): p. 34-5.

54. Hayes, M.R., et al., *Endogenous Leptin Signaling in the Caudal Nucleus Tractus Solitarius and Area Postrema Is Required for Energy Balance Regulation.* Cell Metabolism, 2010. **11**(1): p. 77-83.

55. Chou, S.H. and C. Mantzoros, *20 years of leptin: role of leptin in human reproductive disorders.* J Endocrinol, 2014. **223**(1): p. T49-62.

56. Sienkiewicz, E., et al., *Long-term metreleptin treatment increases bone mineral density and content at the lumbar spine of lean hypoleptinemic women.* Metabolism, 2011. **60**(9): p. 1211-21.

57. Broberger, C., et al., *The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 15043-8.

58. Dalamaga, M., et al., *Leptin at the intersection of neuroendocrinology and metabolism: current evidence and therapeutic perspectives.* Cell Metab, 2013. **18**(1): p. 29-42.

59. Greenblatt, M.B., et al., *The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice.* J Clin Invest, 2010. **120**(7): p. 2457-73.

60. Li, J., *JAK-STAT and bone metabolism.* Jakstat, 2013. **2**(3): p. e23930.

61. Moon, H.S., et al., *Identification and saturable nature of signaling pathways induced by metreleptin in humans: comparative evaluation of in vivo, ex vivo, and in vitro administration.* Diabetes, 2015. **64**(3): p. 828-39.

62. Welt, C.K., et al., *Recombinant human leptin in women with hypothalamic amenorrhea.* N Engl J Med, 2004. **351**(10): p. 987-97.

63. Foo, J.P., et al., *The effect of leptin replacement on parathyroid hormone, RANKL-osteoprotegerin axis, and Wnt inhibitors in young women with hypothalamic amenorrhea.* J Clin Endocrinol Metab, 2014. **99**(11): p. E2252-8.

64. Chou, S.H., et al., *Leptin is an effective treatment for hypothalamic amenorrhea.* Proc Natl Acad Sci U S A, 2011. **108**(16): p. 6585-90.

65. Halaas, J.L., et al., *Weight-reducing effects of the plasma protein encoded by the obese gene.* Science, 1995. **269**(5223): p. 543-6.

66. Pelleymounter, M.A., et al., *Effects of the obese gene product on body weight regulation in ob/ob mice.* Science, 1995. **269**(5223): p. 540-3.

67. Swerdloff, R.S., R.A. Batt, and G.A. Bray, *Reproductive hormonal function in the genetically obese (ob/ob) mouse.* Endocrinology, 1976. **98**(6): p. 1359-64.

68. Galvez, B.G., N. San Martin, and C. Rodriguez, *TNF-alpha is required for the attraction of mesenchymal precursors to white adipose tissue in Ob/ob mice.* PLoS One, 2009. **4**(2): p. e4444.

69. Hamrick, M.W., et al., *Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice.* J Bone Miner Res, 2005. **20**(6): p. 994-1001.

70. Hamrick, M.W., et al., *Leptin deficiency produces contrasting phenotypes in bones of the limb and spine.* Bone, 2004. **34**(3): p. 376-83.

71. Qian, H., et al., *Brain administration of leptin causes deletion of adipocytes by apoptosis.* Endocrinology, 1998. **139**(2): p. 791-4.

72. Hamrick, M.W., et al., *Injections of leptin into rat ventromedial hypothalamus increase adipocyte apoptosis in peripheral fat and in bone marrow.* Cell Tissue Res, 2007. **327**(1): p. 133-41.

73. Bartell, S.M., et al., *Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice.* Journal of Bone and Mineral Research, 2011. **26**(8): p. 1710-1720.

74. Ducy, P., et al., *Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass.* Cell, 2000. **100**(2): p. 197-207.

75. Takeda, S., et al., *Leptin regulates bone formation via the sympathetic nervous system.* Cell, 2002. **111**(3): p. 305-17.

76. Iwaniec, U.T., et al., *Central leptin gene therapy corrects skeletal abnormalities in leptin-deficient ob/ob mice.* Peptides, 2007. **28**(5): p. 1012-9.

77. Steppan, C.M., et al., *Leptin is a potent stimulator of bone growth in ob/ob mice.* Regulatory Peptides, 2000. **92**(1–3): p. 73-78.

78. Ferron, M. and J. Lacombe, *Regulation of energy metabolism by the skeleton: osteocalcin and beyond.* Arch Biochem Biophys, 2014. **561**: p. 137-46.

79. Laharrague, P., et al., *High expression of leptin by human bone marrow adipocytes in primary culture.* Faseb j, 1998. **12**(9): p. 747-52.

