Bone markers and cytokines in response to low-impact, high-intensity exercise

by

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Abstract

A low-impact, high-intensity interval exercise (HIE) bout was used to determine whether an association exists between cytokines and bone turnover markers following an acute bout of exercise. Twenty-three recreationally active males (21.8±2.4yr) performed a single HIE bout on a cycle ergometer at 90% relative intensity. Venous blood samples were collected prior to exercise, 5-minutes, 1-hour, and 24-hours post-exercise, and were analyzed for serum levels of pro-inflammatory (IL-6, IL-1α, IL-1β, and TNF-α) and anti-inflammatory cytokines (IL-10) and markers of bone formation (BAP, OPG) and resorption (NTX, RANKL). Significant effects were observed with all bone markers, especially 5-minutes post-exercise with BAP, OPG, and RANKL increasing from baseline (p<0.05). Significant effects were also observed for IL-1α, IL-1β, IL-6, and TNF-α (p<0.00, p=0.04, p=0.03, p<0.00). In addition, post-exercise changes in NTX, BAP, and OPG were significantly correlated pro- and anti-inflammatory cytokines, suggesting that an interaction exists between the immune and skeletal response to exercise.
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List of Abbreviations

ActR1 and ActR2 = activin receptor type-1 and type 2
Akt = protein kinase B (PKB)
AP-1 = activator protein-1
BAP = bone alkaline phosphatase
BMD = bone mineral density
BMI = body mass index
BMP = bone morphogenic proteins
BW = brisk walking
c-Fms = colony stimulating factor-1 receptor
CTx = carboxy-terminal collagen cross-linking propeptide
CY = cycling
Dpd = deoxypyridinoline
EE = eccentric exercise
ELISA = enzyme-linked immunosorbent assays
Fzd = Frizzled family
FGF = fibroblast growth factor
Grb-2 = growth factor receptor bound protein-2
GSK3 = glycogen synthase kinase-3β
HIE = high intensity interval training exercise
ICTP = carboxyterminal cross-linked telopeptide of type I procollagen
IFN-γ = interferon gamma
IGF = insulin-like growth factor
IKK = IκB kinase
IκB = inhibitory NF-κB protein
IL-1α = interleukin 1a
IL-1β = interleukin 1b
IL-6 = interleukin 6
IL-8 = interleukin 8
IL-10 = interleukin 10
IL-12 = interleukin 12
JAK = Janus kinase
LEF = lymphoid enhancer-binding factor 1
LPS2 = phospholipase
LRP = lipoprotein-receptor related protein
MAPK = mitogen-activated protein kinase
M-CSF = macrophage colony-stimulating factor
MET = metabolic equivalents
MIP-1 and MIP2 = monocyte chemoattractant protein-1 and 2
MITF = microphthalmia transcription factor
NFATc1 = nuclear factor of activated T-cells c1
NF-κB = nuclear factor
NTx = amino-terminal cross-linking propeptide
OC = osteocalcin
OPG = osteoprotegerin
PDGF = platelet-derived growth factor
PI3K = phosphatidylinositol 3-kinase
PICP = carboxyterminal propeptide of type I procollagen
PINP = aminoterminal propeptide of type I procollagen
Pyr=pyridinoline
RANK = receptor activator of NF-κB
RANKL = RANK ligand
RPM = rotations per minute
RU = running
Runx2 = runt-related transcription factor-2
SEM = standard error of mean
STAT = signal transducer and activator of transcription
SOCS = suppressors of cytokine signalling proteins
TCF = T cell-specific transcription factor
TGF-β = transforming growth factor-β
TR = treadmill running
TRAF6 = TNF receptor associated factor-6
TRAP5b=tartrate-resistant acid phosphatase (isoenzyme 5b)
TNF = tumor necrosis factor
Wnt = Wingless tail ligands
Chapter 1: Literature Review

1.1 Bone Cells

Bone remodeling is a multicellular process. Bone resorption and bone formation are conducted through the communication of several cell types, however the two key cells that are responsible for these functions are the osteoclasts (bone resorption cells) and osteoblasts (bone formation cells). Both osteoclasts and osteoblasts undergo several morphological phases before they differentiate into their active resorption and formation phase. These cells originate from different precursors, perform different functions on bone tissue, and are activated by separate molecular pathways, yet they are well-coordinated not only by local signals but also by each other to maintain the structure of bone (Kular et al., 2012).

1.1.1 Osteoclast differentiation and proliferation

Osteoclasts are large multinucleated cells formed by the fusion of mononuclear progenitor cells that originate from the monocyte family. The differentiation of osteoclasts, also known as osteoclastogenesis, is controlled by contact-mediated signaling between osteoblasts and osteoclasts (Blair et al., 2002). As seen in Figure 1, osteoclastogenesis is initiated when a hematopoetic stem cell from the bone marrow or spleen is stimulated to generate mononuclear cells (monocytes or macrophages), this stimulus arises from factors produced by marrow stromal cells, osteoblasts, or T-lymphocytes (Kular et al., 2012; Robling et al., 2006). These mononuclear cells become committed preosteoclasts and are introduced into the blood stream (Figure 1). The
functional state in which the osteoclast precursors are found in the peripheral circulation before they reach their resorptive site is known as the motile phase. During this phase, motile osteoclasts are flattened and non-polarised cells. Morphologically, motile osteoclasts are characterized by membrane protrusions, called lamellipodia, and podosome complexes containing actin (Kular et al., 2012). The movement of the motile osteoclasts is facilitated by the lamellipodia formation.

The end of the motile phase is marked when the osteoclasts exit the peripheral circulation at or near the site to be resorbed and fuse with one another to form a multinucleated immature osteoclast (Figure 1). This functional state in which the osteoclasts exert their bone resorbing function is known as the resorptive phase. At this phase, resorptive osteoclasts attach to the bone surface and subsequently undergo cytoskeletal reorganization, after which they become polarized (Kular et al., 2012). Polarization results into development of morphological changes and formation of domains. The lamellipodia developed in the non-motile state disappear and the osteoclasts develop a large dome-shape and ruffled border. The domains that are acquired during this state are the sealing zone, functional secretory domain and basolateral membrane. These domains are important for the resorptive function of the osteoclast, which begins as soon as the cells reach osteoclast maturity (Robling et al., 2006). Once peripherally attached to the matrix of the bone, the osteoclast engages in bone resorption by forming a tightly sealed compartment and pumping out $\text{H}^+$ ions that cause extracellular acidification (Blair et al., 2002). This leads to calcium resorption as well as the digestion of collagen fragments such as amino-terminal cross-linking propeptide (NTx) and carboxy-terminal collagen cross-linking propeptide (CTx) (Banfi et al., 2012).
1.1.2 Osteoblast differentiation and proliferation

Unlike osteoclasts, osteoblasts are derived from mesenchymal stem cells of the bone marrow and periosteum (Figure 1) (Datta et al., 2008). Mesenchymal stem cells require specific stimuli in order for them to differentiate into osteoblast precursors; otherwise they may also differentiate into adipocyte, myocytes, or chondrocytes (Robling et al., 2006). In order for mesenchymal stem cells to differentiate into the osteoblast lineage, the expression of transcription factors, such as the runt-related transcription factor-2 (Runx2) is required (Robling et al., 2006).

Similarly to osteoclasts, osteoblasts also have separate functional phases: the preosteoblast, mature osteoblast, bone lining cell, and osteocyte phases (Sims & Gooi, 2008) (Figure 1). Upon the activation of the osteoblast transcription factors, the mesenchymal stem cells differentiate into committed preosteoblasts (Sims & Gooi, 2008). Despite the fact that preosteoblasts lack the ability to produce mineralized tissue, these cells are very similar to mature osteoblasts in function and morphology. Like osteoblasts, preosteoblasts secrete type 1 collagen (PICP), which is the basic building block of bone, in addition to bone alkaline phosphatase (BAP), which is an essential enzyme for bone deposition (Robling et al., 2006). Preosteoblasts reside along the bone surface at sites of active bone formation until they differentiate into mature osteoblasts.

Mature osteoblasts are cuboidal in shape, produce type 1 collagen and BAP, and also form bone. Bone formation is carried out by osteoblasts through the deposition of unmineralized matrix (Robling et al., 2006). The new bone is organized into multicellular units of osteoblasts connected by gap junctions, called osteons. Each osteon retains collagen strand orientation and the individual cells remain alive and interconnected.
during the life of the functional bone unit. Osteoblasts that remain on the surface of bone are called bone-lining cells (Kular et al., 2012). These cells are quiescent and are important in initiating the formation of the remodelling compartment. By releasing a digestive enzyme called collagenase, bone lining cells digest unmineralized matrix in preparation for the underlying mineralized matrix to be resorbed by osteoclasts (Sims & Gooi, 2008). The alternative fate of osteoblasts, which is also final functional stage, is the differentiation into osteocytes. At one point the osteoblasts become completely engulfed by the matrix that they themselves synthesize and subsequently become osteocytes (Figure 1) (Datta et al., 2008).

Osteocytes are terminally differentiated cells that are found dispersed throughout the matrix. Around 95% of the bone cells in the adult skeleton are osteocytes (Bloomfield, 2001). These cells have extensive cell processes that extend outwards via minute channels in the bone matrix called canaliculi, which connect with adjacent lacunae (Bloomfield, 2001). Although osteocytes seem inactive compared to osteoblasts and osteoclasts, they happen to play a very fundamental role in bone remodelling. Osteocytes use their dendritic cell processes to communicate with other osteocytes, osteoblasts, and osteoclasts via gap junctions. This elaborate network of communication allows osteocytes to partly regulate bone formation and bone resorption by producing chemokines that influence osteoblast and osteoclast activity (Sims & Gooi, 2008). In addition, recent research has shown that osteocytes also act as mechanosensory cells that are responsible for initiating the responses of osteoblasts and osteoclasts to mechanical loading (Datta et al., 2008).
More current research has focused in determining the signaling pathways that modulate bone remodeling. The differentiation of osteoclasts and osteoblasts involves spatiotemporal coordination of interaction among diverse endocrine, paracrine, and autocrine factors (Datta et al., 2008).

**Figure 1 Differentiation of osteoblasts and osteoclasts:** (A) The differentiation of mesenchymal cells into cells of the osteoblastic lineage is dependent on Runx2 and osterix expression. Mesenchymal cells differentiate into preosteoblasts via the Wnt signaling and BMP pathways. Towards osteoblast maturation, BMP is also critical for promoting osteoblastic bone formation. Eventually osteoblasts become engulfed by the matrix they produce, which stimulates their differentiation into osteocytes (B) Osteoclasts originate from cells of the monocyte and macrophage lineage. Monocytes and macrophages (which differentiate from hematopoietic stem cells) express the c-Fms receptor, which is the binding site for M-CSF. M-CSF signaling is crucial for the generation of preosteoblasts and also for the expression of RANK in monocytes and macrophages. The expression of RANK allows these cells to respond to RANKL (secreted by osteoblasts), which promotes osteoclastogenesis. RANKL is also responsible for stimulating osteoclast bone resorption.

### 1.2 Bone Signaling Pathways

More current research has focused in determining the signaling pathways that modulate bone remodeling. The differentiation of osteoclasts and osteoblasts involves spatiotemporal coordination of interaction among diverse endocrine, paracrine, and autocrine factors (Datta et al., 2008).
1.2.1 Osteoclast signaling

Osteoclast proliferation and differentiation is heavily reliant on the expression of the nuclear factor of activated T-cells c1 (NFATc1), which happens to be the master transcription factor of osteoclastogenesis (Shinohara & Takayanagi, 2007). Previous studies have shown that signaling cascades responsible for osteoclastogenesis eventually increase the expression of NFATc1, which attaches to its promoter site in the nucleus. The importance of NFATc1 in these signaling pathways has been demonstrated by studies in which NFATc1-deficient embryonic stem cells were not able to differentiate into osteoclasts (Shinohara & Takayanagi, 2007). Recently, it has been shown that NFATc1 is activated by multiple pathways. The two pathways that have been researched extensively are the macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB (RANK) pathways, as presented in Figure 1 and Figure 2 (Shinohara & Takayanagi, 2007).

In the M-CSF signaling pathway, M-CSF binds to the plasma membrane of osteoclast precursor cells through a specific colony stimulating factor-1 receptor (c-Fms), which is a member of the tyrosine kinase superfamily (Shinohara & Takayanagi, 2007). c-Fms becomes autophosphorylated and attracts phosphatidylinositol 3-kinase (PI3K) and growth factor receptor bound protein-2 (Grb-2), which subsequently leads to the activation of Akt and mitogen activated protein kinase (MAPK). The activation of Akt and MAPK lead to the phosphorylation of the microphthalmia transcription factor (MITF), which translocates into the nucleus and forms a complex with NFATc1. This complex binds to its promoter site of osteoclast-specific genes that are critical for osteoclast differentiation and survival (Shinohara & Takayanagi, 2007).
Figure 2 Osteoclastogenesis signaling: Osteoclast proliferation and differentiation is reliant on the expression of NFATc1, which can be activated by (A) M-CSF and (B) RANKL and possibly other (C) unknown ligands (Shinohara & Takayanagi, 2007).

Osteoclast differentiation is also jointly regulated by the RANK ligand (RANKL) pathway (Figure 2). Briefly, RANKL (which is secreted by osteoblasts) binds to its type 1 transmembrane receptor RANK, leading to the recruitment of TNF receptor associated factor-6 (TRAF6), which activates MAPK and inhibitor κ B kinase (IKK). MAPK activates activator protein-1 (AP-1), which translocates into the nucleus and interacts with NFATc1 thereby further promoting the expression of osteoclast-specific genes. IKK also
promotes osteoclast differentiation by activating another dimeric transcription factor complex NF-κB which in turn also translocates into the nucleus to stimulate osteoclastogenesis (Shinohara & Takayanagi, 2007).

1.2.2 Osteoblast signaling

The differentiation of osteoblasts has been elucidated in previous studies to be mediated by the expression of Runx2 and Osterix, which are key osteoblast differentiation transcription factors (Datta et al., 2008). The importance of Runx2 and Osterix expression in bone development has been demonstrated in earlier studies using Osterix -/- and Runx2 -/- mice that showed similar resulting phenotypes; both mice presented a lack of endochondral and intramembraneous ossification due to the absence of osteoblast differentiation (Datta et al., 2008). Runx2 acts on mesenchymal stem cells by promoting their differentiation into cells of the osteoblastic lineage and also by inhibiting the differentiation of mesenchymal stem cells into adipocytes and chondrocytes. Osterix is believed to act downstream of Runx2 in promoting osteoblast differentiation. The expression of both Runx2 and Osterix is activated by two main factors: Wingless tail ligands (Wnts) and bone morphogenic proteins (BMPs). Both factors activate signaling pathways that jointly promote Runx2 and Osterix expression thereby promoting osteoblast proliferation and differentiation, as seen in Figure 1 and Figure 3 (Toulis et al., 2011).

