Acute Effects of Greek Yogurt Consumption on Circulating Bone Turnover and Metabolism Markers Following High-intensity Interval Cycling in Young- to Middle-aged Premenopausal Females with Overweight and Obesity

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A Thesis submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of
Master of Science

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August 2025

Abstract

This thesis compared the bone turnover and metabolism responses of post-exercise Greek yogurt (GY) consumption and an isoenergetic carbohydrate pudding (CP) following high-intensity interval cycling (HIIC) in young- to middle-aged inactive premenopausal females with overweight and obesity. We hypothesized that GY would attenuate post-exercise bone resorption and/or amplify formation compared to CP. Using a randomized crossover design, participants completed an acute HIIC bout followed by the consumption of either GY or CP. Blood samples were collected at pre-exercise, post-exercise, 1hr and 3hr postprandially, and 24hr post-exercise. Bone turnover and metabolism markers RANKL, OPG, PTH, OPN, CTX, SOST, OC, and IGF-1 were measured from serum. Interactions were observed whereby OPG and OC were higher at 1hr and 3hr postprandially, respectively, and PTH was lower throughout the postprandial period in GY versus CP. Thus, GY consumption favourably modulated the acute post-exercise bone turnover and metabolism response in these females.

Acknowledgements

I would like to thank my supervisor, **Dr. Andrea Josse**, for her guidance and support over the last two years, and for giving me the opportunity to be part of such an incredible lab group. We faced our fair share of challenges getting the Luminex up and running, and I truly appreciate how available and supportive you were throughout, always ready to help and answer my many questions. I would also like to thank my committee member, **Dr. Nota Klentrou**, for sharing her expertise on bone markers and providing valuable advice on the assays. Sincere thanks to our collaborators at the University of Toronto. To **Dr. Jenna Gillen**, I appreciate the opportunity to spend time in your lab and gain experience with data collection for the PEN study. **Alexa**, thank you for being so inclusive and for letting me take part in so many aspects of the study.

A huge thank you to all my lab mates who were always there for a shared laugh and have also become great friends. **Joel**, working on DRIVE with you as a first-year master's student was such a valuable learning experience, thank you for teaching me and for having the confidence in me to help finish up data collection. To **Emily**, thank you for always being able and willing to answer my questions, offering the calming presence I so often needed, and for our shared love of long walks and croissants. **Nick**, thank you for being such a genuinely kind human, it was comforting to have someone at the same stage, working on a similar project, to bounce ideas off of. And last but not least, to **Monika**, thank you for always encouraging a break and a treat (whether we deserved one or not), and for sticking it out with Nick and me through all those long days in the lab.

To my **Mom and Dad**, thank you for always supporting and encouraging me. **Dad**, thank you for being my personal chauffeur and for treating me to my favourite coffee when I've had a rough day. **Mom**, I definitely got my attention to detail and workaholic tendencies from you, thank you for always understanding how I feel and encouraging me to keep pushing forward.

To my sister **Natalie**, thank you for sitting outside with me for hours on end while I worked away at this thesis, your quiet company meant more than you know.

Thank you to my best friends who patiently listened as I explained the trials and tribulations of research. To **Madelaine**, thank you for always being willing to have a work party with me, even throughout the summer months after you had already graduated. **Rachel**, my travel buddy, our

trips to Montreal were a much-needed reset. Thank you both for helping me take my mind off things during our (almost) weekly adventures. To **Frances**, even from across the country, you're always checking in and somehow always know what to say to make me laugh. And to **Daniela**, thank you for encouraging me from the very beginning, from getting involved with research at Mac to now.

Looking back, I've learned that research is never a solo pursuit, and I feel incredibly lucky to have had such a strong support system every step of the way. Thank you to everyone who made this chapter of my life so meaningful — I won't forget it.

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List of Abbreviations

BMD	Bone mineral density
cOC	Carboxylated osteocalcin
CP	Carbohydrate pudding
CTX	C-terminal telopeptide of type I collagen
CVs	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assays
FDR	False discovery rate
GY	Greek yogurt
HIIC	High-intensity interval cycling
HIIR	High-intensity interval running
HRR	Heart rate reserve
HRmax	Heart rate maximum
HSCs	Hematopoietic stem cells
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
LMM	Linear mixed model
LRP	Lipoprotein-related protein
MSCs	Mesenchymal stem cells
NCPs	Non-collagenous proteins
net iAUC	Net incremental area under the curve
OB	Obesity
OC	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OW/OB	Overweight/obesity
P1NP	Procollagen type 1 N-terminal propeptide
PBM	Peak bone mass
PPO	Peak power output
PTH	Parathyroid hormone

RANKL	Receptor activator of nuclear factor-κB ligand
RCF	Relative Centrifugal Force
RDA	Recommended dietary allowance
REML	Restricted maximum likelihood
RPE	Ratings of perceived exertion
RPM	Revolutions per minute
SEM	Standard error of the mean
SOST	Sclerostin
tAUC	Total area under the curve
TNF-α	Tumour necrosis factor-alpha
unOC	Undercarboxylated osteocalcin
VO_2max	Maximal oxygen uptake
VO_2peak	Peak oxygen uptake
Wnt	Wingless-related integration site

Chapter 1: Introduction

Bone mass accrual predominates during childhood, adolescence, and early adulthood, with peak bone mass (PBM) achieved by approximately 20 years of age[1,2]. This type of bone growth, in which the bone develops in length and width to reach adult form, is known as bone modeling[2,3]. On average, a higher PBM is achieved in males, and the slope of the age-related decline tends to be slower and shallower compared to females[1,2]. Bone tissue also undergoes remodeling across the lifespan, a process by which older or damaged bone is broken down ("bone resorption") and replaced with newer and stronger tissue ("bone formation")[4]. The bone cells primarily involved with this process are osteocytes (mechanosensors: sense load and microdamage), osteoclasts (bone resorption), and osteoblasts (bone formation: deposition and mineralization)[4]. Upregulation and/or downregulation of these processes determine net bone turnover rates and bone mass[4]. Following the achievement of PBM, bone turnover should be tightly coupled (i.e., resorption=formation), resulting in bone mass remaining relatively stable throughout adulthood[4]. However, various physiological conditions can uncouple this process, favouring resorption. For instance, it has been suggested that low-grade systemic inflammation (i.e., increased circulating cytokines including interleukin-6 [IL-6] and tumour necrosis factor-alpha [TNF-α]) associated with higher adiposity in overweight and obesity (OW/OB) can modify skeletal microstructure and quality *via* dysregulated bone-adipose tissue 'crosstalk', leading to greater bone fragility and fracture risk[5]. This may be especially detrimental to females as menopause triggers unique physiological shifts (i.e., decreased estrogen, increased adiposity, and alterations in systemic inflammation) that can accelerate bone loss (i.e., a greater and steeper slope) (**Figure 1**), resulting in an increased risk of osteopenia and/or osteoporosis with increased age[1,2,6]. Indeed, data indicate that females may be up to four times more likely to develop

osteoporosis than males[7]. Thus, developing strategies to optimize bone mass maintenance (highlighted in **Figure 1**) between these two critical periods (i.e., PBM achievement and menopause) within the lifespan is of utmost importance, especially among females with OW/OB, where negative bone health outcomes (i.e., higher fragility and fracture risk) may be exacerbated.

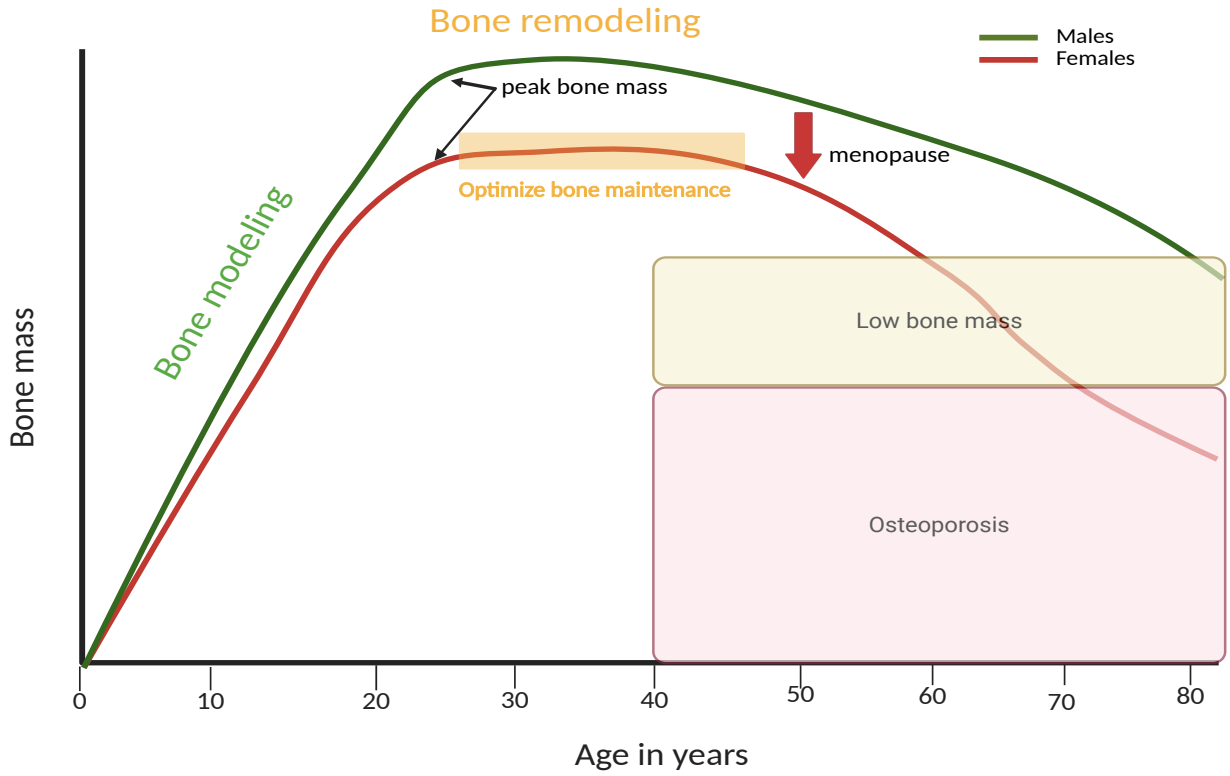


Figure 1: Bone mass across the lifespan for males and females detailing bone growth before the achievement of PBM, bone mass maintenance after PBM, and declines with aging (i.e., pronounced reduction with menopause). Adapted from Weaver et al.[1] Created with BioRender.com.

Regular exercise is a well-established strategy to improve bone health across the lifespan[6,8]. High-intensity interval exercise has often been reported to be time efficient, well-tolerated, and enjoyable among females with OW/OB[9–11]. Despite conflicting evidence in terms of the effect of non-weight bearing cycling on bone health (i.e., lower bone mineral density in trained cyclists)[12–14], research conducted in young and healthy premenopausal females has found similar bone turnover and metabolism responses between acute high-intensity interval running

([HIIR] weight-bearing/high-impact) and cycling ([HIIC] weight-supported/lower-impact)[15,16]. This may relate to the fact that HIIC also involves working against resistance, which increases muscle force and induces mechanical stress on the bone tissue[17]. HIIC also reduces the risk of injury associated with novel treadmill use[18], may minimize discomfort (i.e., joint pain) in those with OW/OB[19], and can increase cardiorespiratory fitness in young females with OB[11]. Given these factors, HIIC may be an ideal exercise modality for such individuals for safety and enjoyability, while also having a positive impact on health outcomes. While chronic exercise can result in favourable adaptations by modifying net bone turnover to increase bone formation and/or decrease bone resorption, contributing to bone mass maintenance, acute exercise stimulates bone turnover, beginning with resorption[12,20]. This is where post-exercise nutrition may play a vital role. Dairy products, for example, contain bone-supporting nutrients such as protein, calcium, vitamin D (if fortified), potassium, magnesium, zinc, and phosphorous, and may thus modulate the bone turnover and metabolism response post-exercise[12,21,22]. Indeed, higher dairy consumption in combination with exercise has yielded beneficial bone turnover and metabolism effects such as decreased resorption and/or increased formation in young and healthy females[23–25], young and healthy males[26], and females with OW/OB[27–29]. Despite this, individuals often gravitate towards carbohydrate dense snacks post-exercise[30,31]. This likely relates to the priority of rehydrating and replenishing glycogen stores, which is more critical among endurance athletes than those who are untrained[32]. On the other hand, Greek yogurt (GY) is a semi-solid food that contains bone-supporting nutrients and bacterial cultures[33], has a reduced carbohydrate and higher protein content, and consequently may offer more benefit to bone and glucose handling post-exercise than carbohydrate. Therefore, GY may serve as an ideal post-exercise snack for females with OW/OB who are at risk of metabolic dysregulation and bone fragility.

Chapter 2: Objectives and Hypotheses

2.1 Objectives

The overall objective of this thesis was to compare the acute effects (up to 24 hours) of Greek yogurt (GY) consumption versus an isoenergetic carbohydrate pudding (CP) on markers of bone turnover and metabolism (receptor activator of nuclear factor-κB ligand [RANKL], osteoprotegerin [OPG], parathyroid hormone [PTH], osteopontin [OPN], c-terminal telopeptide of type I collagen [CTX], sclerostin [SOST], osteocalcin [OC], and insulin-like growth factor 1 [IGF-1]) following a single bout of high-intensity interval cycling (HIIC) in young- to middle-aged inactive premenopausal females with OW/OB.

The detailed objectives include:

A. To assess the influence of GY or CP following HIIC on the absolute concentrations of the aforementioned bone markers (pre-exercise to 24hr post-exercise).
B. To compare the influence of GY and CP on each marker's cumulative physiological response (post-exercise to 24hr post-exercise) using net incremental area under the curve (net iAUC) calculations.

2.2 Hypothesis

It was hypothesized that the consumption of GY post-exercise would positively impact markers of bone turnover and metabolism following acute HIIC compared to the isoenergetic CP.

Specifically, GY in comparison to CP would:

A. Modulate the post-exercise bone turnover and metabolism response *via*:
    i. Attenuating of RANKL, PTH, OPN, CTX, and SOST.

ii. Amplifying of OPG and IGF-1.

iii. Attenuating or amplifying OC, depending on its role in relation to other markers, as it reflects overall bone turnover.

B. Reduce the net iAUC for resorption responses (RANKL, PTH, OPN, CTX, SOST [inhibitor of formation], and OC if increased in relation to resorption) and augment the net iAUC for formation responses (OPG [inhibitor of resorption], IGF-1, and OC if increased in relation to formation).

Chapter 3: Literature Review

3.1 Bone Structure, Composition, and Remodeling

Bone tissue is comprised of the extracellular matrix (organic [proteins] and inorganic [minerals]) and cellular matrix (bone cells: osteoblasts, osteoclasts, osteocytes), and has 3 compartments: cortical bone, trabecular bone, and bone marrow[34].

3.1.1 Extracellular Matrix – Inorganic and Organic

The extracellular matrix is approximately 60% inorganic material, with organic material and water providing the remaining 30% and 10%, respectively[35]. Hydroxyapatite crystals of calcium and phosphate [$Ca_{10}(PO_4)_6(OH)_2$] make up the majority of the inorganic material[35,36]. The rest of the inorganic matrix is composed of other ions, such as magnesium, sodium, and potassium[36]. The organic matrix, also known as the osteoid, is predominantly made up of type I collagen (~90%)[35,36]. Type I collagen is a critical structural component of bone, serving as a scaffold for mineral deposition and providing an anchor for non-collagenous proteins (NCPs), which make up the rest of the organic matrix (i.e., osteocalcin [OC] and osteopontin [OPN])[37]. These NCPs are essential for maintaining bone structure as they are involved in signalling in the bone remodeling cycle through their interactions with osteoblasts and osteoclasts[38].

3.1.2 Bone Compartments – Cortical, Trabecular, and Bone Marrow

The skeleton is comprised of two types of bone, cortical (also known as compact bone) and trabecular (also known as cancellous or spongy bone) bone (as outlined in **Figure 2**)[34]. A third component of the bone tissue is the bone marrow, which plays a central role in bone turnover and metabolism by supplying the precursor cells for osteoblast and osteoclast differentiation (key steps in the bone remodeling cycle)[34]. The cortical bone is located on the

outer surfaces of the bone, mainly at the shaft (diaphysis) of long bones such as the femur and humerus, and is strong and dense in structure[34]. The trabecular bone is comprised of spongy networks of trabecular plates and rods, and is lighter and less dense than cortical bone[34]. The trabecular bone tissue network is typically found within the ends of long bones (epiphyses), within the vertebral bodies, in flat and short bones such as the bones of the wrist and ankle, and interspersed within the bone marrow compartment[39]. Both types of bone are vascularized and contain osteocytes, osteoblasts, and osteoclasts[40]. The vasculature plays a vital role in delivering oxygen and nutrients to the bone cells and facilitates communication between the bone cells to coordinate bone remodeling[40].

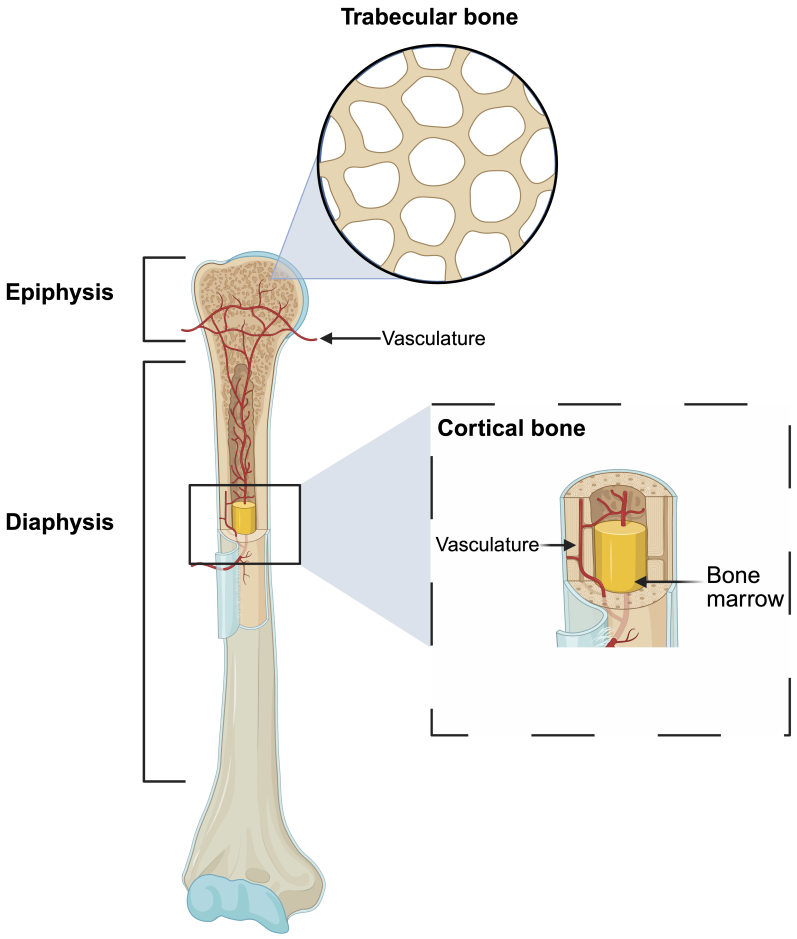


Figure 2: Bone structure including epiphysis and diaphysis, and compartments including the cortical bone, trabecular bone, and bone marrow. Created with BioRender.com.

3.1.3 Cellular Matrix and The Bone Remodeling Cycle

Bone is a dynamic organ that continuously undergoes turnover (i.e., formation and resorption) at varying rates across the lifespan as well as under different conditions (i.e., fed, rested, during and following exercise, etc.)[41,42]. In adulthood, following the achievement of peak bone mass (PBM), bone mass is generally preserved through an equilibrium (or coupling) between bone formation and resorption to remodel older tissue with newer and stronger tissue, until age-related bone loss begins to occur (i.e., accelerated bone mass loss with the onset of menopause in females)[1,43]. In adulthood, an uncoupling of formation to resorption can result in cortical porosity, trabecular bone thinning, and the eventual development of osteoporosis[1,44]. Thus, maintaining bone mass equilibrium in young to middle adulthood is vital to combat bone mass declines with aging, especially in females, as discussed in the Introduction section. In healthy adults, a remodeling cycle can take anywhere from 2 to 9 months, with the average turnover rate being 10% per year[3,45]. The bone remodeling cycle is comprised of 5 stages: activation, resorption, reversal, formation and mineralization, and termination (summarized in **Figure 3**)[44].

3.1.3.1 Activation

When mechanical stress and microdamage occur at a specific area of the bone structure, osteocytes which act as mechanosensors, sense these signals and activate the bone remodeling cycle through the release of receptor activator of nuclear factor-κB ligand (RANKL) to facilitate osteoclastogenesis (osteoclast differentiation) and osteoclast recruitment[44]. Osteoclasts are differentiated from hematopoietic stem cells (HSCs) in the bone marrow[46]. First, HSCs become monocytes or macrophages, then osteoclast progenitors, followed by pre-osteoclasts and osteoclasts, and finally active osteoclasts with the help of additional RANKL binding to its

receptor RANK on osteoclasts[44]. Osteoblastogenesis (osteoblast differentiation) is also stimulated, although to a lesser extent than osteoclastogenesis, as formation occurs at a later stage[47]. Upon activation, inactive osteoblasts (bone lining cells) form a bone remodeling unit by lifting off the surface of the bone tissue, resulting in organized pockets/canopies, where remodeling will occur[44] (as outlined in **Figure 3**). Remodeling (and resorption) can also be activated metabolically through the signalling of hormones like parathyroid hormone (PTH), which act to mobilize calcium from the bone tissue (at non-specific sites) in order to restore calcium homeostasis in the circulation[44]. Activation is essential for ensuring that bone remodeling in healthy individuals is only initiated when necessary[44].

3.1.3.2 Resorption

Following activation, the resorption process begins[44]. Active osteoclasts anchor to bone tissue with the help of OPN, creating a 'sealing zone'[44]. Through this mechanism, an acidic environment is created whereby osteoclasts dissolve the mineral component of the bone tissue (*via* hydrochloric acid) and the organic portion (*via* proteolytic enzymes such as cathepsin K)[40,44], releasing aspects of the matrix into the circulation. Following the degradation of the tissue, to ensure that excess resorption does not occur, factors such as estrogen, osteoprotegerin ([OPG], a decoy receptor for RANKL), and calcium can indirectly induce osteoclast apoptosis[44].

3.1.3.3 Reversal

Before bone formation, there is a reversal or transition phase[44]. This stage is needed to signal and prepare the bone surface for deposition of the new matrix tissue[44]. First, there is the deposition of the cement line, which allows osteoblasts (i.e., bone building cells) to adhere to the bone surface during formation[44,48] (**Figure 3**). The transition of resorption to formation may occur due to the interaction between a receptor on osteoclasts and its respective ligand on

osteoblasts[44]. Insulin-like Growth Factor 1 (IGF-1) has also been postulated to have a potential role in this coupling process[45,47].

3.1.3.4 Formation

Out of the bone remodeling stages, bone formation takes the longest[44]. This process can be divided into two stages: matrix deposition and mineralization[44].