80. Snyder, A.C., et al., *Bone mineral content of elite lightweight amenorrheic oarswomen.* Hum Biol, 1986. **58**(6): p. 863-9.

81. Cohen, B., et al., *Effect of exercise training programme on bone mineral density in novice college rowers.* Br J Sports Med, 1995. **29**(2): p. 85-8.

82. Morris, F.L., et al., *Compressive and Shear ForceGenerated in the Lumbar Spine of Female Rowers.* Int J Sports Med, 2000. **21**(07): p. 518-523.

83. Young, K.C., et al., *Rowing performance, body composition, and bone mineral density outcomes in college-level rowers after a season of concurrent training.* Int J Sports Physiol Perform, 2014. **9**(6): p. 966-72.

84. Lariviere, J.A., T.L. Robinson, and C.M. Snow, *Spine bone mineral density increases in experienced but not novice collegiate female rowers.* Med Sci Sports Exerc, 2003. **35**(10): p. 1740-4.

85. McDonnell LK., H.P., Nolte V., *Rib Stress Fractures Among Rowers - Definition, Epidemiology, Mechanisms, Risk Factors and Effectiveness of Injury Prevention Strategies.* Sports Med, 2011. **11**(41): p. 883-901.

86. Lombardi, G., et al., *Bone and energy metabolism parameters in professional cyclists during the Giro d'Italia 3-weeks stage race.* PLoS One, 2012. **7**(7): p. e42077.

87. O'Kane, J.W., et al., *Sport-related differences in biomarkers of bone resorption and cartilage degradation in endurance athletes.* Osteoarthritis Cartilage, 2006. **14**(1): p. 71-6.

88. Sansoni, V., et al., *Bone turnover response is linked to both acute and established metabolic changes in ultra-marathon runners.* Endocrine, 2016.

89. Zanker C.L. and S. I.L., *Responses of bone turnover markers to repeated endurance running in humans under conditions of energyy balance or energy restriction.* Eur J Appl Physiol, 2000. **83**: p. 434-440.

90. De Souza, M.J., et al., *The presence of both an energy deficiency and estrogen deficiency exacerbate alterations of bone metabolism in exercising women.* Bone, 2008. **43**(1): p. 140-8.

91. Nusse R., V.H., *Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome.* Cell, 1982. **31**: p. 99-109.

92. Habas, R. and I.B. Dawid, *Dishevelled and Wnt signaling: is the nucleus the final frontier?* Journal of Biology, 2005. **4**(1): p. 1-4.

93. Komekado, H., et al., *Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a.* Genes Cells, 2007. **12**(4): p. 521-34.

94. Franch-Marro, X., et al., *In vivo role of lipid adducts on Wingless.* J Cell Sci, 2008. **121**(Pt 10): p. 1587-92.

95. Zoltewicz, J.S., et al., *Wnt signaling is regulated by endoplasmic reticulum retention.* PLoS One, 2009. **4**(7): p. e6191.

96. Kang, D.W. and S. Min do, *Positive feedback regulation between phospholipase D and Wnt signaling promotes Wnt-driven anchorage-independent growth of colorectal cancer cells.* PLoS One, 2010. **5**(8): p. e12109.

97. Willert, K. and R. Nusse, *Wnt Proteins.* Cold Spring Harbor Perspectives in Biology, 2012. **4**(9): p. a007864.

98. Gregory, C.A., et al., *How Wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow.* Ann N Y Acad Sci, 2005. **1049**: p. 97-106.

99. Karner, C.M. and F. Long, *Wnt signaling and cellular metabolism in osteoblasts.* Cell Mol Life Sci, 2016.

100. Spencer GJ., U.J., Etheridge SL., Arnett TR.. Genever PG, *Wnt signalling in osteoblasts regulates expression of the receptor activator of NFκB ligand and inhibits osteoclastogenesis in vitro.* Journal of Cell Science, 2005. **119**: p. 1283-1297.

101. Holmen, S.L., et al., *Essential role of beta-catenin in postnatal bone acquisition.* J Biol Chem, 2005. **280**(22): p. 21162-8.

102. Spencer, G.J., et al., *Wnt signalling in osteoblasts regulates expression of the receptor activator of NFκB ligand and inhibits osteoclastogenesis in vitro.* Journal of Cell Science, 2006. **119**(7): p. 1283-1296.

103. Li, C., et al., *Disruption of LRP6 in osteoblasts blunts the bone anabolic activity of PTH.* J Bone Miner Res, 2013. **28**(10): p. 2094-108.

