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INFLUENCE OF EXERCISE ON THE TEMPORAL RESPONSE OF SERUM OSTEOCALCIN FOLLOWING NUTRIENT INTAKE

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INFLUENCE OF EXERCISE ON THE TEMPORAL RESPONSE OF SERUM OSTEOCALCIN FOLLOWING NUTRIENT INTAKE

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Abstract

Osteocalcin, also known as bone γ-carboxyglutamic acid, is an osteoblast-specific protein that has long be utilized in research as a marker of bone formation. Recently, osteocalcin has been recognized as a major regulator of glucose metabolism and insulin sensitivity. Osteocalcin deficient mice were shown to have decreased insulin secretion and insulin sensitivity and injections of recombinant osteocalcin reversed these dysfunctions. Furthermore, osteocalcin has been associated with obesity and insulin resistance in human adults. Osteocalcin has also been shown to transiently decrease following ingestion of a glucose load, while acute and long-term exercise has been shown to elevate osteocalcin in adults. Few studies investigating the temporal effects of glucose and/or exercise on osteocalcin levels have been published. Determining how OC responds to exercise and nutrients may help the understanding of how exercise influences glucose metabolism and lead to OC becoming a potential therapeutic target for type 2 diabetes. Therefore the purpose of this study is to examine the effects of a glucose load with or without a prior exercise bout on serum osteocalcin in overweight young men. Fifteen overweight young men were recruited. Subjects reported to the lab on three separate occasions. On two separate trials, conducted on in randomized order and separated by at least 4 days, serum carboxylated osteocalcin, undercarboxylated osteocalcin, glucose and insulin were measured during a standard 75-gram, 2-hour oral glucose tolerance test (OGTT). During one visit the subjects rested prior the glucose drink, while on the other visit aerobic interval exercise (EX) was performed just prior to ingestion of the glucose. There were no significant differences between the OGTT and EX trials in baseline serum cOC (32.24±21.32 vs 26.86±15.68 ng/mL, respectively),

serum uOC (8.48±3.23 vs 9.03±3.68 ng/mL, respectively) or serum tOC (40.72±20.88 vs 35.90±17.98 ng/mL, respectively). There were also no significant differences between the OGTT and EX trials for cOC AUC (3466±2238 vs.3345±1873 ng min/mL, respectively; p=0.470), uOC AUC (932±385 vs. 989±422 ng min/mL, respectively; p=0.295), or tOC AUC (4399±2319 vs. 4334±2018 ng min/mL, respectively; p=0.727). Additionally, exercise did not alter the blood glucose or insulin concentrations compared to the resting trial. There were no significant associations between measurements of cOC, uOC or tOC and glucose or insulin at baseline or the post-glucose AUC. This study is the first to report the temporal response of serum OC within 2 hours of glucose ingestion. The results of the study reveal that a neither a bolus dose of glucose, nor exercise preceding the glucose drink altered serum cOC, uOC or tOC in sedentary, overweight young men. Additionally, there was no relationship between postprandial changes in serum cOC, uOC or tOC with glucose or insulin with or without a prior exercise bout. Therefore, OC does not appear to play a role in glycemic control in overweight but otherwise healthy young men.

Chapter I: Introduction

The Economic Costs of Diabetes report of 2007 states that for every five U.S. dollars spent on health care, one dollar is spent caring for someone with diagnosed diabetes and one out of every ten is spent on health care costs attributed to diabetes [1]. Since the early 1960s, the prevalence of adult obesity in the United States increased from 13.4 to 35.7% and in 2008 over two thirds (68%) of US adults were considered overweight (body mass index, BMI > 25) [2]. In 2009-2010, ~17% of children and adolescents in the United States were considered obese [3]. Since type 2 diabetes (T2D) is often preceded by development of obesity, both of which have increased prevalence, an urgent public health need exists to develop novel strategies to prevent and/or treat obese populations at high risk of developing diabetes. This is especially important in the younger population where obesity can track into adulthood [4].

While skeletal muscle has long been known to regulate energy metabolism, adipose tissue and bone have recently become appreciated for their roles in regulating inter-organ nutrient metabolism. The interaction of bone-specific proteins and glucose metabolism was recently demonstrated in studies of a mouse model harboring an osteoblast-specific protein knockout that contained a side-effect of abnormally large visceral fat pads [5]. The protein of interest in that study was osteocalcin (OC), also known as bone γ-carboxyglutamic acid (Gla) protein (BGP), is an osteoblast-specific protein found in circulation and is one of the ten most abundant proteins found within the human body so elucidation of its effects, especially beyond the bone, is essential to the understanding of human physiology [6]. Since OC was long thought to represent

osteoblast-specific activity, this abundant protein has frequently been measured as a marker of bone formation [7]. However, with the recent work by Ducy and Karsenty [8], the metabolic regulatory properties of OC have become increasingly apparent. For example, OC knockout mice have elevated blood glucose and lower serum insulin levels and also develop insulin resistance and have decreased β-cell proliferation [8]. OC is produced by the osteoblasts and undergoes post-translational modification in a vitamin-K dependent process so that there are two distinct forms found within the body, carboxylated (cOC) and undercarboxylated (uOC) [9]. While both isoforms are found in circulation and have been associated with glucose metabolism in human cross-sectional research, animal models show that uOC is the isoform actively involved in insulin secretion and sensitivity [8].

Recently it has been reported that obese people and/or those with T2D, both of which are likely to demonstrate glucose intolerance and insulin resistance, have decreased serum OC [10-13]. While it is established that both acute and chronic exercise results in increased insulin sensitivity and postprandial glucose disposal, the exact mechanism by which this occurs is not yet clear [14]. It has been proposed that a circulating protein (or proteins) is required for the post-exercise enhancement of insulin sensitivity [15]. Since OC is one of the most abundant proteins found in the circulation, is decreased in those with dysfunctional glucose metabolism, and exercise has the ability to directly affect the tissue from which OC is released (bone, due to loading stimulation), it is reasonable to propose that OC may be a serum factor that mediates improved insulin sensitivity following exercise.

While longitudinal studies have shown that serum OC can be influenced by weight fluctuations, exercise, and nutrition in adults, there has not been sufficient research conducted to determine the acute effects of nutrition and/or exercise on circulating OC. Since the prevalence obesity and T2D has risen there is need for successful intervention strategies and reliable serum markers to prevent and treat insulin resistance. OC may be a potential mediator of insulin action that could serve as a marker of diabetes risk or a potential target for treatment. However, initial studies to characterize how OC and uOC vary with acute nutrient intake and exercise have not been performed. Therefore, the purpose of this study was to examine the changes in serum OC of overweight young men in an acute (2 hours) time period following ingestion of glucose and the influence of exercise preceding glucose ingestion.

Research Questions

1. Do circulating cOC, uOC, or total OC concentrations decrease from baseline (fasting) levels in an acute time period (<2 hours) following ingestion of a glucose load in overweight young men?
2. Can an acute bout of aerobic interval exercise prior to ingestion of a glucose load blunt the decline in postprandial circulating cOC, uOC, or total OC?
3. Are the changes in insulin and/or glucose following ingestion of a glucose load related to the fasting concentrations and/or postprandial changes in cOC, uOC, or total OC?

4. Does an acute bout of aerobic interval exercise influence the relationship between postprandial changes in serum insulin and/or glucose and postprandial changes in serum cOC, uOC, or total OC?

5. Do the components of body composition (i.e. fat mass, lean mass, bone mineral density) correlate with baseline values or postprandial changes in cOC, uOC, or total OC?

Hypotheses

1. It is hypothesized that circulating cOC, uOC, and total OC concentrations will decrease following glucose ingestion in overweight young men.

2. It is hypothesized that an acute bout of aerobic interval exercise will attenuate the postprandial decline in cOC, uOC, and total OC.

3. It is hypothesized that postprandial changes in insulin and/or glucose will associated with baseline concentrations and/or postprandial changes in cOC, uOC, and total OC.

4. It is hypothesized that an acute bout of aerobic interval exercise will alter the relationship between postprandial changes in serum insulin and/or glucose and postprandial changes in serum cOC, uOC, and total OC.

5. It is hypothesized that body composition measures, specifically fat mass and bone mineral density, will be significantly associated with baseline values and postprandial changes in cOC, uOC, and total OC.

Limitations

1. The study population was limited to overweight to obese class I but otherwise healthy, young adult males. This inclusion criteria means that the results can only be applied to this narrow population. It is unknown if the menstrual cycle, age or metabolic disease would result in alterations to osteocalcin in response to glucose with or without exercise.
2. Osteocalcin was the only bone turnover protein measured therefore the subjects' bone remodeling phase was not fully characterized. The measurement of additional bone turnover markers would indicate if the exercise protocol was able to induce bone formation. Additionally, undercarboxylated osteocalcin is released in an absorption-dependent manner and thus measurement of bone resorption would have been useful to determine the effect of bone formation and resorption on the isoforms of osteocalcin.
3. Hemoglobin and hematocrit were not measured during the exercise visit therefore it is unknown if plasma volume changes occurred. Exercise often results in a shift of water out of the vascular space, resulting in hemoconcentration of some of the circulating proteins independent of a treatment effect of exercise per se. Although this type of concentration shift is generally small for the type of short-term exercise in moderate temperature and humidity performed in the current study, applying the volume correction described by Dill and Costill [16] would have been useful to confirm the magnitude of effect.

Assumptions

1. Subjects were honest in answering all questions on health status and exercise readiness questionnaires. The inclusion criteria for this study stated that subjects must be overweight to class I obese but otherwise apparently healthy. Withholding of any information about personal or family health history or exercise readiness could have placed the subject at increased risk for cardiovascular complications during the exercise bout. Additionally, osteocalcin has been shown to be decreased diabetes and it is speculated that persons with lower levels of osteocalcin could have a greater response to exercise so it was important for subjects to be free of diabetes. Fasting glucose samples revealed that no subjects fit the criteria for diabetes so the results were not altered by this factor.
2. Subjects adhered to the same diet and exercise regimen in the three days prior to each trial visit. Osteocalcin is carboxylated in a vitamin K-dependent manner and therefore a change in diet would affect the carboxylation status of osteocalcin. However, given the short timeframe that this study was completed, it is unlikely that any additional dietary vitamin K would have impacted the results. Additionally, exercise can stimulate bone formation and alter glucose metabolism in the following days. An increase in exercise regimen in the days preceding a study visit could result in increased osteocalcin and/or improved glucose tolerance during the study visits.

Abbreviations

OC – Total osteocalcin

cOC –Carboxylated osteocalcin

uOC – Undercarboxylated osteocalcin

BGP – Bone γ-carboxyglutamic acid protein

BMI – Body mass index

HA – Hydroxyapatite

Esp – Embryonic stem cell protein tyrosine phosphatase

OST-PTP – Osteotesticular protein tyrosine phosphatase

GTT – Glucose tolerance test

OGTT – Oral glucose tolerance test

InsR – Insulin receptor

HOMA-IR – Homeostatic model assessment-insulin resistance

T2D – type 2 diabetes

Chapter II: Review of Literature

Synthesis and Release of Osteocalcin

Osteocalcin (OC) is expressed almost exclusively by osteoblasts; however, OC mRNA has been measured in the ovaries, testes, prostate, skeletal muscle, thyroid and adipose tissue [17, 18]. Initially, OC is formed as a 95-99 residue pre-pro-OC and subsequent cleavage of the 23-residue prepeptide results in pro-OC [19]. Pro-OC then undergoes further modification through vitamin K-dependent γ-carboxylation at residues 17, 21, and 24 [20]. Fully carboxylated OC (cOC) has a high binding affinity to hydroxyapatite (HA) and calcium [20-22]. While this binding affinity of cOC suggests that it would be fully incorporated into the bone matrix, its presence in serum concentration demonstrates that some cOC is released into circulation. Approximately one third of newly synthesized total OC is released into circulation [23] but the concentration varies with sex, age, body weight, and height [24]. While most circulating OC is in the cOC form, there is also circulating OC that is not completely carboxylated (undercarboxylated, uOC). Originally, uOC was thought to be a result of vitamin K deficiency [25] or use of the medication, warfarin, or other anticoagulants [26]. Serum OC concentration fluctuates with a circadian rhythm in adult humans [27], with highest levels during nocturnal hours, and trough levels occurring around noon-time. Likewise, a study conducted on seven children (Tanner stage I-III) reported that children display a diurnal variation in serum OC with peak levels occurring in the morning hours and troughs in the early afternoon hours [28].

Discovery of Osteocalcin as a Metabolic Factor

While conducting research on bone physiology, the research team led by Gerard Karsenty generated an *Osteocalcin*-/- (*Ocn*-/-) mouse model to determine the effect on bone formation [5]. The results revealed that OC had an influence on bone formation, but Karsenty and colleague Patricia Ducy also noticed that these mice had an abnormally high amount of visceral adipose tissue [8]. This keen observation provided the first evidence that the skeleton may play a role in whole-body energy metabolism. Since osteoblasts have a scarcity of cell-specific genes, Karsenty and colleagues began a search for an osteoblast-specific gene that could regulate energy metabolism. They created mutant mice with the embryonic stem cell protein tyrosine phosphatase (*Esp*) gene inactivated [8]. The *Esp* gene (also termed *Ptprv*) encodes for a receptor-like protein tyrosine phosphatase termed osteotesticular protein tyrosine phosphatase (OST-PTP) [29]. The *Esp*-/- pups had a severe reduction in blood glucose levels prior to milk ingestion that was maintained through adulthood, even following random feedings [8]. The reduction in blood glucose was explained by an increase in serum insulin in both newborn and adult *Esp*-/- mice. Insulin secretion in response to an intraperitoneal (IP) injection of glucose (glucose tolerance test, GTT) was also increased in the mutant mice and consequently glucose tolerance was increased. Histological and immunochemical analyses of the pancreases of the *Esp*-/- mice revealed increases in insulin content, size and number of islets, and β-cell mass [8]. Therefore, these results showed for the first time that a gene expressed primarily by bone has a large impact on whole body glucose metabolism.