3.1.3.4.1 Matrix deposition

Following the reversal stage, osteoblastogenesis predominates[44,47]. Osteoblasts are differentiated from mesenchymal stem cells (MSCs) within the bone marrow[46]. From these MSCs, adipocytes, chondrocytes, and myocytes can also be differentiated[49]. The osteoblast differentiation pathway is facilitated by the activation of the Wingless-related integration site (Wnt) signaling pathway, which allows for the transcription of genes required for osteoblastogenesis[44]. From there, MSCs are differentiated into osteoblast precursors/osteoprogenitor cells, then pre-osteoblasts, and finally active osteoblasts[44]. Active osteoblasts adhere to the cement line and begin to synthesize and secrete the osteoid matrix (type 1 collagen)[44] (**Figure 3**). OPG continues to play a role in attenuating resorption by binding RANKL[47].

3.1.3.4.2 Mineralization

After the deposition of type 1 collagen, the bone is mineralized[40,50]. During primary mineralization, osteoblasts facilitate the binding of the newly synthesized osteoid to hydroxyapatite crystals and other minerals of the inorganic matrix (*via* the release of OC, an NCP)[44]. Secondary mineralization involves more deposition and organization of these components[40,50]. Importantly, the mineralization process is dependent on systemic calcium and phosphate homeostasis, as the bone acquires these minerals from the circulation and acts as a

reservoir for other bodily sources[44]. Thus, high circulating levels of PTH, which releases calcium stores from bone, can result in mineralization deficits and bone mass loss[44]. In addition, mineralization can be inhibited by local factors/NCPs such as OPN[44,47].

3.1.3.5 Termination/Quiescence

Termination or quiescence occurs as the final stage of the remodeling cycle. In this final stage, osteoblasts either undergo apoptosis, become bone lining cells, or differentiate into osteocytes by embedding themselves in the bone matrix[44]. Osteocytes play an important role in terminating remodeling by secreting sclerostin (SOST), which inhibits osteogenesis (bone formation)[44].

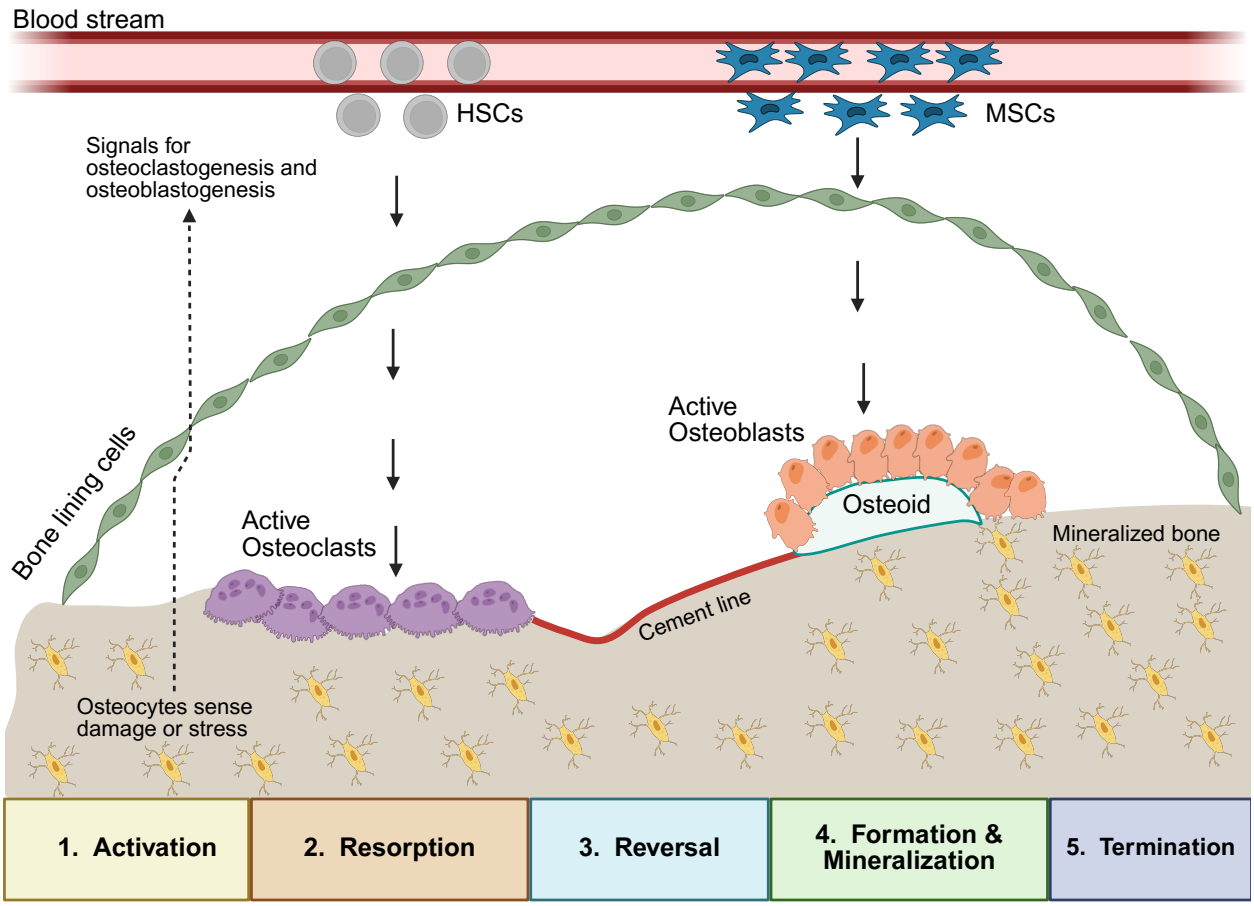


Figure 3: Bone remodeling stages occurring in the bone remodeling unit from Activation to Termination. Adapted from Kenkre & Bassett[44]. Created with BioRender.com.

3.2 Bone Turnover and Metabolism Markers and Their Response to Acute Exercise

Exercise training (especially in exercises/sports involving high-impact and unaccustomed loading) is known to elicit osteogenesis (bone formation)[51]. With acute exercise, the metabolic (i.e., lower circulating calcium levels) and mechanical stimulation (i.e., physical stress) on the bone tissue typically results in an immediate increase in bone turnover and metabolism, leading to osteoclast activation and bone resorption (the first two stages of the bone remodeling cycle)[12]. This is reflected in a systemic increase in markers indicative of bone resorption[12,52]. The duration and magnitude of the acute bone resorption response likely depends on the duration and mode of exercise, fitness (trained vs. untrained), health status (i.e., normal weight vs. having overweight/obesity [OW/OB]), and sex and age of the individual, among other factors[53,54].

3.2.1 RANKL/RANK/OPG Axis/Pathway

Increased expression of RANKL and its binding to its receptor RANK on osteoclasts facilitates osteoclastogenesis and increases bone resorption (outlined in **Figure 4A**)[44,46]. The primary source of RANKL is the osteocytes, which, upon sensing of loading and microdamage, stimulates the remodeling cycle (activation and subsequent resorption) by releasing this factor[44]. OPG, a glycoprotein that is secreted mainly by osteoblasts, acts as a decoy receptor for RANKL to prevent its binding to RANK receptors on osteoclasts (outlined in **Figure 4B**), indirectly blunting osteoclastogenesis and resorption, allowing for the upregulation of osteoblastogenesis[44]. OPG concentrations have been found to increase immediately post-exercise[16,20,53,55–57]. In some studies conducted in young males, OPG levels remained elevated for up to 4hr post-exercise[53,55,56]. In premenopausal females, while impact does not seem to have an effect on OPG concentrations (same response in high-impact high-intensity interval running [HIIR] vs. lower-impact high-intensity interval cycling [HIIC], at 90% heart rate maximum [HRmax]), a time

effect was found, reflecting an increase at 5min and 1hr post-exercise compared to pre-exercise, although significance disappeared with post hoc testing[16]. In another study conducted in this population, no change in OPG concentrations were observed following plyometric and resistance exercise[24]. Thus, it is possible that the OPG response may be more transient or not as strong in premenopausal females. In order to determine the effectiveness of OPG in blunting resorption through the RANKL/RANK/OPG axis, the RANKL response must also be assessed. However, many of the aforementioned studies did not measure RANKL[53,55,56,58], as serum levels are often difficult to detect[53]. In studies where RANKL was measured, an increase immediately post-exercise in both young males and premenopausal females following HIIR and HIIC was observed, with a return to pre-exercise levels by 1hr post-exercise[16,57]. The quick return to pre-exercise in RANKL suggests an early compensatory effect or coupling of OPG and RANKL. A compensatory mechanism can also be seen following plyometric and resistance exercise in premenopausal females, where RANKL has been found to decrease immediately post-exercise, with an increase in the OPG/RANKL ratio (despite no increase in OPG itself as previously mentioned)[24]. In the acute post-exercise period, resorption is typically transiently upregulated as microdamage is sensed and RANKL release is stimulated[20], thus, a prompt return to pre-exercise levels in RANKL and/or increases in OPG may signify a compensatory response to attenuate resorption processes in young and healthy adults.

3.2.2 Parathyroid Hormone (PTH) and Calcium Homeostasis

Bone tissue plays a critical role in mineral metabolism as a reservoir for calcium, ensuring that circulating levels are homeostatic[44]. When calcium levels are low within the systemic circulation (below the normal range of 2.2 to 2.7 mmol/L[59]), PTH is released from the parathyroid glands to degrade the bone matrix (at non-specific sites) and free up calcium stores

from the bone tissue[44]. One way this is achieved is through activating RANKL and downregulating OPG, this facilitates the binding of RANKL to RANK (as outlined in **Figure 4A**), resulting in the activation of osteoclastogenesis, subsequent bone resorption, and calcium mobilization[44]. Acute exercise typically triggers a rise in PTH, due to decreased serum ionized calcium[60]. Indeed, many studies have shown a transient increase in PTH during and immediately following exercise[16,20,53,55,56,60–70], with concentrations returning to pre-exercise levels (or decreasing below it) within 2hr post-exercise[16,55,56,58,60,61,63,66–68]. However, a secondary increase has been observed at 2hr and 4hr[53] and 24hr post-exercise[61] in certain cases. Acute exercise can be expected to stimulate PTH in the short term and may promote acute bone resorption following exercise in young and healthy adults.

3.2.3 Osteopontin (OPN)

OPN is released by osteoblasts and is the 2nd most abundant NCP found within the bone matrix[37]. During turnover, OPN facilitates osteoclast adhesion to bone surfaces[37] (**Figure 4A**). This protein also plays a role in preventing excessive bone formation/mineralization and is integral in binding/forming connections between mineralized components and collagen fibrils[37,46]. However, at abnormally high levels, OPN has also been found to be detrimental to the bone structure (i.e., impaired mineralization, excess resorption, bone loss) and overall health through its pro-inflammatory role[71]. Indeed, studies have found OPN to be upregulated in osteoporosis in postmenopausal females[72–76]. In addition, OPN is often overexpressed in the adipose tissue of individuals with obesity[77,78]. In these inflammatory conditions, osteoclast activity and binding (*via* OPN) is overly stimulated, resulting in increased bone resorption[79–83]. Since OPN plays a role in resorption, it is reasonable to assume that OPN would respond to exercise where acute bone turnover (and resorption) is upregulated. In a study performed in

otherwise healthy young males with OW/OB, OPN was found to decrease immediately post-exercise (in both continuous moderate cycling and HIIC) and returned to pre-exercise levels by 25hr post-exercise[84]. This goes against what was hypothesized in that study; however, it was suggested that the decline in circulation may be reflective of the sequestration of OPN to the peripheral tissues to facilitate turnover, as this study found an increase in other proteins related to tissue remodeling[84]. To date, no other studies have assessed the transient changes in OPN in the context of bone following acute exercise. However, long-term studies have found decreases in resting concentrations of OPN following exercise training in young premenopausal females with[85] and without OW/OB (with a corresponding increase in bone mineral density [BMD])[86]. More research is required to assess the post-exercise OPN response and whether these long-term adaptations coincide with the bone response to an acute bout of exercise in premenopausal females.

3.2.4 C-terminal telopeptide of type 1 collagen (CTX)

During bone resorption, active osteoclasts digest type 1 collagen (which makes up ~90% of the organic matrix) and release fragments of this protein, known as CTX (as outlined in **Figure 4A**), into the circulation[46]. Although previous research suggests that high-impact exercise typically elicits higher circulating CTX levels[20,53,58,61,87], acute cycling (lower-impact, non-weight bearing) exercise of high-intensity or over prolonged periods have been shown to acutely increase CTX post-exercise[20,23,52,60,62–65,70,88]. Of note, this body of research has largely been conducted in young males[52,56,58,61–66,70,87,88]. In understudied populations (i.e., premenopausal females), post-exercise CTX is not as well characterized. In fact, studies conducted in such populations have found no change in CTX following an acute bout of plyometric exercise[89], as well as high-intensity interval exercise (running [HIIR] and cycling [HIIC])[15]. For example,

following HIIR (high-impact) and HIIC (lower-impact) in young premenopausal females, CTX remained unchanged across time and between modalities[15], suggesting no effect of exercise or impact on this marker. Similar results were found following an acute bout involving HIIR (~80-90% heart rate reserve [HRR]) in young- to middle-aged premenopausal females with overweight only[90]. Overall, CTX may be expected to remain unchanged or increase transiently following high-intensity aerobic exercise in young adults, although more research in premenopausal females is needed.

3.2.5 Sclerostin (SOST) and Wnt Pathway

A major regulator of osteoblastogenesis and bone formation is the β-catenin–dependent-canonical Wnt pathway[44]. This pathway is activated when Wnt binds to the frizzled receptor and a coreceptor, lipoprotein-related protein (LRP) 5 or 6, which prevents the degradation of β-catenin and allows it to accumulate, a process that is required for the transcription of genes involved in osteoblastogenesis[44,91]. Additionally, the Wnt pathway inhibits MSCs differentiation into adipocytes and plays an indirect role in preventing the binding of RANKL to RANK by producing OPG[44,91]. This pathway (and subsequent osteoblastogenesis) is inhibited by the binding of SOST, a glycoprotein secreted by osteocytes (as outlined in **Figure 4A**), to LRP 5 or 6[44]. In terms of acute exercise, many studies have found a transient increase in SOST immediately post-exercise in young and healthy adults[15,16,20,53,66,88,89,92], largely returning to pre-exercise levels by 1hr post-exercise[15,16,66,88,89,92]. These studies involved a wide variety of exercises such as HIIR, HIIC, and moderate-to-vigorous running. Furthermore, HIIC and HIIR have been found to induce an increased post-exercise SOST response with no differences between modalities[15,16,88,92], suggesting an effect of high-intensity exercise, regardless of impact. This transient increase may help facilitate the initial bone turnover and metabolism response as

exercise-induced microdamage and mechanical stress is sensed by the osteocytes, which release SOST to transiently blunt osteoblastogenesis, indirectly promoting bone resorption in the acute post-exercise period.

3.2.6 Osteocalcin (OC)

OC is released by mature osteoblasts and is the most abundant NCP in the organic matrix[37,46]. This protein contributes to the organization of the bone matrix by binding calcium in hydroxyapatite crystals, helping to regulate mineral deposition and crystal formation[93] (as outlined in **Figure 4B**). While OC has often been classified as a marker of bone formation as it is released by osteoblasts and plays a role in mineralization, the carboxylation status determines the effects of OC as a regulator of bone turnover/resorption and energy metabolism (undercarboxylated version [unOC; more abundant]) versus bone formation (carboxylated version [cOC]), with total OC being the sum of both[94]. For example, unOC has been found to be released during bone turnover and to be correlated with the bone resorption marker CTX[95] and a higher risk of fracture among older females[96], while cOC is correlated with osteoblast number and bone formation[97]. Thus, this marker, especially when measured as total OC, reflects bone turnover rather than exclusively formation[91,95]. In young and healthy adults, total OC has been found to significantly increase immediately following high-intensity exercise[16,20,53,55,65,97], with some studies reporting a return to pre-exercise levels by 1hr post-exercise[16,55,65]. In terms of HIIR and HIIC, OC increased immediately post-exercise, returning to pre-exercise levels by 1hr in both modalities in premenopausal females[16]. Again suggesting little effect of impact, but an effect of intensity on circulating total OC. Hiam et al.[97] found increases in total and unOC 5mins following HIIC in young adults, while cOC remained unchanged. The increase in total and unOC likely relates to the transient increase in bone turnover (beginning with resorption) post-exercise.

Therefore, increases in total OC immediately[16,53,55,65,97], are likely indicative of exercise-induced bone turnover, perhaps *via* higher bone resorption. When taking all these studies into consideration, OC may be expected to transiently increase following exercise. However, total OC, should be interpreted along with other markers to gain a more comprehensive understanding of what is occurring at the level of the bone tissue.

3.2.7 Insulin-like Growth Factor 1 (IGF-1)

IGF-1 is the most abundant growth factor in the bone tissue and is typically stored in an inactive form[98–100]. During bone remodeling, in response to bone resorption, active IGF-1 is released from the bone matrix and signals the differentiation of MSCs into osteoblasts to promote type 1 collagen production and bone formation (**Figure 4B**)[99,100]. Following the achievement of PBM, IGF-1 also plays a role in maintaining bone mass equilibrium in adulthood, ensuring that resorption is coupled to formation[99,100]. In terms of acute exercise, studies involving high-intensity cycling bouts have found increases in IGF-1 within the first 15-40mins post-exercise, returning to pre-exercise (or decreasing below pre-exercise levels) well within the first hour following exercise[101–107]. By contrast, one study involving HIIC did not find an increase in IGF-1; this may relate to the long rest periods (5mins) between 30 sec high-intensity bouts[108]. Furthermore, IGF-1 seems to be less responsive in those with obesity compared to lean individuals, where moderate intensity cycling (65%HRR) showed no change in IGF-1 levels post-exercise, despite an increase in lean individuals[105]. Overall, HIIC may be expected to transiently increase IGF-1 in young and healthy adults.

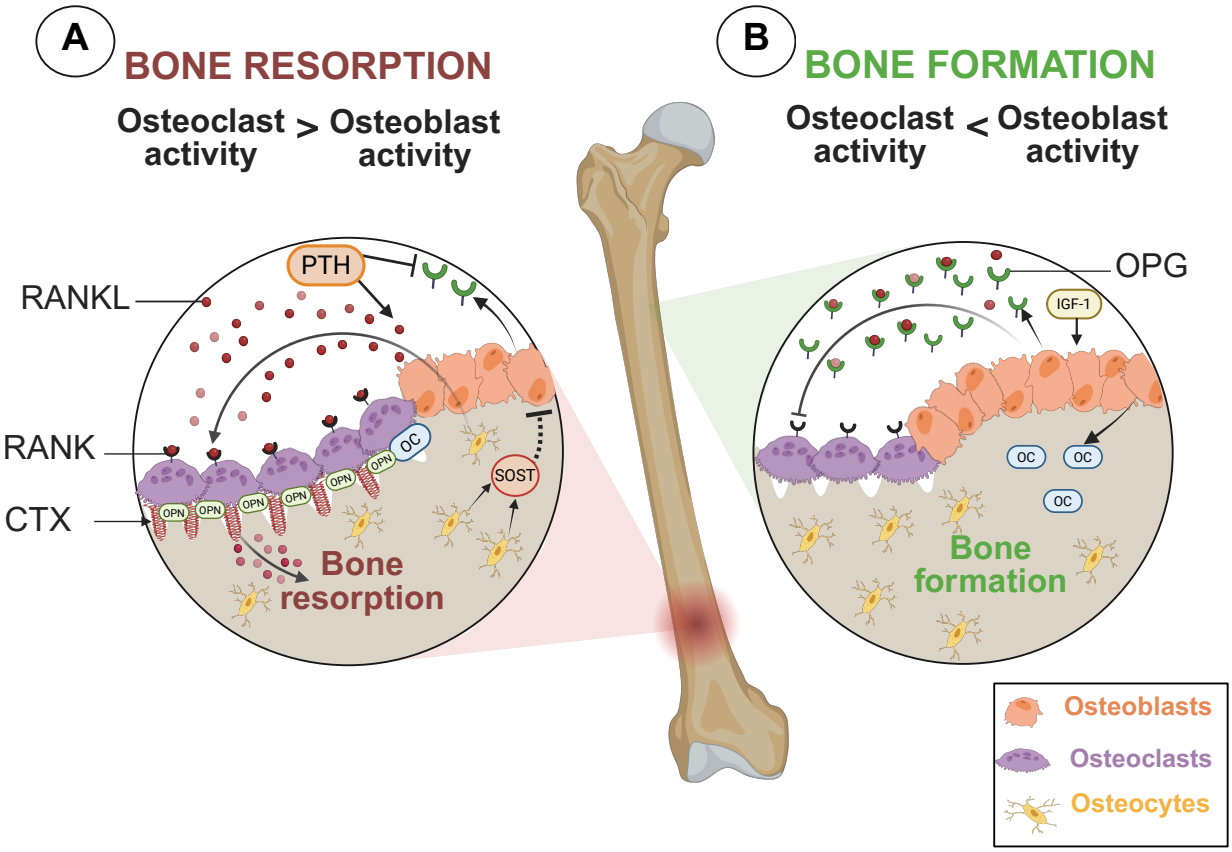


Figure 4: General overview of the mechanisms of bone turnover and metabolism markers. **A** depicts the binding of RANKL to its receptor RANK on osteoclasts, OPN helping to adhere osteoclasts to the bone surface, PTH facilitating RANKL production and blunting OPG production, and SOST blunting osteoblast activity (and the production of OPG), indirectly promoting osteoclast activity and bone resorption. The outcome of resorption is shown through the breaking down of OC and type 1 collagen protein (released as CTX). **B** depicts the production of OPG from osteoblasts, which binds to RANKL and prevents its binding to RANK and thus inhibits osteoclast activity, the production of OC from osteoblasts during bone formation and mineralization, and the role of IGF-1 in stimulating osteoblast activity and bone formation. Created with BioRender.com.

3.2.8 Summary of Bone Turnover and Metabolism Marker Responses to Acute High-Intensity Exercise

A summary of the direction and timing of the bone turnover and metabolism response following acute high-intensity exercise (i.e., plyometrics, and running or cycling exercises 70-95% HRR/HRmax/VO_2max [maximal oxygen uptake]) is outlined below in **Table 1**.

Table 1: Summary of the bone turnover and metabolism response following an acute bout of high-intensity exercise, without the influence of post-exercise nutrition in young adults.

Bone marker	Acute response to exercise
RANKL	• Increase immediately post-exercise[16,57]. • Return to pre-exercise by 1hr post-exercise[16,57].
OPG	• Increase immediately following exercise[16,20,53,55–57]. • Can return to pre-exercise levels by 1hr post-exercise[57] or remain elevated for 1-4hr[53,55,56].
PTH	• Increase immediately post-exercise[16,20,53,55,56,60–70]. • Return to or decreasing below pre-exercise levels within 15mins-2hr[16,55,56,58,60,61,63,66–68]. • In some cases a secondary increase can occur at 2-4hr[53] and 24hr post-exercise[61].
OPN	• Research is very limited, 1 study found a decrease immediately post-exercise, returning to pre-exercise levels by 25hr post-exercise[84].
CTX	• Increase[20,53,56,58,60,61,63,65,70,87,88] or no change[15,89,90] post-exercise. • Peak between 5mins – 2hr post-exercise[20,53,56,58,60,61,63,65,70,87,88].
SOST	• Increase immediately following exercise[15,16,20,53,66,88,89,92]. • Return to pre-exercise by 1hr post-exercise[15,16,66,88,89,92].
OC	• Increase immediately following exercise[16,20,53,55,65,97]. • Return to pre-exercise levels by 1hr post-exercise[16,55,65].
IGF-1	• Increase within 40mins post-exercise[101–104,106,107] or no change[108] post-exercise. • With the majority returning to pre-exercise levels within 1hr post-exercise[101–103,106,107].