104. Glass, D.A., 2nd, et al., *Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation.* Dev Cell, 2005. **8**(5): p. 751-64.

105. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**.

106. De Boer J., W.H., Van Blitterswijk C. , *Effects of wnt signaling on proliferation and differentiation of human mesenchymal stem cells.* Tissue Engineering, 2004. **10**.

107. Bennett, C.N., et al., *Regulation of osteoblastogenesis and bone mass by Wnt10b.* Proc Natl Acad Sci U S A, 2005. **102**(9): p. 3324-9.

108. Jackson, A., et al., *Gene array analysis of Wnt-regulated genes in C3H10T1/2 cells.* Bone, 2005. **36**(4): p. 585-98.

109. Clement-Lacroix, P., et al., *Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice.* Proc Natl Acad Sci U S A, 2005. **102**(48): p. 17406-11.

110. Banerjee, C., et al., *Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex.* J Cell Biochem, 1997. **66**(1): p. 1-8.

111. Komori, T., et al., *Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts.* Cell, 1997. **89**(5): p. 755-64.

112. Geoffroy, V., et al., *High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage.* Mol Cell Biol, 2002. **22**(17): p. 6222-33.

113. Baniwal, S.K., et al., *Runx2 promotes both osteoblastogenesis and novel osteoclastogenic signals in ST2 mesenchymal progenitor cells.* Osteoporos Int, 2012. **23**(4): p. 1399-413.

114. Martin, A., et al., *Estrogens Antagonize RUNX2-Mediated Osteoblast-Driven Osteoclastogenesis Through Regulating RANKL Membrane Association.* Bone, 2015. **75**: p. 96-104.

115. van Lierop, A.H., et al., *Van Buchem disease: clinical, biochemical, and densitometric features of patients and disease carriers.* J Bone Miner Res, 2013. **28**(4): p. 848-54.

116. Balemans, W., et al., *Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST).* Hum Mol Genet, 2001. **10**(5): p. 537-43.

117. Semenov, M., K. Tamai, and X. He, *SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor.* J Biol Chem, 2005. **280**(29): p. 26770-5.

118. Tamai K., S.M., Kato Y., Spokony R., Liu R., Katsuyama Y., Hess F., Saint-Jeannet JP., and X. He, *LDL receptor related proteins in Wnt signal transduction.* Nature, 2000. **407**: p. 530-535.

119. Poole, K.E., et al., *Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation.* FASEB J, 2005. **19**(13): p. 1842-4.

120. Amrein, K., et al., *Sclerostin and its association with physical activity, age, gender, body composition and bone mineral content in healthy adults.* J Clin Endocrinol Metab, 2012. **97**.

121. Perez-Campo, F.M., et al., *Osterix and RUNX2 are Transcriptional Regulators of Sclerostin in Human Bone.* Calcif Tissue Int, 2016. **99**(3): p. 302-9.

122. Yang, F., et al., *Sclerostin is a direct target of osteoblast-specific transcription factor osterix.* Biochem Biophys Res Commun, 2010. **400**(4): p. 684-8.

123. van Bezooijen, R.L., et al., *Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation.* J Bone Miner Res, 2007. **22**(1): p. 19-28.

124. Spatz, J.M., et al., *The Wnt Inhibitor Sclerostin Is Up-regulated by Mechanical Unloading in Osteocytes in Vitro.* J Biol Chem, 2015. **290**(27): p. 16744-58.

125. Stolina, M., et al., *Temporal changes in systemic and local expression of bone turnover markers during six months of sclerostin antibody administration to ovariectomized rats.* Bone, 2014. **67**: p. 305-13.

126. Zhang, D., et al., *Sclerostin antibody prevented progressive bone loss in combined ovariectomized and concurrent functional disuse.* Bone, 2016. **87**: p. 161-8.

127. Li, X., et al., *Progressive increases in bone mass and bone strength in an ovariectomized rat model of osteoporosis after 26 weeks of treatment with a sclerostin antibody.* Endocrinology, 2014. **155**(12): p. 4785-97.

128. Dawson-Hughes, B., et al., *Serum sclerostin levels vary with season.* J Clin Endocrinol Metab, 2014. **99**(1): p. E149-52.

129. Mirza, F.S., et al., *Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women.* J Clin Endocrinol Metab, 2010. **95**.

130. Ardawi, M.S., et al., *Determinants of serum sclerostin in healthy pre- and postmenopausal women.* J Bone Miner Res, 2011. **26**(12): p. 2812-22.