An increase in circulating insulin alone may not be effective in decreasing blood glucose, since T2D is characterized by a decrease in hepatic and peripheral insulin sensitivity, typically in the presence of elevated, compensatory, circulating insulin concentration [30]. However, *Esp*-/- mice had both an elevated fasting insulin concentration, and an increase in insulin sensitivity compared to wild-type (WT) mice when measured by an insulin tolerance test (ITT) [8]. Hyperinsulinemic euglycemic clamps confirmed that the mutant mice required a 50% increase in glucose infusion rate to maintain euglycemia compared to their WT littermates, further establishing the increased insulin sensitivity in the *Esp*-/- mice [8]. The increased infusion rate was due to an increase in glucose uptake of the muscle and white and brown adipose tissue. Adiponectin, a protein secreted by white adipose tissue, has been shown to increase insulin sensitivity [31] and *Esp*-/- mice showed an increased gene expression in fat and serum levels of adiponectin [8]. This suggested that bone can communicate with adipose tissue to affect insulin action in insulin-sensitive tissues such as skeletal muscle.

Esp-/- mice were also studied using two different models of obesity: goldthioglucose (GTG) induced and high-fat diet (HFD) induced obesity. The inactivation of *Esp* was able to correct the metabolic abnormalities typically seen in these forms of obesity. While injection of GTG into WT and *Esp*-/- mice resulted in hyperphagia, only the WT+GTG mice gained significant body weight compared to those not injected with GTG. Knockout of *Esp* was also able to spare the mutant mice from the resultant increase in fat mass, serum triglyceride concentration, and glucose and insulin intolerance. Similar metabolic protection occurred in the HFD-induced obesity model with *Esp* inactivation [8].

Another series of experiments by the same investigators using a gain-of-function approach by overexpression of the osteoblast-specific *Esp* gene, further demonstrated the role osteoblastic expression of *Esp* on whole-body glucose metabolism. *Esp* overexpression resulted in an opposite metabolic phenotype to that seen in the knockout experiments. Overexpression of *Esp* within the osteoblasts resulted in decreased β-cell proliferation and mass, decreased insulin concentration following feeding, along with impaired insulin secretion in response to glucose injection, and decreased adiponectin concentration [8]. Co-culturing osteoblasts with pancreatic islets from WT mice increased *Insulin* expression with a greater increase occurring in *Esp*-/- osteoblasts [8]. Co-culture of WT osteoblasts with WT adipocytes increased *Adiponectin* expression within the adipocytes and, in support of the earlier evidence that *Esp*-/- mice had increased serum adiponectin concentration, *Esp*-/- osteoblasts were able to double this increase in expression. It was determined that the increases in *Insulin* and *Adiponectin* expression were a result of a molecule that is secreted by the osteoblasts since the expression of these genes were increased even when separated during co-culture.

Since *Ocn*-/- mice had been recognized previously as being abnormally fat, subsequent research addressed whether OC is a molecule secreted from osteoblasts that regulates glucose homeostasis under the control of *Esp* [8]. The serum of *Ocn*-/- mice had decreased insulin and adiponectin while their serum glucose and triglyceride concentration was increased. These mice also demonstrated impaired glucose tolerance and insulin secretion. Additionally, islets size and number and β-cell mass and proliferation were decreased while their adipocyte number and fat mass were both

increased. Thus, *Ocn*-/- mice were phenotypically opposite of those mice with inactivation of *Esp*.

Similar to the co-culture studies of *Esp*-/- osteoblasts, *Ocn*-/- osteoblasts were put in co-culture with WT islets or adipocytes; however, unlike the WT or *Esp*-/- osteoblasts, *Ocn*-/- osteoblasts were unable to induce expression of either *Insulin* or *Adiponectin* in the islets or adipocytes, respectively [8]. Overexpression of *Osteocalcin* was able to increase the expression of *Insulin* and *Adiponectin*; moreover, addition of recombinant *Osteocalcin* to fibroblasts co-cultured with β-cells was able to induce expression of *Insulin* in these cells. These culture studies were supported by in vivo experiments subjecting *Ocn*-/- mice to a GTT where half of these mice were injected with glucose only and half received glucose plus 20ng of OC [8]. As discussed earlier, the *Ocn*-/- mice were glucose and insulin intolerant; however, those injected with recombinant OC were able to tolerate the glucose load similar to WT mice and insulin secretion was significantly increased compared to those mice injected with the vehicle treatment. This finding demonstrated that OC plays a primary role in glucose metabolism.

Since *Esp*-/- and *Ocn*-/- mice display opposite metabolic phenotypes, it was hypothesized that these two genes must lie along the same metabolic pathway. In fact, deletion one allele of the *Ocn* gene in *Esp*-/- mice reversed the metabolic advantages that were shown in previous experiments [8]. Interestingly, the serum levels of OC were unaffected by *Esp* knockout, which led to the proposal that OST-PTP may affect osteocalcin activity and not production. To address this possibility, Karsenty's group examined whether the effect of OST-PTP on OC was to promote the post-translational

γ-carboxylation of OC. They took advantage of the binding affinity that cOC has to HA and discovered that, following incubation with HA, 16% less OC from *Esp*-/- mouse serum was bound to HA than OC measured in the serum from WT mice [8]. To further establish that it was the undercarboxylated form of OC that regulated glucose metabolism, WT osteoblasts were treated with warfarin, known to inhibit γ-carboxylation [32], and this treatment resulted in a decrease in the amount of OC bound to HA [8]. These warfarin-treated osteoblasts were more potent in inducing the expression of *Adiponectin*. Also, only uOC, produced by bacteria, was able to induce expression of *Insulin* and *Adiponectin* in islets and adipocytes, respectively [8]. Summarizing their findings, the authors stated since *Esp*-/- did not develop obesity or diabetes and were resistance to glucose intolerance and insulin resistance that the skeleton may act as a key regulator of energy metabolism and contribute to the development of the metabolic syndrome.

So throughout the study by Lee et al. [8], Karsenty and colleagues had shown that the bone was a key regulator of glucose metabolism through their ingenious *Esp*-/- mouse model since *Esp* is an osteoblast specific gene. Subsequently, through their co-cultures of osteoblasts with islet and adipocyte cells, they established that there must be an osteoblast specific protein that is being released from these *Esp*-/- cells influencing adiponectin and insulin. They turned back to their observation of *Ocn*-/- mice being abnormally fat and established that OC was the protein responsible. Further investigation showed that *Esp* did not regulate the production of OC but was responsible for reduced carboxylation of OC, which in turn increased the ability of OC to improve glucose metabolism.

A key characteristic of hormonal regulation is that an endocrine organ secretes hormones to act on other tissues and also receives information from the affected tissue as part of a feedback loop. Since they were able to show that OC secreted from osteoblasts was able to affect insulin release by the pancreas, Karsenty's research group began to examine if insulin could then affect the osteoblasts, and thereby form a feedback loop. The interaction of insulin with its receptor (InsR) results in a phosphorylation of the receptor. Building off of the previous studies of *Esp*-/-, Karsenty and colleagues conducted experiments that determined that OST-PTP was able to dephosphorylate, meaning deactivate, InsR and that *Esp*-/- osteoblasts showed increased phosphorylation of the InsR [33]. They were then able to generate a mouse model with an osteoblast-specific knockout of InsR and these mutant mice had decreased insulin secretion in response to a glucose challenge, increased postprandial blood glucose, and glucose and insulin intolerance. This mutant model demonstrated that insulin signaling within the osteoblast had a significant effect on whole-body glucose homeostasis. Following these observations, Karsenty and colleagues proposed that, since OST-PTP has an influence on insulin signaling and OC activity within the osteoblasts, these two must lie in the same metabolic pathway as well. To prove this hypothesis they showed that mice with impaired insulin signaling in osteoblasts due to the loss of one allele of *InsR* had decreased uOC levels and that *Esp*-/- mice had an increase in uOC and increased insulin signaling in osteoblasts [33]. Additionally, they found that *InsR*-/- mice had a decrease in bone resorption markers while the *Esp*-/- mice had an increase in these markers. Combining these observations and the knowledge that bone resorption results in an acidic pH in the bone matrix, which can decarboxylate

proteins [34], the authors theorized that bone resorption may regulate the carboxylation of OC. To test this hypothesis, the authors incubated fully carboxylated OC in a solution that had a pH 4.5 to replicate the pH of resorption lacunae and this resulted in a 2-fold increase in decarboxylation compared to cOC incubated in a pH 7.5. Additionally, they cultured osteoclasts on cortical bone slides and reported that when osteoclast differentiation was triggered there was a 2-fold increase in the uOC/OC ratio [33]. Subsequently, the authors used a mouse model that had a decreased ability to acidify the bone extracellular matrix and found that these mice showed similar metabolic characteristics as *Ocn*-/- mice. Importantly, the *Esp* gene in mice is just a pseudogene in humans, so there is concern that some results may not be fully generalizable. However, the finding that the insulin receptor was a substrate for the protein encoded by *Esp* (OST-PTP) was used to identify that the human protein involved in insulin signaling in the osteoblasts was protein-tyrosine phosphatase 1B (PTP1B) [33]. The same authors also reported that human patients with a defect in bone resorption had decreased concentration of circulating uOC and decreased serum insulin following feeding [33]. Therefore, the major findings of this study were that the osteoblast secretes OC, osteoclast activity decarboxylates OC, insulin signaling in the osteoblasts is a key regulator in whole body glucose homeostasis, and PTP1B is the human protein within the intracellular insulin signaling pathway that can regulate OC activity [33].

To confirm an in vivo role for OC in glucoregulation, the same research group first implanted 8-week old WT mice with pumps that delivered OC in amounts of 0.03, 0.3, 3 and 30 ng/h for 4 weeks [35]. Ferron et al. established that mice receiving OC in

doses or 0.3 and 3 ng/h had significantly lower blood glucose and increased insulin levels than those receiving a vehicle. They also reported that the mice receiving 0.3 or 3 ng/h had greater tolerance to glucose and insulin sensitivity than mice receiving vehicle. Subsequently, they implanted mice with pellets that released 3 ng/h of OC and fed them a high-fat diet for 8 weeks to examine if this exogenous dose of OC could counteract the negative metabolic effect seen in diet-induced obesity. They reported that the mice receiving OC treatment gained significantly less weight, had smaller fat fad, normal triglyceride levels, had improved glucose tolerance and were more insulin sensitive than mice fed a high-fat diet without OC treatment [35]. Based on the results from their previous study, Ferron et al. [36] subsequently investigated whether daily injections with recombinant OC at doses of 3 or 30 ng/g/day would improve glucose handling by 8-week old WT mice. While mice injected with OC did not differ in body weight during the 16 weeks of treatment, following 5 weeks of treatment, and continuing throughout the treatment period, the 30 ng/g/day dose resulted in reduced blood glucose compared to mice receiving a vehicle injection. Glucose tolerance tests performed at both 4 and 8 weeks of treatment revealed that both doses were able to improve glucose clearance (in a dose-dependent manner) and insulin sensitivity following 4 weeks of treatment. Following the full 16 weeks of treatment with OC the WT mice had, dose-dependent increases in insulin secretion to a glucose challenge and increased β-cell mass. These results established that the 30 ng/g/day dose was the most effective for improving glucose metabolism. To further establish the therapeutic effect of exogenous OC, Ferron et al. [36] put 8-week old mice on a high-fat diet for 8 weeks to induce insulin resistance and dysglycemia. Following the 8-week high-fat diet the

mice were injected with 30 ng/g/day for 8 weeks. Injection of OC resulted in partial normalization of FPG and the glucose intolerance compared to mice injected with a vehicle following just a month of treatment. Additionally, insulin sensitivity was increased and fasting serum insulin decreased by 70% in mice receiving OC compared to the vehicle-injected mice following 8 weeks of treatment. Interestingly, while food intake and physical activity were similar in the vehicle- and OC-injected mice, the mice injected with OC had significantly lower body weight and fat mass that was attributed to increased energy expenditure and heat production [36]. These results suggested that OC was able to reduce body weight gain resulting from a high-fat diet by increasing energy expenditure. Morphological analysis of the skeletal muscle of OC injected mice revealed greater mitochondrial mass compared to vehicle injected mice, which provides a plausible mechanism through which excess energy could have been dissipated rather than accrued as stored lipids [36].

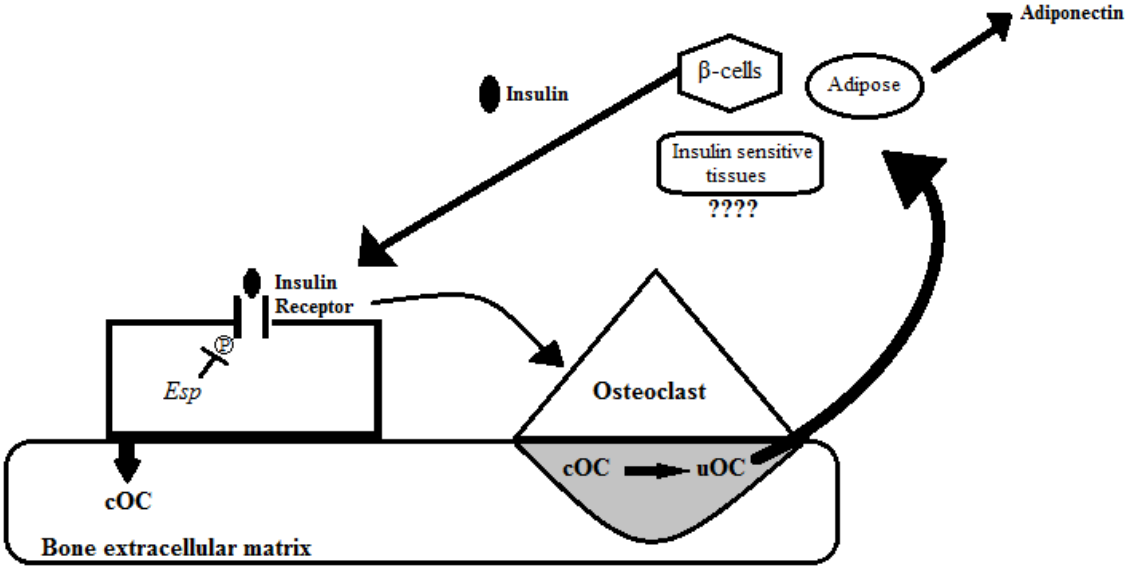
This series of experiments done by Karsenty and colleagues established that OC plays a major role in the regulation of glucose metabolism and energy expenditure and that insulin plays a key role in the bioavailability of OC via a feedback loop . In a recent review [37], Karsenty stated:

> "Altogether, the metabolic functions of osteocalcin and their regulation by leptin and insulin reveal how intertwined energy metabolism and bone (re)modelling are. They also underscore the importance of the interplay between osteoblasts and osteoclasts for glucose metabolism and indicate that the regulation of osteocalcin activity occurs at both the transcriptional and post-translational levels."

