THE POSTPRANDIAL EXCURSION OF
TOTAL AND UNDERCARBOXYLATED OSTEOCALCIN CONCENTRATION
FOLLOWING LOW VERSUS HIGH COMPLEX CARBOHYDRATE
MEALS IN THE HEALTHY WEIGHT ADULT

By

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A THESIS

Presented to the Graduate Programs in Human Nutrition

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of Master of Science

in

Clinical Nutrition

16 April 2009

School of Medicine
Oregon Health & Science University

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LIST OF ABBREVIATIONS

μL	Microliter
AUC	Area under the curve
CHO	Carbohydrate
cOC	Carboxylated osteocalcin
CV	Coefficient of Variation
DASH	Dietary approaches to stop hypertension
DXA	Dual energy x-ray absorptiomery
ELISA	Enzyme linked immunosorbant assay
g	Gravitational fields
iAUC	Incremental Area Under the Curve
iOC	Intact osteocalcin
IV	Intravenous
mL	Milliliters
ng	Nanograms
nm	Nanometers
OCTRI	Oregon Clinical and Translational Research Institute
OHSU	Oregon Health & Science University
OST-PTP	Osteotesticular protein tyrosine phosphatase
PBS	Phosphate buffered saline
PGC-1 α	Peroxisome proliferator activated receptor gamma coactivator one alpha
POD	Peroxidase
PTH	Parathyroid hormone

SEM	Standard error of the mean
St Dev	Standard deviation
tOC	Total osteocalcin
ucOC	Undercarboxylated osteocalcin
UCP-1	Uncoupling protein 1
UCR	Undercarboxylated osteocalcin ratio, equivalent to undercarboxylated osteocalcin :intact osteocalcin

AKNOWLEDGEMENT

I would like to thank everyone who made this research possible. I would like to thank the Oregon Clinical and Translational Research Institute for allowing me to execute this project. Without the work of the Bionutrition Department, the Clinical Research Nurses, the Scheduling Department and the Core Lab, this project would have been impossible. Many individuals went above and beyond their duties to make this project a success, and I appreciate all of their efforts. I would particularly like to thank Aaron Clemons, of the Core Lab for his support and guidance through my chemical analyses.

I would like to thank Mike Lasarev, for his guidance and expertise in statistics.

I would like to thank the research participants who gave up their time and energy to participate in this project.

I would like to thank Sarah Bergman and Melissa Kumagai, co-investigators on this study. Without their hard work, support and friendship, my thesis experience would not have been possible. I have greatly enjoyed working on this team for the last two years, and will miss them both terribly, both personally and professionally.

I would like to thank my peers, Hebah Bensalamah, Julie Feifers and Svetlana Zubkova; their understanding, support and friendship throughout the past two years of courses, research and life have been invaluable.

I would like to thank my family and friends for supporting me in my decisions and listening to "scientific garble" at all hours of the day, long distance, as I tried to figure out this project.

I would like to thank my committee members Dr. Melanie Gillingham and Dr. Robert Klein for their patience, guidance and support throughout this process.

I would like to thank Dr. Diane Stadler, for her guidance and mentorship throughout my time here at OHSU. I thank her for sparking my interest in osteocalcin, for her hours of support and for being a role-model in research, academia and life that I hope to live up to one day.

Lastly, I would like to thank Dr. Tracy Ryan-Borchers, my advisor, for taking me on as a student, for her guidance and support throughout the past two years, and for all the work she has done with my thesis. Her positive attitude has truly been sanity-saving, and I appreciate all that she has done for me throughout my dietetic internship and research experience.

ABSTRACT

Background: Osteocalcin, a protein secreted during bone formation has been shown to impact glucose homeostasis. It has been theorized that the undercarboxylated osteocalcin may act in an endocrine fashion to positively modulate adiponectin to decrease fat stores. Undercarboxylated osteocalcin may also increase the proliferation of β-cells in the pancreas and thus insulin secretion. The carboxylated form of osteocalcin has been shown to increase bone strength and decrease the risk of osteoporosis. Previous studies have examined the effects of single nutrients on the circulating concentration of this protein, and found a correlation between dietary composition and carboxylation status and excursion of osteocalcin concentration. The purpose of this study is to characterize the effects of low carbohydrate (LC) versus high complex carbohydrate (HC) meals on total osteocalcin and undercarboxylated osteocalcin concentrations in the acute postprandial state.