131. Leupin, O., et al., *Control of the SOST bone enhancer by PTH using MEF2 transcription factors.* J Bone Miner Res, 2007. **22**(12): p. 1957-67.

132. Bellido, T., et al., *Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis.* Endocrinology, 2005. **146**(11): p. 4577-83.

133. Silvestrini, G., et al., *Effects of intermittent parathyroid hormone (PTH) administration on SOST mRNA and protein in rat bone.* J Mol Histol, 2007. **38**(4): p. 261-9.

134. Rhee, Y., et al., *Resorption controls bone anabolism driven by parathyroid hormone (PTH) receptor signaling in osteocytes.* J Biol Chem, 2013. **288**(41): p. 29809-20.

135. Polyzos, S.A., et al., *Serum sclerostin levels positively correlate with lumbar spinal bone mineral density in postmenopausal women – the six-month effect of risedronate and teriparatide.* Osteoporos Int, 2012. **23**.

136. Roggia, C., et al., *Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13960-5.

137. Roggia, C., et al., *Role of TNF-alpha producing T-cells in bone loss induced by estrogen deficiency.* Minerva Med, 2004. **95**(2): p. 125-32.

138. Cenci, S., et al., *Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-gamma-induced class II transactivator.* Proc Natl Acad Sci U S A, 2003. **100**(18): p. 10405-10.

139. Gao, Y., et al., *Estrogen prevents bone loss through transforming growth factor beta signaling in T cells.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16618-23.

140. Kim, W., et al., *Increased sclerostin levels after further ablation of remnant estrogen by aromatase inhibitors.* Endocrinol Metab (Seoul), 2015. **30**(1): p. 58-64.

141. Komori, T., *Regulation of osteoblast differentiation by Runx2.* Adv Exp Med Biol, 2010. **658**: p. 43-9.

142. Halson, S.L., et al., *Immunological responses to overreaching in cyclists.* Med Sci Sports Exerc, 2003. **35**(5): p. 854-61.

143. Ramson, R., et al., *The influence of increased training volume on cytokines and ghrelin concentration in college level male rowers.* Eur J Appl Physiol, 2008. **104**(5): p. 839-46.

144. Main, L.C., et al., *Impact of training on changes in perceived stress and cytokine production.* Res Sports Med, 2009. **17**(2): p. 121-32.

145. Smith, L.L., *Cytokine hypothesis of overtraining: a physiological adaptation to excessive stress?* Med Sci Sports Exerc, 2000. **32**(2): p. 317-31.

146. Pedersen, B.K. and M.A. Febbraio, *Muscle as an endocrine organ: focus on muscle-derived interleukin-6.* Physiol. Rev., 2008. **88**: p. 1379-1406.

147. Schnyder, S. and C. Handschin, *Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise.* Bone, 2015. **80**: p. 115-125.

148. Brunelli, D.T., et al., *Monitoring of immunological parameters in adolescent basketball athletes during and after a sports season.* J Sports Sci, 2014. **32**(11): p. 1050-9.

149. Nemet, D., et al., *Cytokines and growth factors during and after a wrestling season in adolescent boys.* Med Sci Sports Exerc, 2004. **36**(5): p. 794-800.

150. Eliakim, A. and D. Nemet, *Exercise training, physical fitness and the growth hormone-insulin-like growth factor-1 axis and cytokine balance.* Med Sport Sci, 2010. **55**: p. 128-40.

151. Eliakim, A., *Endocrine Response to Exercise and Training-Closing the Gaps.* Pediatr Exerc Sci, 2016. **28**(2): p. 226-32.

152. Sartorio A., et al., *Elite volunteer athletes of different sport disciplines may have elevated baseline G levels divorced from unaltered levels of IGF-1 and GH-*

dependent bone and collagen markers: a study on-the-field. J. Endocrinol. invest, 2004. **27**: p. 410-415.

153. Ubertini, G., et al., *Young elite athletes of different sport disciplines present with an increase in pulsatile secretion of growth hormone compared with non-elite athletes and sedentary subjects.* Journal of Endocrinological Investigation, 2008. **31**(2): p. 138-145.

154. Antonelli, G., et al., *Measurement of free IGF-I saliva levels: Perspectives in the detection of GH/IGF axis in athletes.* Clinical Biochemistry, 2007. **40**(8): p. 545-550.

155. Hecksteden, A., et al., *Blood-Borne Markers of Fatigue in Competitive Athletes – Results from Simulated Training Camps.* PLOS ONE, 2016. **11**(2): p. e0148810.

156. Jahreis, G., et al., *Influence of intensive exercise on insulin-like growth factor I, thyroid and steroid hormones in female gymnasts.* Growth Regul, 1991. **1**(3): p. 95-9.