3.2.9 Modulation of the Post-exercise Bone Turnover and Metabolism Response

Although the post-exercise bone turnover and metabolism response (specifically bone resorption, in response to mechanical or metabolic stimuli) plays a critical role in facilitating bone repair and adaptation to exercise (*via* breaking down microdamage resulting from exercise), attenuating its longer-term response and/or increasing bone formation in the hours following exercise may be beneficial in supporting osteogenesis and overall bone mass[12]. As seen in **Table 1** above, many studies see transient increases in post-exercise bone turnover and metabolism markers, including RANKL, OPG, PTH, CTX, SOST, OC, and IGF-1 immediately post-exercise. SOST, RANKL (its decoy receptor OPG), OC, and IGF-1 may experience a coupling of resorption to formation *via* a prompt rise and then return to pre-exercise levels within the first hour post-exercise; however, CTX and PTH may remain elevated for longer (as shown in **Table 1**), which can uncouple this response and drive a prolonged resorptive effect in the acute bout. It would therefore be advantageous to implement strategies aimed at modulating the bone turnover and metabolism response, with the goal of attenuating resorption while maintaining higher levels of formation regulators such as OPG (inhibitor of resorption) and IGF-1 in the hours and days following exercise, particularly in those at risk for bone fragility. One way that this may be accomplished is through post-exercise nutrition[12].

Carbohydrate supplementation (and consumption of energy in general) post-exercise is common and has been found to support bone metabolism to some extent[12,109,110]. Combined protein and carbohydrate supplementation has also been found to attenuate CTX in the 1 – 3hr post-exercise period following treadmill running at 75% VO_2max[61]. Despite this, at 3hr and 4hr post-exercise, PTH was found to increase and did not return to pre-exercise levels by 24hr post-exercise[61], suggesting that this supplement may not provide adequate bone benefit, possibly

because it lacks other nutrients, such as calcium, which could result in a longer-term blunting of this marker. Furthermore, dietary calcium is often needed in combination with protein to elicit positive effects on bone health[111]. Thus, calcium and protein may be required to prolong the suppression of CTX and PTH, potentially further decrease RANKL, SOST, and OPN, and/or increase OPG and IGF-1 post-exercise. Dairy products contain both protein and calcium, as well as other key bone-supporting nutrients, which, when consumed post-exercise, may additionally optimize the post-exercise bone turnover and metabolism response.

3.3 Bone-Supporting Nutrients and the Dairy Matrix

Dairy products such as milk, yogurt, and cheese provide a large proportion of bone-supporting nutrients including, calcium, protein, phosphorus, zinc, magnesium, potassium, and vitamin D (if fortified), compared to other foods[21,22]. For example, a serving of 2% milk contains 300 mg of calcium, which is 30% of the recommended dietary allowance ([RDA] 1,000mg/day[112]) for those aged 19 to 50 years. In terms of protein, plain 2% GY provides 16 g, which, for someone weighing 68 kg (RDA: 0.8g/kg of body weight/day for those 19 years of age and over[113]), this provides approximately 30% of the RDA. Of note, dietary protein can increase circulating IGF-1 levels, which in turn stimulates osteoblastogenesis, type 1 collagen production, and supports bone formation[21]. In addition, increased dietary calcium intake can decrease circulating PTH levels[21]. This plays a role in reducing osteoclast activity and preventing PTH-mediated bone resorption[44]. These positive benefits are likely amplified by the broader dairy matrix (i.e., the combination of these nutrients together) rather than the individual nutrients themselves. For instance, the physical composition (solid - cheese, liquid - milk, semi-solid - yogurt) and quantity and quality of macro- and micronutrients, as well as other bioactive compounds, fats, and carbohydrates (i.e., lactose) found within dairy wholefoods uniquely

interact together to influence digestion, absorption, and bioavailability of nutrients and thus their uptake and utilization by bone for microstructural improvements (outlined in **Figure 5**)[21,114]. Fermented dairy products (i.e., GY) also contain bacterial cultures, which, in addition to promoting a healthy gut microbiota, have also been found to be beneficial for bone health through their ability to facilitate intestinal calcium absorption and immune system modulation, which can reduce bone resorption[21,114]. Indeed, yogurt and/or milk consumption as part of the habitual diet is correlated with increased circulating IGF-1 levels[115], and decreased PTH and CTX levels, as well as higher BMD at the hip and lumbar spine[116]. Thus, not only can the consumption of these nutrients through a singular food be more accessible and affordable compared to individual nutrient supplementation, but the dairy matrix itself also provides additional benefit through the interactions within its components[114,117]. Recently, Hilkens et al.[118] assessed the effects of isoenergetic breakfasts containing varying amounts of dairy (no dairy, 1 dairy product, and 2 dairy products) on circulating bone turnover and metabolism markers in young and healthy adults. CTX was found to decrease in all 3 groups 180mins postprandially[118]. However, in the dairy groups, CTX continued to decline at 240mins and remained below baseline at 300mins postprandially compared to the no dairy group[118]. PTH total area under the curve (tAUC) was also significantly lower in the dairy groups compared to the no dairy group[118]. This provides evidence suggesting that young and healthy individuals can benefit from the addition of dairy products into their habitual diets to support bone turnover and metabolism *via* attenuated bone resorption in the acute postprandial period. Furthermore, dairy consumption provides the bone tissue with key nutrients and building blocks to facilitate formation, which may help to offset the acute post-exercise resorptive response or promote a coupling of these processes (resorption=formation).

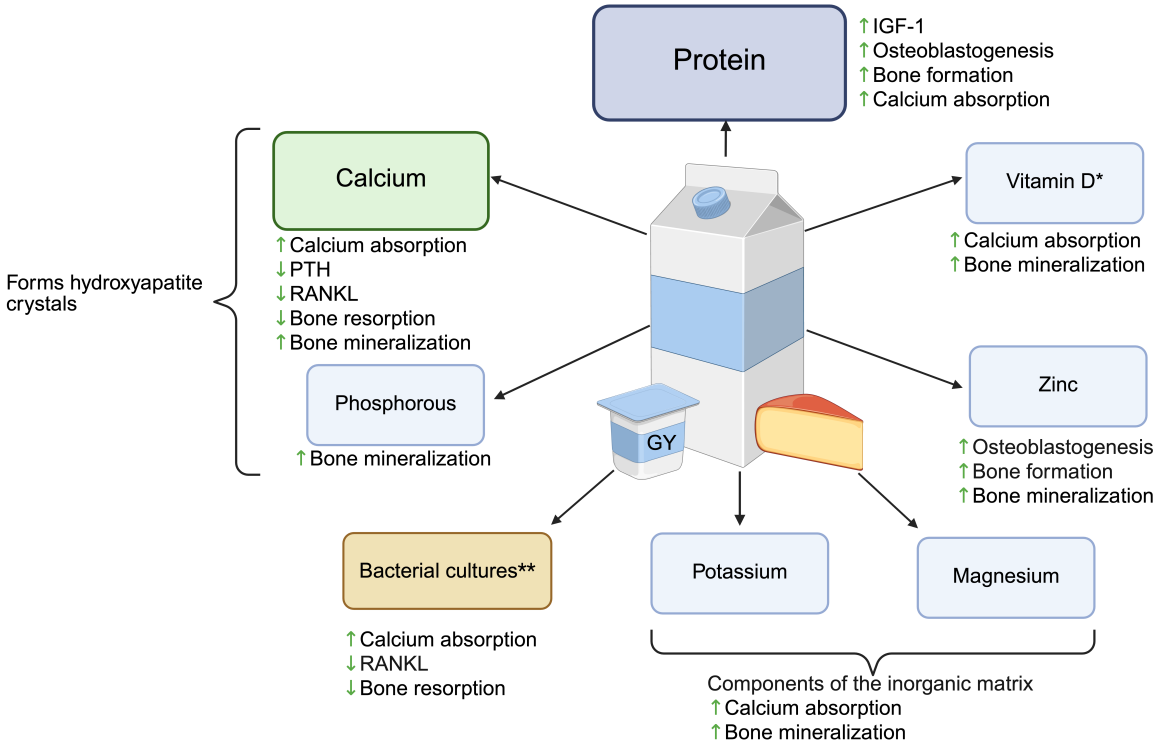


Figure 5: The Dairy Matrix, bone-supporting nutrients, and their effects on bone turnover and metabolism. Abbreviations: GY (Greek Yogurt), * if fortified, **if fermented. Information summarized from Rizzoli[33] and Palacios[119]. Created with BioRender.com.

3.4 The Bone Turnover and Metabolism Response to Exercise and Dairy Consumption in Young and Healthy Adults

3.4.1 Short-term Exercise and Dairy Consumption

Short-term/acute studies assessing the impact of dairy on the bone turnover and metabolism response to exercise are sparse, with only three studies being conducted to date, two of which involve young athletes (i.e., trained female adolescent soccer players and young premenopausal female cyclists). In a crossover trial, Klentrou and colleagues compared the differential effects of GY and isoenergetic carbohydrate pudding supplementation during a 5-day soccer training camp (90mins/day of high volume and intensity soccer training) on bone turnover and metabolism markers in adolescent female soccer players[120]. While total OC, CTX, OPG,

RANKL, and OPG/RANKL ratio remained unchanged across time and between arms, absolute concentrations of unOC decreased in the GY arm but not in the carbohydrate arm, potentially reflecting lower bone turnover and resorption with GY supplementation[120]. This short-term study compared rested pre-training and post-training (after the 5-day camp) bone turnover and metabolism concentrations, which does not reflect how these markers fluctuated following a single bout of exercise. In addition, this population is in a period of the lifespan marked by bone growth, thus such responses are likely to differ among adults who have already achieved PBM. In premenopausal female cyclists, Haakonsen et al.[23] assessed the impact of a pre-exercise calcium rich dairy meal (~1350mg calcium, incorporating a serving of oats cooked with fortified milk, yogurt, and an additional serving of milk) versus a control meal (~50mg calcium, consisting of oats, fruit, and mixed nuts) on bone turnover and metabolism markers following an acute cycling bout. The calcium rich dairy meal or control meal was consumed 2hr prior to prolonged, high-intensity stationary cycling (60% of baseline maximal aerobic power for 80mins followed by 10mins cycling at the highest intensity possible)[23]. Given the long duration of the exercise, cyclists also consumed carbohydrate gels during exercise[23]. Both PTH and CTX were found to increase immediately post-exercise; however, this response was attenuated and was further decreased at 40mins post-exercise in the calcium rich dairy meal group versus the control meal group, as well as at 190mins post-exercise for CTX only[23]. This study demonstrated that a calcium-based meal consumed pre-exercise may attenuate the post-exercise bone resorption response and suggests that calories alone (control meal; ~50mg of calcium) may not provide enough bone-supporting nutrients (notably calcium) to improve acute bone turnover and metabolism outcomes following a prolonged high-intensity cycling bout. These two studies provide some evidence of favourable modulation of bone turnover and metabolism with dairy

supplementation; however, given the populations studied (trained individuals and adolescent athletes), these responses are likely not entirely reflective of the general adult population. To date, only 1 study has been conducted evaluating the acute effects of post-exercise dairy consumption in young and healthy untrained premenopausal females. Prowting et al.[24] conducted a crossover trial involving plyometric and resistance exercise (198 impacts; 3-4 sets, 8-12 reps at 75% 1-repetition max) along with the consumption of skim milk or an isoenergetic carbohydrate drink immediately and 75mins post-exercise. While no trial effect or time x trial interaction was observed in the absolute or relative concentrations of OC, SOST, OPG, RANKL, or OPG/RANKL ratio, relative CTX (% change) AUC was found to be lower in the milk arm compared to the carbohydrate arm[24]. Therefore, post-exercise milk consumption positively impacted bone turnover and metabolism *via* the blunting of CTX compared to carbohydrate in premenopausal females. Overall, these results provide evidence to suggest that dairy consumption in the acute period around an exercise bout can modulate the post-exercise bone turnover and metabolism response (*via* attenuating resorption), in young premenopausal females (trained and untrained). More research is required in other populations, including those with overweight and obesity (OW/OB).

3.4.2 Long-term Exercise and Dairy Consumption

Two studies have compared the effects of 12 weeks of resistance training and post-exercise dairy supplementation versus carbohydrate on circulating markers of bone turnover and metabolism. These studies supplied dairy either in the form of milk in young premenopausal females or GY in young adult males and found a decrease in fasted/rested PTH[25] as well as a greater increase in the bone formation marker procollagen type 1 N-terminal propeptide (P1NP)[26] post-intervention compared to the carbohydrate group, respectively. In addition, in the

study conducted in young adult males with GY supplementation, while CTX decreased in both groups post-training, a trend (p=0.062) was seen between groups, whereby carbohydrate had higher CTX one week into the study, with no change in the GY group[25]. This suggests a potential initial blunting effect of bone resorption with GY in the early stages of the training intervention[25]. Overall, chronic dairy consumption post-exercise may result in positive bone health adaptations through either reductions in mechanisms of bone resorption (PTH) or a net bone formation effect (P1NP) in young and healthy adults.

Taken together, the modulation of the acute and longer-term bone turnover and metabolism response with dairy consumption post-exercise, among females especially, may help optimize bone mass maintenance following the achievement of PBM and thus may improve bone health before the onset of menopause. Such improvements would be beneficial for those at risk for additional poor bone health outcomes, including females with OW/OB.

3.5 Impact of Obesity on Bone Health in Premenopausal Females

Body mass and body fat levels can affect bone metabolism and overall bone health. However, this relationship is nuanced and not completely understood. Within the literature, low body weight/'underweight' (body mass index [BMI] <18.5 kg/m^2) is consistently related to low bone mass and higher risk of fracture compared to those of 'normal weight' (BMI of 18.5 kg/m^2 to 24.9 kg/m^2)[121–127]. Research has previously reported 'overweight' (BMI of 25 kg/m^2 to 29.9 kg/m^2) and 'obesity' ([OB] BMI ≥ 30 kg/m^2) to be positively related to bone mineral density [BMD] or bone mineral content[128–135], and to be inversely related to bone turnover[136,137] and bone loss[138–141]. Furthermore, younger individuals with obesity (OB) tend to have higher muscle/lean mass compared to their lean counterparts[142–144]. It has been hypothesized that the increased mechanical strain and force supplied by the muscle from excess weight, loads the bone

tissue, and may manifest in a higher BMD[142]. This anatomical/physical connection between muscle (acting as pulleys) and bone (acting as levers) also stimulates the hormonal/physiological interplay between these two tissues as signalling molecules and factors are released from bone (osteokines) and muscle (myokines), which have synergistic effects resulting in either catabolism (degradation/resorption) or anabolism (growth/formation)[145,146]. One such example is IGF-1, which can be released from both tissues and relates to muscle growth and bone formation[145,146]. Adipose tissue plays a role in mediating the bone-muscle 'crosstalk' through the secretion of its own cytokines (i.e., adipokines like interleukin-6 [IL-6], and tumour necrosis factor-alpha [TNF-α])[146]. While it has been postulated that excess adiposity provides padding to protect bones from fracture[147], adipokines like TNF-α and IL-6, released from the adipose tissue, can result in bone tissue resorption[146]. As previously discussed, osteoblasts, myocytes, and adipocytes are derived from the same mesenchymal stem cells (MSCs). In a suboptimal environment (i.e., poor nutrition, sedentary lifestyle/decreased physical activity, and low-grade inflammation [in conditions like OB]), an increase in pro-inflammatory cytokines suppresses MSC osteoblastogenesis and promotes adipogenesis[148]. As a result, excess adiposity can contribute to bone loss and further fat accumulation (i.e., bone marrow fat infiltration, as well as accumulation around organs [abdominal fat]), which further maintains chronic low-grade inflammation[148]. This negative bone-adipose tissue 'crosstalk' can modify the structure and function of bone tissue by accelerating bone turnover (i.e., increasing osteoclast activity and bone resorption), uncoupling bone resorption from formation, resulting in increased bone fragility, ultimately increasing the risk of fracture, and assisting in the pathogenesis of diseases, such as osteopenia or osteoporosis[5,79,149]. The influence of OB on select bone turnover and metabolism markers is further outlined in **Table 2**. Therefore, despite potentially higher 'quantity' and density of bone

tissue, i.e., higher BMD due to higher lean/muscle mass and mechanical loading, the quality of the bone tissue (i.e., bone microstructure) is likely negatively impacted by excess adiposity, especially when coupled with an unhealthy metabolic profile (i.e., increase in pro-inflammatory adipokines and cytokines, abdominal fat, and bone marrow fat infiltration)[149]. This phenomenon is also known as the 'obesity paradox'[5] which is characterized by a U-shaped relationship between bone fragility risk and BMI/body mass. The right end of the curve specifically pertains to OW/OB, characterized by increased adiposity and its associated pro-inflammatory profile, which increases bone fragility risk among these individuals[79,121,149,150] (outlined in **Figure 6**). Given that females generally achieve a lower PBM (vs. males) and have a higher propensity to lose bone mass and strength following menopause, maintaining bone mass in young to middle adulthood is of utmost importance. Furthermore, the condition of OW/OB may be especially detrimental to females during this critical period. Indeed, in premenopausal females with OB (body fat > 30%), a negative correlation has been found between fat mass and BMD, thus any bone benefit achieved from a larger absolute muscle mass is likely lost with increasing adiposity[151]. In addition, premenopausal females with OB have been found to have lower relative bone strength, and lower relative bone mass and area, despite subjecting their bones to higher absolute forces[152]. Overall, it seems that chronic low-grade inflammation via TNF-α and IL-6, abdominal fat, and excess bone marrow fat infiltration that tends to accompany OB can increase bone fragility through weakening of the bone microstructure, despite presentation of a higher BMD in some cases[5]. It is therefore crucial to maintain a healthy body weight (i.e., 'normal weight/BMI' and healthy body composition) or adopt healthy lifestyle modification strategies (e.g., dairy consumption and exercise) to combat bone fragility, maintain bone mass equilibrium

(resorption=formation), and improve bone microstructure in females with OW/OB in young to middle adulthood, to attenuate osteoporosis risk later in life[3,153].

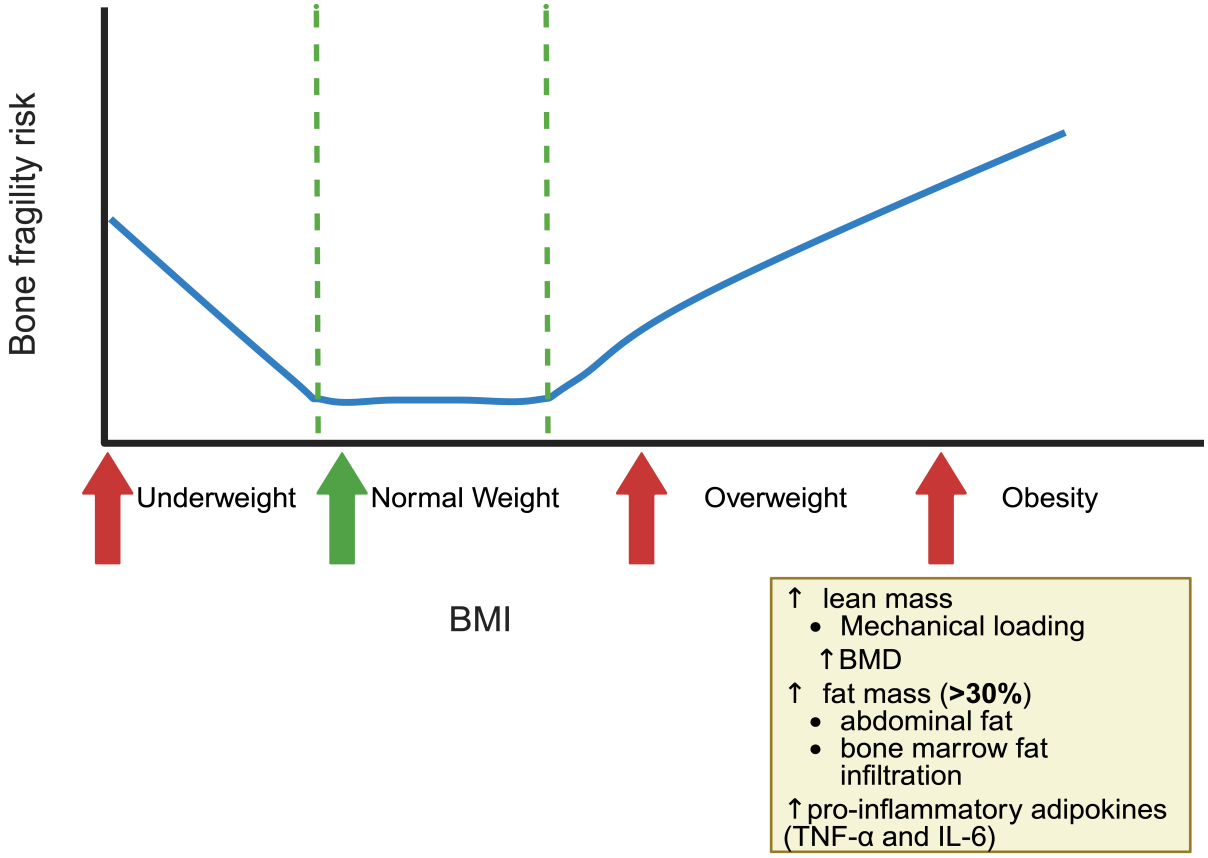


Figure 6: U-shaped relationship between bone fragility risk and BMI. Shows increased fragility risk with underweight, lowest risk with normal weight, and increasing risk with OW and OB. Despite higher BMD in some cases likely attributed to greater absolute muscle/lean mass, higher body fat percentage especially when localized in the abdominal area and within the bone marrow can result in a pro-inflammatory environment through the release of TNF-α and IL-6 , ultimately increasing bone fragility risk[5]. Adapted from De Laet et al.[121] Created with BioRender.com.

Table 2: Summary of the relevant mechanisms and actions of bone turnover and metabolism markers, as well as the influence of obesity (OB) on these markers.