Significance: The results of this study may provide further insight into the impact of dietary composition on the protein osteocalcin. If diet is shown to modulate concentration and/or form of this protein, novel individualized medical nutrition therapy may prove instrumental in the treatment of obesity, osteoporosis and diabetes.

Methodology: Ten healthy men and women from the Portland area completed a randomized crossover feeding trial consisting of two, three day run-periods, followed by a day of HC or LC dietary intervention. On intervention days participants consumed either HC or LC breakfast and lunch meals and provided blood samples while fasting and then every hour postprandially. Plasma was analyzed for total (intact) and undercarboxylated osteocalcin concentrations using ELISA. Incremental

area under the curve (iAUC) using the trapezoidal method was calculated for intact osteocalcin, undercarboxylated osteocalcin and the undercarboxylated : intact osteocalcin ratio (UCR).

Results: Following the HC breakfast meal, circulating intact osteocalcin concentration decreased for the first 2 hours, followed by a rise towards baseline before lunch. After the HC lunch meal, circulating intact osteocalcin concentration was more stable in pattern. Following the LC meals, intact osteocalcin was fairly stable throughout the day. Absolute undercarboxylated osteocalcin concentration was significantly higher after the HC breakfast than the LC breakfast for the first 5 hours of the day [iAUC 3.24 ± 2.25ng/mL* $hr_{0800\text{-}1300}$ vs. -1.55 ± 2.15 ng/mL* $hr_{0800\text{-}1300}$, (as mean ± standard deviation, $\bar{X}$ ± SD), p=0.02] and for the duration of the sample collection (iAUC- 7.01 ± 4.51 ng/mL* $hr_{0800\text{-}1730}$ vs. –1.77 ± 4.55 ng/mL* $hr_{0800\text{-}1730}$, $\bar{X}$ ± SD, p= 0.03). Significant differences in iAUC were not seen in total circulating intact osteocalcin concentrations, absolute undercarboxylated osteocalcin concentrations following the lunch meal, or the UCR at any time interval. Trends showed a higher intact osteocalcin excursion and UCR following the LC lunch versus the HC lunch. The trends in patterns of change exhibited for undercarboxylated and circulating intact osteocalcin concentration suggest that significant differences may exist and may be better captured if studied over a longer period of time.

Funding Disclosure: This sub-study of the Energy Balance Study was funded by the OHSU Graduate Programs in Human Nutrition and grants from the NIH: National Center for Complementary and Alternative Medicine (1 R21 AT002753-01) and the National Center for Research Resources (1U L1RR024140).

CHAPTER 1: SIGNIFICANCE, AIMS AND HYPOTHESES

Significance

In 2000 the Department of Health and Human Services published Healthy People 2010 goals, with 28 focus areas chosen to increase quality of life and decrease health disparities in the United States population. Three of these focus areas, accounting for more than 10% of focus topics, are diabetes, nutrition and overweight, and arthritis, osteoporosis and chronic back pain [1]. Recent research on the bone protein osteocalcin suggests that this one protein may be capable of impacting all three of these focus areas.

Manipulation of the post-translational modification and overall synthesis of osteocalcin may prove therapeutic for glycemic control, obesity, and osteoporosis through differential mechanisms. Individual macro and micronutrients have been shown to affect bone turnover and osteocalcin synthesis and carboxylation [2-5].

If nutritional therapies can manipulate these mechanisms, preventative treatment can be made available to the American public. Based on familial and lifestyle risk factors, patients and health care providers can choose a diet that best reduces the risk of obesity and diabetes or osteoporosis. Dietary treatment is a more holistic approach to health that is economically advantageous compared to pharmacological treatment, and offers individuals greater personal control over their wellbeing.

Specific Aim

The primary specific aim of this study was to measure the plasma concentrations of total and undercarboxylated osteocalcin, while fasting and for 9.5 hours after consumption of low or high complex carbohydrate meals.

Hypotheses

The following hypotheses were tested:

- **Hypothesis I-** High complex carbohydrate meals containing high amounts of fruits, vegetables and select vitamins and minerals will increase circulating intact osteocalcin concentration postprandially versus low carbohydrate, high fat meals with a lesser concentration of micronutrients.

- **Hypothesis II-** High complex carbohydrate meals will result in a lower ratio of undercarboxylated osteocalcin to carboxylated osteocalcin concentration postprandially versus low carbohydrate meals containing high amounts of fat and protein.