157. Roemmich, J.N. and W.E. Sinning, *Weight loss and wrestling training: effects on growth-related hormones.* J Appl Physiol (1985), 1997. **82**(6): p. 1760-4.

158. Eliakim, A., et al., *Changes in circulating IGF-I and their correlation with self-assessment and fitness among elite athletes.* Int J Sports Med, 2002. **23**(8): p. 600-3.

159. Filaire, E., et al., *Effects of 16 weeks of training prior to a major competition on hormonal and biochemical parameters in young elite gymnasts.* J Pediatr Endocrinol Metab, 2003. **16**(5): p. 741-50.

160. Steinacker, J.M., et al., *Training of junior rowers before world championships. Effects on performance, mood state and selected hormonal and metabolic responses.* J Sports Med Phys Fitness, 2000. **40**(4): p. 327-35.

161. Jurimae, J., et al., *Bone metabolism in elite male rowers: adaptation to volume-extended training.* Eur J Appl Physiol, 2006. **97**(1): p. 127-32.

162. Nindl, B.C., et al., *Circulating bioactive and immunoreactive IGF-I remain stable in women, despite physical fitness improvements after 8 weeks of resistance, aerobic, and combined exercise training.* J Appl Physiol (1985), 2010. **109**(1): p. 112-20.

163. Laughlin, G.A. and S.S. Yen, *Hypoleptinemia in women athletes: absence of a diurnal rhythm with amenorrhea.* J Clin Endocrinol Metab, 1997. **82**(1): p. 318-21.

164. Jurimae, J., et al., *Peripheral signals of energy homeostasis as possible markers of training stress in athletes: a review.* Metabolism, 2011. **60**(3): p. 335-50.

165. Jurimae, J., J. Maestu, and T. Jurimae, *Leptin as a marker of training stress in highly trained male rowers?* Eur J Appl Physiol, 2003. **90**(5-6): p. 533-8.

166. Simsch C., L.W., Peterson KG>, Baur S., Liu Y., Hackney AC., Lehmann M., Steinacker JM, *Training intensity influences leptin and thyroid hormones in hihgly trained rowers.* Int J Sports Med, 2002. **23**: p. 422-427.

167. Baylor, L.S. and A.C. Hackney, *Resting thyroid and leptin hormone changes in women following intense, prolonged exercise training.* Eur J Appl Physiol, 2003. **88**(4-5): p. 480-4.

168. Jurimae, J., P. Purge, and T. Jurimae, *Adiponectin and stress hormone responses to maximal sculling after volume-extended training season in elite rowers.* Metabolism, 2006. **55**(1): p. 13-9.

169. Jurimae, J., P. Purge, and T. Jurimae, *Effect of prolonged training period on plasma adiponectin in elite male rowers.* Horm Metab Res, 2007. **39**(7): p. 519-23.

170. Ramson, R., et al., *The effect of 4-week training period on plasma neuropeptide Y, leptin and ghrelin responses in male rowers.* Eur J Appl Physiol, 2012. **112**(5): p. 1873-80.

171. Lakhdar, N., et al., *Effects of intense cycling training on plasma leptin and adiponectin and its relation to insulin resistance.* Neuro Endocrinol Lett, 2013. **34**(3): p. 229-35.

172. Desgorces, F.D., et al., *Leptin, catecholamines and free fatty acids related to reduced recovery delays after training.* European Journal of Applied Physiology, 2004. **93**(1): p. 153-158.

173. Desgorces, F.D., et al., *Leptin response to acute prolonged exercise after training in rowers.* European Journal of Applied Physiology, 2004. **91**(5): p. 677-681.

174. Ishigaki, T., et al., *Plasma leptin levels of elite endurance runners after heavy endurance training.* J Physiol Anthropol Appl Human Sci, 2005. **24**(6): p. 573-8.

175. Desgorces, F.D., et al., *Relationships between leptin levels and carbohydrate intake during rowing training.* J Sports Med Phys Fitness, 2008. **48**(1): p. 83-9.

176. Roupas, N.D., et al., *Salivary adiponectin levels are associated with training intensity but not with bone mass or reproductive function in elite Rhythmic Gymnasts.* Peptides, 2014. **51**: p. 80-5.

177. Desgorces, F.D., et al., *Leptin, catecholamines and free fatty acids related to reduced recovery delays after training.* Eur J Appl Physiol, 2004. **93**(1-2): p. 153-8.