Wnt ligands are lipid-modified, cysteine-rich glycoproteins that play an important role in osteoblast proliferation and differentiation (Toulis et al., 2011). These proteins activate three distinct intracellular signaling pathways, including the Wnt/catenin-β
pathway, also known as the canonical Wnt pathway (Figure 2). This pathway is initiated when Wnt ligands interact with a member of the Frizzled family (Fzd) and the co-receptor lipoprotein-receptor related protein (LRP) 5 or 6 on the osteoprogenitor cells. This interaction leads to the formation of a receptor complex (Wnt-Fzd and LRP5/6), which inhibits the phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK3). In the absence of Wnt ligands, β-catenin is phosphorylated by GSK3, which promotes the ubiquitination and proteosomal degradation of β-catenin (Datta et al., 2008). However, in the presence of Wnt ligands, the degradation of β-catenin is inhibited thereby resulting in its accumulation in the cytoplasm and its translocation into the nucleus. Upon entering the nucleus, β-catenin binds to T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) transcription factors and activates the transcription of downstream genes including Runx2 (Toulis et al., 2011). In addition to having receptors for Wnt ligands, osteoprogenitor cells and osteoblasts have receptors for BMPs (Figure 3). BMPs are members of the transforming growth factor-β (TGF-β) family that have osteoinductive properties (Datta et al., 2008). There are various forms of BMPs not all of which are limited to bone. The main BMPs that act on osteoblasts are BMP-2, BMP-4, and BMP-6. BMPs act on osteoprogenitor cells by binding to two receptors that have intrinsic serine/threonine kinase activity: activin receptor type I (ActR1) and activin receptor type II (ActR2) (not shown in Figure 3) (Toulis et al. 2011). The ligand binds to the assembly of a heterooligomeric receptor complex, in which the ActR2 phosphorylates
Figure 3 Osteoblastogenesis signaling: A. Canonical Wnt pathway. The binding of Wnt to LRP (in the active state) leads to downstream events that result into the activation of osteoblast transcription factors such as Runx2 and Osterix. B. BMP pathway. BMP binds to ActR (also known as BMPR) promote the expression of osteogenic genes through mitogen-activated protein kinase (MAPK) activation (Yavropoulou & Yovos, 2007).

and activates the ActR1. ActR1 in turn phosphorylates a family of receptor substrates, known as the Smad proteins (Smad-1, Smad-5, and Smad-8), which in turn dissociate and form a complex with Smad-4. The Smad complex migrates into the nucleus where it drives gene transcription of osteogenic genes (Zhang et al., 2009). In addition to Smad activation, BMPs can promote the expression of osteogenic genes through mitogen-activated protein kinase (MAPK) activation (Kular et al., 2012).
1.3 Communication between Osteoclasts and Osteoblasts

Throughout this section, the activities and signaling pathways of osteoclasts and osteoblasts have been discussed as independent mechanisms. However, it is important to note that the activities of these cells in an *in vivo* setting, and sometimes *in vitro*, are far from independent (Sims & Gooi, 2008). Osteoclasts and osteoblasts are closely coupled and there exists a great amount of overlap between the signaling pathways and biological markers that stimulate their activities.

One such example of their coupled coordination is the production of osteoprotegerin (OPG) by osteoblasts, which is the decoy receptor of RANK (Sims & Gooi, 2008). OPG acts an inhibitor of the RANK pathway by binding to RANKL, which prevents RANKL from attaching to designated osteoclast receptors. This mechanism allows osteoblasts to regulate osteoclast resorption to prevent an excessive loss of bone matrix. Simultaneously, osteoblasts can also express RANKL and M-CSF to stimulate osteoclastogenesis when needed (Sims & Gooi, 2008). Osteoblasts also secrete chemoattractants such as osteocalcin (OC) and collagen-1 that control the movement of osteoclast precursors to the bone surface (Kular et al., 2012). Other cells of the osteoblast-lineage may also influence osteoclast activity, such as osteocytes. Osteocyte apoptosis has been postulated to release prostaglandins and cytokines that initiate bone resorption, thereby confirming a physiological link between osteocytes and osteoclasts (Sims & Gooi, 2008).

Osteoclasts also regulate osteoblasts activity, though not the same extent as osteoblasts. Osteoclasts regulate osteoblast activity by secreting ligands that influence pathways responsible for osteoblast proliferation and differentiation. An example of
osteoclastic modulation occurs during bone resorption, in which osteoclasts secrete factors into the matrix that stimulate bone formation by osteoblasts. These regulatory factors include insulin-like growth factor (IGF) 1 and 2, acidic and basic fibroblast growth factor (FGF), TGF-β 1, BMPs, and platelet-derived growth factor (PDGF) (Sims & Gooi, 2008). The concentrations of these regulatory factors in the bone matrix correlates with osteoclast activity, thus this will promote bone formation that is proportional to bone resorption (Sims & Gooi, 2008).

The coupled activities of osteoclasts and osteoblasts are very crucial to bone structure, strength, and homeostasis (Datta et al., 2008). In addition to bone maintenance, osteoclasts and osteoblasts communicate with one another and other factors during bone healing that may occur after fracture or disease. An imbalance between either cell population may lead to the development of bone degenerative conditions such as osteoporosis, osteopenia, and other bone metabolism disorders. Thus successful activity of osteoblasts and osteoclasts is dependent on the proper transmission of signals and equipment of messengers that regulate their activities in a harmonious nature. Of great importance to osteoblast and osteoclast regulation are cytokines.

1.4 Cytokines

Cytokines are soluble mediators that regulate the interaction and communication between cells. They are small to medium sized proteins or glycoproteins with molecular weights ranging from 8 to 40,000 Da. Cytokines play central roles in the immune response, hematopoiesis, and inflammation, which are very well established in the literature (Takayanagi, 2007). Cytokine is the general term, however cytokines can also be termed
according to their activity or cellular source. Other names include chemokines (cytokines with chemotactic activities), lymphokines (cytokines secreted by lymphocytes), monokines (cytokines secreted by monocytes), adipokines (cytokines secreted by adipocytes), myokines (cytokines secreted by myoblasts), and interleukins (cytokines secreted by leukocytes) (Pederson, 2011; You & Nicklas, 2008; Zhang & An, 2007).

Cytokines may exhibit functions that are autocrine (acting on the same cells that secrete them), paracrine (acting on nearby cells) or endocrine (acting on distant cells) (Zhang & An, 2007). Originally, cytokines were thought to be specific in function (Akira et al., 1990). However, research with a variety of cell types has demonstrated that cytokines are able to mediate a vast array of different biological functions. In fact, most cytokines are distinguished by their multifunctionality, or pleiotropy. One cytokine may produce a variety of actions depending on the source of its secretion, target tissue, and sequence of secretion in an event (Dinarello, 1998). Simultaneously, there is large extent of redundancy with cytokines as many of their functions overlap with one another. Numerous cytokines share the same target cell and mediate the same or similar processes (Akira et al., 1990).

1.4.1 Cytokines and inflammation

Cytokines are mainly only produced during noxious events, such as inflammation. Inflammation is a nonspecific reaction of the immune system that is required for functional host defense mechanisms, and can be triggered by various cell stressors such as microbial infection, disease, injury, and trauma (Dinarello, 1997). This complex process involves host cells, blood vessels, and other proteins in order to eliminate the source
of inflammation (Kumar et al., 2012). Furthermore inflammation can be acute, lasting from a few minutes to a few days, or chronic, from days to possibly years. Generally, inflammation is characterized by the recruitment of leukocytes and plasma proteins to the site of injury or infection. Both leukocytes and plasma proteins, such as clotting factors and kininogens, assist with the elimination of microbes and dead tissue and mediate inflammation. In addition to these factors, endothelial cells within the vascular walls and cells of the extracellular matrix (ECM) also assist in the induction of inflammation and the resolution (Kumar et al., 2012). Endothelial cells may aggravate the onset of inflammation by releasing inflammatory mediators such as nitric oxide, prostaglandin, and histamine. Other cell types such as fibroblasts, dendritic cells, mast cells and macrophages can also synthesize inflammatory mediators. In addition, these cells also secrete cytokines, which are crucial for inflammatory processes.

There are two classifications for cytokines with respect to inflammation: pro-inflammatory cytokines and anti-inflammatory cytokines. The concept of this classification is based on cytokine induced synthesis of mediator molecules that are up regulated during inflammation (Dinarello, 2000). Pro-inflammatory cytokines up-regulate mediators that aggravate inflammation, such as adhesion molecules, nitric oxide, prostanglandin, and platelet activating factors (Dinarello, 2000). Anti-inflammatory cytokines attenuate inflammation by controlling the inflammatory response and stimulating inhibitors of inflammatory pathways (Dinarello, 2000). Of importance to this paper are the activities and signaling pathways of the anti-inflammatory cytokine interleukin 10 (IL-10), and four pro-inflammatory cytokines: interleukin 1 alpha (IL-1), interleukin beta (IL-1 β ), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6).
The selection of these cytokines is based on the established literature that mainly focuses on the levels of IL-10, IL-1α, IL-1β, TNF-α and interleukin IL-6 in association to acute bouts of exercise.

1.4.2 Interleukin-1

IL-1 is a family of highly inflammatory cytokines that includes the peptides IL-1α and IL-1β. They are two distinct proteins with varying degrees of inflammatory effects. IL-1α consists of 271 amino acids and has a molecular weight of 31 kDa (Akira et al., 1990; Dinarello, 1991). The main producers of IL-1α are monocytes and macrophages, however it can also be produced by keratinocytes, endothelial cells, T cells, B cells, astrocytes, microglia and fibroblasts (Akira et al., 1990). IL-1α is first synthesized in a precursor form known as proIL-1α, and is subsequently converted into its active form by cleavage of an intracellular protease calcium dependent cysteine protease, known as caspase, or by the influx of calcium into the cell (Gross et al., 2012; Miller et al., 1994). The active form of IL-1α is bound to nuclear DNA, where it is released upon cell death to bind to a membrane bound receptor on nearby cells (Luheshi et al., 2011; Rider et al., 2011). The binding of IL-1α upon necrotic cell death triggers a pro-inflammatory response that is characterized by a neutrophilic influx, followed by an influx of monocytes (Rider et al., 2011). IL-1α also induces the transcription of phospholipase A2 and cyclooxygenase, which are genes that up regulate the synthesis of inflammatory leukotrienes and prostanglandins (Dinarello, 1997).
IL-1 β has similar action to IL-1 α, including the up regulation of chemokines and other inflammatory cytokines such as IL-6 and TNF-α (Dinarello, 2011). IL-1 β is 269 amino acids long and has a molecular weight of 31 kDa (Akira et al., 1990). Similarly to IL-1 α, it is produced by monocytes, macrophages, and dendritic cells, as well as B lymphocytes and natural killer cells (van de Veerdonk & Netea, 2013). The inactive form of IL-1 β, which is proIL-1 β, is also processed by caspase; however it can also be converted into the active form by acute phase proteins such as proteinase-3 (van de Veerdonk & Netea, 2013). Following the synthesis of the active cytokine in the cytoplasm, IL-1 β is transported by exocytosis and enters the circulation where it acts on target cells such as fibroblasts, endothelial cells, and keratinocytes (Tosato & Jones, 1990).

1.4.3 Tumor Necrosis Factor Alpha

TNF-α, also known as cachectin, is a highly inflammatory cytokine that is involved in both systemic and local inflammation. It was first named TNF-α for its ability to destroy tumors by obliterating their vessels or by direct attack on the cells (Zhang & An, 2007). It is expressed ubiquitously in tissue, which may explain why its pro-inflammatory effects are very prominent compared to other cytokines (Northoff & Berg, 1991). TNF-α is released by a variety of cells, including activated keratinocytes, dendrocytes, monocytes, macrophages, mast cells and activated B and T cells (Weinberg et al. 2006). It exists in both soluble and membrane bound form, with molecular weights of 17 kDa and 26 kDa (Weinberg et al., 2006). There are two receptors for TNF-α, p55 and p75, both of which
are similar to some extent. The binding of TNF-α to p55 and p75, which can exist as membrane bound receptors or in soluble form in extracellular fluid, leads to increased expression of other cytokines and cell adhesion molecules, and the emigration of lymphocyte and neutrophils (Dinarello, 1997; Opal & DePalo, 2000). The difference between the two receptors is that p55 stimulates the cell to undergo apoptosis, while p75 does not (Opal & DePalo, 2000). Similarly to the IL-1 family, TNF-α up regulates the expression of carbon dioxide and nitric oxide synthase inhibitors. In fact, IL-1 and TNF-α overlap in many of their functions that they are considered to be synergistic cytokines.

1.4.4 Interleukin-6

IL-6 is an extensively studied cytokine that is associated with a wide range of functions. It is a single glycoprotein chain of 26 kDa and consists of 212 amino acids (Barton, 1997). IL-6 can be produced by monocytes, macrophages, fibroblasts, endothelial cells, and B cells (Barton, 1997) IL-6 is also considered a myokine as it can be produced by skeletal muscle as well (Pedersen, 2011). The receptor for IL-6 is a membrane bound heterodimer consisting of an alpha and beta subunit. The beta subunit, also known as gp130, is responsible for signal transduction. IL-6 receptors are expressed a variety of cells, including lymphocytes, monocytes, fibroblasts, endothelial cells, and pituitary cells (Barton, 1997).

Findings regarding the role of IL-6 during inflammation are interesting as it can exhibit both pro-inflammatory and anti-inflammatory actions (Akira et al., 1990). In the case of chronic inflammation, such as arthritis, colitis and autoimmune disease, IL-6 is pro-inflammatory and also a key player in mediating the pro-inflammatory response.
Upon persistent inflammation, IL-6 induces neutrophilic apoptosis and phagocytosis, and up regulates the expression of adhesion molecules and other pro-inflammatory cytokines such as IL-8. The main function of IL-6 during chronic inflammation is the recruitment of monocytes to the area of injury or inflammation, which leads to the continuous production of the proinflammatory monocyte chemoattractant protein (MIP)-1 (Gleeson et al., 2011). Thus IL-6 levels are elevated in inflammatory disease in humans. In addition, IL-6 may also be secreted by damaged tissue, as seen with muscle very strenuous exercise (Pederson, 2011).