Bone marker	Primary mechanism	Secreted by/released from	Relevant actions	Influence of Obesity
RANKL	Osteokine; resorption	Osteocytes (main) and osteoblasts	Binds to RANK receptors on osteoclasts to facilitate the differentiation, proliferation, and activation of osteoclasts and subsequent bone resorption[44,46].	The RANKL/RANK/OPG axis has been found to be dysregulated, higher RANKL to OPG, which can lead to a weakening of the bone microstructure in individuals with OB[79,154].
OPG	Osteokine; negative regulator of resorption	Osteoblasts (main) and osteocytes	Acts as a decoy receptor for RANKL to prevent its binding to RANK, therefore blunting bone resorption[44].	↓ OPG in young adults with OB compared to lean counterparts[155–157]. Resorption may be upregulated due to the uncoupling of OPG and RANKL, leading to bone loss[155–157].
PTH	Hormone; mainly resorption	Parathyroid glands	Released in response to low circulating calcium, resulting in resorption to mobilize calcium stores from the bone tissue[44].	↑ PTH levels in those with OB, either due to decreased intestinal calcium absorption or sequestration in the adipose tissue, leading to increased osteoclast activity and bone resorption[158,159].
OPN	Osteokine; mainly resorption	Osteoblasts (main) and osteocytes	2nd most abundant NCP in the bone matrix[37]. Plays a role in resorption by binding osteoclasts to the bone matrix[160] and inhibits mineralization[44,47].	↑ OPN in those with OB[77,161–163], ↑ levels of OPN have been found in the adipose tissue[77].
CTX	Biochemical marker of bone resorption	Extracellular matrix	Type 1 collagen makes up ~90% of the organic matrix, higher levels of its by-product (CTX) is indicative of type 1 collagen breakdown, and thus bone resorption[46].	↓ CTX[158] and lower net turnover, including reduced formation, contributing to a weaker bone microstructure in those with OB[164].
SOST	Osteokine; negative regulator of bone formation	Osteocytes	Inhibits the β-catenin Wnt pathway by binding to LRP 5 or 6, blunting the differentiation of osteoblasts, OPG production, and ultimately bone formation[44].	↑ SOST in individuals with OB[165–168]. Pro-inflammatory environment of adipose tissue can increase release of SOST and indirectly increase bone resorption[169].

OC	Osteokine; marker of formation (via cOC) or turnover/resorption (via unOC); total OC is turnover	Mature osteoblasts	Most abundant NCP in the bone matrix[37,46]. Contributes to the organization of the bone matrix through binding calcium in hydroxyapatite crystals, helping to regulate mineral deposition and crystal formation[93].	↓ total OC[170,171] and lower unOC and cOC[94]. This is thought to relate to higher systemic inflammation resulting in lower bone turnover (including bone formation)[94,170].
IGF-1	Growth factor; mainly formation	Osteoblasts (locally at the bone)	Active IGF-1 signals the differentiation of MSCs into osteoblasts to promote bone formation[99,100].	↓ IGF-1 levels in those with OB[158,172]. Higher bone marrow fat infiltration is inversely correlated with IGF-1 and BMD in OB[173].

3.6 Dairy Consumption and Exercise in Females with OW/OB

To date, two studies have evaluated the bone turnover and metabolism response to increased dairy consumption and exercise training, one in adolescent females and the other in premenopausal females with OW/OB. Josse et al.[29] and Kurgan et al.[27] assessed the effects of a 12-week high dairy diet (4 servings/day; mix of milk, cheese, and GY) versus a low dairy diet (0-2 servings/day) in combination with individualized multimodal exercise training (consisting of plyometrics, aerobic, and resistance exercise). Josse et al.[29] compared resting/fasted bone markers pre-intervention to post-intervention between the high dairy and low dairy groups. Relative CTX and OC (% change) were lower following the intervention in the high dairy group compared to the low dairy group[29], indicating that increased dairy consumption decreased bone turnover *via* resorption in combination with exercise training. Kurgan and colleagues evaluated the acute response to plyometric exercise before and after the 12-week intervention in the same participants to assess how these training adaptations affected the acute response to exercise[27]. At pre-intervention, relative RANKL (% change) increased in response to the acute plyometric exercise bout in the high dairy group only[27]. At post-intervention, there were no differences

between the high dairy group and the low dairy group in the relative RANKL response, indicating that the high dairy group had a blunted RANKL response post-intervention (i.e., a reduction from the initially elevated response), while the low dairy group remained unchanged[27]. In addition, the relative OPG/RANKL ratio followed a similar pattern to that of relative RANKL in that it was lower following acute exercise in the high dairy group compared to the low dairy group at pre-intervention[27]. The high dairy group showed an increase in the OPG/RANKL ratio post-intervention, reflecting recovery from the initially lower value, whereas the low dairy group remained unchanged[27]. While it is unclear why the high dairy group had higher RANKL and a lower OPG/RANKL ratio at pre-intervention versus the low dairy group, the adaptation in the high dairy group only provides some evidence to suggest that exercise training and a high dairy diet can improve the bone turnover and metabolism response to exercise. However, since adolescence is a period in the lifespan marked by rapid rates of bone growth, these results cannot be extended to adult premenopausal females (who have already achieved PBM) with OW/OB.

Josse et al.[28] conducted another study in young premenopausal females with OW/OB, involving a 16-week weight loss lifestyle intervention in which 3 groups completed multimodal exercise training (aerobic activity 5 days/week and resistance exercise 2 days/week). The 3 groups consumed hypocaloric diets differing in protein and dairy content: (1) high protein and high dairy group (30% protein, ~1600 mg/day calcium), (2) adequate protein and medium dairy group (15% protein, ~1000 mg/day calcium), (3) adequate protein and low dairy (15% protein, ~500 mg/day calcium). Following the intervention, CTX increased in the adequate protein and low dairy group, while P1NP increased in the high protein and high dairy group[28]. OPG/RANKL and P1NP/CTX ratios were also increased in the high protein and high dairy group, whereas P1NP/CTX ratio decreased in the adequate protein and low dairy group[28], demonstrating that

higher dairy and protein consumption in combination with exercise training during weight loss can increase formation over resorption, whereas adequate protein and low dairy with exercise training is not able to attenuate the bone resorption response with weight loss. PTH also decreased in the high protein and high dairy and adequate protein and medium dairy groups[28], suggesting that calcium consumption ≥1000mg/day was able to blunt resting PTH-mediated bone resorption. This study provides further evidence to suggest that higher dairy product intakes (and thus higher protein and calcium intakes) cannot only maintain bone health in situations where bone loss typically occurs (i.e., with weight loss) but can also increase bone formation (with exercise) in a population at risk for poor bone health outcomes.

The evaluation of bone remodeling activity through the measurement of bone turnover and metabolism markers in response to exercise and dairy consumption has only been assessed in a handful of studies, as outlined above. Importantly, while long-term exercise training and dairy consumption have led to the beneficial modulation of bone turnover and metabolism markers among females with OW/OB (adolescents[27,29] and premenopausal females undergoing weight loss[28]) as well as acutely in premenopausal females of normal weight[24], no studies to date have evaluated the acute response to post-exercise dairy consumption in premenopausal females with OW/OB. As also mentioned, HIIC and HIIR have been found to elicit the same bone turnover and metabolism response in young premenopausal females[15,16]. Given that high-intensity interval exercise is preferred (time efficient)[9–11], safer[18], more comfortable (HIIC specifically)[19], and has been found to improve cardiorespiratory fitness[11] among those with OW/OB, it may serve as the ideal exercise in such individuals. GY specifically may be an optimal post-exercise supplement as it contains calcium and other bone-supporting micronutrients, as well as a higher protein to carbohydrate ratio, which may be beneficial for glucose handling among those at risk for

metabolic dysregulation compared to other dairy products such as milk. In addition, the bacterial cultures found in GY can increase intestinal calcium absorption and digestion[21,114] and may thus facilitate the modulation of the bone turnover and metabolism response post-exercise.

Chapter 4: Methods

This study was primarily designed to assess the effects of post-exercise nutrition on glycemia in young- to middle-aged inactive premenopausal females with OW/OB. This secondary analysis investigated the effects of post-exercise nutrition (GY vs. CP) on markers of bone turnover and metabolism.

4.1 Participants

Twenty healthy but inactive young- to middle-aged premenopausal females (18-45 years) with OW/OB (BMI $\geq$ 27 kg/m^2) were recruited from the University of Toronto (Ontario, Canada). Posters were placed across the University of Toronto's St. George Campus, as well as on social media (e.g., Facebook), and interested individuals were prompted to fill out an online questionnaire (linked to REDCap) to ensure they met the eligibility criteria outlined in **Table 3**. If the prospective participant appeared to meet the criteria, a member of the research team contacted them to arrange a virtual introduction. This research study was approved by the University of Toronto Research Ethics Board, where data collection and primary analysis (glycemia) were conducted (in the Goldring Centre) and subsequently by the York University Research Ethics Board (REB # 42474), where the bone turnover and metabolism marker analysis (secondary analysis) took place (in the Farquharson Life Sciences Building). This study met all standards of Canada's Interagency Panel on Research Ethics for conducting human research.

Table 3: Eligibility Criteria.

Inclusion Criteria	Exclusion Criteria
▪ BMI ≥ 27 kg/m^2 ▪ Waist circumference ≥88cm ▪ ≤2 structured exercise sessions/week ▪ Stable weight for the past 6 months (±2kg) ▪ Experiencing regular menstrual cycles ▪ Non-smoker	▪ Allergy to dairy foods or lactose intolerance ▪ History of cardiovascular or metabolic disease ▪ The use of medication for managing blood glucose or lipid metabolism ▪ Current use of oral contraceptives or use within the last 3 months ▪ Irregular menstrual cycles (<21 days or >35 days) ▪ Pregnant or lactating ▪ Recreational tobacco or cannabis use ▪ Inability to perform exercise ▪ Actively engaging in a low-carbohydrate diet (e.g., ketogenic, Atkins)

Abbreviations: BMI: Body mass index.

4.2 Baseline Measurements and Familiarization Sessions

4.2.1 Virtual Introduction

Following the completion of the online form, individuals who appeared eligible were asked to attend a virtual introduction session *via* Zoom. This meeting consisted of reviewing study objectives, protocols, and associated risks. Potential participants were then asked to review the consent form, ask any relevant questions, and provided informed consent by signing the form. Physical activity levels and ability to perform exercise safely were assessed through the International Physical Activity Questionnaire and the CSEP Get Active Questionnaire, respectively. The first in-person session was then scheduled for those who appeared eligible thus far and agreed to participate.

4.2.2 Baseline Testing and Exercise Familiarization

Prospective participants arrived at the lab fasted (~10-12 hours). As a final step to confirm eligibility, waist circumference, height, and weight were measured to ensure that participants met the waist circumference and BMI cutoffs outlined in the inclusion criteria

(**Table 3**). If all inclusion criteria were satisfied at this point, the participant was formally enrolled in the study. Enrolled participants then underwent a body composition assessment using the BodPod (Cosmed USA Inc., Concord, CA) to determine whole-body fat mass, fat-free mass, and percent body fat. Next, participants performed an incremental cycling exercise test (Velotron, Racermate cycle ergometer, Seattle, WA) outlined in **Figure 7** to determine peak oxygen uptake (VO_2peak) and to inform the maximal heart rate (HRmax) and peak power output (PPO) targets for the HIIC performed during the acute metabolic trials. Participants then completed a familiarization session, which included a shorter bout of HIIC (4x1-min intervals) in order to familiarize them with the HIIC exercise bout that they would be required to perform during the metabolic trials. Participants were also asked to complete a 3-day food diary to allow for the determination of their habitual energy intake (using ASA 24[174]).

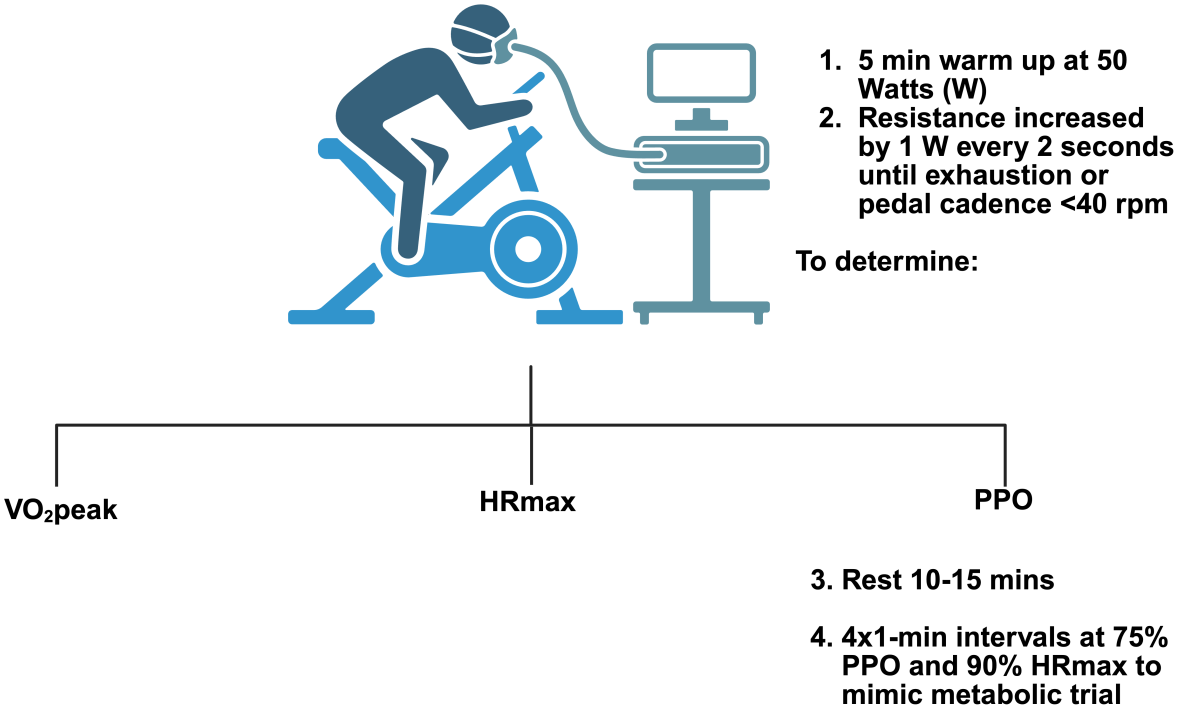


Figure 7: Incremental exercise testing and familiarization session. Abbreviations: rpm: revolutions per minute. Created with BioRender.com.

4.3 Acute Metabolic Trials

This study utilized a within-subject, randomized crossover design in which participants completed two acute metabolic trials in random order: 1) HIIC + GY, and 2) HIIC + CP. Each of the two metabolic trials occurred over a 2-day period (**Figure 8**). In the days leading up to the metabolic trials, participants were instructed to maintain habitual physical activity levels and were also provided with standardized meals (outlined below) to be consumed on both the day prior to and Day 1 of the metabolic trials. They were also instructed to only consume caffeine with lunch, both during (Day 1) and the day before each metabolic trial. During this time, participants also received their continuous glucose monitor, in accordance with the primary outcome of the study (not relevant to the analysis presented in this thesis). On the morning of Day 1 and Day 2 (between 7:30 am and 9 am), participants arrived at the lab following a 10-12hr overnight fast before completing the HIIC + GY or HIIC + CP (Day 1) and the 24-hr blood sample (Day 2), respectively. The two arms of the study (HIIC + GY and HIIC + CP) were conducted during the early- to mid-follicular phase (days 3-11) of the menstrual cycle, and a 4-week washout period separated the trials.

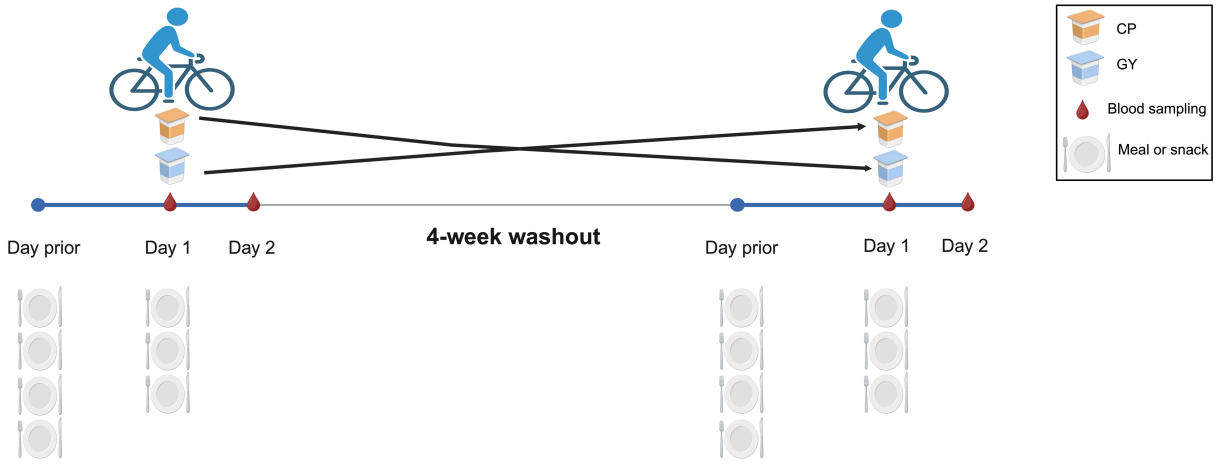


Figure 8: Study timeline including the day prior to and days of the acute metabolic trials (Day 1 and 2). Also outlines the randomized crossover design and 4-week washout period. Created with BioRender.com.

4.3.1 Standardized Diet

Total daily energy requirements for each participant were estimated using the Mifflin-St. Jeor equation and multiplied by a factor of 1.3 to account for activities of daily living, yielding total daily energy expenditure[175]. The estimated total daily energy expenditure and the mean energy intakes of the 3-day food diary (assessed during baseline testing, outlined above) were averaged together to ensure that standardized meals were truly representative of each participant's habitual energy intake and needs. Five meals, two snacks, and one post-exercise supplement were provided prior to and during each metabolic trial. Meals consisted of ~55% carbohydrate, ~30% fat, and ~15% protein; each meal provided ~28% of total daily energy requirements, and afternoon snacks contributed the remaining 15% of total daily energy requirements. Meal and snack composition was as similar as possible between participants and consisted of foods typical of the western diet, including frozen ready meals, fruits, vegetables, sandwiches, and granola bars. The day prior to each metabolic trial, participants consumed 3 meals and 1 snack at home, with instructions to consume breakfast at ~9:00am, lunch at ~1:00pm, afternoon snack at ~4:15pm, and dinner at ~7:30pm. On Day 1 of the metabolic trial, participants arrived at the lab fasted for HIIC + GY or HIIC + CP and blood sampling. At the end of Day 1 (after the 3hr blood draw), participants were provided with lunch, a snack, and dinner to be consumed at the same times as detailed above. Adherence to the standardized diet was evaluated virtually, with participants submitting a photo to a study investigator immediately following each meal.

4.3.2 Exercise Protocol

The exercise bout for GY and CP occurred on Day 1 and consisted of the same HIIC exercise protocol for both trial arms. Fasting blood samples were collected, and then HIIC

commenced using the same cycle ergometer as the incremental exercise test and familiarization session (**Figure 7**). First, participants completed a 3-min warm-up at 50 W, followed by the HIIC bout consisting of 10x1-min cycling bouts at 75% PPO and 90% HRmax interspersed with 1-min of active recovery cycling (at lower intensity) (19mins total), similar to previous studies[15,16,176]. Lastly, participants completed a 3-min cool down at 50 W, making the total exercising time 25mins. Heart rate was measured with a Polar H7 monitor (Polar Electro Oy, Kempele, Finland), and the rating of perceived exertion (RPE) was measured using the 20-point Borg RPE Scale[177].

4.3.3 Supplement Protocol

Participants consumed 2 servings of plain 2% GY (Oikos) for the GY trial, and for the CP trial, participants consumed an isoenergetic CP supplement consisting of maltodextrin and sugar-free chocolate pudding mix (flavoured for palatability) (**Table 4**). Both supplements were consumed 15mins post-exercise (**Figure 5**).

Table 4: Nutrient breakdown of post-exercise trial supplements.

	GY	**CP**
Mass (g)	350	102
Energy (kcal)	240	238
Carbohydrates (g)	14	63
Protein (g)	32	0
Fat (g)	7	0
Calcium (mg)	350	0

4.3.4 Blood Sample Collection

Blood samples were obtained using an intravenous catheter placed in a forearm vein on Day 1 (for pre-exercise to 3hr postprandial sampling), and a butterfly needle on Day 2 (for the

24hr post-exercise blood sample) of both metabolic trials. A trained phlebotomist performed all blood draws, and a total of 6mL of blood was collected (using uncoated vacutainers) across the following timepoints: pre-exercise (baseline), immediately post-exercise, 1hr and 3hr postprandially, and 24hr post-exercise (**Figure 9**). After each blood draw, samples rested at room temperature for 30 to 60mins in order to clot, before being centrifuged (Eppendorf Centrifuge 5804 R, Eppendorf Canada, Mississauga, ON) for 15mins at 4°C at 1500 Relative Centrifugal Force (RCF). Serum was aliquoted into Eppendorf tubes and stored at -80°C until data collection was complete.

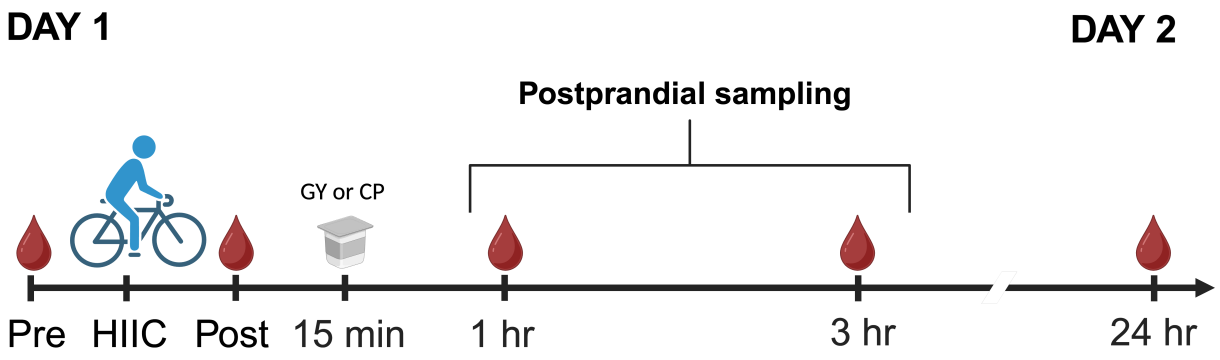


Figure 9: Timeline for Day 1 & 2 for each acute metabolic trial (GY and CP), indicating the time of blood collection, HIIC, and post-exercise supplement. Created with BioRender.com.

4.4 Blood Sample Analysis

Using the Luminex 200 (Luminex Corp., Austin, TX, USA), OPG, PTH, OPN, SOST, and OC were measured in duplicate with microbead multiplex assay kits (Human bone magnetic bead panel, cat# HBNMAG-51K-05; EMD Millipore Corporation, Etobicoke, ON), RANKL was measured in duplicate with microbead single plex assay kits (Human RANKL magnetic bead – single plex, cat# HRNKLMAG-51K-01; EMD Millipore Corporation, Etobicoke, ON). Using a BioTek Synergy LX plate reader (Agilent Technologies, Santa Clara, CA, USA), CTX and IGF-1 were measured with enzyme-linked immunosorbent assays (ELISAs) (Human CTX-1 ELISA

Kit, cat# NBP2-69073, Novus Biologicals, Toronto, ON; Human IGF-I/IGF-1 Quantikine® ELISA Kit, cat# DG100B, R & D Systems, Minneapolis, MN, USA). Belysa analysis software (EMD Millipore Corporation, Etobicoke, ON) was used to optimize standard curves, which allowed for consistent calculations of concentrations across plates. Average intra-assay coefficients of variation (CVs) were 13.8% for RANKL, 4.9% for OPG, 5.7% for OC, 4.2% for SOST, 4.9% for PTH, 5.6% for OPN, 3.4% for IGF-1, and 13.9% for CTX. In cases where the duplicate CV was above 15%, the value that closely aligned with the mean pattern of change of the participant was used for analysis (115/3022 singlets were removed, 3.8%). This process was completed in a blinded fashion to avoid bias.