CHAPTER 2: INTRODUCTION AND BACKGROUND

In 2007, a novel paper was published out of Columbia University, giving evidence that a protein secreted during bone formation and long used as a clinical marker of bone health may act as an endocrine hormone. This protein, osteocalcin, received attention for its potential role in glycemic control and fat catabolism, and has been studied in vitro and in vivo in animals and humans. Intact osteocalcin exists in two forms; fully carboxylated and undercarboxylated. These forms are associated with unique roles in the body. Carboxylated osteocalcin is associated with improving bone mineral density while undercarboxylated osteocalcin is postulated to increase insulin secretion and sensitivity and to up-regulate the adipocyte derived cytokine, adiponectin. Carboxylation of osteocalcin is dependent on Vitamin K, and empirical evidence suggests that other nutritional factors may also influence the total excursion and carboxylation of this protein. This thesis experiment explores the nutritional impact of whole meals of varying nutrient content on the total excursion and carboxylation of osteocalcin.

The effects of two popular types of diet disparate in macronutrient and micronutrient composition were evaluated in a novel, acute, randomized, crossover trial to assess the effect of each diet on circulating total and undercarboxylated osteocalcin concentrations.

CHAPTER 3: OSTEOCALCIN

Osteocalcin Characteristics

Osteocalcin is a low molecular weight, non-collagenous protein secreted from osteoblasts, odontoblasts and hypertrophic chondrocytes[6]. This protein is found in the bone and dentin, and is detectable in blood. Recent studies suggest that osteocalcin may play a role as an endocrine hormone[7]. Two forms of this protein exist; one form is the carboxylated form in which glutamic acid residues have undergone post-translational modification to become γ-carboxy glutamic acids, and the other form is the undercarboxylated form in which one or more of these glutamic acid residues have not undergone post-translational modification. This carboxylation reaction is dependent on the presence of vitamin K[4]. In the past, osteocalcin was thought of as an anchor to hydroxyapatite in bone, positively contributing to bone mineral density. Other studies have reported that osteocalcin is not involved in bone formation[8]. Because osteocalcin is secreted from osteoblasts and takes part in a vitamin K dependent reaction, it has been employed as a marker of bone formation and vitamin K status. Additionally, total osteocalcin status has been inversely correlated with osteoporosis and fracture risk[9]. High levels of undercarboxylated osteocalcin are a risk factor for osteoporosis and fracture risk regardless of bone mineral density[10]. Osteocalcin has been most studied in the total form, but recent investigations have highlighted the necessity to differentiate between undercarboxylated osteocalcin and carboxylated osteocalcin in research investigations. These two forms of the protein are postulated to play unique roles in the body, with undercarboxylated osteocalcin given recent attention for a possible hormonal role in maintaining glucose homeostasis and increasing fat catabolism [4, 5, 7]. The role of

carboxylated osteocalcin in vivo has not been verified. Preliminary evidence shows that various macronutrients and micronutrients influence bone turnover. Protein, fat and carbohydrate have all been shown to impact bone turnover and circulating osteocalcin concentration . The role of vitamin K in the post-translational carboxylation of this protein has been well documented. With the potential of undercarboxylated osteocalcin playing a role in glucose homeostasis, diet must be investigated as a modulator of excursion and carboxylation of osteocalcin.

Structural Characteristics of Osteocalcin

Osteocalcin is synthesized as a prepromolecule of 98 residues, consisting of three parts. A 23 residue signal peptide is cleaved from the parent molecule in the translation process, a 26 residue signal peptide marks osteocalcin for carboxylation, and the remaining 49 residue peptide is the active form[11]. Osteocalcin may be carboxylated at three glutamic acid residues located at amino acids 17, 21 and 24 of the 49 residue chain. All three or fewer than three of the glutamic residues may be carboxylated. The carboxylation reaction occurs post-translationally in the golgi apparatus[12]. Osteocalcin is considered fully carboxylated when all three glutamic acids residues are carboxylated. This carboxylation reaction is catalyzed by the enzyme γ-glutamyl carboxylase which oxidizes vitamin K hydroquinone (a reduced form of the vitamin) to vitamin K 2, 3 epoxide while concurrently adding carbon dioxide to the glutamic acids of the amino acid chain[12]. The carboxylation of the glutamic residue to γ-carboxy glutamic residues of the osteocalcin molecule is shown in Figure 1. Gamma-glutamyl carboxylase catalyzes the

reaction, where the reduced form of vitamin K, vitamin K hydroquinone, is oxidized to vitamin K epoxide while carbon dioxide and oxygen are added to the glutamic acid residue, giving off water[11,14]. This process, commonly referred to as a "gla process", increases the affinity of the protein for the highly positively charged hydroxyapatite in the bone[4, 7]. The carboxylation reaction can occur at amino acids 17, 21 and 24 of the osteocalcin molecule.