Subsequently, Karsenty and Ferron [37] proposed several future directions of research on OC and its role as a regulator of glucose metabolism. While their research team led the way in establishing the bone as a key factor in energy homeostasis and the role that

OC plays in metabolism, much of their research conducted in mice needs to be verified in humans. Human studies will help establish whether the results from studies of mice are generalizable among species and the role of OC in human glucose regulation

Figure 1. Proposed release and action of OC. Adopted from Ferron et al. [33].



Associative studies of Osteocalcin in Humans

Obesity was long thought to have a protective effect on bones [38-42]; however, growing evidence shows that obesity has may actually be associated with low bone mineral content and density, as well as an increased risk for osteoporosis, osteopenia, and non-spinal fractures [43-46]. Early cross-sectional research showed that OC was associated with improved glucose metabolism and insulin sensitivity [10, 11, 47-49]. For example, in older adults (age >65), serum OC was inversely associated with fasting plasma glucose (FPG), insulin, homeostatic model assessment-insulin resistance (HOMA-IR), C-reactive protein, BMI and body fat [12]. These authors also reported that, over a 3 year period, average serum OC was inversely related to the increase in

FPG, which suggests that higher level of OC may be a marker of decreased diabetes risk in older people. In support of these findings, Kindblom et al. [11] showed an inverse correlation with fasting OC and BMI, fat mass, FPG and HOMA-IR in a group of >1,000 elderly Swedish men. In those men, plasma OC was a significant predictor of blood glucose, even when controlling for serum insulin, triglycerides, and body composition. In the subset of men with diabetes in that study, approximately 15% of group, circulating OC was 20% lower than the men without diabetes. The Japanese lab of Ippei Kanazawa also conducted a series of cross-sectional studies that revealed that total serum OC was positively associated with insulin sensitivity and secretion while negatively correlated with FPG, HOMA-IR and hemoglobin A_{1C} (HbA_{1C}) in men and women with T2D [48]. Consistent with the hypothesis that uOC is the active isoform of OC in regulating glucose metabolism the same authors also reported that uOC was negatively associated with FPG and HbA_{1C} while the ratio uOC/OC was positively correlated with BMI, and visceral and subcutaneous fat area in men with T2D [49]. Furthermore, serum OC was positively correlated with brachial-ankle pulse wave velocity (baPWV) and intima-media thickness (IMT), which are measures of atherosclerotic risk, in men with T2D [10].

In agreement with the studies in middle-aged or elderly people, recent research in younger populations supports that OC is associated with multiple components of glucose metabolism and insulin resistance [50, 51]. For example, a study of young adults (17-22 years) showed that total OC, but not uOC, was inversely associated with BMI, waist circumference and systolic blood pressure [52]. A few larger cohort (N=84-1768) studies that included people who were 19-82 years old reported that total OC was

negatively associated with visceral fat area [51, 53] and improved glucose tolerance and insulin secretion and sensitivity [54]. In addition, Lucey et al. [50] reported that young (age 20-40 years) women with less lean body mass (LBM) had lower total OC along with high C-reactive protein (CRP), fasting glucose, insulin and HOMA-IR compared to similar size women. Additionally, women in the lowest tertile of total OC were highest in CRP and men in the lowest tertile of uOC had double the concentration of CRP than men in the higher tertiles. These results combined show that even in young adults OC and uOC may play a role in glucose metabolism, insulin resistance and inflammation associated with obesity.

Over the past four decades obesity prevalence in adolescence has been on the rise and this sharp rise requires researchers and practitioners to develop effective lifestyle interventions that incorporate exercise, nutrition or both, to prevent or reverse the metabolic dysfunction that often accompanies obesity. Establishing a marker of insulin resistance that is responsive to changes in diet and physical activity may be valuable to understand the mechanisms of obesity and inactivity-induced insulin resistance and to monitor effects of specific interventions. Further study of OC may serve either of those purposes. In support of the results from studies with adults, Boucher-Berry et al. [55] reported serum OC level was associated with components of body composition and biochemical markers in 106 middle school children. Total serum OC was inversely related to BMI, percent body fat and fat mass, while being positively associated with FPG in the total group of children. Pollock et al. [13] compared serum OC and OGTT measures of 41 overweight, prediabetic children compared to 99 children who were also overweight but had normal glucose measures. While there were

no differences reported between the groups for BMI percentile, percent body fat, physical activity levels, or subcutaneous abdominal adipose tissue, the prediabetic children had significantly lower total OC (15%) and uOC (28%). Additionally, although not significant, children with prediabetes had a decreased percentage of OC that was decarboxylated. Results from multivariate analysis, using age, sex, race and visceral adipose tissue as covariates, revealed that cOC was positively associated with insulin sensitivity and negatively associated with fasting insulin in the children with prediabetes while uOC was positively associated with both insulinogenic index and disposition index. These results suggest that while cOC may influence glucose homeostasis, uOC may play more of a role in β-cell dysfunction [13].

Not all studies with children are in agreement, however. Rochefort et al. [56] found that fasting concentrations of serum OC and uOC did not differ between obese and non-obese prepubertal children. Furthermore, in a study of 497 healthy German children, Flemming et al. [57] showed that while serum OC was associated with pubertal development and peaked at Tanner stage 3, there was no significant association with BMI. The only significant difference in OC between overweight/obese children and their normal weight counterparts occurred in early puberty (combined Tanner stages 1 and 2). However, the authors reported a significant positive correlation between serum OC and waist-to-hip ratio, specifically in those children in late puberty (combined Tanner stages 4 and 5) [56].

These results indicate that OC may be a marker of dysfunctional glucose metabolism in elderly and young adults and it could also be an effective early marker in children and adolescents. While the exact mechanisms by which OC may affect

glucose metabolism in humans and the isoform that is most active within the glucoregulatory pathway in humans is yet to be established, the cross-sectional results support a need for further investigation about whether and how OC responds to nutritional and physical activity interventions. Such studies should result in better understanding about the potential role of changes in circulating OC or uOC and glucose metabolism in humans.

Table 1. Summary of literature from human studies that report association between OC and selected metabolic risk factors.

	OC	uOC	cOC
Obesity (BMI)	Negative (4 studies)	Inconclusive	Negative (1 study)
Type 2 diabetes	Negative (3 studies)	Negative (1study)	Unknown
Fasting glucose	Negative	Negative (2 studies)	Unknown
Fasting insulin	Negative	Negative (2 studies)	Unknown
HOMA-IR	Negative	Inconclusive	Negative (1 study)
Insulin sensitivity	Positive	Positive	Unknown

Effects of Exercise or Nutrients on Osteocalcin

The reduction in cardiovascular risk associated with an increase in physical activity has been well documented. One of the physiological responses to exercise is enhanced insulin sensitivity and glucose uptake [14]. While many mechanisms for this response have been postulated, the underlying regulatory events have not yet been elucidated. Interestingly, perfused muscles of rats [14] and muscle extracted from rats following exercise [58] showed increased insulin sensitivity; however, muscles that were washed and then contracted *in vitro* did not show the same enhancement in insulin sensitivity [15]. This suggests that a circulatory protein may potentiate insulin action on skeletal muscle. Given its influence on insulin secretion and sensitivity and that exercise

increases bone formation [reviewed in 59, 60], it has been hypothesized that OC may be the missing serum protein that can link exercise to changes in insulin sensitivity [61].

The effects of exercise on serum OC and the association that these changes may have on glucose metabolism have not been as well documented in the literature at this time. However, measurement of serum OC as a marker of bone formation following exercise has been well characterized. Nishiyama et al. [62] reported that athletic individuals had increased serum OC compared to those with lower physical activity. Additionally, 30 minutes of running at ~50% maximal workload resulted in a significant increase (58%) in serum OC in less athletic individuals immediately following exercise, while the athletes actually had an 11% reduction in serum OC. Conversely, 60 minutes following cessation of exercise, the athlete group had a 17% increase from baseline (32% from immediately after exercise) in serum OC whereas the non-athlete group only showed an 11% increase from baseline (-30% from immediately following exercise) [62].

Additionally, in recreationally trained young males, a low-intensity cycling bout (45 minutes at 55% VO_2max) resulted in no changes in serum OC 4 hours following the cessation of exercise while both higher intensity cycling (15 minutes at 85% VO_2max) and strength exercise (5 sets of 8 repetitions at 85% 3RM) resulted in a decrease in OC at the 4-hour post-exercise time point [63]. Rong et al. [63] reported that the absolute concentration of OC wasn't increased following the low-intensity cycling bout, although there was a small relative increase when compared to the control (no exercise), in which OC declined post-exercise. Since the study began at 0700-0800, the control condition of their study showed the diurnal decrease at noon stated previously;

therefore, when comparing the exercise conditions to the control condition, the high-intensity cycling and strength exercise resulted in no effect on OC while low-intensity cycling was able to maintain steady OC concentrations, counteracting the typical early afternoon trough [63].

Maïmoun et al. [64] had 7 male cyclists perform 50 minutes of cycling at either 15% below or 15% above their ventilatory threshold (VT). Contrary to the findings of Rong et al. [63], the longer duration of the high-intensity bouts conducted in this study resulted in increased serum OC [64]. Cycling at -15% of VT resulted in a 7.8% increase in serum OC (not significant); while an intensity of +15% of VT resulted in a significant 12% increase immediately upon cessation of exercise. Interestingly, the authors reported that serum OC returned to baseline within a 15-minute recovery period following exercise. Taken together these data suggest that exercise intensity may play an important role in OC production.

Not all reports on serum OC following exercise are in agreement however; Tosun et al. [65] examined serum OC in young women immediately and 15 minutes following either brisk walking or similar exercise (60-85% age-predicted maximal heart rate) carrying a 5kg weight in a backpack. The within-subject statistical analysis revealed that there was no significant difference between the exercise bouts and the resting measurements of serum OC. Additionally, a similar non-weighted exercise stimulus was unable to affect circulating OC at 1, 8, 16, 24, or 32 hours post-exercise in 10 healthy young (age 20-35) men [66]. The combination of the above results could indicate that serum OC is affected by the training status of the individual and the

intensity of the exercise stimulus involved. Also, post-exercise variations in serum OC could differ depending on the timing of the obtainment of the serum sample.

In addition to the above acute studies utilizing OC as a marker of bone formation, Hinton et al. [67] studied 19 overweight subjects during a 6 week weight loss intervention examining changes in OC with aerobic exercise. Their results revealed that while weight was reduced by ~5%, normally resulting in decreases in bone formation [68, 69], the addition of aerobic exercise was able to neutralize these theorized decreases, and increase OC by 18.5% while increasing bone alkaline phosphatase (BAP), another marker of bone formation [67].

While the previous studies investigated changes in OC in response to exercise, most were examining serum OC only as a marker of bone turnover. Fernández-Real et al. [47] conducted three separate studies to examine the associations of OC on people without diabetes and if weight loss, either through diet alone or diet and exercise, has a significant effect on these concentrations. Their cross-sectional study of healthy, middle-aged people supported the findings suggested earlier that circulating OC was positively associated with insulin sensitivity (calculated by HOMA-IR). The authors also examined the effect of weight loss achieved either with diet alone (-500 kcal/day) or diet with the addition of a structured resistance exercise program on circulating OC and insulin resistance in 26 obese female subjects. While weight loss (~7-8%) alone had no effect on serum OC, a structured resistance training program along with diet resulting in a similar degree of weight loss resulted in increased serum OC [47]. Corresponding to these increases in serum OC for the diet plus resistance training group were decreases in visceral adipose tissue (r= -0.53) and increases in leg strength (r=

0.69). While the diet plus resistance training group experienced increases in leg strength and no change in thigh muscle mass, the diet only group saw a decrease in thigh muscle mass. For the entire group the changes in visceral adipose tissue was determined to be the greatest predictor of change in OC in multiple regression analysis when the authors controlled for age, BMI, and HOMA-IR; when leg muscle strength was added to the model, both change in visceral adipose tissue and change in leg muscle strength accounted for 30% of the variation in OC [47]. In addition to these studies, Fernández-Real and colleagues examined if double the weight loss (~17%) could result in greater changes to circulating OC. In the 20 obese (average BMI = 38.0) subjects studied, they did report a significant increase in serum OC; however, the increase in OC showed no significant associations with changes in insulin resistance, measured by the quantitative insulin sensitivity check index (QUICKI) [47]. Fernández-Real and colleagues showed that moderate (~17%) but not slight (~7%) diet-induced weight loss can increase serum OC levels and this finding may indicate that there is a threshold of weight-loss that is needed to show the changes in bone turnover that are induced by weight loss that have been previously reported [reviewed in 70]. The most intriguing finding in their studies seemed to be that resistance exercise training and diet resulting in similar slight weight loss can also significantly increase circulating OC. A possible explanation for this is that increased total serum OC is only indicative of increased bone turnover and that uOC has been more closely linked with changes in glucose metabolism [8] so the percentage of uOC could differ between the diet only weight loss and the diet plus exercise induced weight loss.

In addition, Levinger et al. [71] investigated the changes in serum OC following either an acute aerobic or an acute resistance exercise bout and how these changes may associate with changes in glucose metabolism. They studied 28 middle-aged, obese men, randomly assigned to perform either 45-minutes of cycling at a heart rate corresponding to 70-75% VO_2max or an acute power exercise bout, 2 x 5 sets 70-75% 1RM leg press with a jumping routine completed between each five sets. Both OC and uOC was significantly increased by the acute aerobic exercise bout, while the acute power exercise bout showed no significant increase in either OC or uOC. These authors also performed a sub-analysis of subjects with T2D and found that exercise, pooled aerobic and power, was able to increase uOC by 4.4% and this change in uOC was positively correlated with changes in blood glucose concentrations following the exercise bout [71].

Since insulin has been shown to have an effect on bone remodeling [33] and insulin signaling in mice has been shown to increase OC activity, it would be reasonable to believe that factors increasing insulin would in turn effect circulating OC. An oral glucose tolerance test (OGTT) is a common clinical test conducted to examine the glucose clearance rate by the body following ingestion of 75 grams of glucose, typically done to test for possible T2D [72]. Paldánius et al. [73] recently examined the effect of an OGTT on circulating OC concentrations in 23 healthy subjects. Following (2 hours) the consumption of 75 grams of glucose, serum OC decreased by 32.1% and cOC decreased by 34.4%. The authors also reported that these changes in serum OC and cOC were not associated with changes in insulin or glucose at this 2-hour time point [73].