4.5 Statistical Analysis

Before statistical analysis, markers were assessed for normality using skewness and kurtosis z-scores, and scores ± 3 were considered for transformation. Absolute concentrations of CTX were not normally distributed and were thus log-transformed to improve normality. Net incremental area under the curve (iAUCs) for the OPG/RANKL ratio, CTX, and PTH were not normally distributed, so nonparametric tests were performed. Data were also treated for outliers (± 2 SD; 10 datapoints/marker) if a datapoint fell outside of this range, it was replaced with the mean ± 2 SD value (i.e., the extreme 2SD limit).

Linear mixed-effects models (LMMs) were conducted on the absolute concentrations of bone turnover and metabolism markers (log-transformed values for CTX) to assess main fixed effects of time (pre-exercise, post-exercise, 1hr and 3hr postprandially, as well as 24hr post-exercise) and trial (GY, CP), as well as their interactions (time x trial) (Objective A). Subject ID, subject ID x time, and subject ID x trial were included as random effects. If significant interactions or main effects were found, post hoc analyses (paired t-tests) were performed with a

false discovery rate (FDR) (two-stage step-up Benjamini, Krieger, and Yekutieli method) correction for multiple comparisons. In cases where blood samples were not obtained (11 out of 200 samples across the study), the mixed model used restricted maximum likelihood (REML) to account for missing data by using all available observations to estimate fixed effects without imputing missing values.

Paired t-tests were conducted on the net iAUCs of OPG, RANKL, SOST, OC, OPN, and IGF-1 (normally distributed) and Wilcoxon matched-pairs signed rank tests for OPG/RANKL ratio, CTX, and PTH (not normally distributed) to determine the overall net response of each marker (relative to baseline/pre-exercise) for GY vs. CP from post-exercise until 24hr post-exercise (Objective B). One-sample t-tests were used to compare the mean net iAUC for each trial to a hypothetical value of zero, which represents no change from baseline/pre-exercise across the measured time-period. Paired t-tests were also used to compare the average %HRmax and %PPO achieved during HIIC in both trial arms (GY or CP) and the average menstrual cycle day in which the HIIC was completed for GY vs. CP.

Significance was considered to be reached at $p<0.05$, and a trend was considered to be reached at $p<0.1$. Corrected p-values from the FDR (i.e., q-values) are reported throughout the Results section as post hoc test p-values. Statistical analyses were performed on Prism version 10.5.0 for macOS (GraphPad software, San Diego, California, USA).

Chapter 5: Results

5.1 Participants

Baseline characteristics are shown in **Table 5**. Nine participants completed the GY trial before the CP trial, while the remaining eleven participants completed the CP trial before the GY trial. Day 1 of each trial arm (HIIC + GY or HIIC + CP) was conducted on the $5^{th} \pm 1$ day of the menstrual cycle. Trials were separated by 28 ± 13 days.

Table 5: Participant baseline characteristics.

Characteristic	Value
Age (y)	36.3 ± 5.2
Height (m)	164.0 ± 5.8
Weight (kg)	104.4 ± 22.5
BMI (kg/m^2)	38.7 ± 7.6
Waist circumference (cm)	104.7 ± 14.5
Fat mass (kg)	48.8 ± 16.6
% fat mass	46.0 ± 5.8
Fat free mass (kg)	56.6 ± 11.3
% fat free mass	54.0 ± 5.8
Relative VO_2 peak (ml/kg/min)	20.2 ± 4.9
PPO (W)	178.5 ± 28.1
HRmax (bpm)	175.0 ± 10.0

Note: all values are mean ± SD; n=20. Abbreviations: BMI: body mass index; HRmax: heart rate maximum; PPO: peak power output; VO_2peak: peak oxygen uptake**.**

5.2 Exercise Data

In both the GY and CP trials, all twenty participants completed all 10 intervals of the HIIC bout. There were no significant differences in the average %HRmax (GY: 94.5 ± 6.5%; CP: 93.1 ± 5.6%; p=0.220) or average %PPO (GY: 71.6 ± 6.4%; CP: 71.8 ± 6.2%; p=0.480) achieved across the 10 intervals between GY and CP.

5.3 Dietary Data

The table below (**Table 6**) outlines the average energy and macronutrient breakdown of standardized meals, including the post-exercise trial supplements (GY and CP) on Day 1 of the metabolic trials. The only difference between trials was the consumption of the post-exercise supplement.

Table 6: Average dietary intake during the GY and CP trials on Day 1 (i.e., day of exercise) including the provided post-exercise supplement.

Dietary intake	GY	CP
Energy intake (kcal)	1969 ± 301	1967 ± 301
Protein (g)	97 ± 12	65 ± 12
Carbohydrate (g)	254 ± 47	303 ± 47
Sugar (g)	72 ± 24	125 ± 24
Fat (g)	66 ± 10	59 ± 10
Calcium (mg)	840 ± 86	490 ± 86
Potassium (mg)	2036 ± 500	1586 ± 500
Phosphorus (mg)	1227 ± 174	898 ± 174
Magnesium (mg)	272 ± 53	233 ± 53
Iron (mg)	13 ± 3	13 ± 3
Vitamin D (IU)	51 ± 7	17 ± 7

Note: all values are mean ± SD; n=20.

5.4 Issues with Blood Sampling and Analyses

Eleven blood samples were missed throughout the trial as blood could not be obtained during blood draws. Certain markers in some samples were undetectable (i.e., below the detection limit of the assay) and were thus excluded from the analysis, leading to a lower n for CTX (n=18) and OPN (n=19). For one participant, there was not enough sample for the pre-exercise timepoint in the CP trial for IGF-1 analysis. This led to an n of 19 for the net iAUC for IGF-1.

5.5 Bone Turnover and Metabolism Marker Responses

5.5.1 Absolute Concentrations of Bone Turnover and Metabolism Markers

For RANKL, there was a main effect of time ($p<0.001$; n=20; **Figure 10A**), with no trial effect ($p=0.883$), or time x trial interaction ($p=0.872$). Post-hoc analysis showed that all timepoints were significantly different from each other. RANKL increased immediately post-exercise, decreased below pre-exercise at 1hr postprandially, reached its lowest point at 3hr postprandially, and remained suppressed at 24hr post-exercise (post hoc tests, all $p<0.05$).

For OPG, there was a main effect of time ($p<0.001$; n=20; **Figure 10B**), a trending trial effect ($p=0.057$), and a time x trial interaction ($p=0.001$). Following the post-exercise rise, OPG changed differently at 1hr postprandially in GY compared to CP. In the GY trial, OPG returned to pre-exercise levels by 1hr postprandially (post hoc test, $p=0.168$). In the CP trial, OPG levels decreased below pre-exercise at 1hr postprandially (post hoc test, $p=0.004$). At this timepoint, OPG was significantly higher in GY than in CP (+29.34 pg/mL, post hoc test, $p<0.001$). At 3hr, OPG returned to or remained at pre-exercise levels in CP and GY, respectively (post hoc tests, $p>0.050$). At 24hr post-exercise, while OPG remained unchanged in the GY trial (vs. pre, post hoc test, $p=0.433$), it increased above pre-exercise levels in the CP trial (post hoc test, $p=0.004$), revealing a significant difference between trial arms at this timepoint (+17.30 pg/mL in CP vs. GY, post hoc test, $p=0.017$).

For the OPG/RANKL ratio, a main effect of time was observed ($p<0.001$; n=20; **Figure 10C**), with no trial effect ($p=0.957$), or time x trial interaction ($p=0.284$). OPG/RANKL did not change from pre- to post-exercise (post hoc test, $p=0.161$); however, it increased at 1hr postprandially (vs. pre, post hoc test, $p=0.004$), reaching its highest point at 3hr postprandially

(vs. pre, post hoc test, p=0.004), and returned to pre-exercise levels by 24hr post-exercise (post hoc test, p=0.075).

For PTH, a significant time effect (p<0.001; n=20; **Figure 10D**), a significant trial effect (p=0.001), and a time x trial interaction (p=0.008) were observed. In GY, following the transient post-exercise rise, PTH decreased below pre-exercise levels at 1hr postprandially (post hoc test, p<0.001), and stayed below pre-exercise levels at 3hr postprandially (post hoc test, p=0.016). At 24hr post-exercise, PTH rose above pre-exercise (post hoc test, p<0.005) in the GY trial. In the CP trial, following the post-exercise rise, PTH levels decreased but remained above pre-exercise levels at 1hr postprandially (post hoc test, p=0.007), at 3hr postprandially (post hoc test, p=0.003), and 24hr post-exercise (post hoc test, p<0.001). PTH was higher at 1hr (+25.66 pg/mL, post hoc test, p<0.001) and 3hr postprandially (+26.01 pg/mL, post hoc test, p=0.001) in CP compared to GY (post hoc tests, p<0.001).

For OPN, a main effect of time (p=0.001; n=19; **Figure 10E**) was observed, with no trial (p=0.701) or time x trial interaction (p=0.397). OPN levels decreased from pre-exercise to post-exercise (post hoc test, p<0.001) and at 1hr postprandially (reaching its lowest point by this time) (post hoc test, p<0.001). At 3hr postprandially, OPN rose back to post-exercise levels (post hoc test, p=0.429) and returned to pre-exercise levels by 24hr post-exercise (post hoc test p=0.235).

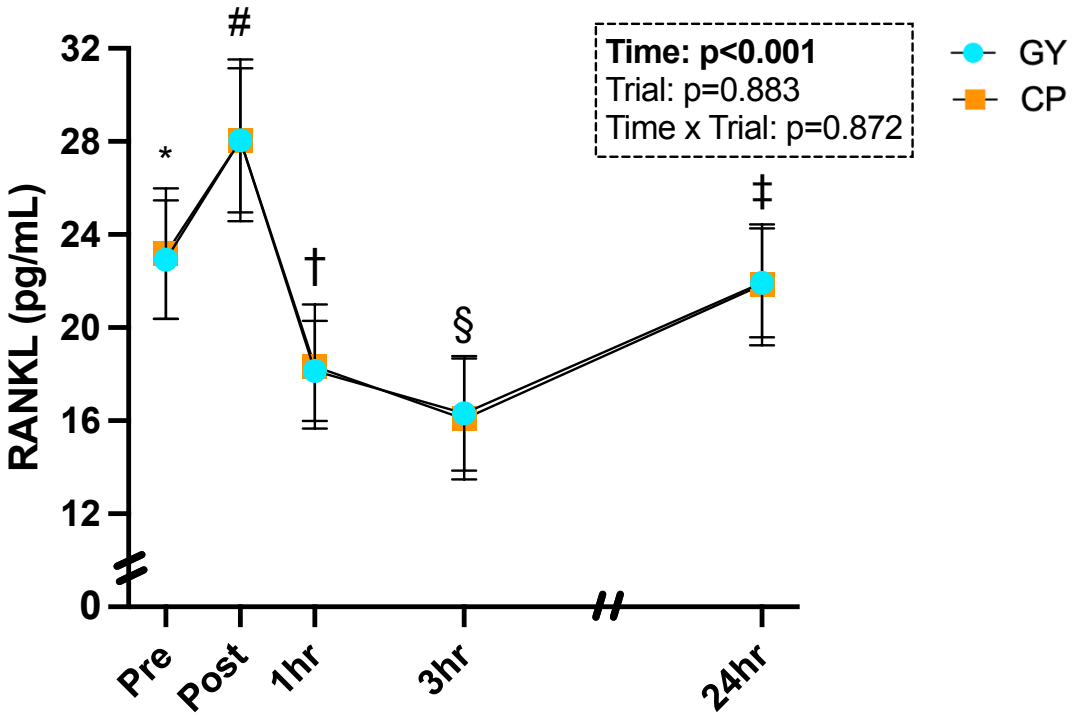
For CTX, there was a main effect of time (p<0.001; n=18; **Figure 10F**), with no trial (p=0.209) or time x trial interaction (p=0.676). Following the post-exercise rise, CTX levels decreased at 1hr and 3hr postprandially (vs. post, post hoc tests, p=0.001) but remained elevated above pre-exercise at 1hr (post hoc test, p=0.001) and 3hr postprandially (post hoc test, p=0.033). CTX returned to pre-exercise levels by 24hr post-exercise (post hoc test, p=0.061).

For SOST, a main effect of time was observed ($p<0.001$; n=20; **Figure 10G**), with no trial effect ($p=0.876$) or time x trial interaction ($p=0.381$). Following the post-exercise rise, SOST reached its lowest point at 1hr postprandially, decreasing below pre-exercise (post hoc test, $p=0.002$), and returned to pre-exercise levels by 3hr postprandially (post hoc test, $p=0.117$). At 24hr post-exercise, SOST was significantly higher than pre-exercise (post hoc test, $p=0.003$).

For OC, a main effect of time ($p<0.001$; n=20; **Figure 10H**) and a time x trial interaction ($p=0.021$) were observed, although the trial effect ($p=0.180$) was not significant. In the GY trial, following the post-exercise rise, OC decreased below pre-exercise at 1hr postprandially (post hoc test, $p=0.002$). While a similar response was seen in the CP trial at 1hr postprandially (vs. pre, post hoc $p=0.002$), at 3hr postprandially, OC further decreased in the CP trial (vs. 1hr postprandially; post hoc test, $p=0.009$). In the GY trial, OC levels remained below pre-exercise at 3hr postprandially (vs. pre, post hoc test, $p=0.042$), although this value trended higher than 1hr postprandially (post hoc test, $p=0.071$). OC was significantly higher in GY than in CP (+969.4 pg/mL, post hoc test, $p=0.031$) at this timepoint (3hr postprandially). OC did not return to pre-exercise levels by 24hr post-exercise in either GY (post hoc test, $p<0.001$) or CP (post hoc test, $p=0.002$).

Lastly, for IGF-1, a main effect of time ($p<0.001$; n=20; **Figure 10I**) was observed with no effect of trial ($p=0.129$) or time x trial interaction ($p=0.488$). Following the post-exercise rise, IGF-1 concentrations decreased back to pre-exercise levels at 1hr (post hoc test, $p=0.165$) and 3hr postprandially (post hoc test, $p=0.130$) and remained at pre-exercise levels at 24hr post-exercise (post hoc test, $p=0.088$).

A


Time: p<0.001
Trial: p=0.883
Time x Trial: p=0.872
GY
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RANKL (pg/mL)
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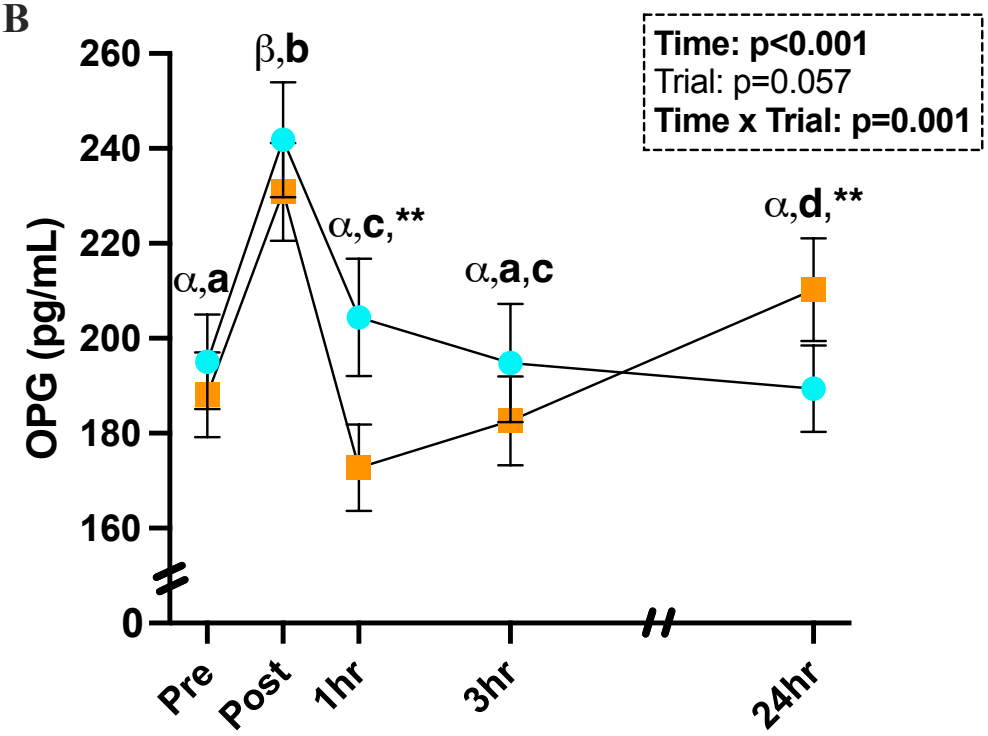
B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24hr

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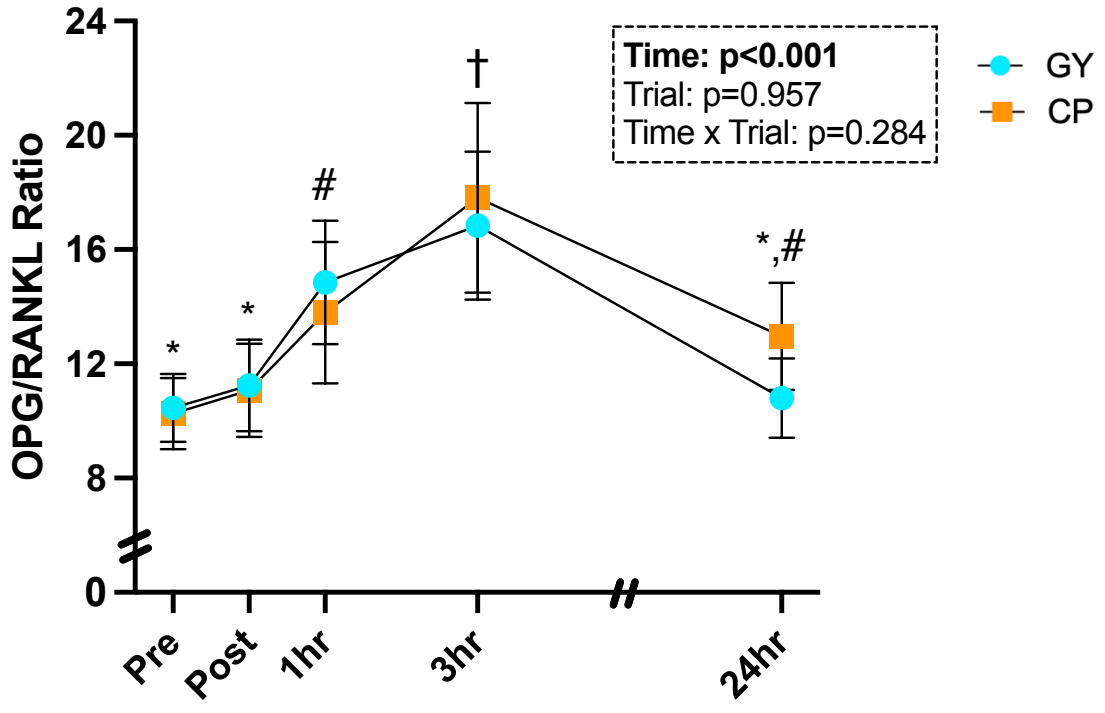
OPG/RANKL Ratio
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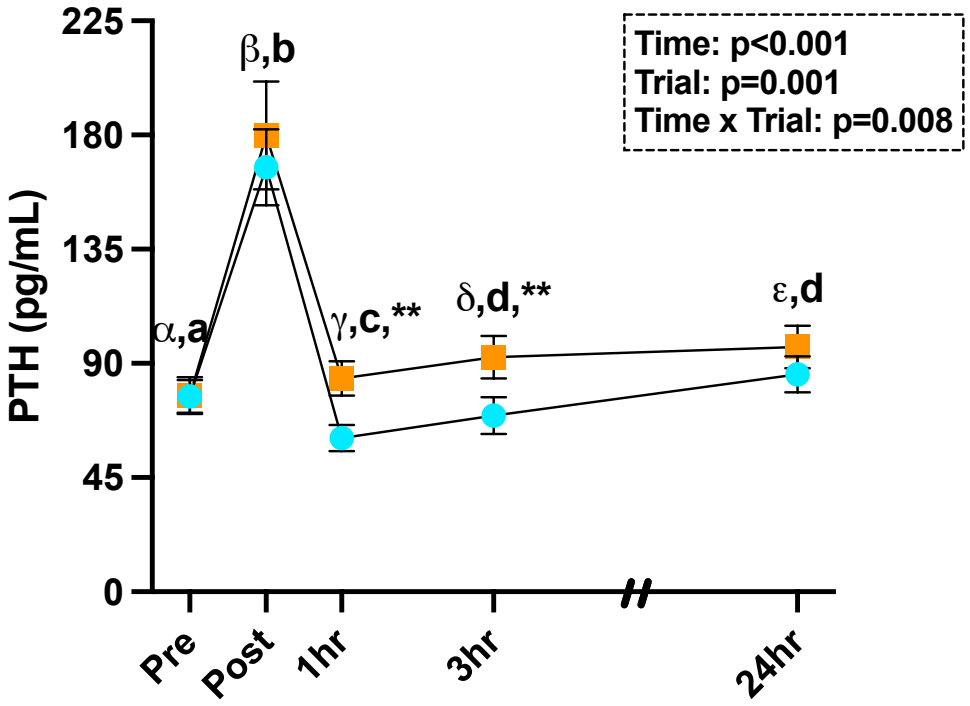
Time: p<0.001
Trial: p=0.957
Time x Trial: p=0.284
GY
CP

D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δ,d,**
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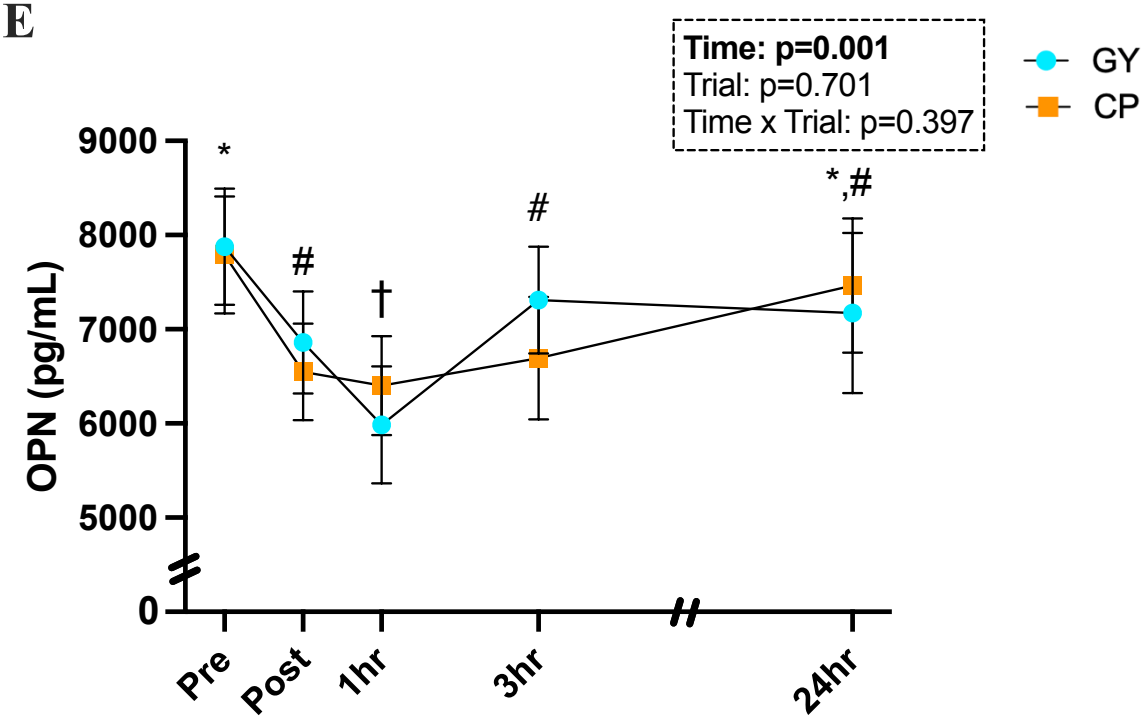
Trial: p=0.001
Time x Trial: p=0.008