Carboxylated osteocalcin consists of two anti-parallel alpha-helical domains (between residues 16-25 and 30-41), connected by a beta turn[11]. A disulphide bond between residues 23 and 29 stabilizes the molecule[11]. Osteocalcin has a half life of 20 minutes in adults, and is cleared from the body through the kidneys[6].

In addition to carboxylation status, several structural analogs of osteocalcin exist in vivo. Total osteocalcin includes intact osteocalcin and fragmented osteocalcin. Intact osteocalcin refers to osteocalcin that has an intact disulfide bond and includes both undercarboxylated osteocalcin and carboxylated osteocalcin. Fragmented osteocalcin includes osteocalcin molecules that have been degraded by osteoclasts and do not have intact disulfide bonds. From this point on total osteocalcin will refer to total intact osteocalcin.

Carboxylated Osteocalcin

Although it is commonly accepted that carboxylated osteocalcin plays a distinct role in bone formation and increasing bone mineral density, there are inconsistent reports. Carboxylated osteocalcin, like other vitamin K dependent proteins, is a negatively

charged molecule and thus has a high affinity for the positively charged calcium ion. This property allows osteocalcin to bind with hydroxyapatite, a molecule with high amounts of calcium, to form structural support in the bone. It is the chemical structure of carboxylated osteocalcin that increases its affinity for calcium. The three carboxylated glutamic acid residues are located on the same surface of the alpha helix[15]. Two carboxylated osteocalcin molecules sandwich 5 calcium ions[15]. The calcium ions, which are part of the hydroxyapatite molecule, increase bone mineralization. This has been seen via 1H-NMR spectroscopy[15]. In an in vitro experiment, hydroxyapatite was exposed to non-cross linked collagen and minerals, and either osteocalcin or a placebo. This trial showed that hydroxyapatite in the presence of osteocalcin resulted in normal mineralization of the collagen fibers[15]. In the absence of osteocalcin, mineral clusters were "needle like" and loosely bound to collagen[15]. Vitamin K is found in plants as phylloquinones, in animal products as menaquinones and is produced from bacteria in the intestine as menaquinones. A trial examining serum phylloquinone concentration in elderly Japanese women showed that women with insufficient concentrations of phylloquinones had increased undercarboxylated osteocalcin concentrations, resulting in an increased vertebral fracture risk independent of bone mineral density[16]. No correlation between intact osteocalcin and bone mineral density has been shown.

Other studies have reported that bone mineral density is independent of osteocalcin synthesis. This was shown through an animal model that was transgenically developed to lack the osteocalcin gene. It was found that these osteocalcin knockout mice actually had increased bone formation and mineralization compared to wild type

controls[17]. This experiment was later confirmed by another study group suggesting that osteocalcin is not required for bone mineralization[18].

Undercarboxylated Osteocalcin

The carboxylation of osteocalcin is of great importance when considering recent reports that undercarboxylated osteocalcin may play a dual role of increasing beta cell proliferation of the pancreas, increasing insulin secretion and up-regulating adiponectin which increases insulin sensitivity [7]. If this is confirmed, osteocalcin would be unique to all other hormones in the body as it would synergistically increase both insulin production and insulin sensitivity[7]. Undercarboxylated osteocalcin leaves the bone and travels to the pancreas and adipose tissue. In the pancreas, undercarboxylated osteocalcin increases proliferation of β-cells and insulin secretion[7]. Adiponectin is an adipocytokine found in inverse proportion to fat mass. This adipocytokine increases glucose uptake, lipid catabolism and energy expenditure, and decreases gluconeogenesis, resulting in increased insulin sensitivity in adipocytes and myocytes. Exogenous administration of adiponectin in an animal model has been shown to increase insulin sensitivity and promote weight loss[19]. Overall undercarboxylated osteocalcin affects target organs to improve glycemia and decrease fat mass[20].