Simultaneously, IL-6 also exhibits anti-inflammatory effects indirectly during acute inflammation as seen with injury, infection or trauma. The anti-inflammatory actions of IL-6 in this case are mostly focused towards inhibiting the production of pro-inflammatory cytokines such as interferon gamma (IFN-γ), IL-8, and MIP-2. In addition, it also inhibits the synthesis of other major inflammatory mediators such as prostaglandins, nitric oxide, and phospholipase (LPS2) as well as inflammatory processes such as apoptosis and the recruitment of monocytes and adhesion molecules to the site of injury (Barton, 1997). At the same time, IL-6 also down-regulates the synthesis of IL-1 and TNF-α in a negative feedback fashion, thereby attenuating their inflammatory effects (Opal & DePalo, 2000).
1.4.5 Interleukin-10

Perhaps the most studied anti-inflammatory cytokine is IL-10 (Opal & DePalo, 2000). It is a homodimer of 18.5 kDa consisting of 160 amino acids (Opal & DePalo, 2000). It is produced by T helper cells, lymphocytes, mast cells, eosinophils, monocytes, macrophages and keratinocytes (Lalani et al., 1997). The receptor for IL-10 is a membrane bound receptor of 90-110 kDa. The binding of IL-10 to its receptor inhibits the expression monocyte and macrophage derived pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1, IL-6, IL-8 and IL-12 as well as other pro-inflammatory mediators such as the macrophage and granulocyte colony stimulating factor (Opal & DePalo, 2000). It also prevents the synthesis of pro-inflammatory cytokines by natural killer cells and neutrophils and promotes the degradation of messenger mRNA of pro-inflammatory cytokines (Opal & DePalo, 2000). Furthermore, IL-10 inhibits the recruitment of monocytes and leukocytes to the site of inflammation and down regulates the production of nitric oxide and prostaglandins, thereby attenuating tissue damage caused by inflammation. IL-10 also reduces the pro-inflammatory response by regulating the production of pro-inflammatory cytokine receptors. For example, IL-10 attenuates the expression of TNF-\(\alpha\) receptors and promotes the shedding of TNF receptors into systemic circulation, and promotes the production of IL-1 receptor antagonist, ultimately minimizing the pro-inflammatory responses of TNF-\(\alpha\) and IL-1. (Lalani et al., 1997; Opal & DePalo, 2000).
1.5 Cytokine Signal Transduction

Over the past decade a number of proteins have been identified that are necessary for the signaling of pro-inflammatory and ant-inflammatory cytokines. With respect to the cytokines discussed in this paper, two signaling pathways are relevant, the NF-κB pathway, which is activated by IL-1α, IL-1β, and TNF-α, and the JAK/STAT pathway, which is activated by IL-6 and IL-10. Both signaling pathways are presented in Figure 4.

1.5.1 NF-κB

NF-κB is a transcription factor that is usually contained in the cytoplasm of most cells through the interaction with inhibitory NF-κB proteins (IκB). The activation of NF-κB is a crucial step in the development of inflammation and it can be initiated by pro-inflammatory cytokines, such as IL-1 or TNF-α. These cytokines activate their distinct receptors, which subsequently lead to the activation IKK via phosphorylation (Delhase et al., 1999; Hanada & Yoshimura, 2002) (Figure 4). The activation of IKK leads to the phosphorylation of IκB at serine residues, which leads to its degradation by 26S proteasome, thereby releasing NF-κB dimers from the cytoplasmic NF-κB-ΙκB complex, allowing them to translocate into the nucleus where they activate transcription of target genes that encode for additional pro-inflammatory cytokines, including IL-1α, IL-β and TNF-α (Baldwin, 1996). This mechanism also regulates the production of adhesion molecules, chemokines, growth factors, and inducible enzymes cyclooxygenase-2 and nitric oxide synthase.
1.5.2 JAK/STAT

Other cytokines, including IL-6 and IL-10, regulate inflammation through the JAK/STAT pathway (Figure 4). When a cytokine binds to the distinct receptors, the subunits of the receptor homodimerizes, which results the activation of intracellular tyrosine kinase named JAK kinase (Hanada & Yoshimura, 2002). The receptor is then phosphorylated by JAK kinase; this creates docking sites for SH2-containing signaling molecules, amongst them are signal transducers and activators of transcription (STAT) (Figure 4). STAT is a transcription factor usually contained within the cytoplasm in monomeric inactive forms (O'Farrell et al., 1998). STAT is phosphorylated by JAK, which triggers its dimerization and translocation into the nucleus (O'Farrell et al., 1998). Upon entering the nucleus, STAT promotes the transcription of target genes that regulate inflammation. Along with inflammatory regulators, the JAK/STAT pathway also increases the transcription of suppressors of cytokine signaling (SOCS) proteins, which thereby act as negative feedback regulators.

Interestingly the JAK/STAT pathway has been found to either promote or inhibit inflammation, specifically with the STAT3 protein (O'Farrell et al., 1998). When activated by IL-6 during chronic inflammation, STAT3 promotes inflammation by hyperplasia of epithelial cells and the survival of T cells and also enhances the production of other pro-inflammatory cytokines (Hanada & Yoshimura, 2002). However when stimulated by IL-10, it has been shown to provide anti-inflammatory effects via the inhibition of macrophage activation and proliferation (O'Farrell et al., 1998).
**Figure 4 Cytokine signaling:** NF-κB and JAK/STAT pathways. These pathways are important in regulating pro-inflammatory and anti-inflammatory processes. (A) NF-κB is activated by IL-1-α, IL-1β, while the (B) JAK/STAT pathway is activated by IL-6 and IL-10 (Hanada & Yoshimura, 2002).

### 1.6 Interaction between bone and cytokines

Until the 1970s, the skeletal system and immune system were always viewed as separate entities, with no means of interaction aside from the spatial proximity that exists between immune cells residing in the bone marrow (Schett, 2011). However, clinical research in arthritis patients in which pro-inflammatory cytokines are correlated with increased bone resorption showed that there is association between bone and cytokines (Korczowska et al., 2013). This has lead to the establishment of a new discipline that examines the interactions between the skeletal and immune systems, known as osteoimmunology (Takayanagi, 2012).
Research in osteoimmunology examines the association between cytokines and bone cells. The first relationship that was established between these two systems lies in the bone marrow (Takayanagi, 2012). Hematopoietic stem cells, which reside in the bone marrow, give rise to both myeloid progenitors that differentiate into osteoclasts and lymphoid progenitors that give rise to immune cells that produce cytokines, such as T cells and macrophages (Danks & Takayanagi, 2013). Thus, bone regulates the development of cytokine progenitor cells by providing the niche for hematopoietic cell development.

Most of the interaction between bone and cytokines stems from cytokine regulation of bone cell activities. This interaction was originally linked to inflammatory diseases that involve bone such as arthritis, osteoporosis, and osteopenia, in which the degree of inflammation is proportional to bone loss, bone turnover, and the development of progressive bone erosions (Lee et al., 2008; Schett, 2011). These inflammatory diseases are characterized by an increase of circulating IL-1, IL-6 and TNF-α, all of which have been shown to promote bone resorption mainly by up-regulating the expression of RANKL, which is biomarker of osteoclastogenesis (Takayanagi, 2012). All three cytokines up-regulate the production of RANKL from mesenchymal cells, T cells, fibroblasts, and osteoblasts which act on osteoclast precursor cells to promote osteoclastogenesis and bone resorption (Schett, 2011; Takayanagi, 2012). These cytokines can also promote osteoclastogenesis independently of RANKL; TNF-α promotes osteoclast formation by up-regulating the expression of M-CSF and OSCAR on osteoclast precursors (Both M-CSF and OSCAR pathways are illustrated in Figure 2), IL-1 increases prostanglandin synthesis in bone, which stimulates resorptive activity, and IL-
IL-6 promotes the differentiation of myloid cells into osteoclasts (Kudo et al., 2003; Lee et al., 2008; Schett, 2011). Interestingly, IL-6 can produce variable effects on osteoclastogenesis depending on whether it is bound to a soluble receptor complex (IL-6/R). The main action of IL-6 on osteoclastogenesis has been shown to occur in the form of IL-6/R, which promotes osteoclastogenesis and RANKL production. Although IL-6 mainly promotes bone resorption, it can also bind directly on mesenchymal cells to stimulate osteoblast differentiation (Steeve et al. 2004; Schett 2011). On the contrary, pro-inflammatory cytokines such as TNF-α have also been shown to inhibit osteoblast activity and induce osteoblast apoptosis (Walsh et al., 2006). The effects of pro-inflammatory cytokines on bone have also been examined in animal studies in which molecules necessary for cytokine signaling transduction such as IκB and NF-κB have been genetically disrupted. In mice, the elimination of IKKB and NF-κB, both of which are regulatory molecules for IL-1 and TNF-α signaling, resulted in decreased osteoclast differentiation and bone resorption (Takayanagi, 2007). In summary, these studies show that pro-inflammatory cytokines have the capacity to modulate bone physiology through various mechanisms, mainly through promoting osteoclastogenesis.
Figure 5 Interaction between bone and cytokines: The effects of IL-1, IL-6, TNF-α and IL-10 have been shown to be targeted on osteoblasts, osteoclasts, and their precursors. The net effect of IL-1, TNF-α, and IL-6 is osteoclastogenesis, in which the predominant mechanism is RANK/RANKL pathway. These cytokines may also stimulate other factors, such as M-CSF and PGE2 in order to promote osteoclast differentiation. Although IL-6 can promote osteoblast differentiation directly, it mainly promotes osteoclastogenesis after binding to a soluble receptor and forming IL-6R. At early stages of osteoblast differentiation, IL-10 inhibits osteoblastogenesis while simultaneously inhibiting and enhancing RANKL and OPG production respectively.

In addition to pro-inflammatory cytokines, IL-10 has also been shown to influence bone physiology by regulating osteoclast activities. Using bone marrow cultures, IL-10 has been shown to inhibit osteoclastogenesis and bone resorption (Hong et al., 2000; Lee et al., 2008). IL-10 regulates osteoblastic regulation of osteoclasts by inhibiting and enhancing the production of RANKL and OPG respectively in preosteoblasts (Lee et al., 2008). IL-10 has no effects on the activities of mature osteoblasts, however it can modulate bone formation in the preosteoblastic stages in
which IL-10 inhibits BAP synthesis (Vlaesslear et al., 1994). Although IL-10 has not been shown to promote osteoblast differentiation or bone formation, the beneficial effects this anti-inflammatory cytokine has on bone come from its ability to reduce bone resorption and lower pro-inflammatory cytokines which promote resorptive activity (Caeteno-Lopes et al., 2009; Hong et al., 2000).

1.7 Exercise

Many complex interactions exist between the bone and immune system. Bone cells and cytokines are very dynamic and responsive to multiple factors. Some factors that regulate bone metabolism and cytokine levels are endogenous factors, such as hormones and protein factors, and others are exogenous factors such as nutrition, disease, injury, and exercise. It is very well established that exercise is an important regulator of bone turnover and inflammation (Banfi et al., 2012). Studies conducted in humans, animals, and cell cultures have shown the effects of exercise and mechanical stimulation on bone health and inflammatory status.

1.7.1 Exercise and bone

Exercise is a very important behavior that influences bone health. Through exercise, peak bone mass is increased during childhood and the rate of bone loss is decreased in the elderly (Maimoun & Sultan, 2011). It is also a preventative measure against osteoporosis. Overall, studies assessing the effects of exercise on bone health have reported improved bone mineral density (BMD), bone strength, and a lower risk of fracture (Maimoun & Sultan, 2011).
Some forms of exercise may cause more profound effects on bone than others. This is due to several factors that are manifested in the form of exercise, such as the mechanical load of the exercise (ie. high impact or low impact), intensity (high intensity or low intensity), and duration of exercise. High impact exercise, such as weight bearing and plyometric exercises, lead to an increase in BMD at sites affected by strains and provide more osteogenic effects (Guillemant et al., 2004; Maimoun et al., 2006). On the other hand, endurance and low-impact exercises have been shown to have no beneficial or even deleterious effects on bone density (Guillemant et al., 2004). In fact, no gain in BMD has been demonstrated in athletes (ie. cyclists) in the absence of high mechanical strain (Maimoun et al., 2006). However, this is not to say that low-impact exercise, such as cycling, does not have any beneficial effects on bone. In addition to bone quantity (BMD), another equally important determinant of bone strength is bone quality, which reflects the structure of bone (ie. trabecular spacing, trabecular number). No previous studies have assessed the effects of cycling or other forms of low-impact exercise on bone quality, therefore it remains inconclusive whether cycling has an effect on bone health. However, it is very unlikely that cycling does not stimulate bone turnover since it is generally known to stimulate systemic changes, such as endocrine (Pederson & Hoffman-Goetz, 2000) and immune (Pederson & Hoffman-Goetz, 2000), which in combination with a small degree of mechanical strain, may lead to beneficial outcomes on bone.

Many studies that assess the effects of exercise on bone health utilize imaging techniques such as dual-energy X-ray absorptiometry (DEXA) (Maimoun et al., 2006). Although this is the gold standard assessment for determining BMD, it is also
disadvantageous as it poses radiation risk to the individual and lacks a sensitive measure for the short-term effects of exercise on bone (Banfi et al., 2012). Thus, a more practical method of assessing bone health has emerged by utilizing biochemical factors such as bone formation and resorption markers (Banfi et al., 2012). These markers provide evidence of bone turnover and also a prediction of the cellular activities of osteoblasts and osteoclasts in bone formation and resorption. Table 1 lists common markers of bone formation and resorption that can be measured in urine or serum.

Table 2 presents numerous studies that have utilized biochemical assays to understand the acute effects of exercise on bone with a focus on low-impact exercise. Most of these studies have used cycling as a form of low-impact exercise due to minimal weight bearing and strain involved, however brisk walking has been used as well (Tosun

<table>
<thead>
<tr>
<th>Table 1: Bone turnover markers of formation and resorption</th>
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<tbody>
<tr>
<td><strong>Marker</strong></td>
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<tr>
<td><strong>Bone Formation</strong></td>
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<tr>
<td>BAP</td>
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<tr>
<td>OC</td>
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<td>PICP</td>
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<tr>
<td>PINP</td>
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<tr>
<td>OPG</td>
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<tr>
<td><strong>Bone Resorption</strong></td>
</tr>
<tr>
<td>Pyr</td>
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<tr>
<td>Dpd</td>
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<tr>
<td>ICTP</td>
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<tr>
<td>Ntx</td>
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<tr>
<td>TRAP5b</td>
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<tr>
<td>RANKL</td>
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</table>

BAP=bone alkaline phosphatase; CTx=carboxyterminal cross-linking telopeptide of type I collagen; NTx= aminoterminal cross-linking telopeptide of type I collagen; Dpd=deoxypiridinoline; ICTP=carboxyterminal cross-linked telopeptide of type I procollagen; NTx= aminoterminal cross-linking telopeptide of type I collagen; PICP = carboxyterminal propeptide of type I procollagen; PINP = aminoterminal propeptide of type I procollagen; Pyr=pyridinoline; OC=osteocalcin; TRAP5b=tartrate-resistant acid phosphatase (isoenzyme 5b); RANKL= receptor activator of NF-kB ligand; OPG= osteoprotegerin
et al., 2006). The literature pertaining to the response of bone markers to high-intensity, low-impact exercise is discrepant, with some studies reporting an exercise-induced response in bone markers and others showing no significant changes (Tosun et al., 2006). Guillemant et al. (2004) and Hermann et al. (2007) reported significant increases in CTX (bone resorption marker) shortly after cycling at an intensity of at least 75% VO$_2$max for at least 50 minutes. CTX returned to baseline 24 hours post-exercise. Similarly, ICTP (bone resorption marker) was shown to increase 30 minutes after cycling at 80% of VO$_2$max, thereby implying an increase of bone resorption. Guillement et al. (2004) also measured BAP (bone formation marker) but found no significant change after exercise. However, Maimoun et al. (2006) and Rudberg et al. (2006) reported an increase in BAP immediately post-exercise, as well as 30 minutes post-exercise. Furthermore, other markers of bone formation such as OC and PICP have been shown to increase immediately and up to one hour post-exercise (Rudberg et al., 2000; Tosun et al., 2006; Wallace et al., 2000). All of these markers seem to return to pre-exercise levels 1 to 24 hours after exercise. Taken together, the increase of bone resorption and bone formation markers after exercise indicates that high-intensity and low-impact exercise stimulates bone turnover that ceases a few hours after the exercise.