178. Desgorces, F.D., et al., *Leptin response to acute prolonged exercise after training in rowers.* Eur J Appl Physiol, 2004. **91**(5-6): p. 677-81.

179. Prouteau, S., L. Benhamou, and D. Courteix, *Relationships between serum leptin and bone markers during stable weight, weight reduction and weight regain in male and female judoists.* Eur J Endocrinol, 2006. **154**(3): p. 389-95.

180. Soot, T., T. Jurimae, and J. Jurimae, *Areal bone density in young females with different physical activity patterns: relationships with plasma leptin and body composition.* J Sports Med Phys Fitness, 2007. **47**(1): p. 65-9.

181. Yano, K., et al., *Immunological Characterization of Circulating Osteoprotegerin/Osteoclastogenesis Inhibitory Factor: Increased Serum Concentrations in Postmenopausal Women with Osteoporosis.* Journal of Bone and Mineral Research, 1999. **14**(4): p. 518-527.

182. Herrmann, M. and W. Herrmann, *The assessment of bone metabolism in female elite endurance athletes by biochemical bone markers.* Clin Chem Lab Med, 2004. **42**(12): p. 1384-9.

183. West, S.L., J.L. Scheid, and M.J. De Souza, *The effect of exercise and estrogen on osteoprotegerin in premenopausal women.* Bone, 2009. **44**(1): p. 137-144.

184. Scott, J.P., et al., *The effect of training status on the metabolic response of bone to an acute bout of exhaustive treadmill running.* J Clin Endocrinol Metab, 2010. **95**(8): p. 3918-25.

185. Wieczorek-Baranowska, A., A. Nowak, and Ł. Pilaczyńska-Szcześniak, *Osteocalcin and glucose metabolism in postmenopausal women subjected to aerobic training program for 8 weeks.* Metabolism, 2012. **61**(4): p. 542-545.

186. Bergström, I., et al., *Physical training increases osteoprotegerin in postmenopausal women.* Journal of Bone and Mineral Metabolism, 2012. **30**(2): p. 202-207.

187. Davenport, C., et al., *The effect of exercise on osteoprotegerin and TNF-related apoptosis-inducing ligand in obese patients.* European Journal of Clinical Investigation, 2012. **42**(11): p. 1173-1179.

188. Tibana, R.A., et al., *Two Consecutive Days of Crossfit Training Affects Pro and Anti-inflammatory Cytokines and Osteoprotegerin without Impairments in Muscle Power.* Frontiers in Physiology, 2016. **7**: p. 260.

189. Maïmoun, L., et al., *Peripubertal female athletes in high-impact sports show improved bone mass acquisition and bone geometry.* Metabolism, 2013. **62**(8): p. 1088-1098.

190. Marques, E.A., et al., *Response of bone mineral density, inflammatory cytokines, and biochemical bone markers to a 32-week combined loading exercise programme in older men and women.* Arch Gerontol Geriatr, 2013. **57**(2): p. 226-33.

191. Marques, E.A., et al., *Effects of resistance and aerobic exercise on physical function, bone mineral density, OPG and RANKL in older women.* Experimental Gerontology, 2011. **46**(7): p. 524-532.

192. H. Esen, et al., *Do walking programs affect C-reactive protein, osteoprotegerin and soluble receptor activator of nuclear factor-kappaβ ligand?* Turk. J. Biochem. Turk. Biyokimya Dergisi, 2009. **34**: p. 178-186.

193. Ardawi, M.S., A.A. Rouzi, and M.H. Qari, *Physical activity in relation to serum sclerostin, insulin-like growth factor-1, and bone turnover markers in healthy premenopausal women: a cross-sectional and a longitudinal study.* J Clin Endocrinol Metab, 2012. **97**(10): p. 3691-9.

194. Lombardi, G., et al., *Sclerostin concentrations in athletes: role of load and gender.* J Biol Regul Homeost Agents, 2012. **26**(1): p. 157-63.

195. Fazeli, P.K., et al., *Sclerostin and Pref-1 have differential effects on bone mineral density and strength parameters in adolescent athletes compared with non-athletes.* Osteoporos Int, 2013. **24**(9): p. 2433-40.

196. Grasso, D., et al., *Bone-muscle unit activity, salivary steroid hormones profile, and physical effort over a 3-week stage race.* Scand J Med Sci Sports, 2015. **25**(1): p. 70-80.

197. Zagrodna, A., et al., *Sclerostin as a novel marker of bone turnover in athletes.* Biol Sport, 2016. **33**(1): p. 83-7.