E


Time: p=0.001
Trial: p=0.701
Time x Trial: p=0.397
GY
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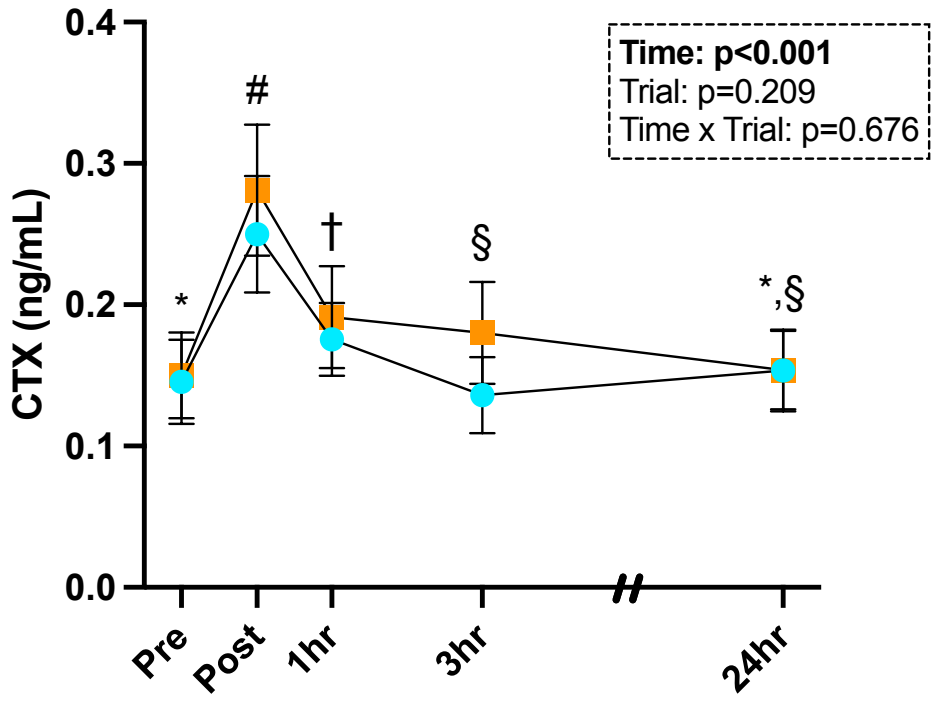
Time: p<0.001
Trial: p=0.209
Time x Trial: p=0.676
CTX (ng/mL)
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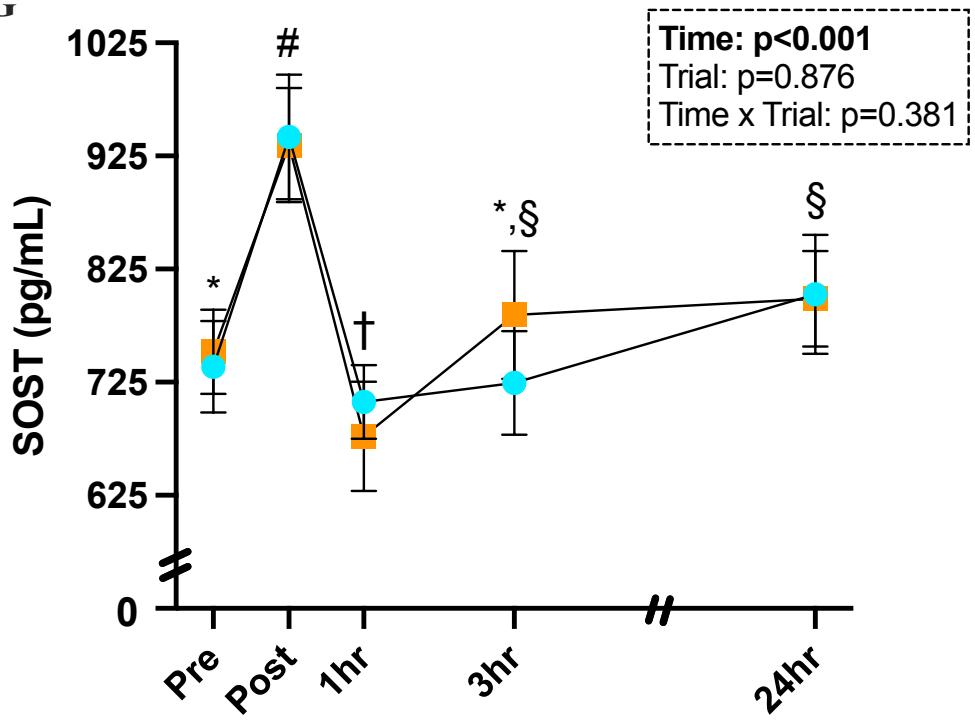
Time: p<0.001
Trial: p=0.876
Time x Trial: p=0.381
SOST (pg/mL)
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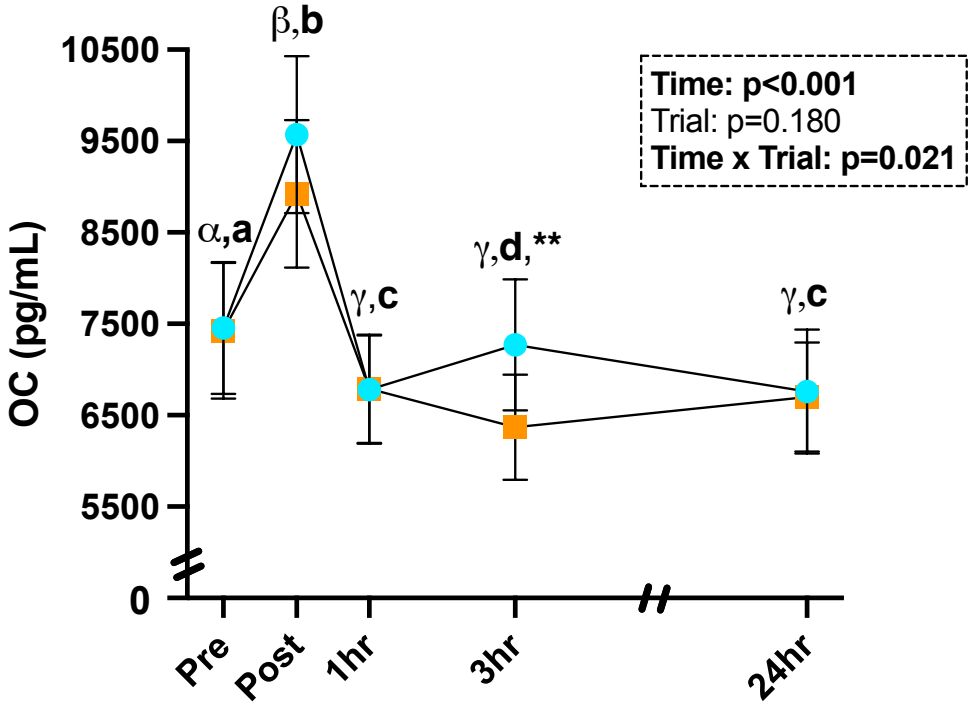
Pre
Post
1hr
3hr
24hr

H


Time: p<0.001
Trial: p=0.180
Time x Trial: p=0.021
OC (pg/mL)
10500
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Post
1hr
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24hr

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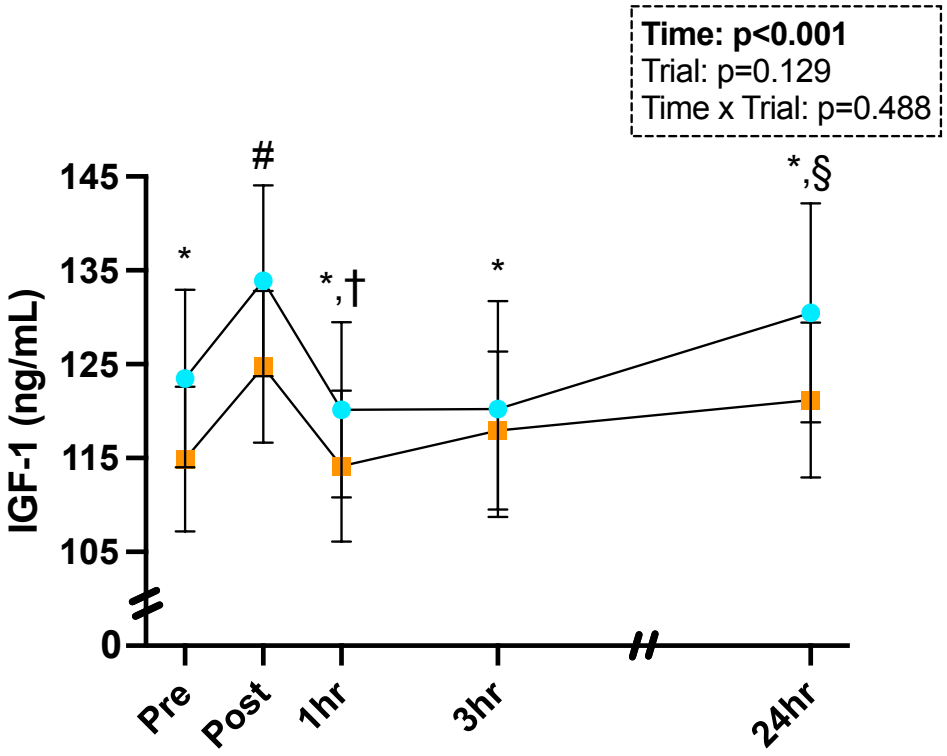
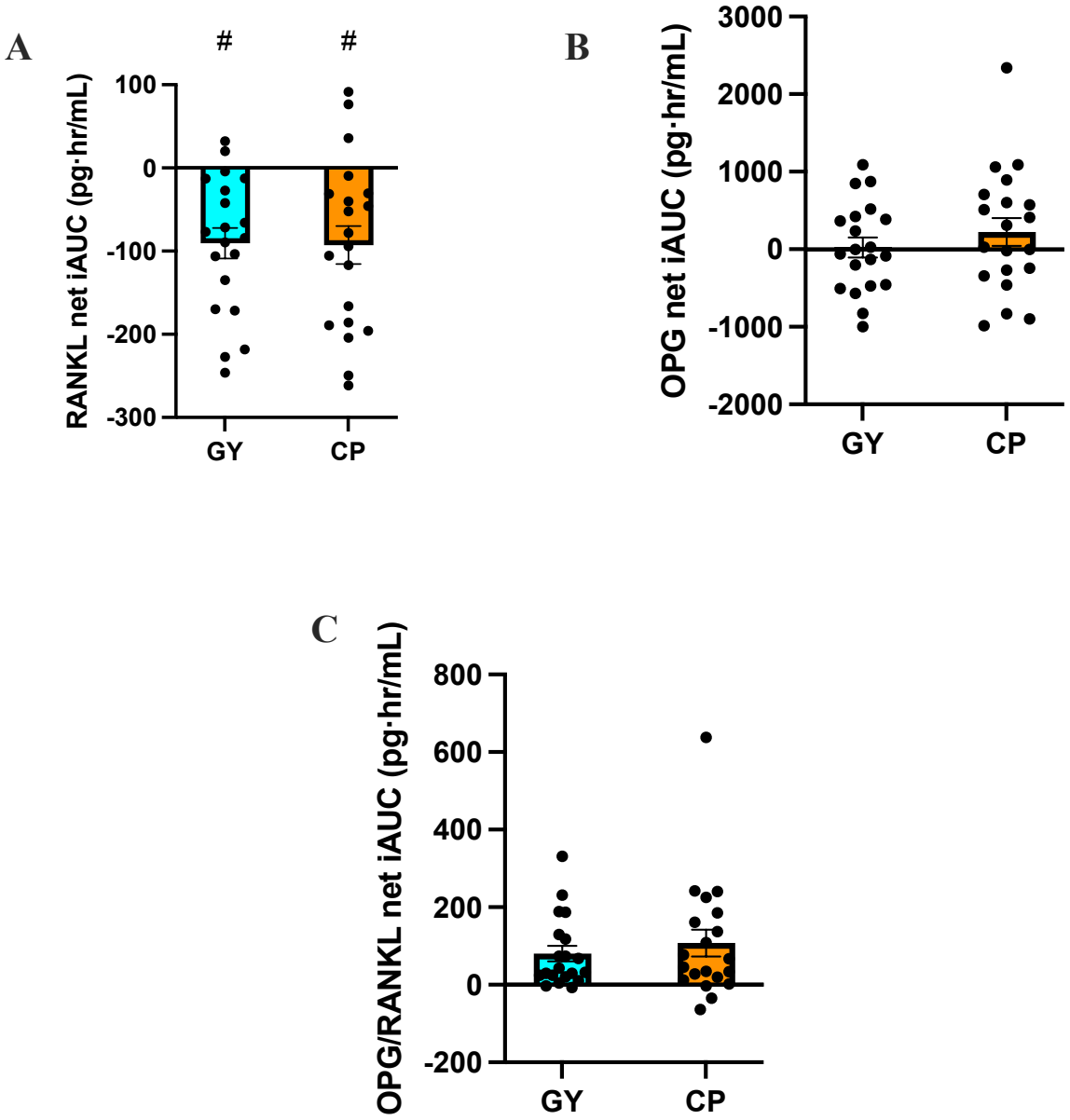


Figure 10: Absolute concentrations over time of RANKL (A), OPG (B), OPG/RANKL ratio (C), PTH (D), OPN (E), CTX (F), SOST (G), OC (H), IGF-1 (I) in the GY and CP trials. Values are mean ± SEM. Main time effects are denoted by symbols *, #, †, §, ‡, whereby a different symbol denotes a significant difference between timepoints ($p<0.05$). For time x trial interactions, differences across time within GY are denoted by Greek letters (α,β,γ,δ,ε) and within CP by bolded English letters (**a,b,c,d,e**); different letters indicate significant within-trial differences over time ($p<0.05$). ** (double asterisk) signifies a difference between GY and CP at a given timepoint ($p<0.05$) (B, D, H).

5.5.2 Net Incremental Area Under the Curve (net iAUC) For Markers of Bone Turnover and Metabolism

The net iAUC for PTH was significantly different between CP and GY, with a greater increase observed in CP vs. GY (n=20, p<0.001, **Figure 11D**). There were no differences between trials for net iAUC for any other marker; RANKL (n=20, p=0.939, **Figure 11A**), OPG (n=20, p=0.226, **Figure 11B**), OPG/RANKL ratio (n=20, p=0.349, **Figure 11C**), OPN (n=19, p=0.978, **Figure 11E**), CTX (n=18, p=0.932, **Figure 11F**), SOST (n=20, p=0.785, **Figure 11G**), OC (n=20, p=0.322, **Figure 11H**), IGF-1 (n=19, p=0.958, **Figure 11I**). Within a trial, net iAUC was significantly reduced from baseline (pre-exercise) in GY and CP for RANKL (GY, p=0.001; CP, p=0.001) and OC (GY, p=0.001; CP, p=0.008). Despite no difference between GY and CP for net iAUC for CTX, CP was significantly increased from pre-exercise (p=0.008) and GY trended differently from pre-exercise (p=0.054). PTH also showed a significant increase from pre-exercise for mean net iAUC in CP (p<0.001) but was not different from pre-exercise in GY (p=0.123). There were no differences in net iAUC from pre-exercise for any other marker (OPG, OPG/RANKL ratio, OPN, SOST or IGF-1).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OPG net iAUC (pg·hr/mL)
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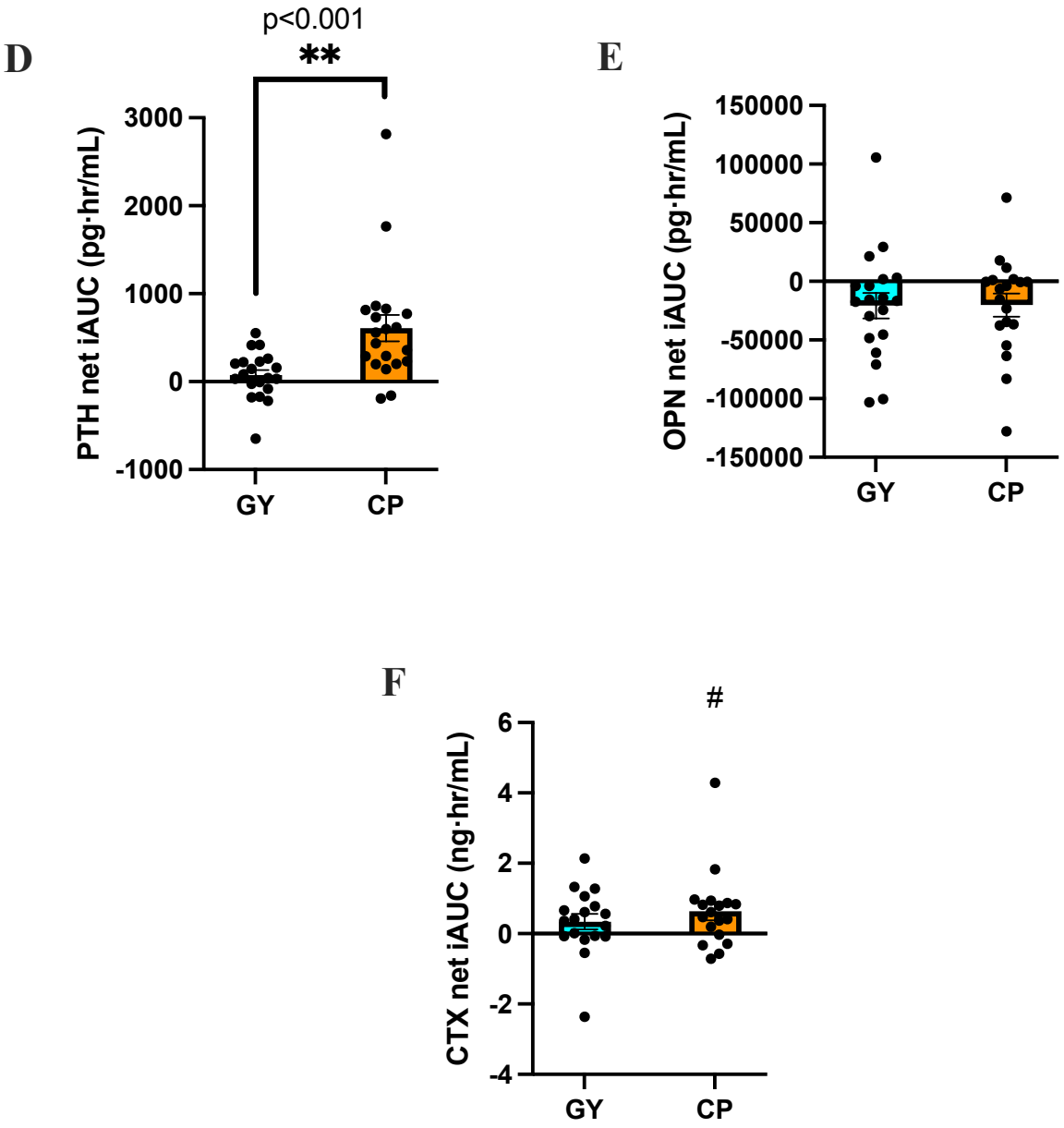
D
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PTH net iAUC (pg·hr/mL)
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GY
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OPN net iAUC (pg·hr/mL)
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100000
50000
0
-50000
-100000
-150000
GY
CP
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CTX net iAUC (ng·hr/mL)
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GY
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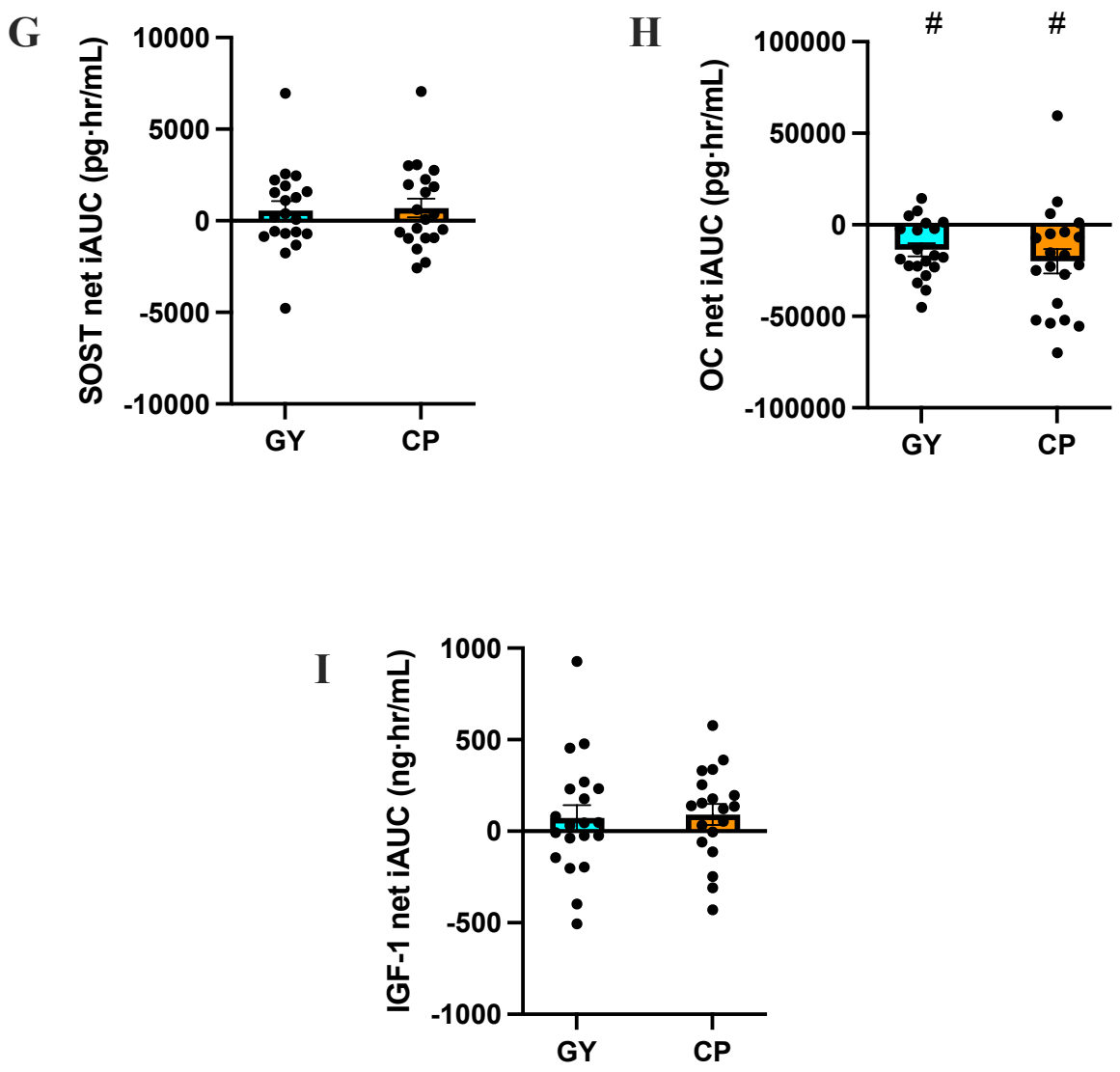

Figure 11: Net iAUC for absolute concentrations of RANKL (A), OPG (B), OPG/RANKL ratio (C), PTH (D), OPN (E), CTX (F), SOST (G), OC (H), IGF-1 (I) in the GY and CP trials. Values are mean ± SEM (A-I). ** signify a difference between GY and CP ($p<0.05$) (D). # signifies a significant reduction or elevation from baseline (pre-exercise) ($p<0.05$) (A, F, H).