Previous studies have suggested that people with the lowest basal level of OC have the greatest increase in OC following exercise [74]. Since other studies have shown that overweight individuals have decreased serum OC compared to their normal weight counterparts, this suggests that overweight people may experience the biggest change in OC in response to an exercise bout. Consistent with this possibility, obese prepubertal children that underwent a 6-month training program consisting mostly of aerobic activities (i.e. cycling, rowing, jumping) demonstrated an increased whole-body bone mineral density (WB-BMD) compared to comparable children without training [56]. Additionally, Rochefort et al. reported increases in serum OC (27%) and serum uOC (37.7%) following 6 months of physical training by obese children. These results also indicated a 3.4% increase in the percentage uOC/OC from 39.3% to 42.7%. In another study obese children had lower OC compared to normal weight peers and completed a year-long weight loss intervention involving of exercise, behavior modification and nutrition therapy [75]. The weight-loss program resulted in increased serum OC, which was related to decreased HOMA-IR, BMI and leptin. In a subset of the obese children, the authors pointed out that 29 of the 60 achieved substantial weight loss, "defined as a reduction in BMI standard deviation score of ≥ 0.5, because only with a reduction of > 0.5 in SDS-BMI was an improvement in insulin resistance measured in obese children" [75]. Children who had substantial weight loss also had a significant increase in OC, while those without substantial weight loss did not demonstrate a significant elevation in OC. The children with substantial weight loss also had significant reductions in HOMA-IR and leptin concentration following the weight loss program [75].

Table 2. Summary of studies on OC responses to exercise in humans.

	OC	uOC	cOC
Acute exercise	↑	↑	Unknown
Exercise training	Inconclusive	Unknown	Unknown

In summary, OC is produced by the osteoblasts and is one of the most abundant proteins found within the circulation. It is released into circulation in two distinct forms determined by the carboxylation status, fully carboxylated and undercarboxylated. It was recently discovered that OC plays a role in glucose metabolism by increasing insulin secretion by the β-cells and increasing insulin sensitivity of the peripheral tissues. Serum OC increases following an acute exercise bout and these changes may be influenced by differing intensity, duration and mode of exercise along with the training status and sex of the population studied. Additionally, longer duration interventions have shown that weight loss achieved by increases in physical activity result in increased circulating OC and the changes in OC are inversely associated with reduction in HOMA-IR. While exercise has been shown to increase circulating OC, glucose intake has been shown to decrease serum OC in an acute timeframe following ingestion.

Chapter III: Methods

Subjects

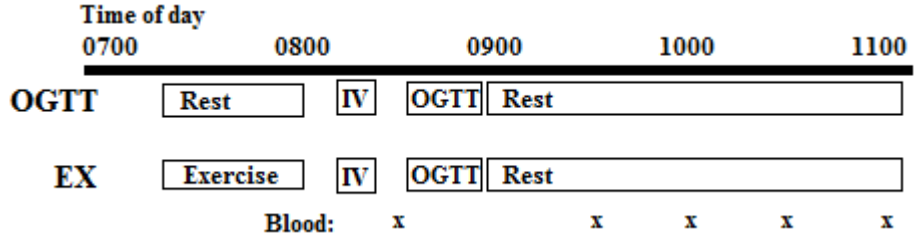
15 overweight or obese young men (18-25 years old) males were recruited to participate. Eligibility inclusion criteria include the following: 1) subjects were overweight as defined by a BMI 25-35 kg/m^2, 2) subjects were not physically active for > 1 hour/week, 3) subjects did not have any cardiometabolic disorders including diabetes, heart disease, arrhythmias, thyroid disease, or hypogonadism, 4) subjects were free of any diseases known to result in decreased bone mineral density.

Research Design

A two-way [intervention (resting vs. exercise) x time (0, 30, 60, 90, 120 minutes)] repeated measures design was utilized to determine the acute effects of exercise or nutrition on OC and glucose metabolism. All subjects reported to the lab on 3 separate occasions [screening, oral glucose tolerance test (OGTT), exercise prior to OGTT (EX)] with at least four days between visits. During the first visit, subjects completed the informed consent forms and were informed of all the requirements to participate in the study. Subjects were then verbally and medically screened to ensure that they met the inclusion criteria. These steps were followed by measurement of body composition using Dual Energy X-Ray Absorptiometry (DXA) and a maximal aerobic capacity test (VO_2max). During each subsequent visit, subjects arrived at the laboratory between 0800 and 0830 following a 10-hour fast and completed one of the two testing protocols in predetermined random order. During the OGTT visit, subjects rested in a

seated or supine position and an intravenous catheter was inserted in a forearm or hand vein and kept patent with saline infusion for serial blood sampling. A blood sample was drawn and designated as the first time point (T0). Following T0, subjects consumed a 75 gram oral glucose load (296 mL; Thermo Scientific, Middletown, VA) within 5 minutes and subsequent blood collections occurred again 30, 60, 90, and 120 minutes after completion of the drink (T30, T60, T90, and T120, respectively). During the EX visit, subjects completed a 5-minute warm-up followed by 4 blocks of exercise consisting of 4 minutes at ~90% maximal heart rate (HRmax, actual: 78-89%) with 3 minute active recovery at a workload eliciting ~65% HRmax, and a 6-minute cool-down (36 minutes total, average HR for entire workout: 77 ± 5% max). A high-intensity interval exercise bout was chosen due to previous research showing greater long-term improvements in insulin signaling utilizing this method compared to moderate continuous moderate exercise [76]. Within 10 minutes of cessation of exercise, subjects had an intravenous catheter inserted in a forearm or hand vein and underwent the same procedures as the OGTT visit. The timing of each test was adjusted so that the ingestion of the glucose load consistently occurred around 0900 and T30 consistently occurred around 0930 among trials to account for the diurnal variations in serum OC in young adults [27]. Study design is shown in Figure 2.

Figure 2. Study Design.



Variables

The independent variables studied were the interventions performed (resting and exercise). The dependent variables studied were:

Carboxylated Osteocalcin – Serum carboxylated OC was quantitatively determined using a commercially available enzyme immunoassay (EIA) kit (Gla-type Osteocalcin EIA kit, Cat. #MK111, Clontech laboratories, Inc., Mountain View, CA). Briefly, 100 µL of serum samples along with a 6-point serial dilution standard (16-0.25 ng/mL) was added, in duplicate, to a microtiter plate that had each well coated with murine monoclonal antibody to Gla-OC. The plate was incubated at room temperature for 2 hours on an orbital shaker at 150 revolutions per minute. Following incubation, the wells were decanted and washed three times with 400 µL of wash buffer [phosphate-buffered saline (PBS)] using a plate washer (Wellwash 4 MK 2, ThermoFisher Scientific, Inc., Waltham, MA). 100 µL of antibody-POD conjugate (horseradish peroxidase conjugated murine monoclonal antibody to OC) was added to each well using an 8-channel pipette and incubated for 1 hour at room temperature on an orbital shaker. Following this incubation, mixtures were decanted and wells were washed 4 times with the washing buffer. Following wash, 100 µL of substrate solution (hydrogen peroxide and tetramethylbenzidine in a buffered solution) was added to each well using an 8-channel pipette and plate was allowed to incubate at room temperature for 15 minutes on an orbital shaker. Subsequently, 100 µL of stop solution (1N H_2SO_4) was added to each well and mixed to stop the reactions. The plate was mixed by hand and the absorbance was read using a microtiter plate reader (Synergy 2 with Gen5 software, Biotek Instruments, Inc., Winooski, VT) at 450 nm using distilled water as a control to

make zero adjustment. The absorbance of the samples was plotted using the standard curve for quantitative determination of the concentration of carboxylated OC. In order to reduce the bias by inter-assay variability, samples for each subjects (OGTT and EX) were analyzed on the same microtiter plate. Intra- and inter-assay precision 3% and 3%, respectively. The manufacturer reports that this assay had no detectable cross reaction with decarboxylated OC.

Undercarboxylated Osteocalcin – Serum uOC was quantitatively determined using a commercially available enzyme immunoassay (EIA) kit (Undercarboxylated EIA kit, Cat. #MK118, Clontech laboratories, Inc., Mountain View, CA). Briefly, samples were first diluted 1:1 with sample diluent. 240 µL of diluted serum samples along with a 6-point serial dilution standard and blank (dH2O) were added to an untreated microtiter plate. The samples, standards and blank were then transferred (using an 8-channel pipette so to ensure the entire plate was loaded in <5 minutes) to a supplied microtiter plate on which each well was coated with anti-Glu-OC monoclonal antibodies. The plate was incubated at room temperature on an orbital shaker at 150 revolutions per minute for 2 hours. Following incubation, the wells were decanted and washed three times with a washing buffer [phosphate-buffered saline (PBS)] using a plate washer. Plate was decanted and tapped on absorbent cloth following the wash. 100 µL of antibody-POD conjugate (horseradish peroxidase conjugated murine monoclonal antibody to OC) was added to each well using an 8-channel pipette and incubated for 1 hour at room temperature on an orbital shaker at 400-500 revolutions per minute. Following this incubation, mixtures were decanted and wells washed 4 times with the washing buffer using the plate washer. Plate was decanted and tapped on

absorbent cloth following the wash. 100 µL of substrate solution (3,3', 5,5'-tetramethylbenzidine solution) was added to each well using an 8-channel pipette and plate was allowed to incubate at room temperature on an orbital shaker for 10-15 minutes. Subsequently, 100 µL of stop solution (1N H_2SO_4) was added to each well to stop the reactions. Plate was mixed briefly on the orbital shaker at 400-500 revolutions per minute and within 10 minutes the absorbance was read using a microtiter plate reader at 450 nm using distilled water as a control to make zero adjustment. The absorbance of the samples was plotted using the standard curve for quantitative determination of the concentration of uOC. The calculated concentrations were then multiplied by a dilution factor (x2) for the final concentrations. In order to reduce the bias by inter-assay variability, samples for each subjects (OGTT and EX) were analyzed on the same microtiter plate. Intra- and inter-assay precision 8% and 6%, respectively. Manufacturer's measurement of uOC utilizing this EIA kit has been closely ($r = 0.82$) with a hydroxyapatite binding assay. The manufacturer also states that this assay had 5% cross reactivity with human bone OC (most likely carboxylated) and 1.7% cross reactivity with bovine bone OC (most likely carboxylated) so it is likely to overestimate the true concentration of uOC.

Total Osteocalcin – Total serum osteocalcin was calculated by adding the measured carboxylated and undercarboxylated fractions of OC. While this is not a direct measure of total osteocalcin, we utilized EIA kits for cOC and uOC from the same manufacturer (Clontech laboratories, Inc., Mountain View, CA) and the manufacturer states that the cOC kit has no detectable cross reaction with uOC and the

uOC kit has only a 5% cross reactivity with cOC. Additionally, this approach has been utilized by previous authors [52].

Insulin – Serum insulin was measured using an enzyme-linked immunosorbent assay (ELISA) (Cat. #EZHIASF-14K, EMD Millipore, Billerica, MA) according to manufacturer's directions. Briefly, 300 µL of wash buffer (50 mM Tris buffered saline contained 200 µU/mL Tween-20) was added to each well and incubated at room temperature for 5 minutes before being decanted. 80 µL of assay buffer (0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05% ProClin300, and 0.1% Human Serum Albumin) was added to each blank and sample well and 60 µL of assay buffer was added to the standard curve wells and quality control (QC1 and QC2) wells. Then, 20 µL of a matrix solution (treated human serum) was added to the blank, standard and quality control wells. 20 µL of the standard curve, QC1 and QC2 (purified recombinant human insulin in assay buffer), and samples were added to the wells in duplicate. The plate was sealed with adhesive plate sealer film and incubated at room temperature for 2 hours on an orbital plate shaker at 400-500 revolutions per minute. Solutions were decanted following incubation and plate was washed 3 times with 300 µL of wash buffer using the plate washer. Plate was decanted and tapped on absorbent cloth following the wash. Subsequently, 100 µL human insulin ASF detection antibody (pre-titered biotinylated monoclonal insulin antibody) solution was added to each well and plate was sealed and incubated for 1 hour at room temperature with moderate shaking. Following incubation, plate was decanted and washed 3 times with wash buffer as described above. Plate was decanted and tapped on absorbent cloth following the wash. 100 µL of enzyme solution (streptavidin-horseradish peroxidase conjugate) was added to each well and plate was sealed and incubated for 30 minutes at room temperature on an orbital plate shaker.

Solutions were decanted following incubation and plate was washed 5 times with 300 µL of wash buffer using a plate washer. Plate was decanted and tapped on absorbent cloth following the wash. 100 µL of substrate solution (3, 3',5,5'-tetramethylbenzidine in buffer) was added to each well and plate was sealed and incubated at room temperature on the plate shaker for 5 minutes. Following color development, 100 µL of stop solution (0.3 M HCl) was added to each well and plate was shaken by hand until solutions turned yellow. Absorbance was read at 450 nm within 5 minutes using a microtiter plate reader. The absorbance values of the samples were plotted using the standard curve for quantitative determination of the concentration of insulin. The sensitivity of the assay is 0.85 µU/mL and range is 2-200 µU/mL. In order to reduce the bias by inter-assay variability, samples for each subjects (OGTT and EX) were analyzed on the same microtiter plate. Intra- and inter-assay precision was 6% and 2%, respectively.

Glucose – Plasma glucose was measured by the glucose oxidase method (2300STAT Plus, Yellow Springs Instruments, Yellow Springs, OH). Each sample was measured in duplicate and the average of the duplicates was used as the blood glucose concentration. Intra-assay CV was 1%.

Homeostatic Model Assessment–Insulin Resistance (HOMA-IR) – Fasting glucose and insulin values from OGTT visit were used to calculate the fasting insulin resistance. The product of fasting glucose (mg/dL) and fasting insulin (µU/mL) was divided by 405 as previously described [77].

Oral Glucose Insulin Sensitivity (OGIS) – OGIS was calculated as described by Mari et al. [78]. Mari et al. reported that the OGIS calculation is in good agreement with glucose clearance as measured by Hyperinsulinemic-euglycemic glucose clamp and is as effective at establishing differences between groups (lean vs. obese, obese vs.

T2D) or relationships between insulin sensitivity and other physiological variables such as this study [78].

Anthropometry – Subject's height and weight were measured using a stadiometer and electronic scale, respectively. Body mass index (BMI) was calculated using the equation BMI = weight (kg) ÷ height^2 (m). Body composition was measured using Dual Energy X-Ray Absorptiometry (DXA) (Lunar iDXA, GE-Healthcare, Fairfield, CT). Subjects were placed in the supine position with arms close to the side of the body and legs fastened together by a Velcro strap. The scanner then passed over the entire body of the subject and the manufacturer software measured total body mass, total body fat mass, total body lean mass and total body bone mineral content. Using the computer software, the total body masses were separated into regional (arms, legs, trunk) components for use in analysis.