198. Kerschan-Schindl, K., et al., *Changes in Serum Levels of Myokines and Wnt-Antagonists after an Ultramarathon Race.* PLoS One, 2015. **10**(7): p. e0132478.

199. Torstveit, M.K. and J. Sundgot-Borgen, *Low bone mineral density is two to three times more prevalent in non-athletic premenopausal women than in elite athletes: a comprehensive controlled study.* Br J Sports Med, 2005. **39**(5): p. 282-7; discussion 282-7.

200. Nichols, D.L., C.F. Sanborn, and E.V. Essery, *Bone density and young athletic women. An update.* Sports Med, 2007. **37**(11): p. 1001-14.

201. Banfi, G., et al., *Bone Metabolism Markers in Sports Medicine.* Sports Medicine, 2010. **40**(8): p. 697-714.

202. D'Ailly, P.N., J.K. Sluiter, and P.P. Kuijer, *Rib stress fractures among rowers: a systematic review on return to sports, risk factors and prevention.* J Sports Med Phys Fitness, 2016. **56**(6): p. 744-53.

203. Dimitriou, L., et al., *Bone mineral density, rib pain and other features of the female athlete triad in elite lightweight rowers.* BMJ Open, 2014. **4**(2): p. e004369.

204. Barrack, M.T., et al., *Body mass, training, menses, and bone in adolescent runners: a 3-yr follow-up.* Med Sci Sports Exerc, 2011. **43**(6): p. 959-66.

205. Nattiv, A., et al., *American College of Sports Medicine position stand. The female athlete triad.* Med Sci Sports Exerc, 2007. **39**(10): p. 1867-82.

206. Kim, J.H., et al., *Wnt signaling in bone formation and its therapeutic potential for bone diseases.* Ther Adv Musculoskelet Dis, 2013. **5**(1): p. 13-31.

207. Boyce, B.F. and L. Xing, *Functions of RANKL/RANK/OPG in bone modeling and remodeling.* Archives of biochemistry and biophysics, 2008. **473**(2): p. 139-146.

208. Ardawi, M.S., et al., *High serum sclerostin predicts the occurrence of osteoporotic fractures in postmenopausal women: the Center of Excellence for Osteoporosis Research Study.* J Bone Miner Res, 2012. **27**(12): p. 2592-602.

209. Baek, K., et al., *TNF-α Upregulates Sclerostin Expression in Obese Mice Fed a High-Fat Diet.* Journal of Cellular Physiology, 2014. **229**(5): p. 640-650.

210. Saad, C.G., et al., *Low sclerostin levels: a predictive marker of persistent inflammation in ankylosing spondylitis during anti-tumor necrosis factor therapy?* Arthritis Res Ther, 2012. **14**(5): p. R216.

211. Zhang, Y.H., et al., *Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways.* J Biol Chem, 2001. **276**(1): p. 563-8.

212. Gruodytė, R., et al., *The relationships among bone health, insulin-like growth factor-1 and sex hormones in adolescent female athletes.* Journal of Bone and Mineral Metabolism, 2010. **28**(3): p. 306-313.

213. Banister, E., *In: Macdouggal JD, Wenger HA, Green HJ, eds. Physiological Testing of Elite Athletes. Champaign Illinois.* Human Kinetics, 1991: p. 403-422.

214. Schoenau, E., *From mechanostat theory to development of the "Functional Muscle-Bone-Unit".* J Musculoskelet Neuronal Interact, 2005. **5**(3): p. 232-8.

215. Matkovic, V., et al., *Timing of peak bone mass in Caucasian females and its implication for the prevention of osteoporosis. Inference from a cross-sectional model.* J Clin Invest, 1994. **93**(2): p. 799-808.

216. Cadogan, J., et al., *A longitudinal study of bone gain in pubertal girls: anthropometric and biochemical correlates.* J Bone Miner Res, 1998. **13**(10): p. 1602-12.

217. Elgan, C., G. Samsioe, and A.K. Dykes, *Influence of smoking and oral contraceptives on bone mineral density and bone remodeling in young women: a 2-year study.* Contraception, 2003. **67**(6): p. 439-47.

218. Lin, C., et al., *Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/β-Catenin signalling.* J Bone Miner Res, 2009. **24**.

219. Ziegler, S., et al., *Endurance running acutely raises plasma osteoprotegerin and lowers plasma receptor activator of nuclear factor κ B ligand.* Metabolism, 2005. **54**(7): p. 935-938.

220. Maïmoun, L., et al., *In peripubertal girls, artistic gymnastics improves areal bone mineral density and femoral bone geometry without affecting serum OPG/RANKL levels.* Osteoporosis International, 2011. **22**(12): p. 3055-3066.