Other studies, however, reported no significant changes in bone marker after exercise (Kristoffersson et al. 1995; Tosun et al. 2006) including OC, PICP, ICTP and PINP. However, Kristofferson et al. 1995 utilized an exercise protocol lasting 30 sec while the studies described earlier adhered to at least 20 minutes of exercise. Therefore, it is possible that duration is an important factor governing the bone marker response to exercise (Maimoun et al., 2006). In addition, Tosun et al. (2006) investigated the effects
of low-impact exercise in healthy, pre-menopausal women, which indicates that sex may also be a factor in the bone response to exercise.
Table 2: Studies on the effects of acute and low-impact exercise on bone markers.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Exercise Mode</th>
<th>Participants</th>
<th>Method</th>
<th>Outcome Measures</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kristoffersson et al. 1994</td>
<td>CY; 30 sec at 7.5% retardation of body weight</td>
<td>Male athletes, 19-26 y N=7</td>
<td>Serum</td>
<td>OC, PICP, ICTP</td>
<td>No significant changes during are post-exercise</td>
</tr>
<tr>
<td>Rong et al. 1997</td>
<td>CY; Two sessions of acute exercise bouts: 45 min at 55% VO2max and 15 min at 85% VO2max</td>
<td>Healthy males, 20-26 y N=8</td>
<td>Serum</td>
<td>OC, ICTP</td>
<td>No significant difference in OC and ICTP immediately or 1h post-exercise. ICTP decreased 4 h post-exercise at both intensities. OC decreased post-exercise at 85% VO2max. A positive correlation was found between OC and ICTP at all time points.</td>
</tr>
<tr>
<td>Wallace et al. 1999</td>
<td>CY; two 5-min sessions at 1 watt/kg, 2 watt/kg followed by session of 20-min at 80% VO2max</td>
<td>Males with a high level of habitual aerobic activity, 18-40 y N=17</td>
<td>Serum</td>
<td>OC, PICP, PIIIP, ICTP</td>
<td>PICP, PIIIP and ICTP increased 30 min post-exercise. All markers decreased to baseline levels during recovery.</td>
</tr>
<tr>
<td>Rudberg et al. 1999</td>
<td>CY; 24 min at a workload of 120-180 W</td>
<td>Healthy postmenopausal women, 53-61 y N=8</td>
<td>Serum</td>
<td>BAP</td>
<td>BAP increased significantly immediately post-exercise, and returned to baseline 20 min post-exercise.</td>
</tr>
<tr>
<td>Guillemant et al. 2004</td>
<td>CY; 60-min at 80% VO2max</td>
<td>Elite male triathletes, 23-37 y N=12</td>
<td>Serum</td>
<td>BAP, CTX</td>
<td>CTX increased 30 min post-exercise, and began to decrease 2 h post-exercise. No significant changes were</td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Design/Intervention</td>
<td>Participants</td>
<td>Sample</td>
<td>Markers</td>
<td>Results</td>
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<tr>
<td>Maimoun et al. 2006</td>
<td>CY; two 50-min sessions at 15% above (+VT) and below (-VT)</td>
<td>Male cyclists, 20-39 y, N=7</td>
<td>Serum</td>
<td>BAP, CTX, OC</td>
<td>OC increased 30 and 50 min post-exercise at +VT</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>BAP increased for both intensity levels 30 min post-exercise</td>
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<td></td>
<td></td>
<td></td>
<td>CTX increased 30 and 50 min post-exercise at +VT</td>
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<td></td>
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<td>All markers returned to baseline levels during recovery</td>
</tr>
<tr>
<td>Tosun et al. 2006</td>
<td>BW; two sessions of exercise bouts; 30 min walking, followed by walking with weight-lifting (5kg) at submaximal intensity. Sessions were separated by period of 1 week</td>
<td>Healthy and sedentary premenopausal women, 26-33 y N=9</td>
<td>Plasma</td>
<td>OC, PICP, ICTP, PINP, BAP</td>
<td>No significant changes were observed with OC, PICP, ICTP and PINP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAP decreased 24 h post-exercise in walking group</td>
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<td></td>
<td></td>
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<td></td>
<td>BAP increased 24 h post-exercise in walking with weight-lifting group</td>
</tr>
<tr>
<td>Hermann et al. 2007</td>
<td>CY; 60 min at 75%, 95% and 110% VO2max</td>
<td>Male and female athletes (MA, FA) and sedentary controls (MCo, FCo), 17-39 y N=32</td>
<td>Serum</td>
<td>CTX</td>
<td>CTX increased in MA at 95% and 110%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CTX decreased 24 h post-exercise in MA, MCo and FA</td>
</tr>
</tbody>
</table>

**Notes:**
- CY = cycling; BW = brisk walking; BAP = bone alkaline phosphatase; CTX = C-terminal of cross-linking telopeptide of type 1 collagen; OC = osteocalcin; PICP = propeptide of type 1 collagen; PIIP = propeptide of type 3 collagen; ICTP = Pyridinoline cross-linked carboxy-terminal telopeptide of type 1 collagen; VT = ventilator threshold.
1.7.2 Exercise and inflammation

Exercise is a form of physical stress that affects all compartments of the body, including the immune system. Thus cytokines, as regulators of the immune system, are significantly influenced by exercise (Spanoudaki et al., 2010). It is well established in the literature that regular exercise improves inflammatory status and decreases the risk of inflammatory and metabolic conditions (Gleeson et al., 2011). In fact, regular exercise is associated with many anti-inflammatory effects, such as decreased expression of macrophages, decreased adipose tissue infiltration, and decreased production of pro-inflammatory monocytes (Gleeson et al., 2011).

As seen with regular exercise, acute exercise also acts as a physical stressor that stimulates numerous metabolic and physiological changes. The most common anti-inflammatory changes reported after strenuous exercise include decreases in neutrophil, lymphocyte, and macrophage function (Neto et al., 2011; Nieman et al., 2007). Furthermore, acute exercise modifies the circulating levels of many cytokines including IL-1α, IL-1β, IL-6, TNF-α and IL-10. A list of studies on the effects of acute exercise on these cytokines is provided in Table 3. As shown in Table 3, an acute bout of exercise is generally immediately followed by moderate increases in IL-1α, IL-1β and TNF-α, and a substantial increase in IL-6 peaking towards the end of the exercise (Scott et al. 2011; Pedersen & Toft, 2000). These cytokines remain elevated for approximately an hour after exercise before returning to their baseline values. An anti-inflammatory effect is observed with a delayed increase of IL-10 usually occurring at least 1 hour after exercise (Peterson & Pederson 2005). Figure 6 illustrates the cytokine response during high-intensity exercise.
These findings are consistent in both high-impact and low-impact exercise protocols (LaVoy et al., 2013; Nieman et al., 2007; Zaldivar et al., 2006). Studies utilizing high-impact (i.e., treadmill running) and low-impact exercise (i.e., cycling) reported similar increases of TNF-α and IL-6 during or immediately after exercise (Otrowski et al., 1999; Scott et al., 2013; Spanoudaki et al., 2010; Zaldivar et al., 2006). However, the cytokine response seems to differ between exercises that focus on concentric muscle contractions (shortening of muscle fibers) as opposed to eccentric muscle contractions (elongation of muscle fibers). Eccentric contractions are associated with increased muscle damage resulting in a prolonged rise in pro-inflammatory cytokine secretion (Rohde et al., 1997). The effect of eccentric contractions on TNF-α and IL-6 secretion has been demonstrated in exercise studies (Bruunsgaard et al., 1997).

Furthermore, as shown in Table 3, studies reporting significant cytokine variations utilized exercise durations ranging from 3 minutes to 2 hours of exercise (Cannon et al., 1991; Ostrowski et al., 1998; Ullum et al., 1994). This is also supported by a cross-over study that examined the cytokine response after two cycling protocols of different duration (Nieman et al., 2007). One protocol consisted of 2 hours of continuous cycling whereas the other protocol consisted of 10-minute bouts of exercise separated by 3 minutes rest. This study reported similar cytokine responses for both exercise durations thereby indicating the cytokine response may be more sensitive to the intensity of the exercise (with high-intensity exercise producing pronounced changes in cytokine levels) as opposed to exercise duration (Nieman et al., 2007).
Table 3: Studies on the effects of acute exercise on pro-inflammatory and anti-inflammatory cytokines.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Exercise Mode</th>
<th>Participants</th>
<th>Method</th>
<th>Outcome Measures</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canon et al. 1986</td>
<td>CY; 1 h at 60% VO2max</td>
<td>Male, age 20-36 yr n=8</td>
<td>Plasma</td>
<td>IL-1</td>
<td>IL-1 β increased 3 h post-exercise</td>
</tr>
<tr>
<td>Evans et al. 1986</td>
<td>EE (leg); 45 min at 250 W (42% VO2max untrained, 27% VO2max runners)</td>
<td>Untrained men (n=5) and highly trained endurance runners (n=4)</td>
<td>Plasma,</td>
<td>IL-1 β</td>
<td>IL-1 β increased 3 h post-exercise in untrained men</td>
</tr>
<tr>
<td>Dufaux &amp; Order, 1989</td>
<td>RU; 2.5 h</td>
<td>Male, sports students, 20-28 yr n=8</td>
<td>Plasma</td>
<td>TNF-α</td>
<td>TNF-α increased 1 h post-exercise</td>
</tr>
<tr>
<td>Esperson et al. 1989</td>
<td>RU; 5 km</td>
<td>Elite runners n=11</td>
<td>Plasma</td>
<td>TNF-α</td>
<td>TNF-α increased 2 h post-exercise; returned to baseline after 24 h</td>
</tr>
<tr>
<td>Canon et al. 1991</td>
<td>TR; three 15-minute periods 75% VO2max, separated by 5 min rest</td>
<td>Sedentary adult males, 25-65 yr</td>
<td>Cell culture supernatant</td>
<td>IL-1 β, TNF-α, IL6</td>
<td>IL-1 β and TNF-α increased 24 h post-exercise</td>
</tr>
<tr>
<td>Sprenger et al. 1992</td>
<td>RU; 20 km</td>
<td>Long distance runners; males (n=14), females (n=8), 28-54 yr</td>
<td>Plasma, Urine</td>
<td>IL-1, IL-6, TNF-α</td>
<td>TNF-α increased immediately post-exercise</td>
</tr>
<tr>
<td>Ullum et al. 1994</td>
<td>CY; 1 h at 75% VO2max</td>
<td>Healthy, young moderately trained males, 29-39 yr n=17</td>
<td>Plasma</td>
<td>IL-1 α, IL-1 β, IL-6, TNF-α</td>
<td>IL-6 increased during exercise, and returned to/below baseline 1 h post-exercise IL-1 α, IL-1 β, and TNF-α were not detectable in plasma</td>
</tr>
<tr>
<td>Castell et al. 1996</td>
<td>RU; 3.45 h marathon running (43 km)</td>
<td>Male athletes, 20-40 yr</td>
<td>Plasma</td>
<td>TNF-α, IL-6</td>
<td>IL-6 increased immediately and 1 h post-exercise</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Duration</td>
<td>Condition</td>
<td>Exercise Intensity</td>
<td>Participants</td>
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<tr>
<td>Bruunsgaard et al. 1997</td>
<td>CY</td>
<td>1st session of 30 min concentric exercise (normal cycling) at 65% VO2max, 2nd session of EE (reversed cycling) of 30 min at 100-150% VO2max</td>
<td>Healthy young males</td>
<td>Serum</td>
<td>12</td>
</tr>
<tr>
<td>Venkatraman &amp; Pendergast, 1998</td>
<td>Running</td>
<td>40-80% VO2max until voluntary exhaustion</td>
<td>Runners; males (n=10), females (n=10), 32-38 yr</td>
<td>Plasma</td>
<td>IL-6</td>
</tr>
<tr>
<td>Ostrowski et al. 1998</td>
<td>TR</td>
<td>2.5 h at 75% VO2max</td>
<td>Male athletes (n=10)</td>
<td>Plasma</td>
<td>IL-6, IL-1β</td>
</tr>
<tr>
<td>Ostrowski et al. 1999</td>
<td>RU</td>
<td>3.26 h marathon running</td>
<td>Male volunteers, 24-37 yr (n=10)</td>
<td>Plasma</td>
<td>TNF-α, IL-6, IL-1β, IL-10</td>
</tr>
<tr>
<td>Steensberg et al. 2001</td>
<td>Running</td>
<td>2.5 h at 75% VO2max</td>
<td>Male runners, 25-50 yr (n=9)</td>
<td>Plasma</td>
<td>IL-6</td>
</tr>
<tr>
<td>Chan et al. 2004</td>
<td>CY</td>
<td>1 h at 70% VO2max</td>
<td>Active males, 22-26 yr (n=8)</td>
<td>Plasma</td>
<td>IL-1β, IL-6</td>
</tr>
<tr>
<td>Reference</td>
<td>Protocol</td>
<td>Participants</td>
<td>Biomarkers</td>
<td>Changes After Exercise</td>
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<tr>
<td>Zaldivar et al. 2006</td>
<td>Cycling; 30 min at 80% VO2max</td>
<td>Healthy male adults, 18-30y n=11</td>
<td>Serum</td>
<td>IL-1 α, IL-6, IL-10, TNF-α</td>
<td></td>
</tr>
<tr>
<td>Nieman et al. 2007</td>
<td>CY; 1st session of continuous cycling at 64% VO2max for 2 h; 2nd session of intermittent exercise at 64% VO2max separated by 3-min of rest every 10 min</td>
<td>Trained cyclists n=12</td>
<td>Plasma</td>
<td>IL-6, IL-10</td>
<td></td>
</tr>
<tr>
<td>Croft et al. 2009</td>
<td>TR; Five 3-min bouts at 90% VO2max, separated by 3-min active recovery periods</td>
<td>Recreationally active males n=?</td>
<td>Plasma/Serum</td>
<td>IL-6 and TNF - α</td>
<td></td>
</tr>
<tr>
<td>Spanoudaki et al. 2010</td>
<td>CY; 1st session of intermittent cycling for 1 h between 47-120% VO2max, 2nd session of constant cycling for 1 h at 105% VO2max (24 h apart)</td>
<td>Healthy, moderately trained men n=10</td>
<td>Serum</td>
<td>TNF-α, IL-6</td>
<td></td>
</tr>
<tr>
<td>Scott et al. 2011</td>
<td>TR; 3 sessions of 1 h at 55%, 65%, and 75%</td>
<td>Physically active men</td>
<td>Plasma/Serum</td>
<td>TNF-α, IL-1 β, IL-6</td>
<td></td>
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<tr>
<td>Study</td>
<td>Exercise Type</td>
<td>Participants</td>
<td>Methods</td>
<td>Results</td>
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<tr>
<td>LaVoy et al. 2012</td>
<td>Cycling; 1 h at 95% VO2max</td>
<td>Trained male cyclists, 28-38 y n=16</td>
<td>Cell culture</td>
<td>Expression of TNF-α, IL-6, IL-10 by CD8+ T-cells</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-6 increased during exercise at all intensities (at higher concentration with higher intensity)</td>
<td></td>
</tr>
<tr>
<td>Scott et al. 2013</td>
<td>TR; 1 h of continuous running at 65% VO2max, followed intermittent to exhaustion at 70% VO2max</td>
<td>Recreationally active men (n=11) and endurance trained men (n=10)</td>
<td>Plasma/Serum</td>
<td>TNF-α, IL-6, and IL-10 increased 1 h post-exercise</td>
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<td></td>
<td></td>
<td>TFN-α increased during and post-continuous exercise and up to 2 h post-intermittent exercise</td>
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<td>IL-1α increased 1 h post-continuous exercise and remained higher than baseline 24 h post</td>
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<td></td>
<td></td>
<td>IL-1β increased during and immediately after continuous and intermittent exercise</td>
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<td></td>
<td></td>
<td>IL-6 increased throughout/after constant running and up to 24 h post-exercise</td>
<td></td>
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<tr>
<td>Kerasioti et al. 2013</td>
<td>CY; 2 h at 60-65% VO2max, followed by 4 h recovery, and 1 h 60-95% VO2max</td>
<td>Physically active men, 26-30 yr n=9</td>
<td>Plasma</td>
<td>IL-6, IL-10</td>
<td></td>
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<td></td>
<td>IL-6 increased 30 min post-exercise and up to 24 h post-exercise</td>
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</table>

**CY** = cycling; **RU** = running; **TR** = treadmill running; **EE** = eccentric exercise
Based on the current literature, it is apparent that exercise stimulates perturbations in bone turnover and cytokine levels. With respect to low-impact exercise, the duration of exercise may have greater effects on bone turnover in comparison to other factors.
(Maumoun et al. 2006), while the cytokine response may be more sensitive to exercise intensity (Nieman et al. 2007) as observed in Tables 2 and 3.