Blood Sample Collection and Storage

Blood was collected with a 10mL syringe via an intravenous catheter and transferred to serum (red top) and glucose (gray top) tubes. Tubes were inverted 5-10 times (allowing the sodium fluoride to completely dissolve in the glucose tubes) and allowed to sit for at least 30 minutes (at room temperature for serum tubes and 4C for glucose tubes) prior to being centrifuged at room temperature at 1000 RCF for 10 minutes in a fixed angle centrifuge. 500µL of serum and plasma was aliquoted into labeled microcentrifuge tubes for each variable (OC, uOC, insulin and glucose) so to avoid freeze/thaw cycles during analysis. Samples were stored at -80°C until analyses were performed.

Statistical Analysis

The Shapiro-Wilk statistic was determined for each dependent variable at each time point to ensure statistical normality. Dependent variables that were skewed (Shapiro-Wilk statistic $p < 0.05$) were logarithmically transformed for further analysis. A two-way [intervention (OGTT vs. EX) x time (T0, T30, T60, T90, T120 minutes)] ANOVA with repeated measures was used to determine interaction and main effects. When significant interaction effects were found, one-way ANOVAs with Bonferroni corrected pair-wise comparisons were used to determine where specific between-group differences were located and repeated measures ANOVAs were conducted on each treatment to determine where specific within-group differences were located. Additionally, multiple linear regression models were used to determine the association between total OC and uOC, and other selected variables. An alpha of $p \leq 0.05$ was used to determine significance for all statistical tests, and all analyses were performed using Predictive Analytics SoftWare statistics 18 package (PASW; Chicago, IL).

Chapter IV: Results

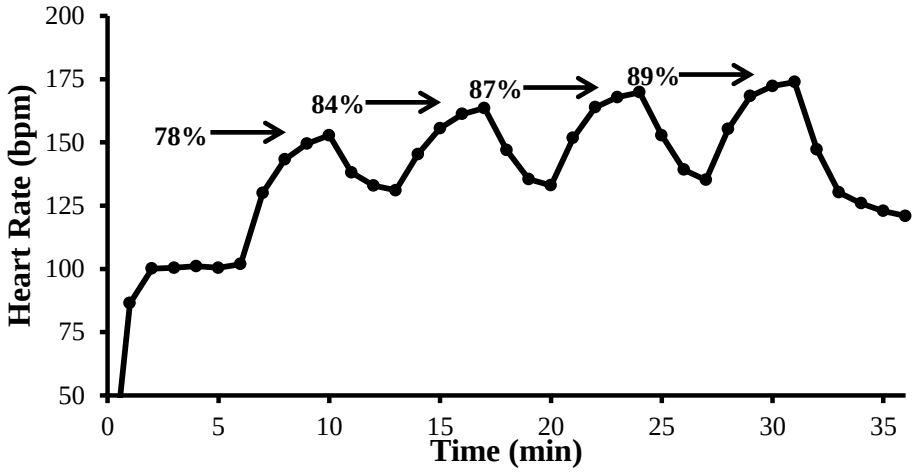
Descriptive Data

Descriptive and anthropometric data for the study subjects are listed in Table 3. The average heart rate for the whole group during the exercise session is depicted in Figure 3. The heart rates achieved at the end of each of the four high-intensity intervals corresponded to 78±10%, 84±6%, 87±5% and 89±6% of maximal HR, respectively. The average heart rate during the 25 minute exercise session (minus the warm-up and cool-down periods) was 151±11 beats per minute (77±5% heart rate max).

Table 3. Subject characteristics.

	Mean		SD
Age (y)	21.40	±	1.68
Height (cm)	180.77	±	7.80
Weight (kg)	95.61	±	14.85
Body mass index (kg/m^2)	29.15	±	3.30
Waist circumference (cm)	92.10	±	9.03
HOMA-IR	3.48	±	4.26
VO_2max (mL O_2/kg/min)	34.90	±	6.50
Maximal heart rate (bpm)	194.87	±	10.74
Body fat (%)	32.23	±	7.66
Fat mass (kg)	30.17	±	10.27
Lean mass (kg)	61.65	±	7.84
Total bone mineral content (kg)	3.59	±	0.50
Total bone mineral density (g/cm^2)	1.32	±	0.10

Figure 3. Average heart rate response during the exercise bout.

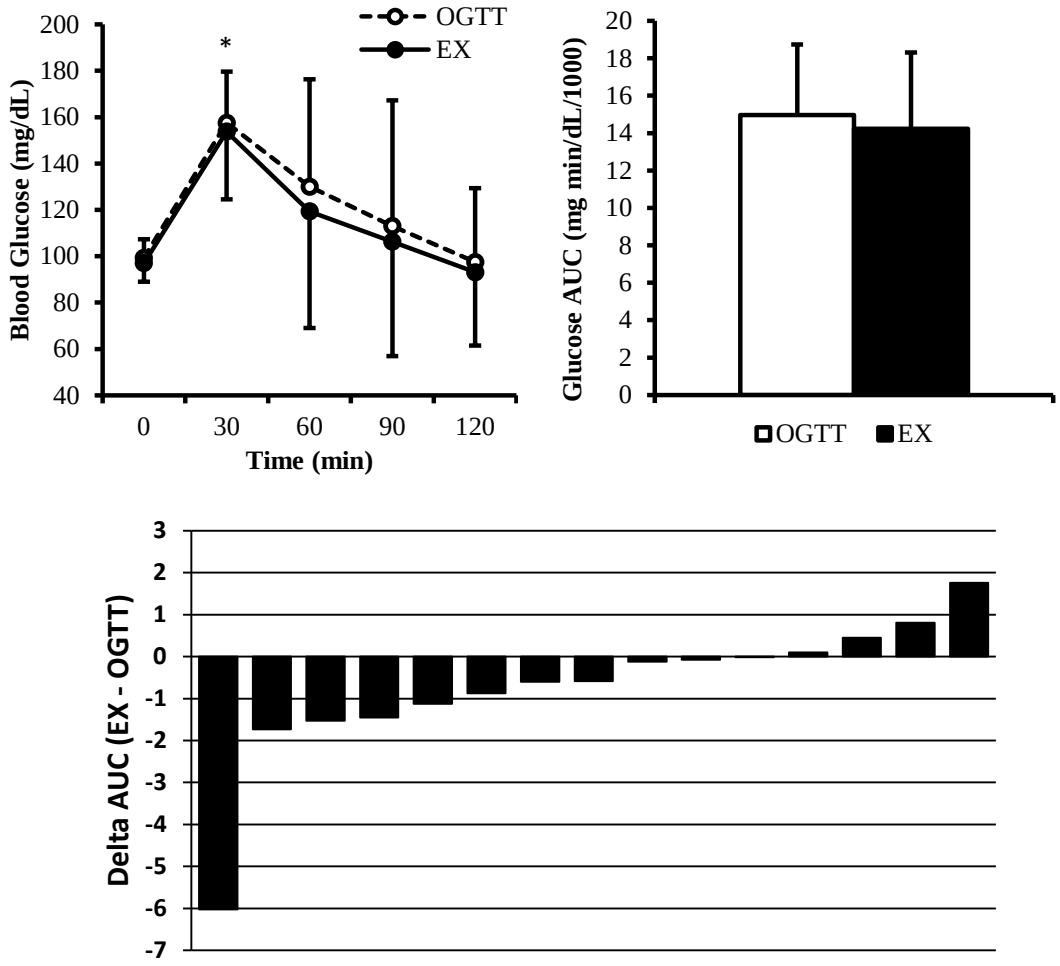


Glucose and Insulin Data

As shown in figure 4, there were no significant differences in baseline blood glucose between the OGTT and EX trials (99±8 vs 97±8 mg/dL, respectively). No significant interaction effects were found between groups over time. There was not a significant group effect; however, a significant time effect was found for glucose ($p<0.001$). Post-hoc analysis revealed that glucose at T30 was significantly greater than all other time points ($p<0.05$ for all) and T60 was significantly greater than T120 ($p=0.012$). Additionally, there were no differences between OGTT and EX for glucose AUC (14.97±3.77 vs 14.23±4.08 mg min/dL/1000, respectively; $p=0.123$).

Glucose AUC following the OGTT was significantly associated with BMI ($r=0.550$, $p=0.34$), waist circumference ($r=0.525$, $p=0.044$), body fat % ($r=0.632$, $p=0.011$) and total fat mass ($r=0.605$, $p=0.017$). Blood glucose at T0 following EX was significantly and negatively correlated with total ($r=-0.631$, $p=0.012$) and leg ($r=-0.619$, $p=0.014$) BMC.

Figure 4. Blood glucose at each time point (left panel) and area under the curve (right panel) for OGTT (open circles and bars) and EX (closed circles and bars). Individual delta glucose AUC response to EX relative to OGTT (bottom panel). Data are mean ± SD. * significantly different than time 0.



There were no significant differences in baseline serum insulin between the OGTT and EX trials (14.22±17.48 vs 15.08±17.84 µU/mL, respectively). Similar to glucose, no significant interaction effects were found between groups over time and no differences were present between groups. A significant time effect was found for insulin ($p<0.001$). As shown in Figure 5, insulin at T30 was significantly elevated compared to T0 ($p<0.001$) and T60 was significantly greater than T0 ($p=0.003$) and

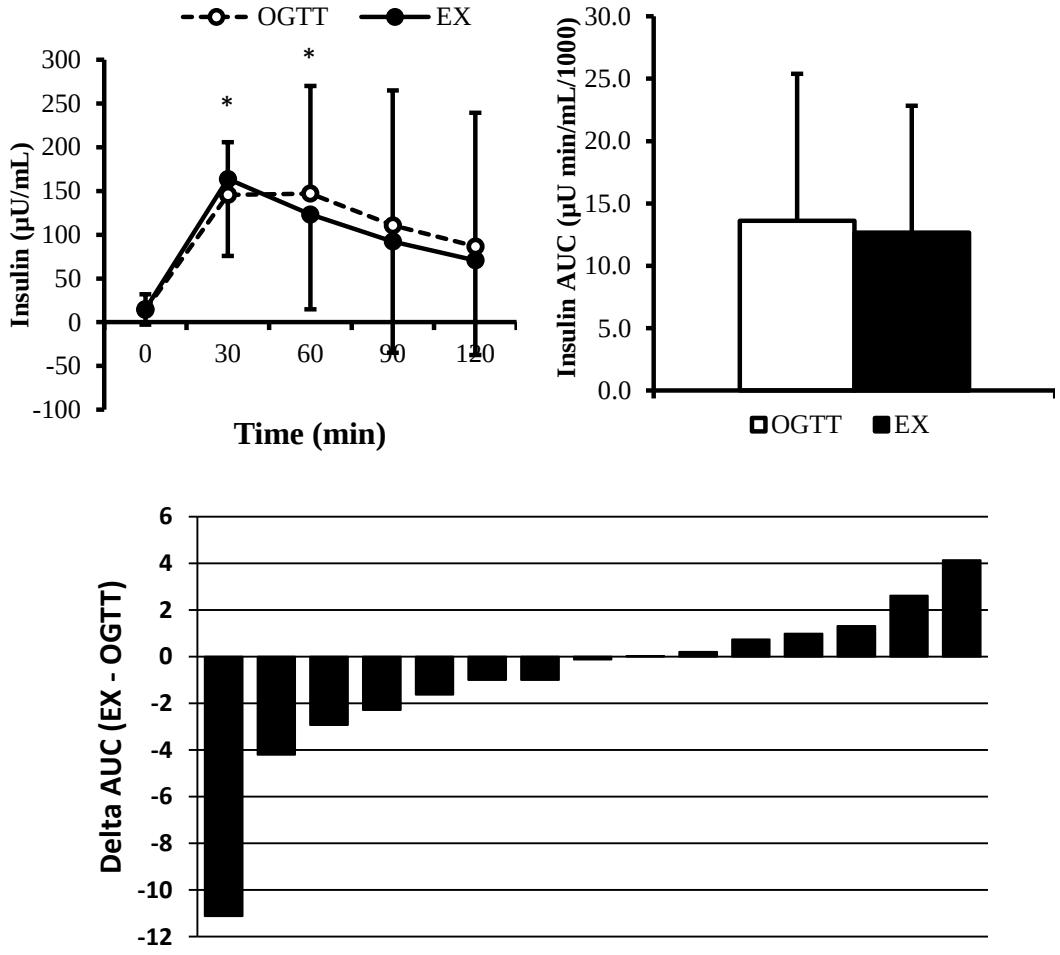
T120 (p=0.002). There wasn't a significant difference between OGTT and EX for insulin AUC (13.62±11.78 VS 12.66±10.17 μU min/mL/1000, respectively; p=0.311). In addition, oral glucose insulin sensitivity was not significantly different between OGTT and EX (Figure 5; 369±73 vs 393±87 mL min/m^2, respectively).

As indicated in Table 4, baseline (T0) serum insulin and insulin AUC during OGTT were significantly associated with weight, BMI, waist circumference, body fat % and total fat mass. Exercise mitigated the associations between T0 serum insulin and BMI, waist circumference and total fat mass. Exercise also diminished the relationship between insulin AUC and BMI. The lack of an association between insulin AUC and BMI following exercise appears to be due to decreased insulin AUC for subjects with higher BMIs. Insulin AUC (both OGTT and EX) was also significantly negatively associated with VO_2max (r=-0.588 and -0.645, respectively; p<0.05).

Table 4. Correlation coefficients for insulin at T0 and insulin AUC for OGTT and EX. Bold indicates significant association.