221. Mallinson, R.J., et al., *Body composition and reproductive function exert unique influences on indices of bone health in exercising women.* Bone, 2013. **56**(1): p. 91-100.

222. Welt , C.K., et al., *Recombinant Human Leptin in Women with Hypothalamic Amenorrhea.* New England Journal of Medicine, 2004. **351**(10): p. 987-997.

223. Chou, S.H., et al., *Leptin is an effective treatment for hypothalamic amenorrhea.* Proceedings of the National Academy of Sciences, 2011. **108**(16): p. 6585-6590.

224. Lamghari, M., et al., *Leptin effect on RANKL and OPG expression in MC3T3-E1 osteoblasts.* J Cell Biochem, 2006. **98**(5): p. 1123-9.

APPENDIX

A. Brock University Ethics Board Clearance

Brock University
Research Ethics Office
Tel: 905-688-5550 ext. 3035
Email: reb@brocku.ca

Bioscience Research Ethics Board

Certificate of Ethics Clearance for Human Participant Research

DATE: 10/15/2015

PRINCIPAL INVESTIGATOR: SPRENGER, Heather - Kinesiology
Brock Investigator Bareket Falk
FILE: 15-067 - SPRENGER

TYPE: Faculty Research STUDENT:
SUPERVISOR:

TITLE: Monitoring Bone Health in High Performance Athletes Throughout the Year Training Plan

ETHICS CLEARANCE GRANTED

Type of Clearance: NEW Expiry Date: 10/31/2016

The Brock University Bioscience Research Ethics Board has reviewed the above named research proposal and considers the procedures, as described by the applicant, to conform to the University's ethical standards and the Tri-Council Policy Statement. Clearance granted from **10/15/2015** to **10/31/2016**.

The Tri-Council Policy Statement requires that ongoing research be monitored by, at a minimum, an annual report. Should your project extend beyond the expiry date, you are required to submit a Renewal form before 10/31/2016. Continued clearance is contingent on timely submission of reports.

To comply with the Tri-Council Policy Statement, you must also submit a final report upon completion of your project. All report forms can be found on the Research Ethics web page at http://www.brocku.ca/research/policies-and-forms/research-forms.

In addition, throughout your research, you must report promptly to the REB:

a) Changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) All adverse and/or unanticipated experiences or events that may have real or potential unfavourable implications for participants;
c) New information that may adversely affect the safety of the participants or the conduct of the study;
d) Any changes in your source of funding or new funding to a previously unfunded project.

We wish you success with your research.

Approved: [redacted]

Sandra Peters, Chair
Bioscience Research Ethics Board

Note: Brock University is accountable for the research carried out in its own jurisdiction or under its auspices and may refuse certain research even though the REB has found it ethically acceptable.

If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of research at that site.

B. Canadian Sports Institute Ethics Board Clearance

CANADIAN SPORT INSTITUTE / INSTITUT CANADIEN DU SPORT

CSIO Research Ethics Board

MEMORANDUM

DATE: September 19th, 2015

TO: Dr. Heather Sprenger, Principle Investigator

Dr. Nota Klentrou, Co-Principle Investigator

Dr. Bareket Falk, Co-Principle Investigator

FROM: Dr. Judy Goss, PhD, Lead of Mental Performance, Research Ethics Board Member

REB #: 2015 – 06

Title: **Monitoring Bone Health in High Performance Athletes Throughout the Year Training Plan**

Congratulations the Canadian Sport Institute Ontario Research Ethics Board (REB) has reviewed your project submission and has granted the project approval. Thank-you for making the necessary corrections. We have accepted the recent revision that was submitted regarding the volume of blood drawn on each athlete per Test Day from 2-3 mL to 10 mL collected.

Your project has been assigned an REB #2015-06. Please refer to your project REB number for future correspondence.

If this project changes in anyway OR if you continue with more data collection beyond the submitted completion date, you have the explicit responsibility to notify the CSIO Research Ethics Board Member assigned to this project at that time in writing.

Research records must be retained for a minimum of 3 years after completion of the research; if the study involves medical treatment, it is recommended that the results are retained for 5 years.

You are responsible for notifying all parties about the approval of this project, including your co-investigators, PSO/NSO coaches, and management. Please be advised that you will need to submit a progress report every six-month until the study is completed and a final report outlining the key findings of the study. **Your six-month progress report will be due on March 18th, 2016**.

Good luck with your research pursuits,

Dr. Judy Goss, PhD, Lead of Mental Performance

jgoss@csiontario.ca, (416) 596-1240 ext. 227