Chapter 6: Discussion

This is the first study, to our knowledge, to assess the bone turnover and metabolism response to an acute high-intensity interval exercise bout (cycling; HIIC) with post-exercise consumption of GY versus isoenergetic CP in young- to middle-aged inactive premenopausal females with OW/OB. All individual bone turnover and metabolism markers displayed an immediate post-exercise rise (RANKL, OPG, PTH, CTX, SOST, OC, IGF-1) or decline (OPN), demonstrating that HIIC was able to stimulate a bone turnover and metabolism response. Main effects of time were observed for RANKL, OPG/RANKL ratio, OPN, CTX, SOST, and IGF-1, suggesting no differential effect of post-exercise supplementation in the form of GY or CP. In terms of the interactions between arms, GY consumption blunted the reduction in OPG and OC concentrations at 1hr and 3hr postprandially, respectively, and reduced PTH concentrations during the entire postprandial period (1hr and 3hr) compared to CP (Objective A). This is also reflected in the net iAUC for PTH, where CP had a greater increase compared to GY (Objective B). Overall, this suggests a more favourable modulation of the bone turnover and metabolism response in some markers with post-exercise GY consumption versus CP.

6.1 Individual Bone Turnover and Metabolism Responses

6.1.1 RANKL/RANK/OPG

6.1.1.1 RANKL

A main effect of time for RANKL was found in the present study ($p<0.001$; **Figure 10A**) whereby concentrations peaked immediately post-exercise, which is consistent with previous research utilizing HIIC or HIIR in young adults[16,57]. In these studies, concentrations returned to pre-exercise by 1hr post-exercise, with no differences at 24hr post-exercise[16,57]. In our study,

RANKL decreased below pre-exercise during the postprandial period (1hr and 3hr) and did not return to pre-exercise levels by 24hr post-exercise (although it was close). RANKL is crucial for stimulating bone turnover by facilitating the activation of osteoclasts[44,46], thus its attenuation through the collective modulation of HIIC and post-exercise nutrition, irrespective of its composition, is indicative of a potential blunting in resorption processes. Additionally, the mean net iAUC displays a marked reduction from pre-exercise in both GY and CP, suggesting a cumulative effect of reduced bone resorption over time. RANKL has also been found to decrease 75mins following resistance and plyometric exercise with milk or carbohydrate consumption in young normal weight premenopausal females[24]. Collectively, this suggests that post-exercise nutritional provision in the form of carbohydrate or dairy (milk or GY), may elicit a similar RANKL decrease at around 1hr post-exercise, regardless of modality in premenopausal females with or without OW/OB, whereas without nutrition, RANKL does not decrease below pre-exercise levels in the post-exercise period.

6.1.1.2 OPG

A time x trial interaction was observed for OPG in the present study (p=0.001; **Figure 10B**). OPG peaked immediately post-exercise in both trials, which is consistent with previous studies[16,53,55–57]. At 1hr postprandially, while OPG decreased below pre-exercise concentrations in CP, this decline was blunted in GY as OPG returned to pre-exercise levels and was 18% higher (+29.34 pg/ml, post hoc test, p<0.001) compared to CP. In Prowting et al.'s study involving post-exercise milk versus carbohydrate supplementation, OPG remained unchanged between trials and across time in normal weight premenopausal females[24]. The maintenance of OPG concentrations in that study could relate to either the exercise performed (plyometrics and resistance exercise) or a compensatory mechanism in young premenopausal females of normal

weight, in an attempt to keep OPG concentrations constant. Taken together, our results could suggest that GY (but not CP) provides an early benefit *via* the maintenance of OPG at pre-exercise levels at 1hr postprandially (and throughout the rest of the GY trial), potentially blunting acute exercise induced bone resorption in premenopausal females with OW/OB.

6.1.1.3 OPG/RANKL Ratio

The OPG/RANKL ratio in the present study showed a main effect of time ($p<0.001$; **Figure 10C**), in which the ratio increased at 1hr and 3hr (peak) postprandially, and then began to return to pre-exercise levels by 24hr post-exercise. Similar results have been observed in a previous study, with the ratio peaking at 75mins post-exercise with milk or carbohydrate consumption[24]. The lack of difference in the OPG/RANKL ratio between GY and CP, but the presence of a difference in OPG between trials suggests that GY and CP may be promoting different temporal compensatory mechanisms resulting in a similar compounding effect over the trials. That is, while GY blunted earlier resorption (*via* higher OPG at 1hr postprandially compared to CP), CP resulted in a later OPG rise (at 24hr post-exercise), whereas RANKL was substantially reduced in both trials following the post-exercise rise. Taken together, these responses suggest an overall acute favouring of OPG over RANKL, regardless of nutritional supplement in a population where the RANKL/RANK/OPG axis may be dysregulated[79,154–157].

6.1.2 PTH

For PTH, a time x trial interaction ($p=0.008$; **Figure 10D**) was observed. The robust post-exercise rise observed in our study is commonly seen in the literature[16,20,53,55,56,60,61,63,65,66,68–70]. During the postprandial period, PTH in CP remained above pre-exercise levels at both timepoints (i.e., 1hr and 3hr) and was 39% (+25.66 pg/mL, post hoc test, $p=0.001$) and 33% (+26.10 pg/mL, post hoc test, $p=0.001$) higher than GY, which decreased below pre-exercise levels at 1hr and

3hr, respectively. PTH has been found to increase and remain elevated above pre-exercise at 3hr and 24hr post-exercise in young and healthy males consuming a combined protein and carbohydrate supplement (devoid of calcium) following high-intensity running[61], similar to what was observed with CP in our study. Previous studies involving calcium infusions or calcium supplementation have been found to reduce circulating PTH levels in the hours following acute exercise[62,63], similar to what was observed with GY in our study. Thus, the rise in PTH following acute exercise in response to increased calcium losses (*via* sweat or other mechanisms), which induces bone resorption to restore calcium homeostasis[44,60,62,63], may be modulated by calcium and other bone-supporting nutrients provided through wholefoods. Indeed, in trained female cyclists a high calcium/high dairy pre-exercise meal was able to attenuate the rise in PTH immediately and 40mins post-exercise compared to a low calcium/low dairy meal[23]. Despite a similar effect, the attenuation of PTH was even more transient in that study compared to ours, which may relate to the fact that dairy products were consumed pre-exercise rather than post-exercise. Furthermore, that study was conducted in trained female cyclists who performed a longer and more intensive cycling bout, which may have resulted in a greater reduction in circulating calcium. The response with GY supplementation in the postprandial period in our study suggests that bone-supporting nutrients (i.e., calcium), were able to attenuate PTH following the acute post-exercise rise. In addition, due to GY's semi-solid nature, it is possible that the calcium was delivered in a more slow and sustained way over time than if it were received from milk. However, this mechanism needs to be further explored.

6.1.3 OPN

A time effect was found for OPN ($p=0.001$; **Figure 10E**) in which, concentrations decreased post-exercise, reaching a nadir at 1hr, and then gradually returned to pre-exercise by

24hr post-exercise. These results are consistent with a previous study that found a decrease in OPN immediately post-exercise (HIIC), returning to pre-exercise levels by 25hr post-exercise in young adult males with OW/OB[84]. The acute decrease in OPN in GY and CP could signify an attenuation effect on bone resorption related processes as OPN plays a role in bone resorption by facilitating the binding of osteoclasts to the bone surface[37,38,78,81]. Indeed, RANKL, which also facilitates bone resorption, was found to decrease below pre-exercise levels at 1hr and 3hr postprandially, similarly to OPN. The reduction in OPN in our study with HIIC and nutrition may be additionally beneficial in females with OW/OB, as OPN is often elevated at rest[71,77,78].

6.1.4 CTX

A main effect of time was found for CTX ($p<0.001$; **Figure 10F**), whereby a peak immediately post-exercise was observed with a gradual return to pre-exercise levels by 24hr post-exercise. While these results are consistent with those previously observed in males, especially following acute cycling[20,27,60–65,88], CTX may be unchanged in premenopausal females of normal weight or with overweight only in the acute post-exercise period[15,89,90]. The results in our study may suggest a differential post-exercise CTX response, indicative of collagen breakdown and bone resorption[46], in premenopausal females with OB compared to premenopausal females of normal weight or with overweight only. In normal weight untrained young premenopausal females, the % change AUC for CTX following plyometric and resistance exercise was significantly lower following milk consumption compared to carbohydrate indicating a blunting of bone resorption[24]. Given this, GY in our study was expected to blunt the CTX response, but this was not observed. A lack of a differential response in our study between GY and CP may relate to the population being studied (normal weight vs. OW/OB), the exercise

being performed (cycling vs. plyometrics and resistance exercise), or the consumption of milk versus GY (which have different digestion and absorption properties).

6.1.5 SOST

We observed a main effect of time for SOST ($p<0.001$; **Figure 10G**), whereby concentrations increased immediately post-exercise, which is congruent with previous studies[15,16,20,53,88,89,92]. SOST typically returns to pre-exercise levels within the first hour post-exercise[15,16,88,89,92]. In our study, SOST decreased slightly below pre-exercise levels at 1hr postprandially and then began to increase again, eventually increasing above pre-exercise levels at 24hr post-exercise, although the magnitude of this rise was small (only 7% increase compared to pre-exercise). As far as post-exercise nutrition is concerned, Prowting et al.[24] also found a decrease below baseline (pre-exercise levels) at 75mins post-exercise followed by an increase above baseline at 24hr post-exercise with both milk and carbohydrate supplementation. This suggests that non-specific nutritional provision (dairy or carbohydrate) post-exercise differentially modulates this negative regulator of bone formation in the post-exercise period, compared to previous studies without nutrition. Therefore, the SOST response seen in our study may relate to the collective effects of HIIC and nutrition, but it likely does not relate to OW/OB or impact *per se* as similar results have been shown in premenopausal females of normal weight with a similar nutritional intervention and a different exercise bout involving loading and impact (plyometrics and resistance exercise) compared to ours which was unloaded, high-intensity cycling.

6.1.6 OC

A significant time x trial interaction was observed for OC ($p=0.021$; **Figure 10H**). While both CP and GY experienced the same post-exercise rise, which is often seen in the

literature[16,20,55,65,97], concentrations declined below pre-exercise levels in both trials by 1hr postprandially. At 3hr postprandially, OC in GY was 14% higher than CP (+969.4 pg/mL, post hoc test, p=0.031), potentially reflecting higher bone turnover. OC has also been found to decrease at 75mins post-exercise in young premenopausal females undergoing plyometric and resistance exercise and post-exercise supplementation of milk or carbohydrate[24]. Although there was no difference in OC between supplements[24], there was no 3hr blood sample, which may have missed a potential differential response between milk and carbohydrate, as seen in our study with GY versus CP. OC returned to pre-exercise levels by 24hr post-exercise in that study, whereas in our study, OC did not return to pre-exercise levels. In fact, we demonstrated that the mean net iAUCs in GY and CP were significantly reduced compared to pre-exercise, suggesting a blunting across the trial periods. This mechanism may relate to OW/OB in general as OC has been found to be lower at rest in adults with OW/OB (indicating lower turnover and formation)[170], as well as at pre-exercise and across the acute post-exercise period in adolescent females with OW/OB compared to their lean counterparts[171]. More research is needed to assess if GY supplementation can be modified (i.e., dosage and/or timing) to amplify the transient 3hr postprandial response and extend these benefits to the 24hr post-exercise period, discussed further in future directions.

6.1.7 IGF-1

A main effect of time was observed for IGF-1 (p=0.0001; **Figure 10I**), whereby IGF-1 peaked post-exercise and returned to pre-exercise levels by 1hr postprandially, which is consistent with other acute high-intensity exercises bouts[101–104,106,107]. IGF-1 is associated with protein intake and has been found to relate to bone formation outcomes, such as type 1 collagen production[21]. However, no difference was observed in our study between GY (high protein) or

CP (devoid of protein). Our results are similar to a previous study conducted in premenopausal females of normal weight where IGF-1 increased immediately post-exercise and returned to pre-exercise levels by 1hr post-exercise following milk or carbohydrate consumption[25]. In another recent study, the post-exercise increase in IGF-1 was blunted in those with OB compared to their lean counterparts following cycling[105]. While post-exercise nutrition such as GY or CP does not change or improve the IGF-1 response compared to studies without nutrition, together, HIIC and post-exercise nutrition yielded a transient increase in IGF-1 in those with OW/OB.

6.2 Putting it together: Modulation of PTH, OPG, and OC with GY versus CP

We observed a favourable modulation of the bone turnover and metabolism response *via* PTH, OPG, and OC with GY supplementation versus CP throughout the postprandial period. Specifically, PTH was reduced with GY from pre-exercise levels at 1hr and 3hr postprandially, resulting in a cumulative reduction in the net iAUC from post-exercise to 24hr post-exercise compared to CP. Mechanistically, PTH plays a role in blunting the production of OPG[44], thus, the simultaneous reduction in PTH and maintenance of OPG at 1hr postprandially in the GY trial may be related to this mechanism (outlined in **Figure 12**). Furthermore, this is supported by the parallel response seen in the maintenance of OC at 3hr postprandially and provides evidence to suggest that a modulation of the turnover response in favour of formation may have occurred. When interpreting the CP response, resorption processes were likely upregulated as PTH was elevated at 1hr and 3hr postprandially with a concurrent suppression in OPG (at 1hr postprandially), so it is plausible that the reduction at 3hr in OC relates to a propensity for decreased formation (as outlined in **Figure 12**). Overall, these differential effects and suggested processes point to a potential transient net resorptive effect in CP, whereas in GY they may relate to a better coupling of resorption to formation *via* reduced resorption in the postprandial period.

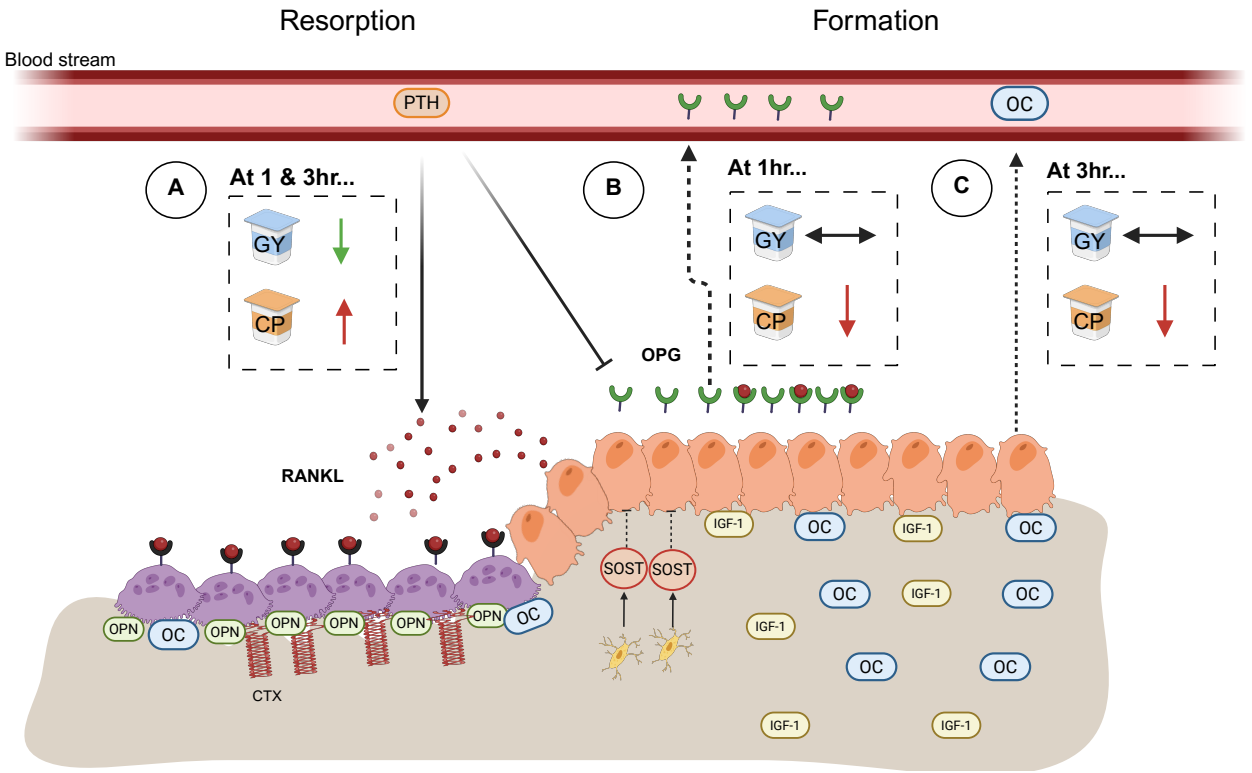

Figure 12: Proposed mechanism of the modulation of bone turnover and metabolism markers (PTH, OPG, OC) by GY vs. CP during the postprandial period (1hr and 3hr). **A** depicts the role of PTH in blunting OPG and increasing RANKL production, we observed an increase in PTH at 1hr and 3hr postprandially in CP compared to a reduction in GY. **B** depicts OPG production from osteoblasts and their role in binding RANKL to blunt resorption processes, we observed a maintenance of OPG in GY compared to a reduction in CP at 1hr postprandially. **C** depicts the release of OC from osteoblasts during bone formation, we observed a parallel effect compared to OPG at 1hr, whereby OC was maintained in GY and reduced in CP at 3hr postprandially. Created with BioRender.com.

6.3 Implications and Future Directions

Females achieve a lower PBM in young adulthood, experience a steeper decline in bone mass loss with age (i.e., the onset of menopause)[1], and are at increased risk of developing osteoporosis[7] compared to males. Thus, the period of the lifespan following the achievement of PBM and before the onset of menopause (i.e., young to middle adulthood) is a critical time for bone mass maintenance[1,43]. Negative bone health outcomes in females can be further exacerbated by OW/OB, which can increase the risk of bone fragility and fractures[5]. Despite these important points, research into how best to preserve bone and promote bone health largely ignores the

demographic of young- to middle-aged premenopausal females with OW/OB. To date, only 1 study has assessed the chronic effects of a higher dairy diet and multimodal exercise as part of a weight loss intervention in premenopausal females with OW/OB, in which bone turnover and metabolism was positively modulated *via* increased resting/fasting levels of P1NP/CTX ratio, OPG/RANKL ratio, and OC, as well as reduced resting/fasting PTH[28]. Our study was the first acute trial to assess similar bone and nutrition outcomes in females with OW/OB. In our study, transient improvements were observed with the maintenance of OPG and OC, and reduction in PTH with GY supplementation in the postprandial period. This suggests that GY supplementation was able to help mitigate decreases in circulating calcium levels that occur with exercise, likely due to its calcium content as well as protein and other micronutrients (i.e., phosphorus, zinc, potassium, magnesium). This may promote bone turnover and metabolism favouring reduced resorption and a better coupling of resorption to formation that supports bone microstructure and remodeling. On the other hand, CP may have been unable to modulate PTH, and higher concentrations of this hormone may have resulted in lower OPG and OC during the postprandial period (indicative of a more resorptive state within bone). Given that females with OW/OB often have higher levels of PTH[159] and lower levels of OC (indicating reduced turnover and bone formation)[170] and OPG[155–157] at rest, this response to GY consumption may be additionally favourable if incorporated consistently over time in an exercise training/healthy lifestyle context.

The dairy matrix consisting of bone-supporting nutrients, bioactive components, peptides, fatty acids, and carbohydrates form complex interactions and work together to improve nutrient absorption[21,114]. This may be additionally beneficial among those with OW/OB as intestinal calcium absorption is often hindered, which may explain higher resting PTH levels[159]. GY was

chosen as the post-exercise dairy supplement in this study because it contains a higher proportion of protein to carbohydrate as well as bacterial cultures which may further support glucose metabolism and intestinal nutrient absorption, however, milk (which has been more thoroughly researched in a post-exercise context previously) contains more calcium per serving and vitamin D compared to GY[21,114]. Additionally, the differential physical composition of these 2 dairy products may affect nutrient digestion and absorption[117]. Indeed, previous studies involving milk consumption pre- or post-exercise have elicited a reduced CTX response in trained female cyclists[23] or untrained premenopausal females[24], respectively. Nevertheless, the present study still demonstrated a favourable bone response, suggesting that GY can be an effective post-exercise supplement to support bone turnover and metabolism in this population.

Many of the studies mentioned throughout this document provided post-exercise supplementation immediately post-exercise, as well as at 1hr post-exercise, and sometimes additionally before bed[24,25,27,29,120]. In the present study, 2 servings were given immediately after exercise, so perhaps spreading out individual servings (or incorporating different dairy products, such as milk) would elicit an amplified response in OPG and OC, a more sustained response in PTH (at 24hr post-exercise) or additional benefit through other markers such as CTX. Additional research evaluating the appropriate dairy food, combination and/or dosage and timing in this population is needed.

In individuals with OW/OB, high-intensity interval exercise is often preferred as it is time efficient[9–11]. HIIC specifically is also favoured as it is more comfortable on the joints[19] and may reduce risk of musculoskeletal injury occurring (particularly in untrained individuals)[18]. As a result, HIIC may be more desirable for long-term adherence among those with OW/OB. Mechanical loading is widely considered a primary stimulus of osteogenesis (bone formation)[51],

despite being non-weight bearing and lower-impact, HIIC involves working against resistance at a high power output (i.e., high relative intensity) which can generate strong muscle contractions that exert mechanical forces on the bone tissue[17]. As detailed throughout the document, HIIR (weight-bearing, high-impact) and HIIC have been found to elicit similar acute bone turnover and metabolism responses in premenopausal females[15,16]. Given that we also observed an acute modulation of bone turnover and metabolism with GY consumption, incorporating dairy supplementation post-exercise into HIIC training may aid in extrapolating these benefits to the long-term, potentially contributing to bone mass maintenance in premenopausal females with OW/OB. Dairy products, including GY, are easily accessible, relatively low cost, and can be safely consumed by a large percentage of the population. So based on our findings and others, dairy foods should be considered a beneficial post-exercise nutritional option to support bone turnover and metabolism. Future training studies should also incorporate the measurement of P1NP (i.e., the reciprocal bone collagen formation marker to CTX) and BMD to better characterize bone health adaptations. Overall, more research is required to determine the optimal exercise and supplementation strategies for improving bone health outcomes among premenopausal females with OW/OB at risk of bone fragility and fracture. The present study provides a piece of the puzzle in advancing this field.