The mechanisms that contribute to the effects of bone turnover and cytokine responses, respectively, have been well established in the literature. Bone turnover has been shown to be primarily influenced by mechanical strain and variations in parathyroid hormone, growth hormone, testosterone, and leptin, all of which are upregulated during exercise (Bouassida et al., 2006; Wallace et al., 2000; Yakar et al., 2002; Hough et al., 2011; Bergnan et al., 2013). The cytokine response during exercise has been attributed mostly to the stimulation of lymphoid organs, production of cytokines in muscle and adipose tissue, and glucocorticoid and catecholamine production by the hypothalamic adrenal axis (Singh et al., 1998; Neto et al., 2009; Steensberg et al., 2002). All of these mechanisms stimulate bone turnover and cytokine production during exercise, however what happens beyond this point between cytokines and bone is unclear due to the lack of literature investigating this relationship. As previously discussed, bone cells and their precursors are capable of responding to cytokines as well as producing them. Therefore it is possible that local and systemic cytokines (which increase with exercise) may interact with bone thereby also manipulating the bone turnover response to exercise. Figure 6 illustrates the possible interaction between cytokines and bone during and before high-intensity exercise with association to time. Based on the sequence of cytokine level variations depicted in Figure 5, the initial pro-inflammatory cytokine response may initially result in greater bone resorption by upregulating RANKL, increasing osteoclast proliferation, and inducing and osteoblast differentiation, all of which have been shown to be induced by TNF-α, IL-6, IL-1α, and IL-1β with in vitro applications (Schett, 2011;
Takayanagi, 2012; Walsh et al., 2006). After the end of the exercise, the increase in pro-inflammatory response by IL-10 may stimulate bone formation by inhibiting RANKL expression (Hong et al., 2000). Based on these postulations, it is expected that low-impact, high-intensity exercise may initially be accompanied by an increase in bone resorption markers, followed by increased bone formation markers in serum. Due to the large discrepancies found in the literature pertaining to high-intensity, low-impact exercise and bone turnover, it is difficult to conclude whether these sequence of events may in fact be the case. Thus more studies are required that investigate the effects of high-intensity, low-impact exercise on bone formation and bone resorption markers. In addition, a comprehensive examination of the changes in cytokines and bone turnover markers with respect to each other is needed to understand the interaction between cytokine and bone in exercise.
Figure 7 Possible associations between cytokines and bone in response to HIE and the possible net effect on bone turnover. The onset of high intensity exercise leads to cytokine production by various compartments in the body. According to Pederson & Toft (2000) the first source of cytokine production during exercise starts from skeletal muscle. Contracting muscle fibers produce IL-6 locally and eventually systemically as IL-6 leaks out from the tissue. Damage in the muscle caused by contractions leads to the recruitment of monocytes and macrophages, which increases the production of IL-1 and TNF-α. TNF-α secretion may also originate from the liver and adipose tissue, both of which are stimulated by circulating LPS levels which increase with exercise (Scott et al., 2011). Another source of TNF-α is monocytes and macrophages that are stimulated of HPA during exercise, however this contribution is minimal given that the secretion of TNF-α by these cells is very low. Finally, increases in IL-10 are stimulated by the HPA as well as lymphoid organs, such as the spleen. Based on previous studies that have assessed the effects of cytokine on bone with in vivo and in vitro studies, it is very likely that the cytokine response during exercise will also govern the magnitude of bone formation and primarily bone resorption.
Chapter 2: Proposed Study

2.1 Rationale

Regular exercise improves bone health by increasing bone mineral density and bone strength (Maimoun & Sultan, 2011). Current dogma is that long-term exercise maintains or improves BMD and lowers the risk of fracture and development of osteoporosis in adults (Korpelainen et al., 2010; Andreoli et al., 2011; Kemmler et al., 2012; Guadalupe-Grau et al., 2009; Cousins et al., 2010; Lofgren et al., 2012). However, the acute bone response to a single bout of exercise is less clear (Maimoun & Sultan, 2011). Recently, short bouts of exercise in the form of high-intensity interval exercise (HIE) have been receiving scholarly attention for their ability to induce a number of metabolic adaptations associated with conventional long-term exercise (Gibala & McGee, 2007). Simultaneously, evidence from in vitro bone models suggests that intermittent exercise (as observed with the intermittent application of exercise-induced systemic factors) may produce anabolic effects on bone (Maimoun et al., 2006). However, the use of HIE as an intermittent form of exercise on bone has not been established. Given the short duration of HIE, the application of this form of exercise may pose greater convenience in today’s predominantly busy lifestyles. Consequently, HIE may be used by healthy and clinical populations to maintain or improve bone health.

Osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) are two key players in bone remodeling, thus examining how biochemical markers of bone formation and resorption respond to acute exercise is an important step in identifying the mechanisms of the bone response to exercise. Furthermore, osteoblasts and osteoclasts
are mediated by many systemic factors such as cytokines. Cytokines, both pro-inflammatory and anti-inflammatory, play a very important role in bone formation and resorption (Takayanagi, 2007). This has been shown by clinical studies in which patients with bone conditions such as arthritis present an imbalance of pro-inflammatory and anti-inflammatory cytokines in association with poor bone health (Schett, 2011; Takayanagi, 2012). Cytokine levels are altered by exercise, resulting in an improved inflammatory profile with the increased levels of anti-inflammatory cytokines outlasting pro-inflammatory cytokine levels (Ullum et al., 1994). However, although markers of bone turnover and cytokines have been assessed following single bouts of different exercise protocols independently, there is a paucity of data regarding whether the cytokine response is associated with the bone turnover response following exercise and especially HIE. The interconnected relationship between bone and cytokines has been well illustrated with various models such as in vitro and in vivo animal studies (Lee et al., 2008; Takayanagi 2007), however it has not been explored in an exercise model. Investigating the association between changes in bone markers and cytokines may be beneficial in understanding how exercise affects each compartment.

Bone is very dynamic and sensitive to mechanical stimulation. In fact, high-impact exercise may stimulate local bone turnover markers from the site of mechanical loading (Bloomfield, 2001). Therefore, in order to characterize the systemic effects of exercise rather than mechanical loading on markers of bone turnover and cytokines and their potential association, a mode of HIE needs to be assessed that is low-impact and utilizes minimal weight-bearing activity. One such exercise is cycling (Guillemant et al., 2004).
2.2 Objectives and Hypotheses

2.2.1 Objectives

The overall objective of this study was to investigate the bone turnover and cytokine response to a single bout of low-impact, high intensity exercise (HIE). The specific objectives were to:

- Examine the magnitude and timing of post-exercise changes in bone formation and bone resorption markers.
- Examine the magnitude and timing of post-exercise changes in pro- and anti-inflammatory cytokines.
- Determine if there is an association between the percent changes of bone turnover markers and cytokines across different time points after exercise.

2.2.2 Hypotheses

The overall hypothesis of this study is that a single bout of HIE would induce a bone turnover and cytokine response similar to prolonged exercise with levels increasing 5min post-exercise and slowly returning to baseline levels 24hr following the exercise. The specific hypotheses were:

- an acute bout of HIE would stimulate an increase in bone turnover markers 5min post exercise with levels slowly returning to baseline levels 24hr following the exercise;
• an acute bout of HIE would lead to increased pro- and anti-inflammatory cytokine levels 5min post exercise with levels slowly returning to baseline levels 24hr following the exercise;
• the post-exercise changes in markers of bone turnover would be associated with changes in the pro- and anti-inflammatory cytokines.

2.3 Implications
By investigating the combined effects of a single bout of HIE on markers of bone turnover and cytokine levels, we could postulate whether a post-exercise association exists between the immune and bone response to low-impact, high intensity exercise. This investigation may pave the way to establish mechanistic studies that further explore these associations and elucidate the mechanisms in which bone and cytokine interact during exercise. Furthermore, exploring the effects of HIE on bone health and inflammatory status may provide evidence to encourage the use of HIE as a mode of maintaining bone health and preventing the development of osteoporotic conditions in both healthy and clinical populations.
Chapter 3: Methods

3.1 Subjects

Twenty-three young males (mean ± SD, age 21.8 ± 2.4 y, weight 76.63± 11.7 kg) were voluntarily recruited from Brock University to participate in the study. The sample size was chosen in accordance with previous studies (outlined in Tables 1 and 2) that utilized cycling as a mode of exercise for assessing levels of bone markers or cytokines. Participants were recruited through class announcements during the academic year. Participants provided informed consent during their first visit and filled out the Medical History Questionnaire and the Godin-Shephard Leisure-Time Exercise Questionnaire. The latter is a simple questionnaire that assesses leisure time exercise behavior, in which the responses are converted into metabolic equivalents (MET) (Godin & Shephard, 1985). No medical conditions were reported and participants refrained from smoking and alcohol consumption prior to and during their visits. These subjects were recreationally active, with a mean weekly physical activity score of 66.1± 28.2 MET. All subjects were asked to refrain from strenuous physical activity 24h prior to HIT trial and between the third and last blood sample collection.

3.2 Exercise Protocol

The study consisted of 3 visits on separate days between 12:30-3:00 PM. During the first visit participants provided informed consent and performed an incremental test on a cycle ergometer to measure their maximum workload. The exercise started with a 5-minute warm-up session of cycling at 80 W, followed by the incremental test in which the resistance was increased by 15 W every minute. Participants cycled until exhaustion, and
maximum workload was recorded. Heart rate was monitored continuously throughout the test in order to record working intensity. On the second visit, participants performed the HIE trial based on the cycle ergometer at 90% of the maximum workload. The trial involved 12 minutes of intermittent cycling consisting of six 1-minute high-intensity cycling intervals separated by six 1-minute active rest periods. During the active rest periods, participants were allowed to cycle in the opposite direction (which eliminates resistance) or pause completely on the cycle ergometer. The HIE trial was initiated with a 4-minute warm-up which consisted of cycling at 25%, 50%, 75% and 100% of their maximum workload per minute. The trial was followed by a cool-down period of 2-3 minutes that consisted of cycling at 70 W.

3.3 Blood Collection

Blood was drawn from the participants at four times points: pre-exercise (baseline), 5min post-exercise, 1h post-exercise and 24h post-exercise. Blood was drawn from the antecubital vein using 21 G BD Vacutainer® with subjects in a seated position. Blood was transferred into clear serum tubes (BD Vacutainer®) and allowed to clot for 60 minutes. The tubes were transferred to a pre-cooled centrifuge (Thermo Scientific®) and were spun at 4°C and 3000 RPM for 15 minutes. Serum was collected from the tubes and stored in plastic Eppendorf® tubes at -70°C.

3.4 Biochemical Assessments

All bone markers were assessed using enzyme-linked immunosorbent assays (ELISA). ELISA is a highly sensitive immunoassay utilizing enzyme-conjugated antibodies, with
antigen or antibodies bound to a solid support. The assay measures changes in enzyme activities proportional to the antigen or antibody concentrations involved in the underlying immune reactions. The results of the bone marker assays were assessed using a Bio-Tek spectrometer and KC4 (3.3) Software, while cytokines were assessed using the Magpix reader and Luminex Software.

3.4.1 NTx (Osteomark® NTx Serum kit, Alere)

All samples and reagents were allowed to equilibrate to room temperature before being used. Working strength solution was prepared by diluting 30X Wash Concentrate 1:30 with deionized water and mixed for a minimum of five minutes. The work strength conjugate solution was also prepared by diluting the Antibody Conjugate Concentration to a 1:101 ratio using Antibody Conjugate Diluent. The solution was mixed gently. The calibrators, controls and specimens were thoroughly mixed and then subsequently diluted to a 1:5 ratio with Specimen Diluent. The samples were then mixed thoroughly and then 100 μl of each sample was pipetted into a 96-well plate using a multichannel pipette. After this step, 100 μl of working strength conjugate solution was pipetted into each microwell. The plate was then incubated at room temperature (20-25°C) for 90 minutes. After the incubation, the microwells were washed manually five times with the working strength wash solution and blotted after the final wash. Once the strips were dry, the 200 μl of Chromagen Reagent/Buffered substrate solution, which was prepared by diluting Chromagen Reagent into the Buffered substrate solution at a ratio of 1:101, was immediately pipetted into the microwells. The plate was then incubated at room temperature for 30 minutes. After incubation, 100 μl of Stop solution was added to each
microwell. The plate was then swirled gently for 15-20 seconds and subsequently incubated for 5 minutes at room temperature. Following incubation, the absorbance of the calibrators, controls and specimens was read using a plate reader at an absorbance of 450 nm. A standard calibration curve was generated using a polynomial curve fitting equation. The minimum detectable concentration measured with the NTx assay is 3.2 nM.

3.4.2 BAP (Ostase® BAP Serum kit, Immunodiagnostic Systems)
All reagents were brought to room temperature and thoroughly mixed prior to use. Wash solution was prepared by diluting 50 ml of Wash Concentrate with 950 ml of distilled water. 50 μl of the zero calibrator, calibrators B-F, controls and specimens were pipetted into the bottom of each assigned well. Then 100 μl of conjugate solution was pipetted into all microwells. The plate was then incubated for 1 hour at room temperature on a horizontal rotator set at 500 rpm. After incubation, 100 μl of Quench reagent was added to each microwell. Within 5 minutes of adding the reagent, the absorbance of the plate was read at 405 nm. A standard curve was generated using a polynomial curve fitting equation. The minimum detectable concentration measured with the BAP assay is 0.7 μg/l.