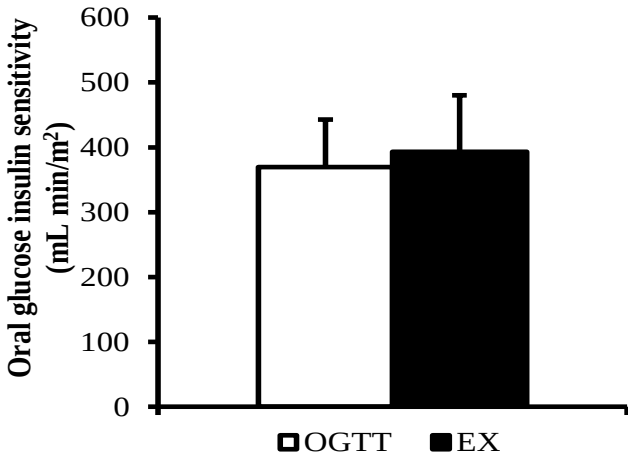
	OGTT		**EX**	
	T0 Insulin	Insulin AUC	T0 Insulin	Insulin AUC
Weight	**0.620**	0.293	0.146	0.170
BMI	**0.717**	**0.541**	0.158	0.432
Waist Circumference	**0.751**	**0.638**	0.473	**0.535**
Body Fat %	**0.602**	**0.623**	**0.630**	**0.628**
Fat Mass	**0.729**	**0.597**	**0.507**	**0.534**
Lean Mass	0.184	-0.217	-0.361	-0.361
Bone Mineral Density	-0.018	-0.320	**-0.526**	-0.402
Bone Mineral Content	<0.001	-0.341	-0.240	-0.385

Figure 5. Serum insulin at each time point (left panel) and area under the curve (right panel) for OGTT (open circles and bars) and EX (closed circles and bars). Individual delta insulin AUC response to EX relative to OGTT (bottom panel). Data are mean ± SD. * significantly different than time 0.



Exercise (393±87 mL min/m^2) marginally improved OGIS compared to rest (369±73 mL min/m^2), but this improvement did not reach statistical significance (p=0.06; Figure 6). OGIS was significantly negatively associated with BMI (r=-0.623, p=0.013), waist circumference (r=-0.636, p=0.011), body fat percentage (r=-0.627, p=0.012) and fat mass (r=-0.632, p=0.011) during the OGTT visit. Exercise eliminated all of these associations suggesting that those with greater adiposity had a greater improvement in insulin sensitivity following exercise.

Figure 6. Oral glucose insulin sensitivity for OGTT (open bar) and EX (closed bar).



Osteocalcin Data

As depicted in Figure 7, there were no significant differences in baseline serum cOC between the OGTT and EX trials (32.24±21.32 vs 26.86±15.68 ng/mL, respectively). There were no significant interaction effects between groups over time, nor were there main effects of group or time. There was also no significant difference between OGTT and EX for cOC AUC (3466±2238 vs.3345±1873 ng min/mL, respectively; $p=0.470$).

As depicted in Figure 8, the difference in cOC AUC between the EX and OGTT trials was significantly negatively associated with lean mass and BMD ($r=-0.600$ and $r=-0.548$, respectively; $p<0.05$). This difference in cOC AUC was significantly correlated with the difference in glucose AUC ($r=0.540$, $p=0.038$).

Figure 7. Serum carboxylated osteocalcin at each time point (top left panel) and area under the curve (top right panel) for OGTT (open circles and bars) and EX

(closed circles and bars). Individual delta cOC AUC response to EX relative to OGTT (bottom panel). Data are mean ± SD

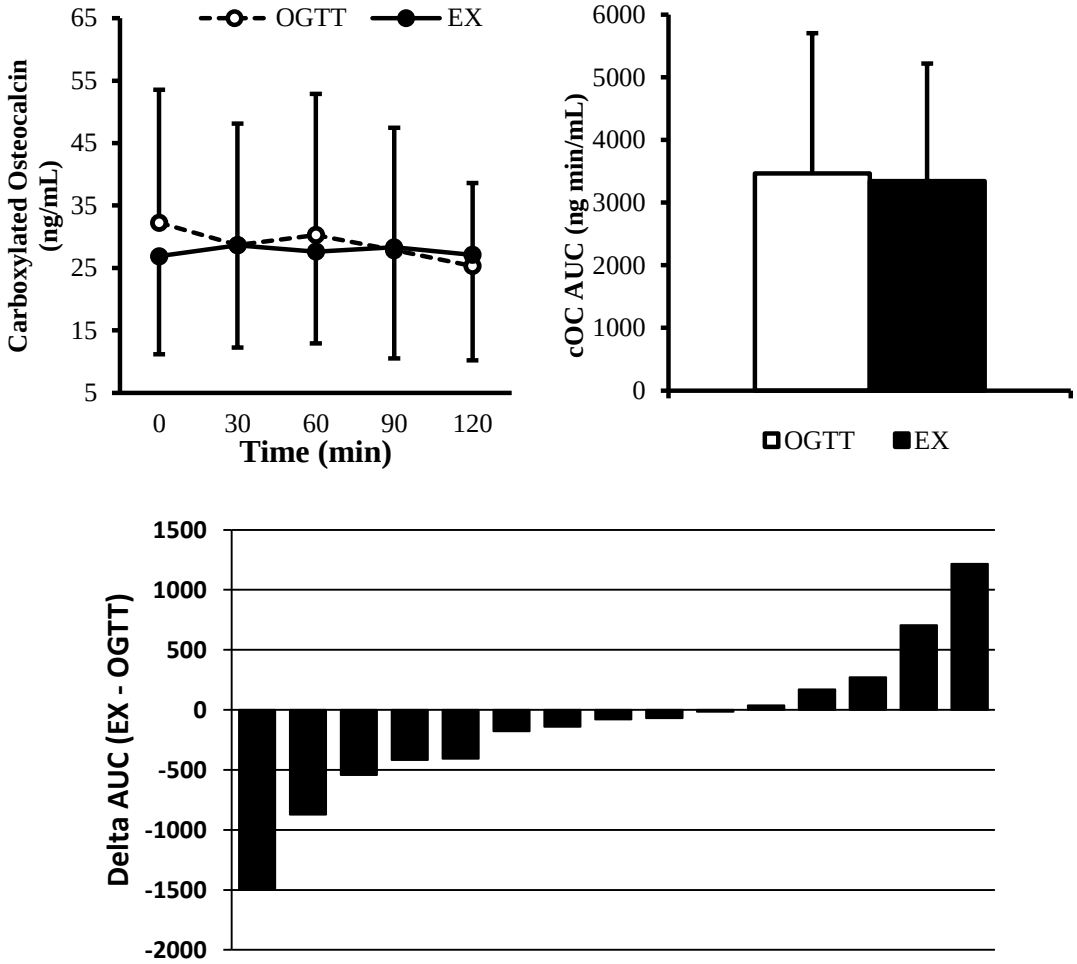
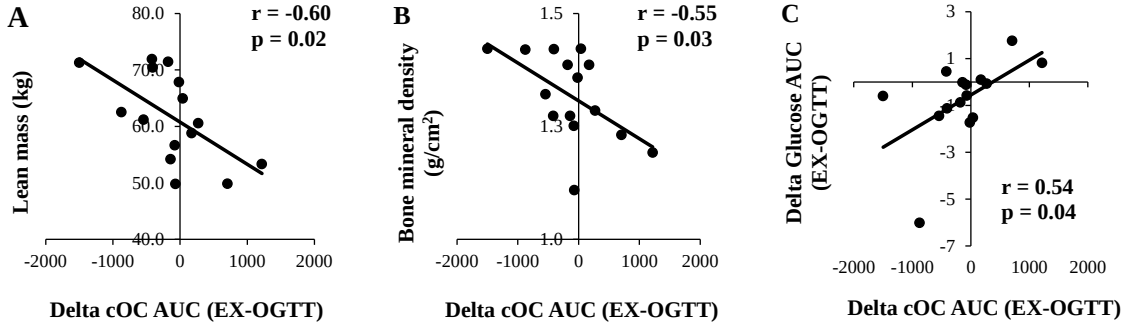


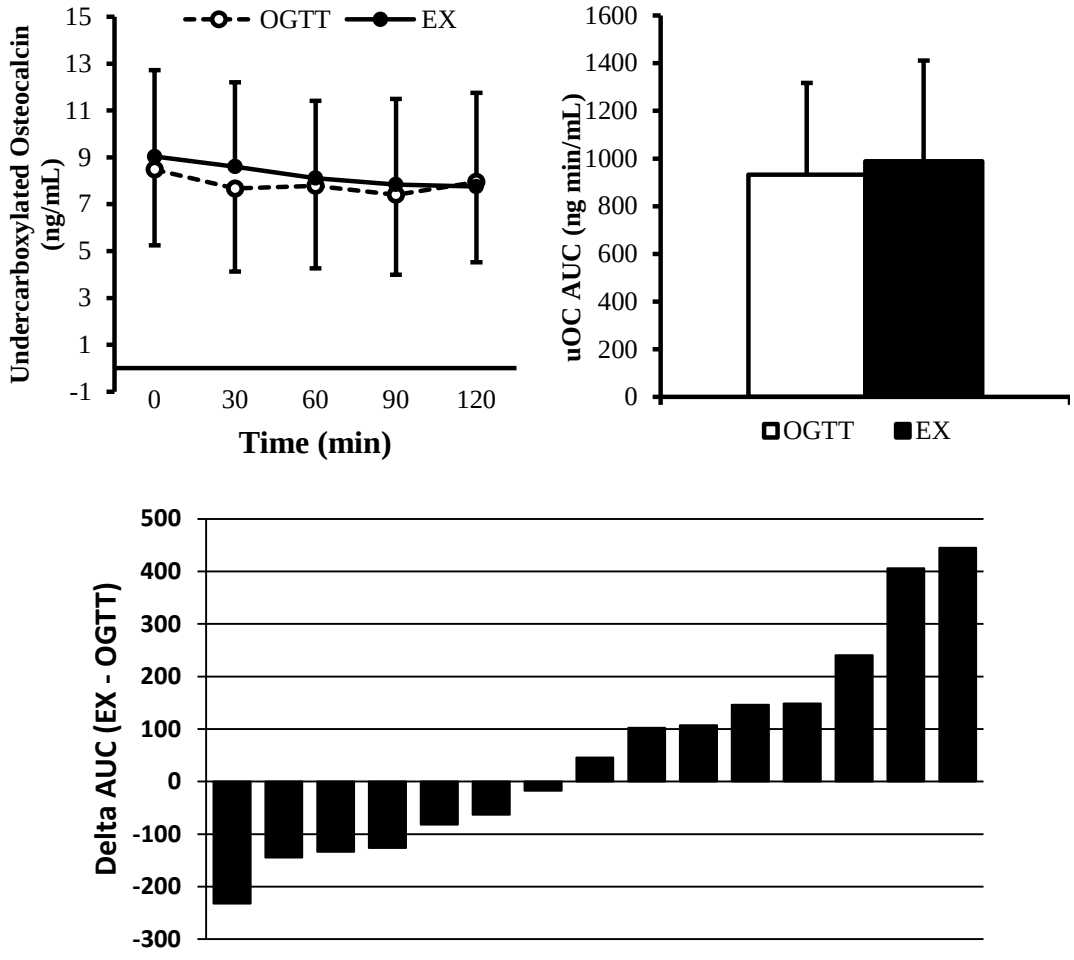
Figure 8. Association of lean mass (A), bone mineral density (B) and delta glucose AUC (C) with delta cOC AUC response to EX relative to OGTT.



As depicted in Figure 9, there were no significant differences in baseline serum uOC between the OGTT and EX trials (8.48±3.23 vs 9.03±3.68 ng/mL, respectively).

There were no significant interaction effects between groups over time, nor were there main group or time effects were. There was also no significant difference between OGTT and EX for uOC AUC (932±385 vs. 989±422 ng min/mL, respectively; p=0.295).

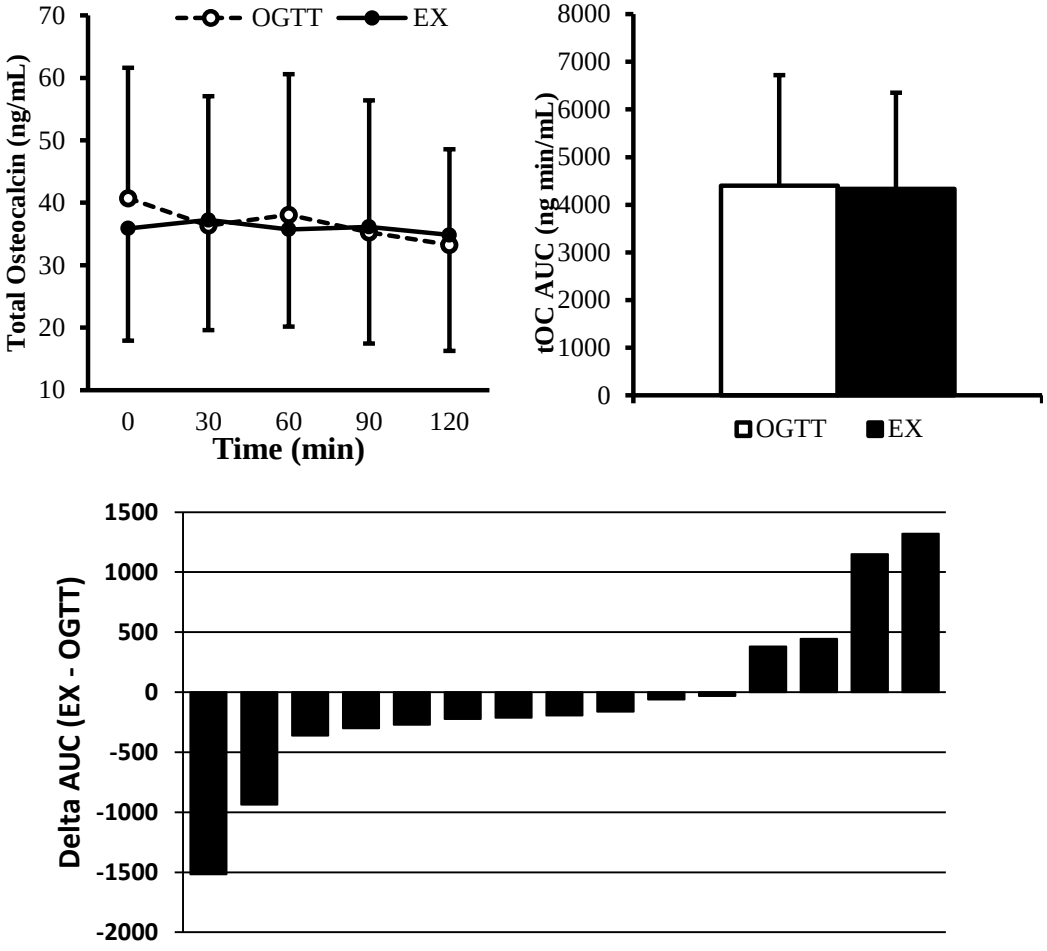
Figure 9. Serum undercarboxylated osteocalcin at each time point (left panel) and area under the curve (right panel) for OGTT (open circles and bars) and EX (closed circles and bars). Individual delta uOC AUC response to EX relative to OGTT (bottom panel). Data are mean ± SD.