6.4 Strengths and Limitations

6.4.1 Strengths

The present study utilized a crossover design, where participants completed both metabolic (GY and CP) trials. Each individual served as their own reference/control which inherently reduced the potential for between-subject variability that is more likely to occur with a parallel design. Furthermore, the 4-week washout period allowed for the minimization of

confounding variables such as repeated bout effects. Since the same HIIC bout was performed in both metabolic trials, the extended period between trials helps to ensure that no bone turnover or metabolic "adaptations" are carried into the second metabolic trial. In addition, to maintain consistency and minimize the potential effects of hormonal shifts throughout the menstrual cycle (although this may not affect these markers[178]), all participants completed both trials in the early- to mid-follicular phase of their cycle. Prior to study participation, participants performed a VO_2peak test to determine their individualized HRmax and PPO for the exercise bout. This allowed us to ensure that high intensity was truly achieved during the HIIC bouts for each participant. Lastly, to be able to best attribute differences between trials to the nutritional provision of the supplements (GY or CP), standardized meals were consumed the day prior to the trial and the day of the trial (Day 1). These meals were prepared by our research team and were standardized to each participant's body weight to meet individual energy needs.

6.4.2 Limitations

Many of the studies outlined throughout this document measured hematocrit to account for plasma volume shifts that may have occurred due to sweating with exercise as this could artificially inflate concentrations of the bone turnover and metabolism markers[179]. Hematocrit was not measured in this study, and this could be a limitation. While the exercise bout was of a high intensity, it was relatively short in duration (25mins total including warm-up and cool-down), we also used a fan to keep participants cool and water was provided between intervals. Together, these factors reduce the likelihood of substantial fluid loss *via* sweating, minimizing the likelihood of influential plasma fluid shifts. Undercarboxylated and carboxylated OC were not measured in the present study. This makes it difficult to fully characterize the turnover mechanisms at play for this marker when we just have a measurement of total OC. However, we

can somewhat infer the effect of total OC by assessing it in relation to the responses of other related markers including PTH and OPG. Lastly, since there was no control exercise arm in this study (i.e., exercise without a supplement), it is hard to tease out the individual effects of HIIC alone and the effects of the supplements alone (indirectly). Any time effects we saw are to be attributed to the collective effects of HIIC and post-exercise nutrition. While a control group with exercise and without nutrition would have been helpful, it was not necessary to answer our initial research question, nor was it feasible.

6.5 Conclusion

This is the first study to evaluate the effects of dairy supplementation following high-intensity interval cycling (HIIC) in young- to middle-aged inactive premenopausal females with OW/OB. We observed transient changes following HIIC in all markers (CTX, OC, OPN, SOST, RANKL, OPG, PTH, and IGF-1), demonstrating that a single bout of HIIC was effective at eliciting a bone turnover and metabolism response in this understudied population. The supplementation of GY post-exercise attenuated the decline in OPG and OC at 1hr and 3hr, respectively, and blunted the PTH response throughout the postprandial period compared to CP. This provides evidence to suggest that post-exercise nutritional provision in the form of GY, a wholefood with bone-supporting nutrients, can acutely modulate bone turnover and metabolism favouring reduced resorption in a novel population at risk for poor bone health outcomes. Further research is warranted to assess if this response is sustained or amplified when HIIC is performed and dairy products are consumed long-term and if these benefits extend to structural outcomes such as improved BMD, bone microstructure, and bone strength.

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Appendices

Appendix A: Recruitment Poster

V2: 12.13.2022

UNIVERSITY OF TORONTO
FACULTY OF KINESIOLOGY & PHYSICAL EDUCATION

EXERCISE AND NUTRITION RESEARCH STUDY FOR WOMEN

Who can participate?

- Women with a body mass index (BMI) ≥ 27kg/m (overweight or obese category)
- Between the ages of 18 - 45 years
- Not exercising regularly (≤2 days per week)

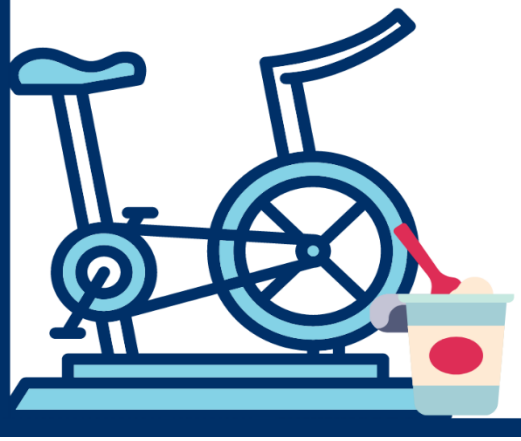
Help us determine how post-exercise snacks influence blood sugar levels following exercise

What's involved?

- Fitness and body composition assessment
- Two exercise trials ~4 weeks apart involving:
  - 25 min of cycling + a post-exercise snack
  - Continuous glucose monitoring and blood draws
  - 3 days of food provided to you, prepared by the study dietitian

This study involves an online introductory visit (~1 hr) and 7 visits to our lab in the Goldring Centre located at 100 Devonshire Pl., Toronto. In-person study visits range from 30 min to 4.5 hr (19 hr total).

You will be compensated for your time.



Scan the QR code above or contact alexa.govette@mail.utoronto.ca

Appendix B: Informed Consent Form

UNIVERSITY OF TORONTO
FACULTY OF KINESIOLOGY & PHYSICAL EDUCATION

Informed consent form to participate in a research study

Study title: Does post-exercise nutrition influence 24-h glycemia following an acute bout of exercise in women?

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Alexa Govette (student)	Kinesiology and Physical Education, University of Toronto		alexa.govette@mail.utoronto.ca

Purpose of Research: A single session of exercise improves blood sugar levels, which lowers risk for diseases like type 2 diabetes. The foods that you eat can also influence your blood sugar levels. The purpose of this research study is to determine if specific post-exercise snacks influence blood sugar levels in the days following exercise.

Description of the Research: As a participant in this study, you will be asked to complete two different exercise and nutritional trials. The two trials are 1) Exercise + Greek Yogurt (GY), and 2) Exercise + a carbohydrate-based Study supplement (SS). Below you will find instructions for completing each trial. The entire study takes approximately 8 weeks, and the two trials will be separated by ~4 weeks (so that they can be performed in the same phase of your menstrual cycle). Throughout the duration of the study, you will be asked to maintain your habitual diet and physical activity patterns, and maintain your current body weight. All in-person visits will take place at the Goldring Centre for High Performance Sport (100 Devonshire Pl, Toronto, ON M5S 2C9).

OVERVIEW OF STUDY VISITS

Visit 1: Introduction (~1 h online)
You will meet a study investigator virtually using a private web link emailed to you. The study will be explained in detail, and you will have an opportunity to ask questions. If you agree to participate, we will determine your eligibility via questionnaires that assess your physical activity levels and general health.

Visit 2: Baseline Testing (~2 h at lab)
You will arrive at the laboratory in the morning after having fasted overnight for 10 h (no food or drink except water). We will measure your height, weight, and waist circumference. You will then be asked to change into a swimsuit and swimcap and sit in an egg-shaped device (called a BodPod) for 1-2 minutes to assess the amount of fat and muscle mass in your body. You will then perform a 10-15-minute fitness test in athletic clothing on a stationary bike. During this fitness test, you will breathe into a mask that allows us to measure how much oxygen you inhale, and we will also measure your heart rate with a chest-worn strap. The resistance on the bike will increase throughout the test, and the test ends when you are no longer able to continue cycling (fatigue). Following this, you will have the opportunity to practice the exercise protocol that you will perform in the exercise + nutrition trials. Upon leaving the laboratory, we will provide you with instructions for how to record all of the food and drinks you will consume for 7 days.

Visits 3-8: Metabolic Trials (4 days each)
In a randomized order, you will complete 2 exercise + nutrition trials (GY and SS) approximated 4 weeks apart in the follicular phase of your menstrual cycle. Each trial takes place over 4 days, but you are not required to come to the laboratory on all days. The only difference between the two trials is the post-exercise snack consumed. **Important:** We will provide you with all of your food to consume during the trial period and ask that you do not consume any other food or drink (including alcohol). If you are a tea or coffee drinker, we can discuss options for incorporating this into Day 0, 2 and 4 of the trials. We also ask that throughout the trial you do not perform any structured exercise outside of the exercise performed in the laboratory. Last, we ask that you maintain your regular sleeping patterns and achieve no less than 7 h time-in-bed each night.

Day 0 of trial (~30 min at the lab): A continuous glucose monitor (CGM) will be attached to your abdomen which measures your blood sugar levels. You will also be given an accelerometer to wear around your hip, which measures your step count and physical activity. Both the CGM and accelerometer will be worn for the entire 4-day trial. At this visit, we will also give you meals to consume at specific times outside of the laboratory starting with the dinner meal on Day 0 and extending over the next 2 days (Day 1 and 2 below). The personalized meals have been prepared by our study dietitian to meet your specific energy needs.

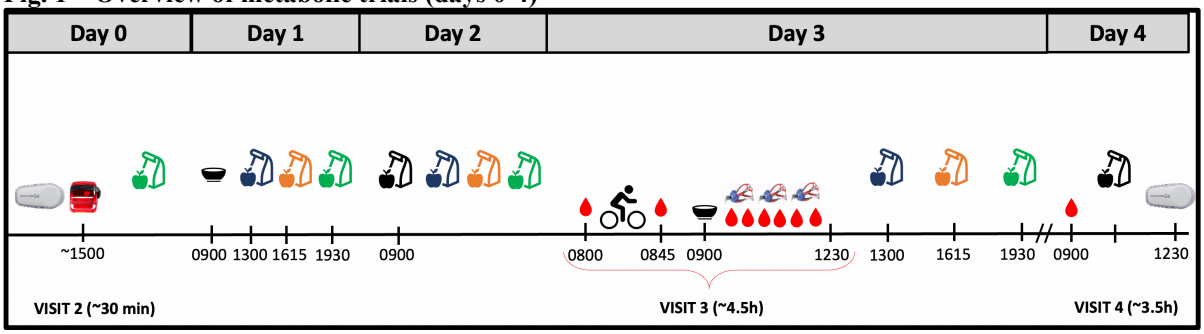
Day 1 of trial (no visit to the lab): This day of the trial is under 'free-living' conditions, meaning you do not need to come to the lab. You will consume the provided breakfast, lunch, snack and dinner meals at specific times throughout the day on your own. You will wear the CGM and accelerometer.

Day 2 of trial (no visit to the lab): This day of the trial is under 'free-living' conditions, meaning you do not need to come to the lab, similar to Day 1. You will consume the provided breakfast, lunch, snack and dinner meals at specific times throughout the day on your own. You will wear the CGM and accelerometer.

Day 3 of trial (~4.5 h at the lab): You will come to the lab in the morning (~7:30-8:00am) without eating or drinking anything after waking up (except water). First, we will insert a venous catheter into a forearm vein and obtain a single blood sample. You will then perform 25 minutes of cycling exercise on a stationary bike. Following exercise, we will obtain a second blood sample from the venous catheter, and you will be seated at table to consume a post-exercise snack (GY or SS). Over the next 3 hours, you will be seated in the lab and can work at a computer to pass the time. During this time, we will obtain a blood sample every 30 min (6 total) and ask you to breathe into a mask for 15-min every hour (3 total). Following (~12:30pm), you will leave the laboratory with standardized meals to consume for the rest of your day outside of the lab (lunch, snack, dinner). You will be wearing the CGM and accelerometer.

Day 4 of trial (~3.5 h at the lab): You will come to the lab in the morning (~9:00am) without having had anything to eat or drink after waking up (except water). We will first obtain a single fasting blood sample. Next, you will consume a standardized breakfast and remain seated in the laboratory for 3 h. You can work on a computer to pass the time. Following (~12:30pm), the investigator will remove the CGM and accelerometer you have been wearing, and you will leave the lab.

Fig. 1 – Overview of metabolic trials (days 0-4)



Detailed Description of Study Procedures:

Anthropometry (height, weight and waist circumference): Your height will be measured with your shoes off using a stadiometer. Your weight will be measured using a standard electronic weighing scale. Your waist circumference will be measured at the umbilicus using a standard, retractable, non-metallic tape measure under the clothing.

Body composition (BodPod): The Bod Pod will involve measurement of your body weight on a standard scale prior to entering an airtight, pressure-controlled pod-like device (with a small window) that is slightly larger than your body. This "pod" will measure how much fat mass and fat-free mass (e.g., total amount of muscle, bone, organs, etc.) you have. The time spent in the "pod" will only be a few minutes and will require you to wear a tight-fitting bathing suit and swimmer's cap.

Fitness test: You will perform an incremental exercise test to exhaustion. Briefly, you will cycle for 2 minutes at 50W, at which point the workload (wattage) will be increased by 1W every 2 seconds (30W per minute). You will be asked to maintain your revolutions per minute (rpm) around 70-80rpm. The test will end when you can no longer continue to cycle against the increasing resistance and your cycling falls below 50rpm. The test usually takes 10-12 minutes. We will measure your heart rate with a chest-worn strap and ask you to wear a mask that collects the air you breathe out (described below).

Collection of air you breathe out: You will breathe normally into a mask that is placed over your nose and mouth either at rest or during exercise. The mask will be attached to tubing and a device called a metabolic cart, which will collect the air that you breathe out. This will tell us how much oxygen you breathe in, and how much carbon dioxide you breathe out, which can be used to determine how much energy you burn and if you are burning carbohydrates or fats as fuel.

7-day food record: You will be asked to complete a 7-day food diary as part of baseline testing. You will need to record everything that you eat and drink, either through a paper food record or a mobile/tablet app called Keenoa. This will allow us to evaluate your nutrient intake and food preferences, which will be used by our study dietitian to prepare your personalized meals for the trials. We ask that the food diary be filled out truthfully and with as much detail as possible. We encourage any questions you have about these diaries, and we will go over how to fill them out with you prior to you filling out your own.

Continuous Glucose Monitor (CGM): A CGM (Dexcom G6) will be worn throughout each 4-day trial to measure your blood sugar levels. The Dexcom G6 CGM is a commercially available sensor system that measures interstitial glucose every 5 minutes. CGM involves inserting a thin, small, flexible filament just beneath the skin on your abdomen beside your belly button (above your stomach), which is attached to a transmitter that sits on the abdomen with an adhesive pad. The transmitter sends data wirelessly to a display device (a compatible smart device).

Venous blood sampling: On Day 3 of each trial, a small, flexible catheter will be inserted into a forearm vein with the guide of a needle. Once the catheter is placed in the vein, the needle is removed. The catheter allows us to take repeated blood samples while you are in the lab, without having to 'poke' you more than once. You are able to move your arm freely while the catheter is in place. We will take a blood sample at 8 time points (~10 ml each) while you are wearing the catheter, for a total of 80 ml (for reference, this is less than 20% of the blood taken during a standard blood donation). After each blood sample, we will flush the catheter with ~1ml of 0.9% saline to prevent a blockage from forming in the tubing of the catheter. When the trial is finished, we will remove the catheter and apply pressure on the site to minimize bleeding. On Day 4 of each trial, a single fasting blood sample (~10 ml) will be obtained from a needle (no catheter is used on this day).

Exercise intervention: The exercise protocol will involve 25 minutes of cycling. Following a 3-min warm-up at a light intensity, you will perform 10 x 1-min cycling intervals at ~90% maximal heart rate interspersed with 1-min of light-intensity cycling. You will perform a 3-min cool-down at a light intensity at the end.

Post-exercise snack: Following exercise, you will consume 350 g of 2% plain Greek yogurt or a study-designed supplement. The study-designed supplement will be similar in texture, consistency and calories as the Greek Yogurt. The Greek yogurt can be sweetened with a non-sugar vanilla flavouring. We will ask you to consume the post-exercise snack within 10 minutes.

Potential Harms, Discomforts or Inconveniences:

Emotional

Although unintended, you may experience some uneasiness or anxiety due to the personal nature of the questions asked on the questionnaires and/or the fitness test or body composition determination (assessment of body fat). We will do our best to minimize these feelings. Body composition and exercise testing will only be supervised by study investigators, and conducted in private. We also welcome any requests you may have to enhance your comfort while participating in this study.

Physical

Venous Blood Sampling: The insertion of a catheter for blood sampling is a common medical procedure and involves minimal risk provided proper precautions are taken. You may experience slight pain, and/or tingling, and/or bruising in the area after the blood samples (venipuncture, and venous IV-catheter). In some instances, you may unexpectedly feel faint or dizzy or lose consciousness if looking at the needle or blood. Also, it is important to know that there is always a risk of infection when the skin is punctured. There is also the remote risk that trauma to the vessel wall could result in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, the investigators have never experienced this. Please inform the researchers if you experience any of the above-mentioned events or are concerned about them.

CGM: You may feel some discomfort (similar to a 'finger flick' on the skin) when the device is placed on the abdomen, however most report that this is a painless procedure. There is a theoretical risk of infection associated with CGM insertion, which will be mitigated considerably by performing this step under sterile conditions. You may experience skin irritation while wearing the monitor as a result of the adhesive pad used to hold the CGM device in place. To minimize the risk of irritation, take precaution when putting clothes on, showering, or putting on a seatbelt, as these activities could move the tape and irritate the skin on the stomach.

Exercise: You will be asked to exercise at a high level that may result in rapid heart rate and breathing, which may present as discomfort. You may stop exercising at any time. The potential risks and discomforts associated with the exercise testing procedures are similar to those associated with any form of physical activity. These include fatigue, fainting, abnormal blood pressure, irregular heart rhythm, and in very rare instances, heart attack, stroke or death (although this has never happened in our studies). You may also experience temporary muscle soreness in the subsequent days. The study team takes all possible precautions to reduce this risk and will not enroll you if they think you are at risk. In the unlikely event that immediate first aid care is needed, an automated external defibrillator (AED) and Emergency First Responder team are both on-site in our building.

COVID-19: Research site is located in the Goldring Centre for High Performance Sport, under the jurisdiction of Toronto public health. We are taking all safety precautions to reduce the risk of spread of COVID-19 and expect you to follow public health directives as well. If you feel that you are from a vulnerable group with respect to COVID-19 effects (e.g., senior, immuno-compromised), please discuss your participation with the research team before consenting. You are under no obligation to participate and nothing bad will happen if you change your mind about participating in the research. Because you are coming onto campus, the following safety protocols must be followed, as per Occupational Health and Safety (see Vaccination Guidance for Human Participant Face-to-face (F2F) Research). We will be collecting personal contact information that we must retain in order to follow up with you and/or conduct contact tracing if you may have been exposed to COVID-19 in coming to the research site. Contact information will be kept separate from data collected through the research study to allow for de-identification of the research data. You maintain your right to withdraw from the study at any time, including research data. If you do withdraw, we will continue to maintain

your contact information and will only give it to Occupational Health if required for contact tracing. We cannot guarantee anonymity as the personal contact information identifies you as a participant.

Trial Compliance Rules

- No structured physical activity during days 0-4 of the metabolic trials (except for study-related exercise)
- No alcohol during days 0-4 of the metabolic trials
- No outside food or drink (except water) during days 1-4 of metabolic trials. The study team will provide you with all of your meals, and you are expected to consume all food provided. If you are a tea or coffee drinker, we can discuss ways to include these beverages on days 0, 2 and 4.
- You will wear the CGM and accelerometer throughout the 4-day metabolic trial.
- You will spend at least 7 h in bed each night throughout the metabolic trial.

Potential Benefits to you:
By participating in this research study, you will: (1) become exposed to a research study; (2) contribute to the advancement of science; and (3) gain knowledge about your own body, health and nutrition. Your own individual results or deidentified group results can also be provided to you upon request.

Reimbursement:
The proposed compensation is meant to adequately reimburse you for any costs incurred (e.g., parking, transportation) and to acknowledge the time and effort you have provided. Compensation will be provided as a total of $400 for full study completion ($200 per completed trial). If you withdraw part way through a trial, you will be compensated at a prorated amount of the days you have completed. There will be no compensation for session 1 or 2 as this includes screening for eligibility purposes.

Confidentiality:
The study investigators are committed to respecting your privacy. Information gathered during this study will be used for research purposes only, including for public presentations and publications. The identity of participants will not be revealed at any time and all collected data will be stored with random study ID only. No persons other than the primary researchers, their research team at the University of Toronto and York University will have access to study data. Paper information will be kept safely for 5 years after publication, at which time, this information will be destroyed. Deidentified biological samples (blood samples) will be kept for 10 years prior to being destroyed. Electronic versions of the data that are anonymous will be kept indefinitely and may be used for related research questions in the future.

Voluntary Participation and Withdrawal: Your participation in the study is completely voluntary and you may choose to stop participating at any time. Your decision not to volunteer, to stop participating, or to refuse to answer particular questions will not influence the nature of the ongoing relationship you may have with the researchers or study staff, or the nature of your relationship with the University of Toronto either now, or in the future. If you decide to stop participating, you may withdraw without penalty, financial or otherwise, and you will still receive remuneration for the time completed. In the event you withdraw from the study, unless you indicate otherwise (and we destroy your samples), your samples and data may be used in our final analysis. Should you wish to withdraw your data after the study, you will have the option to do so before completion of the data analysis. Investigators are also able to withdraw your participation at any time if we consider you ineligible (e.g., change in health status, inability to meet compliance rules). In the event that you are a student in any courses instructed by members of the research team, your decision to participate in or withdraw from this study will have no influence on any aspect of your course involvement (e.g., relationship with instructor, assessment of grades, course standing, etc.).

Research Ethics Board
If you have questions regarding your rights as a research participant, you may contact the Office of Research Ethics at the University of Toronto at 416-946-3273 during business hours. The Research Ethics Boards are also required to do periodic reviews of ongoing research studies. As part of this review, someone may contact you from the Research Ethics Board to discuss your experience in the study

Study Contacts

If you have any questions regarding this study or study procedures, you should contact Dr. Jenna Gillen at 416-946-5620 (office) or by email at jenna.gillen@utoronto.ca

<u>Statement of consent – Participation in the study entitled</u> "Does post-exercise nutrition influence 24-h glycemia following an acute bout of exercise in women?"

My participation in the study has been explained to me, and my questions have been answered to my satisfaction. I understand that I have the right not to participate in this study and the right to withdraw without penalty at any time. The potential harms and benefits (if any) of participating in the research study have been explained to me. I have been assured that data or information relating to me will be kept confidential and no information will be released or printed that would disclose my personal identity without my permission unless required by law. I have been given sufficient time to read and understand the above information.

I consent to participate in the study 'Does post-exercise nutrition influence 24-h glycemia following an acute bout of exercise in women?'

_____ _____

Signature of Participant Date

_____

Printed Name of Participant

_____ _____

Signature of Person Obtaining Consent Date

_____

Printed Name of Person Obtaining Consent