3.4.3 OPG (Human OPG Elisa kit, BioVendor®)
All reagents were brought to room temperature and thoroughly mixed prior to use. Samples were diluted 3x with Dilution Buffer just prior to the assay. After sample preparation, 100 μl of diluted standards, quality controls, dilution buffer, and samples were pipetted into microwells. The plate was incubated for 1 hour at room temperature
while shaking on an orbital plate shaker at 300 rpm. After incubation, the plate was washed three times with Wash solution and blotted at the final wash. Once the plate was dried, 100 µl of Biotin Labeled Antibody was pipetted into each microwell. The plate was incubated again at room temperature for 1 hour while shaking on an orbital plate shaker at 300 rpm. The plate was washed again as previously described. 100 µl of Streptavidin-HRP Conjugate was added into each microwell, and the plate was incubated at room temperature for 30 minutes while shaking on an orbital plate shaker at 300 rpm. After incubation, the plate was washed three times with Wash solution and blotted at the final wash. 100 µl of Substrate Solution was added into each microwell. The plate was then sealed and covered with aluminum foil and incubated for 10 minutes at room temperature without shaking. The colour development was interrupted by adding 100 µl of Stop Solution. The absorbance of the plate was subsequently read by a plate reader at an absorbance of 450 nm. A standard curve was generated using a polynomial curve fitting equation. The minimum detectable concentration measured with the OPG assay is 0.03 pmol/l.

3.4.4 RANKL (Human sRANKL Elisa, BioVendor®)

All reagents were brought to room temperature and were thoroughly mixed before use. Samples were diluted 100x with Dilution Buffer. After sample preparation, 100 µl of Standards, Quality Controls, Dilution Buffer and samples were pipetted into microwells. The plate was incubated for 18 hours at 2-8°C while shaking on an orbital plate shaker at 300 rpm. After incubation, the plate was washed 5 times with Wash Solution, and blotted after the final wash. 100 µl of Streptavidin-HRP Conjugate was added into each
microwell, and the plate was incubated at room temperature for 1 hour while shaking on an orbital plate shaker at 300 rpm. After incubation, the plate was washed three times with Wash solution and blotted at the final wash. The plate was washed 100 μl of Substrate Solution was added into each microwell. The plate was then sealed and covered in aluminum foil and incubated for 25 minutes at room temperature without shaking. The color development was interrupted by adding 100 μl of Stop Solution. The absorbance of the plate was subsequently read by a plate reader at an absorbance of 450 nm. A standard curve was generated using a polynomial curve fitting equation. The minimum detectable concentration measured with the RANKL assay is 0.4 pmol/l.

3.4.5 Cytokines (Milliplex® MAP kit, Millipore).

The cytokines were assessed using the Milliplex® MAP, which is based on Luminex xMAP technology. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of two dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody. This system allows you to detect multiple analytes simultaneously. The Milliplex® MAP kit used in this assay measured cytokine concentrations of IL-10, IL-1α, IL-1β, IL-6 and TNF-α.

All reagents were brought to room temperature and were thoroughly mixed before use. The assay was initiated by adding 200 μl of Wash Buffer into each well of the plate. The plate was sealed and placed on an orbital plate shaker for 10 minutes at room temperature. The Wash Buffer was then decanted and residual amounts were removed by inverting the plate and blotting it on absorbent towels. Then, 25 μl of each Standard and
Control was pipetted into the appropriate wells. 25 μl of Assay Buffer was then added to the sample wells, followed by adding 25 μl of Serum Matrix Solution to the background, standards, and control wells. The samples were then added by pipetting 25 μl of the serum into the appropriate wells. 25 μl of Mixed Beads, that were prepared prior to the assay, were pipetted into all the wells. The plate was then sealed and covered with foil and incubated for 2 hours at room temperature while shaking on an orbital plate shaker at 300 rpm. After incubation, the plate was attached to a plate magnet and inverted to remove well contents. The plate was then washed 2 times and blotted gently on absorbent paper while still attached to the magnet. 25 μl of Detection Antibodies were pipetted into each well, and the plate was subsequently sealed and covered with foil and placed on an orbital plate shaker for 1 hour at room temperature. 25 μl of Streptavidin-Phycoerythrin was pipetted into the wells containing the Detection Antibodies, and the plate was subsequently sealed and covered with foil and placed on an orbital plate shaker for 30 minutes at room temperature. After incubation, the plate was attached to a plate magnet and inverted to remove well contents. The plate was then washed 2 times and blotted gently on absorbent paper while still attached to the magnet. 150 μl of Drive fluid was pipetted in all the wells and the plate was placed on an orbital plate shaker for 5 minutes. The plate was then run on the Luminex software. Cytokine concentrations were determined by analyzing the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic plate curve-fitting method. The minimum detectable concentrations for the cytokines measured with this assay are 1.5 pmol/l of IL-1α, 0.7 pmol/l of IL-1β, 0.4 pmol/l of IL-6, 0.3 pmol/l of IL-10 and 0.3 pmol/l of TNF-α.
3.5 Statistical Analysis

The results were analyzed using SPSS Statistics (Version 21). Descriptive statistics were run to determine normal distribution. All bone marker data were normally distributed, therefore repeated measures analysis was computed to determine significant time effects.

Although cytokine levels were assessed in all samples collected, in some samples not all cytokines were detectable. To control for missing data, a list-wise deletion was applied so that subjects with missing cytokine values were omitted when running the repeated measures model. The final number of subjects that had valid values for all time points and used in the analysis of each cytokine were: n=10 for IL-1α, n=8 for IL-1β, n=9 for IL-6, n=15 for TNF-α, and n=12 for IL-10. Due to this small sample size, the cytokine data were not normally distributed. Thus, non-parametric tests were computed to assess whether there was a significant time effect associated with cytokines and exercise (Friedman Test) and to determine the time points responsible for these effects (Wilcoxon Signed Rank Test).

Finally, the relationship between bone markers and cytokines was assessed. Percent changes from baseline were calculated for all markers and were used to examine the association between cytokines and bone markers using a Pearson correlation. Values are presented as mean ± SEM and significance was accepted at $p<.05$. 
Chapter 4: Results

4.1 Physical Characteristics

The physical characteristics of the subjects are reported in Table 4. Subjects cycled at an intensity of 261.3W± 41.0W at 90% of maximum workload, with heart rate values ranging from 175-185 beats per minute (BPM) throughout working intervals.

| Table 4: Physical Characteristics of Participants (n=23) |
|---------------------------------|-----------------|
| Age (y)                         | 21.8 ± 2.4      |
| Weight (kg)                     | 76.6 ± 11.7     |
| Height (cm)                     | 178.6 ± 6.7     |
| BMI (kg/m²)                     | 23.9 ± 2.5      |
| Percent Body Fat (%)            | 10.5 ± 5.5      |
| Fat Free Mass (kg)              | 68.2 ± 8.9      |
| Physical Activity Score         | 66.6 ± 28.2     |
| BMI= body mass index            |                 |

4.2 Baseline and post-exercise values of bone turnover markers

The baseline and post-exercise values for bone markers are reported in Figures 8-11. There was a significant time effect for all bone turnover markers (p=0.002). At 5min post-exercise, BAP (Figure 9), OPG (Figure 10), and RANKL (Figure 11) significantly increased from baseline values (10.5, 11.8 and 19.7%; p=0.001, p=0.001 and p=0.000, respectively). At 1h post-exercise, only BAP was significantly higher than baseline (9.0%; p=0.010), while the other markers returned to baseline values. At 24h, BAP
remained significantly higher than baseline (10.7%, \( p=0.001 \)) while NTX was significantly decreased from baseline (-12.10%, \( p=0.046 \)) (Figure 8).

**Figure 8:** Serum concentrations of NTX at baseline, 5 minutes, 1 hour and 24 hours post-exercise. *\( p<0.05 \) from all other time points. Values are presented as mean ± SE.

**Figure 9:** Serum concentrations of BAP as baseline, 5 minutes, 1 hour and 24 hours post-exercise. *\( p<0.05 \) from baseline. Values are presented as mean ± SE.
**Figure 10:** Serum concentrations of OPG at baseline, 5 minutes, 1 hour and 24 hours post-exercise. *p<.05 from baseline and 24 h. Values are presented as mean ± SE.

![Graph of Serum OPG concentrations over time](image1)

**Figure 11:** Serum concentrations of RANKL at baseline, 5 minutes, 1 hour and 24 hours post-exercise. *p<.05 from all other time points. Values are presented as mean ± SE.

![Graph of Serum RANKL concentrations over time](image2)
4.3 Baseline and post-exercise values of cytokines

The baseline and post-exercise values of cytokines are illustrated in Figures 12-16. A significant time effect was observed for IL-1α \( (p<0.00) \) (Figure 12), IL-1β \( (p=0.04) \) (Figure 13), IL-6 \( (p=0.03) \) (Figure 15), and TNF-α \( (p<0.00) \) (Figure 16). At 5min post-exercise, IL-1α, IL-1β, IL-6, and TNF-α increased significantly from baseline (40.3%, 41.3%, 70% and 76% respectively). IL-1α, IL-6, TNF-α decreased significantly 1h post-exercise, and IL-1β only began to significantly decrease 24h post-exercise. No significant changes were observed with levels of IL-10 (Figure 14).

**Figure 12:** Serum concentrations of IL-1 α at baseline, 5 minutes, 1 hour and 24 hours post-exercise. *p<0.05 from all other time points. Values are presented as mean ± SE (n=10).
Figure 13: Serum concentrations of IL-1β at baseline, 5 minutes, 1 hour and 24 hours post-exercise. #p<0.05 from 24 h. Values are presented as mean ± SE (n=8).

Figure 14: Serum concentrations of IL-10 at baseline, 5 minutes, 1 hour and 24 hours post-exercise (n=12).
**Figure 15:** Serum concentrations of IL-6 at baseline, 5 minutes, 1 hour and 24 hours post-exercise. ##p<0.05 from 1h and 24h. Values are presented as mean ± SE (n=9).

**Figure 16:** Serum concentrations of TNF-α at baseline, 5 minutes, 1 hour and 24 hours post-exercise. *p<0.05 from all the other time points. Values are presented as mean ± SE (n=15)
4.4 Correlations between bone markers and cytokines

The correlations of percent changes between cytokines and bone markers are reported in Figures 17-20. At 5-minutes post-exercise, a significant correlation was observed between the percent changes in BAP and IL-10 ($r=-0.68$, $r^2=0.47$ $p=0.02$) (Figure 17). At 24-hours post-exercise, significant correlations were also observed between percent changes in BAP and IL-1α ($r=-0.69$, $r^2=0.47$, $p=0.04$) (Figure 18), OPG and IL-10 ($r=0.64$, $r^2=0.41$, $p=0.03$) (Figure 19), and NTX and TNF-α ($r=0.66$, $r^2=0.43$, $p=0.008$) (Figure 20).

![Figure 17: Correlation between percent changes in BAP and IL-10 from pre- to 5-minutes post-exercise (n=11).](image-url)
Figure 18: Correlation between percent changes in BAP and IL-1α from pre- to 24-hours post-exercise (n=9).

Figure 19: Correlation between percent changes in OPG and IL-10 from pre- to 24-hours post-exercise (n=12).
Figure 20: Correlation between percent changes in NTX and TNF-$\alpha$ from pre- to 24-hours post-exercise (n=15).
Chapter 5: Discussion and Conclusions

5.1 Discussion

The purpose of this study was to investigate the combined effects of low-impact HIE on pro- and anti-inflammatory cytokines and bone turnover markers. Young, recreationally active males participated in this study and performed a HIE protocol for a total duration of 12 minutes. It was found that although cycling is low-impact it does stimulate a bone turnover response as seen with the previous literature (Maimoun et al., 2006; Rudberg et al., 1999; Tosun et al., 2006; Wallace et al., 1999). The acute effects of HIE were found to be very similar to the previous literature with an initial rise in pro-inflammatory cytokines, followed by an increase in anti-inflammatory cytokines (Pederson & Toft 2000). Finally, this study was the first of its kind to establish a significant correlation between the changes in bone markers and that of cytokines following high-intensity, low impact exercise. A summary of the findings of this study and the possible net effects on bone are depicted in Figure 21.

It is well documented that regular exercise improves inflammatory status and reduces the risk of inflammatory and metabolic conditions (Gleeson et al., 2011). As discussed earlier, acute exercise is associated with an initial increase in pro-inflammatory cytokines, followed by an increase in anti-inflammatory cytokines (Peterson & Pederson 2000; Pederson & Toft, 2005). Indeed, IL-1α, IL-1β, IL-6 and TNF-α were found to increase significantly at 5 minutes post-exercise, while no significant effects were observed in the levels of IL-10 (Figures 12-16). The cytokine response to exercise has mainly been attributed to the marked increase in IL-6 towards the end of the exercise.
Increases in IL-6 levels may be induced by local muscle production (due to muscle contractions), elevations in IL-1 and TNF-α that act on muscle (due to damage caused by eccentric contractions), and by liver and adipose tissue (Rhode et al., 1997; Steensberg et al., 2002; Bruunsgaard et al., 1997; Scott et al., 2011). A few studies have shown that elevations lipopolysaccharide (LPS) levels associated with exercise stimulates the secretion of TNF-α from liver and adipose tissue (Scott et al., 2011). IL-6 stimulation by the IL-1 and TNF-α leads to the upregulation of their receptor antagonists, namely IL-1 receptor antagonists (IL-1ra) and soluble TNF-α receptors (sTNF-R), which increase immediately after IL-6 to act in a negative feedback mechanism to inhibit the pro-inflammatory effects of IL-1 and TNF-α (Otrowski et al., 1998; Otrowski et al., 1999; Pederson & Toft 2005). Therefore, the marked increase in IL-6 5-minutes post-exercise may actually be critical for reducing the effects of other pro-inflammatory cytokines during exercise. Therefore, based on the cytokine changes that were observed after in the present study, our hypothesis depicted in Figure 7 may closely depict the sequence of cytokine release. It is important to note that increased levels of cytokines or other proteins (including bone turnover markers) could be due to increased production, or due to reduced protein clearance associated with exercise. Whether the observed increases in cytokines or bone markers are due to reduced clearance or increased production is undetermined in this study. It is possible to speculate that changes seen immediately at 5-minutes post-exercise are due to changes in protein clearance or changes in protein stability (reduced protein degradation) while the changes in 1-hour and 24-hour after exercise may be due to both changes in synthesis/production as well as clearance/degradation.
Similar studies have been conducted in the past to evaluate effects of high-intensity and low-impact exercise on bone, however many of the conclusions have been confounded by factors such as the intensity, duration, and type of exercise (Guillemant et al., 2004). To be consistent with the existing literature, this study utilized cycling as a
Figure 21 Reported associations between cytokines and bone in response to HIE and the net effect on bone turnover: An acute response was observed with pro-inflammatory cytokines, were found to increase 5-minutes post-exercise. As it was postulated, the increase in IL-1α, IL-1β, TNF-α and IL-6 were accompanied with increases in RANKL and NTX levels. Interestingly, BAP was also found to increase 5-minutes post-exercise, and continued to increase up to 24-hours post-exercise. This finding was not expected however it may be due to mechanical strain or possibly direct effects of IL-6 on osteoblast precursor cells. OPG was found to increase 5-minutes post-exercise before declining 24-hours after exercise. This decline was associated with a decrease in IL-10.
mode of low-impact exercise. Contrary to high-impact exercise, cycling has been associated with poor outcomes on BMD due to the lack of mechanical loading. In fact, cyclists appear to have lower lumbar spine and hip BMD when compared to age-matched controls (Guillemant et al., 2004). However, BMD does not capture the short-term effects of exercise, thus these reports are not based on the acute effects of cycling. Therefore the use of biochemical markers of bone formation and resorption provides a dynamic and sensitive measure to assess the short-term effects of exercise on bone as seen with this study (Maimoun & Sultan, 2011).