There were no significant differences in baseline serum tOC between the OGTT and EX trials (40.72±20.88 vs 35.90±17.98 ng/mL, respectively). There were no significant interaction effects for tOC between groups over time, nor were there main

group or time effects. There was also no significant difference between OGTT and EX for tOC AUC (4399±2319 vs. 4334±2018 ng min/mL, respectively; p=0.727). Data shown in Figure 10.

Figure 10. Total serum osteocalcin at each time point (left panel) and area under the curve (right panel) for OGTT (open circles and bars) and EX (closed circles and bars). Individual delta tOC AUC response to EX relative to OGTT (bottom panel). Data are mean ± SD.



Chapter V: Discussion

The main findings from this study are that cOC, uOC and tOC are not altered following glucose intake in overweight young men, either with rest or a single bout of aerobic interval exercise before the glucose drink. Additionally, cOC, uOC or tOC were not significantly correlated with either baseline or postprandial concentrations of glucose and insulin, or components of body composition (i.e. fat mass, lean mass, bone mineral content, bone mineral density).

One of the main findings of the current study is that serum cOC, uOC and tOC did not significantly change following glucose intake as was hypothesized and had been previously reported [73, 79]. In a 2012 study, Paldánius et al. [73] reported that a standard 75g oral glucose tolerance test resulted in a 32% and 34% decrease in OC and cOC, respectively, in normoglycemic young adults. The results of the current study reveal only an 18% and 21% decrease in tOC and cOC, respectively. Although that magnitude of change is similar to a more recent study by Paldánius et al [79] in the latter study the change in response to oral glucose reached statistical significance. While the 2012 Paldánius et al. study and the current study reported that subjects were normoglycemic, the current study's subjects had a fasting glucose of 90-115 mg/dL, whereas Paldánius et al. had subjects with glucose concentrations of 68-99 mg/dL. Additionally, at the 120-minute time point, Paldánius et al. reported glucose concentrations of 52-166 mg/dL in their subjects whereas the current study subjects had glucose of 66-202 mg/dL. Since baseline and 120-minute glucose concentrations during the current study were slightly elevated compared to Paldánius et al., this could account for the 13%-14% differences between the current study and those previously

reported. Additionally, Paldánius et al. reported baseline sampling times between 0805 and 1102. Since OC has been shown to have a diurnal variation [27], the results reported by Paldáius et al. could have been influenced by the circadian rhythm. In the current study all baseline samples were collected between 0850 and 0905; therefore it is unlikely that the naturally occurring circadian rhythm influenced our results. However, the average percent change in OC in the current study is similar to that reported in previous studies [80, 81]. Additionally, a prior study suggested that the effect of OC on glucose metabolism may be more evident over a long term observation compared to the acute regulation of a single carbohydrate meal [79]. As in the current study, Paldánius et al. [79] did not find significant associations between postprandial OC and glucose or insulin. In summary, unlike previously published studies, glucose intake did not result in a significant decrease in cOC, uOC or tOC during the current study, which could be explained by the large inter-individual variation in these markers during the current study since the average percent changes were similar to those previously reported.

The decrease in postprandial OC reported previously [73, 79] and the non-significant trend for decline in the current study seem to contradict the insulin feedback loop reported by Karsenty and colleagues [33] as there is a robust transient increase in insulin following the OGTT. However, the duration of insulin exposure needed to stimulate the release of uOC from bone is unknown, and it is also unclear whether the feedback loop observed in vitro is similar in humans [82, 83]. Future studies should examine the effects of long-term or repeated exposure to elevated insulin concentrations

on serum OC to determine whether duration, magnitude or frequency of insulin exposure may alter the insulin-OC feedback loop.

Within the current study, T0 was within 15 minutes after cessation of the exercise bout, but no blood was taken prior to the exercise bout so the actual effect of exercise alone could not be reported. However, since all participant were instructed to repeat their diet and exercise regimen for 3 days prior to each exercise visit and the baseline sampling at the same time of day on each visit, it can be cautiously interpreted that differences in selected outcome values at T0 on the OGTT visit versus EX visit is attributable to the exercise bout. The results of the current study contrast those described by Levinger et al. [71] who reported increased uOC and tOC concentrations following an acute bout of aerobic exercise in middle-aged, obese men. Levinger et al. utilized an exercise protocol consisting of a 45-minute cycling bout performed at a heart rate that corresponded to 70-75% VO_2peak. In response to that exercise session uOC was increased ~6% and tOC >10% at 120 minutes post-exercise compared the resting baseline values. While uOC or tOC was not measured prior to the exercise bout in the current study, uOC was 6.5% greater and tOC was 12% less at T0 following exercise compared to the subjects T0 during the OGTT visit. The differences in the reported findings are likely due to the large difference in age of the two subject pools and the large inter-individual variation in the current study. The subjects in the Levinger et al. study were an average of 53 years old whereas participants in the current study were 21 years old. Previous research has reported that younger males have higher serum OC concentrations compared to middle-aged men [84, 85]. Since it has been previously reported that men with lower basal OC concentrations have an increased response to

exercise [74], the expected lower basal OC concentrations in the middle-aged men studied by Levinger et al. could explain the significant increases they reported. Additionally, while Levinger et al. reported that their subjects had fasting glucose concentration of 123 mg/dL (6.8mmol/L), indicating that the participants had either impaired fasting glucose or diabetes (N = 6 of the 13 participants). In the current study the average fasting glucose was 99 mg/dL; all subjects had either normal or mildly impaired fasting glucose. Since T2D is associated with reduced circulating levels of OC [71, 86] it is possible that people with insulin resistance, such as those in the Levinger et al. study, have larger magnitude of increase in OC in response to exercise. Future investigations should examine the response of serum OC to exercise in subjects with varying degrees of glycemia in order to determine possible influence of OC during and following exercise on glucose regulation.

The lack of effect of the acute exercise bout to alter serum OC in this study is in agreement with previous reports [65, 66, 87, 88]. Tosun et al. [65] reported that serum OC was unchanged in sedentary young women following 30 minutes of brisk walking (60-85% HRmax) with or without a weighted (5kg) backpack. Welsh et al. [66] also observed no change in serum OC following a single bout of brisk treadmill walking in healthy, young men. Interestingly, Nishiyama et al. [62] reported that athletic and nonathletic males had differing responses in OC following aerobic exercise. They revealed that OC response peaks 60 minutes following cessation of exercise in athletic men, but in nonathletic males the peak response in OC occurs immediately following exercise. In our study, there was a large variation in postprandial OC response to exercise that could be related to the training history of the individuals. While all

subjects in our study were sedentary just prior to and during the course of the study, it is likely that they have varying degrees of athletic backgrounds. This difference in training could result in an alteration in the response of OC to the exercise bout and glucose load leading to the large postprandial variation. It could be of interest in future studies to examine young adults with or without previous training history to examine if an athletic background may have long term effects on serum OC and the response of OC to exercise. Additionally, it is possible that the effect of exercise on OC is transient. Maïmoun et al. [64] reported that trained cyclists had an immediate increase in OC following 50 minutes of cycling at 15% above their ventilatory threshold, but this increase was abolished within 15 minutes following exercise. The OGTT in the current study began 10-15 minutes following the cessation of exercise so it is possible that any change in OC occurred within the timeframe prior to the OGTT but following exercise. It is unclear if such a transient response could have an impact on subsequent postprandial glucose or insulin response.

There may be role for exercise volume or intensity as a regulating factor for OC response to physical activity. It was reported that an exercise routine that requiring a negative energy balance of 1200-1500 kilocalories (~2 hours of exercise) resulted in a 24% increase in OC of trained rowers [89]. The authors also reported that OC concentrations measured immediately following exercise were related to the distance covered during the exercise. Therefore, it is possible that the exercise protocol used during the current study was not strenuous enough to elicit an increase in OC. The subjects expended 241-498 kilocalories during the exercise protocol. However, the treadmill activity in the current study was designed to be appropriate for overweight,

untrained young men who were unaccustomed to vigorous exercise and the exercise protocol was completed by all subjects, demonstrating feasibility and generalizability for young adults. Levinger et al. [71] observed an increase in OC following an exercise bout that would have elicited a similar energy expenditure in the current study, albeit at a consistently lower intensity. Still, it is possible that a greater change in OC may have occurred if a longer duration of exercise had been performed by the young men in the current study.

While the results from current study did not reveal an acute change in OC following exercise, previous studies have shown that exercise training involving a similar style of exercise results in increased OC [67, 90]. Long-term aerobic exercise, even with the presence of weight-loss, results in increased OC [67, 90]. However, not all studies are in accordance with these results [91, 92]. The previous studies that have examined the effect of exercise training on OC in a younger population (<50 years old) have generally shown increases in OC; however, when an older population is studied, exercise training has been reported to decrease OC. Nevertheless, there are no studies that have examined the effect of the exercise training on the association between OC and glucose metabolism in young people.

Contrary to expectation, the single session of intermittent high-intensity aerobic exercise did not modify postprandial glucose or insulin response to a standard OGTT. Previous studies have reported that an acute bout of aerobic exercise can have immediate effects on postprandial glucose and insulin response in young adults [93]. Short et al. [93] reported a 6% reduction in glucose AUC and a 20% decrease in insulin AUC 1-hour following a 45-minute moderate intensity exercise bout. In the current

study, there was a 5% reduction in glucose AUC and a 6% reduction in insulin AUC, neither of which were significant. Short et al. used a "mixed meal test" consisting of 45%, 40% and 15% of energy from carbohydrate, fat and protein, respectively. It is possible that the effect of exercise on glucose control following a meal differs from a glucose-only drink, but has not, to our knowledge, been examined. Additionally, Short et al. utilized 3 different exercise modes, 15 minutes each of walking, stationary cycling, and video game boxing, during their 45-minute exercise bout which would activate a larger amount of muscle mass and could result in increased glucose uptake compared to the current study, which utilized only treadmill walking. Insulin sensitivity following a single exercise session has been shown to increase in some [94-97] but not all [98-101] previous studies. This discord in the previously published reports is likely due to the type of insulin sensitivity test (OGTT vs. hyperinsulinemic, euglycemic clamp), time between the exercise bout and glucose test (immediately following vs. 12-48 hours following exercise) and the population studied (age, ethnicity, glycemic status). To our knowledge, Short et al. [93] and the current study are the only studies to examine the effect of a single aerobic exercise session on postprandial glucose and insulin response immediately after an aerobic exercise bout.

Although the exercise bout did not significantly decrease glucose or insulin AUC in the whole group, a comparison of individual responses showed that 11 and 8 subjects had decreases in AUC for glucose and insulin, respectively. The measure of OGIS, which incorporates changes in both glucose and insulin into a single outcome, revealed a stronger effect of exercise compared to the AUC values. Exercise increased OGIS by 6.5% in the current study and although this improvement only produced a

trend (p=0.06), this measure is likely a more accurate representation of the effect of exercise on insulin sensitivity as it has been shown to be in agreement with the more sensitive hyperinsulinemic, euglycemic clamp method [78]. Although in the acute timeframe utilized in this study insulin sensitivity was not significantly improved, it could be hypothesized that repeated exposure to this type of exercise stimulus could lead to long-term benefits in glucose metabolism. As previously stated, short-term effects of exercise on insulin sensitivity are equivocal; however exercise training has been proven to improve insulin sensitivity so it is likely that this study produced expected results for the timeframe it was performed.

In the current study baseline (T0) serum cOC, uOC and tOC concentrations were not associated with fat mass, lean mass, and bone mineral content or density as hypothesized. Several studies have shown negative associations between fat mass and OC across a wide age range [11, 12, 51-53, 55, 102]. These studies have used much larger sample sizes than the current study so in comparison the current study is vastly underpowered. However, not all previous studies have reported an association between OC and adiposity [103]. Given the relatively small sample size and the large variability in OC for the current study, it is difficult to assess the association between OC and fat mass, lean mass, and bone mineral content or density.

Limitations of the current study include a relatively small sample size and low exercise volume compared to some other studies. Given the large inter-individual response in glucose, insulin and OC to both glucose ingestion and exercise during the current study, the sample size utilized may be too small to reveal significant changes in any of the listed variables. However, a priori sample size calculations were done using

previously published studies on OC response to glucose ingestion [73] and aerobic exercise [71] which established that a sample size of 15 was sufficient for the current study. However, there was greater heterogeneity in several outcomes than predicted. This may be attributable to the inclusion of subjects with a relatively wide range in fitness and adiposity, both of which may modify circulating OC at rest and in response to exercise. Those subject characteristics may need to be more tightly controlled or used as covariates in future investigations. Another consideration is the exercise session. The exercise bout resulted in an average estimated energy expenditure of ~330 kilocalories, this is less than the energy expenditure reported in previous studies using young men who were trained athletes [89]. At present it is unclear if OC would increase following exercise performed by young overweight men if the exercise volume was increased, or if several sessions of exercise are needed before changes in OC are elicited. Nonetheless, the exercise bout in the current study was designed to be generalizable to the general population of apparently health, yet untrained young adults. Additionally, the energy expenditure is likely similar to that of a recently published study [71] that did find significant differences in OC following an acute bout of aerobic exercise in an older population.

Future investigations could examine both acute and long-term exercise effects on OC (including total, carboxylated and undercarboxylated) and glucose tolerance in a range of glucose tolerant people. Since it has been previously reported that people with decreased glucose tolerance have lower OC and that people lower basal OC concentrations will have an increased response to exercise, it could be hypothesized that a study covering a range of glucose tolerance may result in differential results between

these groups. Additionally, both aerobic [90] and resistance training [47] have been shown to increase OC. Resistance training is emerging as an effective exercise mode to improve glucose metabolism [104-106]. Thus, future investigations should determine if aerobic training, resistance training, or a combination of both differentially alter serum OC, blood glucose and insulin. It is plausible to predict that resistance training would stimulate more bone formation and subsequent release of OC into circulation. Such a study would aid in delineating the influence of OC on glucose metabolism.

In summary, ingestion of glucose with or without a prior aerobic exercise bout did not result in changes in postprandial OC concentration. OC was not related to postprandial changes in blood glucose, insulin or with any body composition measure. While the exercise bout did not alter postprandial OC, there was also minimal effect on postprandial glucose or insulin; therefore any conclusions that exercise does not influence the relationship of postprandial OC and glucose or insulin should be considered in that context and with restraint. If glucose and insulin were significantly reduced following exercise it is possible that they may also have been an accompanying change in OC. Regardless, these data are the first to examine the postprandial response of serum OC within the 2-hour timeframe of a standard OGTT, and with careful control for potential diurnal variations. Additionally, this study is the first to establish that an acute exercise bout just prior to consumption of glucose does not alter postprandial OC in overweight young men. This novel study lends insight into the transient postprandial associations between blood glucose, insulin and OC concentration and the effect that a single bout of exercise has on those associations. These results could act as a

foundation for future research examining the long-term effects of exercise on the regulation of glucose metabolism by the osseous tissue.