This study is the first of its kind to assess the acute effects of cycling on bone formation markers BAP and OPG and bone resorption markers NTX and RANKL. Comparing to baseline values, all markers exhibited a significant time effect in association to exercise (Figures 8-11). Although there are no reference reports for OPG, NTX, and RANKL, other markers of bone formation and resorption have also been reported to increase immediately and shortly after low-impact exercise such as CTX, OC, PICP, PIIIP, and ICTP (Guillemant et al., 2004; Herrmann et al., 2007; Maîmoun et al., 2006; Wallace et al., 2000).

In this study, OPG and RANKL increased significantly 5 minutes post-exercise, indicating increased osteoblast responsiveness. According to in vitro studies that utilize osteoblast-osteoclast co-culture systems (Steeve et al., 2004), RANKL and OPG are secreted by osteoblasts and their precursors and are responsible for primarily regulating the activities of osteoclasts (Datta et al., 2008; Steeve et al., 2004). Based on the pro-inflammatory cytokine response reported above, it is possible that increases in IL-1, TNF-α and IL-6 are associated with increased osteoclast differentiation by promoting the
production of RANKL and OPG, with a net increase in RANKL (Steeve et al., 2004). However other factors such as hormonal perturbations and mechanical strain may also be involved (Bergnan, 2013).

Bone formation marker BAP also increased 5-minutes post-exercise. A similar finding was also reported by Rudberg et al. (1999), who found BAP increased significantly in postmenopausal women immediately after cycling. This effect may be due to the mechanical strain on bone that is associated with cycling (Tanaka et al., 2006; Rudberg et al., 1999). With respect to in vitro studies, the application of shear stress results in increased mineralization and increased mRNA expression of osteocytes (Bloomfield, 2001; Owan et al., 1997). Similarly, increased blood flow induced by cycling can translate into shear stress on bone, which may increase bone formation (Tanaka et al., 2006). This may explain why BAP increased shortly after exercise. Furthermore, BAP remained elevated 1 hour and 24 hour post-exercise, which is in contradiction with other studies that showed a decline or no change in BAP following low-impact exercise (Banfi et al., 2010; Guillemant et al., 2004; Rudberg et al., 1999). As per Table 2, the only study in which BAP was shown to increase 24 hours post-exercise was by Tosun et al. (2006), who tested brisk walking and weight lifting on bone markers in young women. It was shown that brisk walking alone did not alter BAP levels, however brisk walking accompanied with weight lifting (5kg) lead to an increase in BAP levels 24-hours post-exercise (Tosun et al., 2006). From these findings it can be suggested that the mechanical strain or resistance accompanied with cycling may cause anabolic effects on bone that can be observed hours after the exercise (Tosun et al., 2006).
The only marker that was found to be significantly lower 24 hours post-exercise was the bone resorption marker NTX. This finding is in accordance with the literature as bone resorption markers have generally been reported to decrease a couple of hours after exercise (Herrmann et al., 2007). Taken together with the current findings and particularly the increase in RANKL levels, it appears that primary acute effects of HIE on bone are linked to increased bone resorption.

Given that HIE initially lead to increased bone resorption, followed by increased bone formation, these findings show that this form of exercise stimulates bone turnover. The process of bone turnover is very critical as it not only stimulates both osteoblast and osteoclast activity, but also presents a sign of continuous bone repair and regeneration of bone. The fact that a net bone accrual was not observed in this study (as indicated by bone markers) does not necessarily indicate that cycling or HIE has no benefits to bone. Rather, it further supports the hypothesis that in addition to bone quantity, the quality of bone (ie. the structure of bone) is also an important factor to determine bone strength (Gomez-Bruton et al. 2013). Thus, increased bone turnover in response to low impact exercise may indicate an improved structural adaptation of bone that results in increased overall bone strength.

The main focus of this study was to evaluate the association between cytokines and bone turnover markers. Significant correlations were found between the percent changes of certain markers at the same time points as presented in Figures 17-20. At 5-minutes, it was observed that an increase in BAP levels was negatively correlated with a decline in IL-10 levels. Based on the sequence of cytokine release after exercise, it is expected that BAP levels would be higher prior to production of IL-10. According to in
vitro studies, the incubation of osteoblast precursors with IL-10 leads to a suppression in differentiation and mineralization (Lee et al., 2008). Indeed, the incubation of preosteoblast cultures with IL-10 inhibits BAP expression in preosteoblast cultures (Garcia-Lopez et al., 2005). Based on this association, it is expected that higher levels of BAP will be associated with lower circulating levels of IL-10. Thus it is possible that the effects of shear stress (mechanical strain) on osteoblasts and their precursors cells coupled by low levels of circulating IL-10 attribute for the rapid increase in BAP.

At 24 hours, an increase in BAP levels was negatively correlated with a decline in levels of IL-1 α . Although IL-1 α inhibits osteoblast proliferation, it has only been reported to have stimulatory effects on bone formation and BAP expression in both in vitro and animal research (Hughes et al., 2000; Lee et al., 2010). Based on these studies, it is predicted that a decline in IL-1 α would be associated with a decline in BAP; however this was not the case. Therefore this further supports the postulation that perhaps the increase in BAP was due to other factors such as mechanical strain that induces anabolic effects on bone (Tosun et al., 2006).

Furthermore, at 24-hours we observed a positive correlation between OPG and IL-10. Both OPG and IL-10 decline in comparison to their 5-minute and 1-hour values, which is expected based on Figure 7. IL-10 has been demonstrated by a few studies to reduce bone resorption by downregulating RANKL and by enhancing OPG production (Lee et al., 2008; Hong et al., 2000; Liu et al., 2006). According to animal studies, IL-10 knockout mice present increased bone resorption, and decreased OPG gene expression (Liu et al., 2006). More studies are needed to assess the effects of IL-10 on OPG expression, however our findings may indicate that IL-10 levels may influence OPG
secretion by osteoblasts in combination with the decline in pro-inflammatory cytokines observed 24-hours post-exercise.

A positive correlation was also observed between NTX and TNF-α 24-hours post-exercise. As levels of NTX declined, so did levels of TNF-α, indicating that this cytokine may possibly contribute to the bone resorptive effect of exercise along with other factors such as hormonal perturbations and mechanical strain. The effects of TNF-α on bone resorption have been investigated previously with pharmaceuticals that regulate the activity of this cytokine (Charatcheroenwitthaya et al., 2007). In fact, a similar positive correlation with NTX and TNF-α was observed in a previous study that tested the effects of infliximab, an antagonist of TNF-α, in patients with arthritis (Korczowska et al., 2013). Short-term administration of infliximab lead to a reduction in both serum NTX and TNF-α, both of which were also found to be significantly correlated with each other in that study. A study that used a different TNF-α blocker known as etanercept, also found that short-term administration of this pharmaceutical lead to a decline in NTX levels (Charatcheroenwitthaya et al., 2007). In addition, Korczowska et al. (2013) found that the lower NTX levels were positively correlated with lower levels of the TNF-α receptor 1 (TNF-R1). TNF-R1 also increases immediately after exercise to counterbalance the increased renal clearance of TNF-α, and declines a few hours later (Katsuhiko, 2002). Although TNF-R1 was not measured in this study, it may have also declined. The decline in both TNF-α and possibly TNF-R1 would result in less TNF-α binding on to thereby reducing bone resorption and ultimately NTX production.
5.2 Conclusion

It was shown that low-impact HIE induces a combined bone turnover and cytokine response. As evidenced by the changes in RANKL and NTX, this type of exercise was mostly associated with a bone resorptive effect, which declined 24-hours post-exercise. It was also found that cycling may be associated with increased bone formation through increased BAP production, however the mechanism by which this occurs remains to be elucidated. In accordance to previous literature as well as Figure 6, the exercise used initially stimulated a pro-inflammatory cytokine response followed by an anti-inflammatory cytokine response. Most importantly, this study found that significant correlations existed between bone turnover markers and certain cytokines as hypothesized in Figure 7. Although one correlation needs further investigation, the correlations between BAP, OPG and NTX with IL-1α, IL-10, and TNF-α respectively indicate that cytokines may interact with bone to induce exercise changes on bone formation and bone resorption. Most of the explanations of the effects of the cytokines on bone were based on in vitro and in vivo studies and not exercise trials, however it is possible that the way in which cytokines and bone interacted during exercise is similar to the interactions in these previous studies. Nonetheless, more research is required to further elucidate the mechanisms in which cytokines regulate bone formation and bone resorption during exercise.

Assessing the mechanisms by which cytokines and bone interact during exercise would provide the most reliable evidence of the interdependence of both systems. Although it was not possible to depict mechanisms in this study, the correlations provide the first step into doing so, The correlations observed between the pro- and anti-
inflammatory cytokine response and bone turnover markers predict an interdependence found between the skeletal and immune systems. Further investigation is required to determine whether the correlations between cytokines and bone depict a cause-effect relationship, or whether they present cytokines as additional factors that promote bone turnover during exercise. Indeed, these systems are not separate entities and their interaction may be responsible for beneficial effects of exercise on bone health and inflammatory status.

5.3 Limitations and Future Considerations

Although bone turnover and cytokine responses were detected after exercise, a few limitations are associated with this study. While exercise stimulates the increase of several metabolic factors such as hormones and cytokines, it is also associated with hemoconcentration and plasma volume expansion. This is explained by the redistribution of blood flow to across extracellular and interstitial compartments. This may cause fluctuations in concentrations (Brahm et al., 1997), thereby providing misleading results. Thus, calculating the plasma volume in this study may have been beneficial to support whether the results were indeed reflections of the exercise. However, Brahm et al. (1997) have found that plasma volume does not significantly change osteocalcin levels nor alter this bone marker response. It is possible, therefore, that the plasma volume effect in this study was not significant.

The second limitation of this study was the lack of assessing receptors and receptor antagonists alongside with the other parameters. Although we were able to observe variations in cytokines after exercise, we cannot fully determine the
inflammatory response given that the effect of these cytokines depends on receptor binding. For future consideration, it would be advantageous to also assess the levels receptors of these cytokines as well as their receptor antagonists.

Another limitation of this study was the sample size. Although the sample size was sufficient for the bone marker analysis, the missing data led to a small sample for the cytokine analysis. This was handled using non-parametric statistics for the analysis and significant effects were found for most but not all cytokines.

Finally, the use of nutritional assessments could have been beneficial in this study. Nutrition is an important exogenous factor that may influence both bone health and cytokine dynamics. Both glucose, glycogen and protein have been reported to influence bone markers and cytokines during and after exercise (Chan et al., 2004; Kerasioti et al., 2013).
References


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10.1016/j.bbi.2012.09.006


Miller, A. C., Schattenberg, D. G., Malkinson, A. M., & Ross, D. (1994). Decreased content of the IL1 alpha processing enzyme calpain in murine bone marrow-derived macrophages after treatment with the benzene metabolite hydroquinone. *Toxicology Letters, 74*(2), 177-84


doi:10.1093/emboj/17.4.1006


doi:10.1152/japplphysiol.00164.2004


Tosato, G. , & Jones, K. D.  (1990). Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. *Blood, 75*(6), 1305-10

young women. *Modern Rheumatology / the Japan Rheumatism Association, 16*(5), 300-4


Ullum, H. , Haahr, P. M. , Diamant, M. , Palmo, J. , Halkjaer-Kristensen, J. , & Pedersen, B. K. (1994). Bicycle exercise enhances plasma IL-6 but does not change IL-1 alpha, IL-1 beta, IL-6, or TNF- α lpha pre-mRNA in BMNC. *Journal of Applied Physiology (Bethesda, Md.: 1985), 77*(1), 93-7


Appendices
Certificate of Ethics Clearance for Human Participant Research

DATE: 8/17/2012

PRINCIPAL INVESTIGATOR: KLENTROU, Nota - Kinesiology

FILE: 12-012 - KLENTROU

TYPE: Masters Thesis/Project

STUDENT: Yasmeen Mezil

SUPERVISOR: Nata Klenrou

TITLE: Muscle Activation in Child vs Adults: An Intervention Study

ETHICS CLEARANCE GRANTED

Type of Clearance: NEW

Expiry Date: 8/30/2013

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 8/17/2012 to 8/30/2013.

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 8/30/2013. 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:

Brian Ray, 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.
Appendix B

INVITATION LETTER

The systemic effects of acute exercise on osteoblasts in vitro

Principal Investigators: Dr. Nota Klentrou and Yasmeen Mezil, Faculty of Applied Health Sciences.

We would like to invite you to participate in the present study, which investigates the effects of acute exercise on osteoblasts (bone cells) in adult males using an interdisciplinary approach which involves applied physiology and cell culture techniques.

The purpose of this research project is to examine the growth and maturation of osteoblasts, as measured with a cell culture system, in response to one acute exercise session in moderately to regularly physically active adult males. In other words, we would like to determine the cellular mechanisms of which acute exercise enhances bone status.

This study requires three short visits to the Applied Physiology Lab (30 minutes, 2 hours, 15 minutes). During these visits, participants will fill out two questionnaires on medical history and physical activity and will also perform an exercise protocol on a cycle ergometer for approximately 15 minutes. The exercise will include short warm-up and cool-down intervals. Blood sampling will be conducted as the physiological assessment for this study. Blood samples will be collected from participants prior to exercise and 5 minutes, 1 hour, and 24 hours after exercise.

Participation in this project will allow the opportunity to experience a research facility at Brock and contribute to the advancement of science.

This research is being performed only by Brock University researchers in the Applied Physiology Laboratory and Cairns Family Health and Bioscience Research Complex.

If you have any pertinent questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688-5550 ext 3035, reb@brocku.ca)

If you have any questions, please feel free to contact us.

Thank you,

Principal Investigators:
Dr. Nota Klentrou and Yasmeen Mezil
Faculty of Applied Health Sciences
Brock University
Tel: 905-688-5550 ext: 4538 or 5623
email: nklentrou@brocku.ca or ym07tv@brocku.ca

This study has been reviewed and received ethics clearance through Brock University’s Research Ethics Board [12-012 KLENTROU]
Appendix C

RECRUITMENT POSTER

SEEKING VOLUNTEERS FOR RESEARCH

- If you are a male,
- Between the ages of 18-25 years,
- And is moderatly to regularly physically active...

Then come and visit us!

Live the research experience and participate in a study that aims to determine the effects of exercise on bone!

Principal Investigators: Dr. Nota Klentrou and Yasmeen Mezil
Department of Kinesiology, (905) 688-5550 ext. 4538 and 5623

This study has received clearance from the Brock University Ethics Board (12-012 KLENTROU)

This study requires three short visits to the Applied Physiology Lab. During these visits, participants will fill out two questionnaires on medical history and physical activity and will also perform an exercise protocol on a cycle ergometer for approximately 15 minutes. The exercise will include short warm-up and cool-down intervals. Blood sampling will be conducted as the physiological assessment for this study. Blood samples will be collected from participants prior to exercise and 5 minutes, 1 hour, and 24 hours after exercise.

IF YOU ARE INTERESTED, PLEASE CONTACT:
Yasmeen Mezil, MSc. Candidate
T (905) 688 5550 at X5623 or X6112.
E-mail: ym07tv@brocku.ca
Appendix D

RESEARCH STUDY INFORMATION AND CONSENT

The systemic effects of acute exercise on osteoblasts in vitro

You are being invited to participate in a research study being conducted by the investigators listed below. Prior to participating in this study, please read this form to find out about the purpose and the tests of this study. This study is sponsored by the Faculty of Applied Health Sciences of Brock University.

INVESTIGATORS: DEPARTMENT: CONTACT:
Dr. Nota Klentrou FAHS, Brock University (905) 688-5550 x4538
Yasmeen Mezil FAHS, Brock University (905) 688-5550 x5623
* FAHS = Faculty of Applied Health Sciences

PURPOSE:
The primary objective of this study is to utilize cell culture tools to investigate the systemic effects of exercise on osteoblasts in vitro. The specific objectives of the study are: a) to examine osteoblast proliferation and differentiation upon inducing the cells with post-exercise serum samples, b) to examine bone nodule mineralization as a response to continual incubation of the osteoblasts with post-exercise serum, and c) to investigate the expression of anabolic proteins by osteoblasts as response to serum incubation.

DESCRIPTION OF THE TESTING PROCEDURES:

A. Pre and Post Assessments at Brock University:
All participants enrolled in this study will be asked to visit the Applied Physiology at Brock University for three appointments. In the first appointment, the researcher will sit with the participant to go through the study protocol and to determine the maximum workload he can perform on the ergometer. In addition, the participant will also fill out all necessary questionnaires of the study. In the second appointment, participants will provide a blood sample, and perform the exercise intervention component. On the third appointment (which is on the consecutive day after the appointment) participants will return to have final blood samples collected. The measurements that will be completed by the participants during these visits as well as throughout the cell culture component are listed below:

Measurements:

1. Participant Screening and Medical History Questionnaire: Every participant will be required to complete this questionnaire before completing any other measure. This questionnaire allows us to have a better understanding of the participant’s medical background in order to interpret data properly and to make sure that they are able to participate in all of the required measurements.
2. **Physical Activity Questionnaire for Adults**: This is a brief questionnaire that asks participants to recall their physical activity level in the past week.

3. **Pre-exercise Blood Collection**: 10 mL of blood will be withdrawn from the participants by a certified researcher from each participant prior to exercise. Blood will be collected using a 22G safety needle from the median cubital vein.

4. **Post-exercise Blood Collection**: Blood samples will also be collected 5 min, 1 hr, and 24 hr after the acute exercise intervention (10 mL per draw). Blood will be withdrawn from the participants by a certified phlebotomist. The blood will be collected using a 22G safety needle from the median cubital vein (Total amount of blood drawn for pre-exercise and post-exercise will be 40 mL from each participant, 10 mL per draw).

5. **Serum**: The blood samples will be allowed to clot overnight at 4°C, following a centrifugation at 400 x g. The serum layer will be removed and separated into 2 mL aliquots prior to being frozen at -80°C for later studies.

6. **Enzyme-linked Immunosorbent Assay**: 200 µL of Wash Buffer will be added into each well of a Microtiter Filter Plate. The plate will be sealed and placed on a plate shaker for 10 minutes at room temperature (20-25°C). The buffer will be removed and 25 µL of each Standard will be subsequently added in the appropriate wells, following the addition of 25 µL of Assay Buffer to the sample wells and 25 µL of appropriate matrix solution to the background, standards, and control wells. 25 µL of the serum samples will be added into the appropriate wells. 25 µL of the premixed beads will be added to each well. The plate will be sealed and incubated with agitation overnight at 4°C. After the media is removed, the plate will be washed 2 times with 200 µL/well of Wash Buffer. 25 µL of Detection Antibodies will be added into each well and the plate will be incubated with agitation on a plate shaker for 1 hour at room temperature. 25 µL of Streptavidin-Phycoerythrin will be added to each well containing the 25 µL of Detection Antibodies. Similarly, the plate will be sealed and incubated with agitation on a plate shaker for 30 minutes at room temperature. All of the contents will be subsequently removed by vacuum and 150 µL of Sheath Fluid will be added to all wells. The plate will be run on a Luminex 100™ and fluorescence will be analyzed.

7. **Serum Dilutions**: For each outcome measure assessed in osteoblasts, the serum treatments will be diluted in a growth media to form 10% human serum solution.

8. **Osteoblast Incubation**: Adult human osteoblasts (hOB) will be treated with 10% fetal bovine serum (FBS), 1mM L-glutamine and 100 ng/ml gentamicin at 37°C in a humidified atmosphere containing 5% CO₂. Cells will be passaged when near confluent monolayers are achieved. The cells will be seeded into 96-well plates for the cell proliferation and differentiation assays, and in 10 cm plates for the proteomic analysis. The FBS media will be replaced with 10% human serum to investigate osteoblast proliferation, differentiation, bone nodule mineralization, and proteomic analysis.

9. **Osteoblast Proliferation**: Osteoblast proliferation will be assessed using a thymidine assay, which measures DNA synthesis, and a crystal violet assay, which measures the number of cells present. Following the incubation of osteoblasts in 24-well plates, the cells will be pulse-labeled with 10 µCi/well of
[\textsuperscript{3}H]-thymidine. Cells will then be lysed with 1.5% sodium dodecyl sulfate for 15 min, after which they will be combined with scintillation fluid and the radioactivity will be measured using a liquid scintillation counter. In addition, osteoblasts will also be treated with crystal violet stain solution in 96-well plates. The plate will then be allowed to dry overnight or for a couple of hours. Once the plate is dry, 100 μL of sodium phosphate solution (0.05 M NaH\textsubscript{2}PO\textsubscript{4} in 50% ethanol) will be added to each well. The plate will be gently shaken for 30 minutes and subsequently read at an absorbance of 570 nm after 1 second of low agitation. The relative spectrophotometry readings will be directly proportional to cell numbers.

10. Osteoblast Differentiation: Alkaline phosphatase (ALP) is an enzyme that is activated during osteoblast differentiation. Osteoblasts will be seeded in 10 cm plates and will be incubated for 3-4 days. The treatment medium will be added for a length of 15 min and subsequently the cells will be lysed. The cell lysate containing 2 µg total protein will be added to the assay buffer (2 mM p-nitrophenol phosphate, 1 mM MgCl\textsubscript{2}, 50 mM Tris-HCl/pH 9.2). After 10 min incubation at 37°C, the reaction will be terminated by the addition of NaOH. The absorbance of p-nitrophenol liberated in the reactive solution will be read at 420 nm. The relative ALP activity was defined as nonmoles of p-nitrophenol phosphate hydrolyzed per minute per 1 µg of total protein.

11. Bone Nodule Mineralization: Osteoblasts will be grown in 10% FBS media on 24-well plates. For 35 days, this medium will be fortified with β-glycerophosphate (10 mM) and ascorbic acid (50 µg/ml). At each assay interval (every 7 days), the cells will be rinsed three times with PBS and fixed with 95% methanol for 30 min. Subsequently, the cells will be stained with 1% alizarin red S at pH 6.4 for 5 min and washed with distilled water. Water will be removed aspiration, and the cells will be incubated in PBS for 15 min at room temperature on an orbital rotator. The PBS will be removed, and the cells will be rinsed once with fresh PBS. The counting of mineralized alizarin red S-positive nodules present in each well will be performed using light microscopy.

12. Proteomic Analysis: Osteoblasts will be seeded in 10 cm plates and will be incubated for 3-4 days. 10% human serum will be added for a length of 15 min and subsequently the cells will be lysed. The protein content of the osteoblasts will be assessed using the BioRad assay. A 3x electrophoresis sample buffer will be used to solubilize the lysates. After the addition of the electrophoresis sample buffer the samples will be boiled for 5 min and stored at -20°C. Protein samples will be loaded into wells of a 10% SDS-polyacrylamide gel and separated by electrophoresis at 120V for approximately 2 hours. The samples will subsequently be transferred from gel to a PVDF membrane at 100V for 1 hour. After transfer, the membrane will be washed for 5 min with 25 ml 1xTBS followed by incubation for 1 hour at room temperature in an agitator with 10 ml of 5% (w/v) non-fat dry milk in Tris-buffered saline. Afterwards the membranes will be rinsed with 25 ml of TBS/T and then overnight at 4°C with the primary antibody diluted (1:1000) in primary antibody dilution buffer with gentle agitation. The following primary polyclonal antibodies will be used: Phospho-catenin, Total-catenin, Total RANKL, Phospho-Akt, Total-Akt, Phospho-AMPK, and Total-AMPK. The next
day the membranes will be washed 3x for 5 min with 15 min with 15 ml TBS/T, and the primary antibody will be detected with HRP-conjugated secondary antibody diluted in blocking buffer (1:2000) which will be incubated with the membrane for at least an hour at room temperature. This will be followed by washing the membranes 3x for 5 minutes with TBS/T and the secondary antibody will be detected by ChemiGlo reagent and visualized by FluroChem software via an autodeveloper (ThermoFisher).

B. Acute Exercise Intervention Session:
Participants will be prescribed a high-interval training (HIT) session as prescribed by Gibala & McGee (2007). In this session participants will perform a high-intensity interval training (HIT) program which will involve six 1-minute intervals of all-out maximal cycling against a high braking force on a specialized ergometer, which will be separated by 1-minute recovery periods. Thus the total time for the HIT program should approximately be 15 minutes, including warm-up. The session will take place in the Applied Physiology Lab on a cycle ergometer. The HIT session will only be performed once, in which blood samples will be collected prior to the session and 5 min, 1 hr, and 24 hr after the session.

CONFIDENTIALITY:
All data collected during this study will remain confidential and will be stored in offices and on secured computers to which only the principal and co-investigators have access. You should be aware that the results of this study will be made available to scientists, through publication in a scientific journal, but your name and any personal data will not appear in compiling or publishing these results. Data will be kept for 5 years after the date of publication, at which time all information will be destroyed. Additionally, you will have access to your own data, as well as the group data when it becomes available and if you are interested.

PARTICIPATION & WITHDRAWAL:
You may choose whether to participate in this study or not and may remove your data from the study if you wish. You may also refuse to answer any questions posed during the study and still remain as a participant in the study. The investigators reserve the right to withdraw you from the study if they believe that it is necessary.

RISKS AND BENEFITS:
The only foreseeable risks involved in participation include:
   a) Possible muscle soreness and fatigue within 48 h of the exercise session. If this occurs, it will only be temporary.
   b) The exercise test may cause dizziness, nausea, or fainting. The principle investigators will monitor you during the exercise to make sure that this does not happen and to assist you if you feel any discomfort. In addition, you will be asked to make sure you have consumed enough fluids and a good, rich in carbohydrates meal and fluids approximately 2 hours prior to your arrival at the laboratory.
   c) Blood collection may pose the risk of fainting or the development of an infection.
This risk however will be minimized by professional venipuncture practice.

- Some questionnaires may pose a potential embarrassment. In such a case, you do not need to reply to any question if you do not wish to.

The significance of the proposed research will benefit the scientific and clinical communities by advancing the understanding of the mechanisms induced by exercise which are responsible for enhancing overall health and bone health. It will also allow the researchers to elucidate the systemic factors of which exercise improves bone strength and function. Your participation will allow you to contribute to the advancement of science, become exposed to a research protocol, and gain knowledge about the function of your own body.

**RIGHTS OF RESEARCH PARTICIPANTS:**
You will receive a signed copy of this consent form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies. This study has been reviewed and received clearance from the Brock University Research Ethics Board (12-012 KLENTROU) If you have any pertinent questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688-5550 ext 3035, reb@brocku.ca)

**INFORMATION:**
Please contact Yasmeen Mezil at 905-688-5550 (X5623 or X6112) or Dr. Nota Klentrou at 905-688-5550 (X4538), if you have any questions about the study.

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT. I HAVE ALSO RECEIVED A SIGNED COPY OF THE INFORMATION AND CONSENT FORM. MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION AND I AGREE TO PARTICIPATE IN THIS STUDY.

I UNDERSTAND THAT I HAVE TO EAT A GOOD MEAL RICH IN CARBOHYDRATES AND FLUIDS APPROXIMATELY 2 HOURS PRIOR TO MY ARRIVAL TO THE LABORATORY AND THAT I WILL NEED TO WEAR COMFORTABLE ATTIRE SUITABLE FOR EXERCISING.

___________________________
SIGNATURE of PARTICIPANT

___________________________
DATE

___________________________
PRINTED NAME OF PARTICIPANT

___________________________
DATE
In my judgment the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

SIGNATURE OF INVESTIGATOR

DATE
Appendix E

SUBJECT SCREENING AND MEDICAL HISTORY QUESTIONNAIRE

Name: ________________________________ Date: ________________

Date of Birth: ____________________________

Your responses to this questionnaire are confidential. If you answer “YES” to any of the following questions, please give additional details in the space provided and discuss the matter with one of the investigators. You may refuse to answer any of the following questions.

1. Have you ever had any major joint instability or ongoing chronic pain such as in the knee, back or elbow?  
   YES  NO

2. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?  
   YES  NO

3. Have you taken any medication in the past six months?  
   YES  NO

4. Is there any medical condition with which you have been diagnosed and are under the care of a physician (e.g. asthma, diabetes, anorexia)?  
   YES  NO

5. Do you, or have you in the past, consumed any alcohol on a regular basis?  
   YES  NO

6. Do you, or have you in the past, smoked on a regular basis?  
   YES  NO

7. Are you, or have you in the past, engaged in any extreme diet?  
   YES  NO

8. Do you, or have you in the past, consumed any nutritional supplements (e.g. calcium, multi-vitamin) on a regular basis?  
   YES  NO

9. Do you, or have you in the past, engaged in physical activity on a regular basis?  
   YES  NO

10. Have you had any fractures?  
    YES  NO
Appendix F

GODIN-SHEPHARD LEISURE-TIME EXERCISE QUESTIONNAIRE

Name: ________________________  Date: ____________

1. Considering a 7-day period (a week), how many times on the average do you do the following kinds of exercise for more than 15 minutes during your free-time (write on each line the appropriate number)?

   Times Per Week

   (a) STRENUOUS EXERCISE
      (HEART BEATS RAPIDLY) _________
      (i.e. running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

   (b) MODERATE EXERCISE
      (NOT EXHAUSTING) _________
      (i.e. fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)

   (c) MILD EXERCISE
      (MINIMAL EFFORT) _________
      (i.e. yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)

2. Considering a 7-day period (a week), during your leisure-time, how often do you engage in any regular activity long enough to work up a sweat (heart beats rapidly)?

   1. OFTEN  2. SOMETIMES  3. NEVER/RARELY
          □      □      □