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Appendix A. Informed Consent

Version 1.4, 03 Feb 2013 IRB No: 1720
701-A

CONSENT FORM
University of Oklahoma Health Sciences Center (OUHSC)

Study Title: Influence of exercise on the temporal response of serum osteocalcin following nutrient intake
Study Sponsor: McBride Foundation (Application Submitted)
Principal Investigator: Kevin R. Short, PhD.
Co-Investigators: Kyle Sunderland, MA; Sowmya Krishnan, MD; Rebecca Larson, PhD; David Lantis, MS; John Farrell III, BS
Affiliations: Section of Diabetes/Endocrinology, Department of Pediatrics, University of Oklahoma Health Sciences Center and [2]Department of Health and Exercise Science, University of Oklahoma.

Research studies involve only individuals who choose to participate. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You have been asked to participate because you are 18-25 years of age and not routinely exercising more than 2 days per week.

Why Is This Study Being Done?
This study is being performed to determine if bones may affect blood sugar control and if a single exercise bout can influence the effect.

How Many People Will Take Part In The Study?
A total of 15 young men, ages 18-25 years will take part in this study.

What Is Involved In The Study?
If you agree to participate in the study, you will need to make three planned visits to the research center. You will need to be fasting (no food consumption) for the previous 10 hours and you will need to avoid vigorous exercise for 2 days prior to each visit. For each of the study visits you will need to arrive at the research center between 7:00am and 8:00am. The details of the visits are outlined below:

***Screening visit*:** On this visit a member of the study team will explain the study details and show you several of the tests. You will complete a medical history questionnaire and a questionnaire ensuring your ability to exercise. You will be asked about the potential challenges or concerns you may have about participating. Once you have provided consent and are prepared to continue, you will have your body composition and aerobic fitness measured.

Oral glucose tolerance test trial: After sitting quietly for 20-30 minutes you will be given the glucose drink (sugar and water) to consume within 10 minutes. You will rest quietly for about the next 2 hours while small blood samples are collected.

Exercise then oral glucose tolerance test trial: The trial is similar to the above trial, but includes a 38-minute exercise phase before the glucose drink. After a short rest period, you will begin exercising with a 5-minute warm-up. Next, you will complete 4 blocks of exercise consisting of 4 minutes at a high-intensity (~90% maximal heart rate) with 3 minutes of lower-intensity exercise inbetween. You will then complete a 6-minute cool-down (38 minutes total). The drink following exercise and the blood collection for about 2 hours after the drink will be the same as described above.

Measurements

***Screening visit health status*:** We will record age, height, weight, gender, race, medical history, waist size, family medical history, and physical activity readiness. These medical questionnaires take about 20 minutes and will only be performed once in the study. No genetic testing will be done.

***Body composition test*:** We will use a dual energy X-ray absorptiometry (DEXA) machine to measure the amount of fat and lean tissue in your body. You will lie quietly on a padded table while a scanning arm moves above your body. The length of the scan is about 3-4 minutes. A DEXA scan involves exposure to radiation. This test will be performed once in the study.

***Diet assessment*:** You will keep a record of the foods that you consume for 3 days before the study trials.

***Oral glucose tolerance test*:** Following either a rest period (during drink trial) or within 15 minutes following the exercise bout, we will place an IV (a small flexible plastic tube) into a vein in your arm so that we can collect blood samples during the test. A small blood sample will be taken just before you are given the drink for baseline measures. You will then be given the glucose drink (75 grams of sugar in water) to consume within 10 minutes. You will rest quietly for 2 hours while small blood samples are collected at 30, 60, 90, and 120 minutes later. You may watch television or read during this time.

Exercise fitness test: We will measure your peak aerobic capacity (the amount of oxygen your body can use) while exercising on a treadmill. You will begin by walking or jogging at at a comfortable pace on a 0% (flat) incline. Every 2 minutes the speed or grade of the treadmill will increase until you need to stop. This test is typically completed in 10-15 minutes. You will wear a mouthpiece so that we can monitor the air that you breathe and heart rate monitor on your chest.

Blood tests: The total amount of blood collected during each study trial will be <75 milliliters (about 5 tablespoons, or about 15% of what is collected during a typical blood donation at a blood bank). We will measure the concentration of glucose, insulin (the hormone that controls blood sugar), triglycerides, and a couple of proteins that the bone produces that have been associated with blood sugar control. These measurements will be performed on all of the blood samples for each trial. We will store any remaining samples during the study so that they can be used as a "back-up" in case we have to repeat one of the tests listed above. All of the analyses will be completed within one year after the last participant completes the study. There will be no genetic testing performed on any samples collected in this study.

How Long Will I Be In The Study?

The actual duration you will be in the study will depend on your availability to perform the tests and the schedule of the research team. However, we expect that if you complete all study trials your participation will last at least 2-4 weeks, depending on how much time elapses between study trials.

You may stop participating in this study at any time. However, if you decide to stop participation, we encourage you to discuss your decision with the research team first.

There may be anticipated circumstances under which your participation may be terminated by the investigator without regard to your consent. For example, if he/she feels that it is in your medical best interest,.

What Are The Risks of The Study?
Blood collection: The risks include local bruising, bleeding, temporary color changes of the skin as well as a slight chance of infection at the site of the IV, and becoming lightheaded and fainting during the procedure.

Radiation risk from Body Composition Test: If you participate in this research you will be exposed to radiation from a DEXA scan (a type of X-ray). This radiation exposure is not necessary for your medical care but is necessary to obtain the research information desired (body fat, lean tissue, and bone mineral content in your arms, legs, and trunk). You will not be exposed to this radiation if you choose not to participate in the study.

The amount of radiation from a DEXA scan is less than 1% of the amount of radiation to which people are exposed from natural radiation sources in one year. In addition to any radiographic procedures that are being done as part of this research, you may also be exposed to radiation from procedures that are part of your normal care. The number and frequency of these procedures are based on standard clinical practices. The risk from radiation exposure increases over your lifetime as you receive additional exposure to radiation.

Exercise fitness test: The risks associated with exercise testing are those associated with vigorous exercise and may include shortness of breath, fatigue, and local muscle discomfort. A heart attack can occur during vigorous effort in people who have heart disease. Testing will be discontinued if any unusual or unexpected responses occur. Emergency measures are immediately available in case of cardiac emergency. Such emergencies are rare (1 incident per every 887,000 hours of exercise).

Are There Benefits to Taking Part in The Study?
If you agree to take part in this study, there may be little or no direct medical benefits to you. You will receive information about your body composition (amount of lean and fat tissue) and your exercise fitness. The information collected in this study will be used in the future for help make appropriate lifestyle change recommendations for young adults in order to improve health.

What Other Options Are There?
Instead of being in this study, you have the option to not participate.

What About Confidentiality?
Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

The OUHSC Institutional Review Board may inspect and/or copy your research record for quality assurance and data analysis.

What Are the Costs?
There are no costs to participating in this study. All of the testing procedures are free to you.

Will I Be Paid For Participating in This Study?
Yes, all people in the study will be paid for the screening visit and study trials they finish. You will be paid $20 for completing the screening visit and $50 for completing the first study trial and $60 for the second. Thus, if you complete all 4 of the planned visits you will receive a total of $130.

What if I am Injured or Become Ill While Participating in this Study?
Every effort will be taken to prevent injury to you. In the case of injury or illness resulting from this study, emergency medical treatment is available. However, you or your insurance company may be expected to pay the usual charge for this treatment. No funds have been set aside by The University of Oklahoma Health Sciences Center, the hospital, or the sponsor to compensate you in the event of injury.

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. If you agree to take part and then decide against it, you can withdraw for any reason. Refusal to participate or leaving the study will not result in any penalty or loss of benefits that you would otherwise receive.

You have the right to access the medical information that has been collected about you as part of this research study. However, you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction.

We will tell you about any new information that may affect your health, welfare or willingness to stay in this study.

Whom Do I Call If I have Questions or Problems?
If you have questions, concerns, or complaints about the study or have a research–related injury, you may contact Dr. Kevin R. Short, PhD at 405-271-8001 ext. 43094.

If you cannot reach the Investigator or wish to speak to someone other than the investigator, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

For questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

IRB NUMBER: 1720
IRB APPROVAL DATE: 02/18/2013
IRB EXPIRATION DATE: 02/18/2013

Signature:
By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document. I agree to participate in this study:

Signature of Research Participant	Printed Name of Research Participant	Date

Signature of Person Obtaining Informed Consent	Printed Name of Person Obtaining Informed Consent	Date

Periodically, we may need volunteers for future studies in which you may be able to participate. By checking below, you are stating that it is acceptable for someone to contact you about discussing participation in a different study. Checking yes below in no way obligates you to participate, only to be contacted regarding possible participation.

I consent to be contacted for future studies on myself / my family:

_____Yes _____No

Signature of Research Participant	Printed Name of Research Participant	Date

Signature of Person Obtaining Informed Consent	Printed Name of Person Obtaining Informed Consent	Date

IRB Office Version Date: 09/08/2010

Appendix B. Health History Questionnaire

University of Oklahoma

Influence of exercise on the temporal response of serum osteocalcin following nutrient intake

General Health and History Form

Name: _____ Date:_____

Date of Birth: _____ Phone: _____ Email: _____

Primary Care Physician (Name):_____ Phone:_____

Emergency Contact (Name):_____ Phone:_____

Family History:
Has anyone in your immediate family had any of the following: Please circle **Yes** or **No.**

Heart disease	Yes	No	Diabetes	Yes	No
High blood pressure	Yes	No	Cancer	Yes	No
Stroke	Yes	No	Tuberculosis	Yes	No
Sudden Death (before 50)	Yes	No	Asthma	Yes	No
Epilepsy	Yes	No	Gout	Yes	No
Migraine Headaches	Yes	No	Marfan's Syndrome	Yes	No
Eating Disorder	Yes	No	Sickle Cell	Yes	No

Please explain all Yes responses; denoting relationship and age of onset/occurrence of the family member in question (if known):_____

_____

Personal History:

1. Have you ever been hospitalized? Yes No
   Have you ever had surgery? Yes No
   Are you presently under a doctor's care? Yes No
   Have you ever been diagnosed with a sleeping disorder or clinical depression? Yes No
   Please explain and give dates for all Yes responses: _____
   _____
2. Please list any medications (including antibiotics) you are currently taking and for what conditions:
   _____
3. Please list any known allergies: _____
4. Have you ever had a head injury / concussion? Yes No
   Have you ever been "knocked-out" or unconscious? Yes No
   Have you ever had a seizure, "fit" or epilepsy? Yes No
   Have you ever had a "stinger," "burner" or pinched nerve? Yes No
   Do you have recurring headaches or migraines? Yes No
   Please explain and give dates for all Yes responses:_____
   _____
5. Have you ever had the chicken pox, mumps or measles? Yes No
6. Do you have a history of asthma? Yes No
7. Are you missing an eye, kidney, lung or testicle? Yes No
8. Do you have any problems with your eyes or vision? Yes No
9. Are you being treated for, or ever been diagnosed as having sickle cell anemia? Yes No
10. Have you ever had any other serious medical problems (mononucleosis, diabetes, anemia, etc)? Yes No
11. Are you presently taking or within the past 14 days have you taken any supplements for diet or performance (e.g., creatine, protein, etc.)? Yes No
12. What is the lowest bodyweight you have been at, within the last 6 months? _____

IRB NUMBER: 1720
IRB APPROVAL DATE: 02/18/2013

Highest? _____ What is your ideal weight? _____

13. Do you have trouble breathing, or do you cough during or after exercise? Yes No

14. Have you ever had heat cramps, heat illness or muscle cramps? Yes No

15. Do you have any skin conditions (ex: itching, rashes, acne, rosacea, etc)? Yes No

Please explain all Yes responses for question 5 -15: _____

16. Have you ever fainted during or after exercise? Yes No
    Have you ever been dizzy during or after exercise? Yes No
    Have you ever had chest pain during or after exercise? Yes No
    Have you ever had high blood pressure? Yes No
    Have you ever been told you have a heart murmur? Yes No
    Have you ever had racing of your heart or a skipped heartbeat? Yes No
    Have you ever had an EKG or echocardiogram? Yes No

    Please explain all 'Yes' responses for question 19: _____

17. Have you ever sprained / strained, dislocated, fractured, or had repeated swelling or other injury of any bones or joints? Please explain all Yes responses.

Head / Neck	Yes	No	_____
Shoulder	Yes	No	_____
Elbow & Arm	Yes	No	_____
Wrist, Hand & Fingers	Yes	No	_____
Back	Yes	No	_____
Hip / Thigh	Yes	No	_____
Knee	Yes	No	_____
Shin / Calf	Yes	No	_____
Ankle, Foot & Toes	Yes	No	_____

18. Are you currently participating in another clinical trial or have you participated in another study within the past 30 days? Yes No

19. Do you have a history of drug or alcohol abuse within the prior two years? Yes No

20. Do you regularly use tobacco products (e.g., cigarettes, cigars, dip, snuff)? Yes No

21. Have you ever had or are you currently experiencing or being treated for a bleeding disorder, diabetes mellitus, high blood pressure (systolic > 140 and/or diastolic > 90), tachyarrhythmia, ulcerative colitis, short bowel syndrome, or heart, kidney, liver or thyroid disease? Yes No

    If you answered 'Yes', please explain: _____

22. Do any of the following apply:

    A sudden death in your biological father or brother, or mother or sister prior to age 55 or 65, respectively? Yes No
    Current smoker or have you quit smoking within the past 6 months? Yes No
    Do you take hypertensive medication or have a confirmed systolic or diastolic blood pressure ≥140 or 90 mmHg, respectively? Yes No
    Take lipid lowering medication or have total blood cholesterol ≥200 mg/dL? Yes No
    You have a confirmed fasting blood glucose of ≥100 mg/dL? Yes No
    Have you recently been diagnosed as clinically obese (≥32% body fat)? Yes No
    Are you sedentary? Yes No

Please Sign:
I hereby state that, to the best of my knowledge, my answers to the above questions are correct.

Subject's Signature: _____ Date: _____

IRB NUMBER: 1720
IRB APPROVAL DATE: 02/18/2013