Lee et al. developed knock out animal models to better understand the role of osteocalcin in vivo, using known genetic pathways to increase or suppress osteocalcin synthesis. One model lacks the ESP (also referred to as Ptpvr) gene which encodes the receptor-acting osteotesticular protein tyrosine phosphatase (OST-PTP), found only in

bones and gonads[21]. OST-PTP is highly regulated in bone cells, and plays a role in initiating or inhibiting cell signaling[22]. OST-PTP is thought to play a role in adhesion of molecule to molecule, and for this reason OST-PTP may increase the vitamin K dependent carboxylation of osteocalcin[22]. Lineage of mice lacking the ESP gene showed a significant number of pups dying from hypoglycemia during the weaning process. Further examination showed these pups to have an increased gross pancreatic size and beta cell count, as well as increased insulin levels and sensitivity even though they were hypoglycemic[7]. This effect was found in two knock-out models regardless of whether OST-PTP was removed from the whole body or only the osteoblasts[7]. The increase in beta cell proliferation, insulin secretion, and sensitivity exhibited in the OST-PTP deficient mice acted to protect against obesity and diabetes[7]. These desirable phenotypes were corrected by deleting one allele of osteocalcin, resulting in decreased insulin secretion and sensitivity. In a third model, OST-PTP was transgenically over-expressed in osteoblasts resulting in opposite traits; increased body fat, high blood glucose levels, low circulating insulin concentrations and decreased insulin sensitivity[7]. Osteocalcin expression and serum levels were normal in the ESP knock-out model, impeding the thought that OST-PTP controls osteocalcin [7]. Instead, these findings suggest that OST-PTP and osteocalcin lie in the same regulatory pathway[7] with OST-PTP possibly controlling osteocalcin carboxylation[7]. This evidence strongly supports the premise that undercarboxylated osteocalcin plays a prominent role in carbohydrate homeostasis. OST-PTP is one of hundreds of protein tyrosine phosphatases, but it is suspected that this is the only PTP not conserved from the murine genome to the human genome[23]. Though this particular phosphatase is not found in humans, there are other similar PTPs found in

the human body which may act as OST-PTP does in the murine model. Empirically, humans with Type II diabetes have been shown to have decreased concentrations of total osteocalcin and adiponectin when compared to euglycemic controls[24, 25]. Carboxylated osteocalcin concentration has been correlated with a higher bone mineral density, suggesting that the form of osteocalcin more desirable in humans depends on individual risk factors and life style.

In vitro and in vivo experiments on mice show that differing concentrations of undercarboxylated osteocalcin are needed to affect gene expression[26]. Primary mouse islet cell cultures derived from adult wild type mice in the presence of varying amounts of undercarboxylated osteocalcin were examined for gene expression in vitro. It was shown that as little as 200 fold less than physiologically normal undercarboxylated osteocalcin concentration doubled the expression of insulin genes (Isn1 and Isn2), and concentrations equal to three fold higher than normal physiological levels produced a 6 fold increase in one insulin gene (Isn1) compared to cells not exposed to undercarboxylated osteocalcin[26]. Concentrations of undercarboxylated osteocalcin higher than threefold the physiological concentration did not result in additional increases of insulin gene expression[26]. Expression of genes necessary for β-cell proliferation followed similar patterns to the insulin genes in response to undercarboxylated osteocalcin exposure[26]. Osteocalcin was also administered to a mouse β-cell line resulting in increased expression of insulin and genes associated with β-cell proliferation, suggesting that undercarboxylated osteocalcin acts directly on the pancreas[26]. Mouse adipocytes were examined for gene expression of molecular markers of energy expenditure, and found to have maximal enhanced expression when exposed to at least

half of the normal undercarboxylated osteocalcin concentration in wild type mice[26]. Adiponectin expression was augmented at low levels of undercarboxylated osteocalcin compared to normal physiological conditions[26]. Undercarboxylated osteocalcin is capable of increasing gene expression in adipose tissue at nanomolar concentrations yet is capable of increasing gene expression of the pancreas at picomolar concentrations[26]. This research team also investigated the effects of exogenous undercarboxylated osteocalcin administration in vivo in wild type mice[26]. This trial showed that mice receiving infusions of either 0.3 or 3 nanograms of undercarboxylated osteocalcin per hour demonstrated higher serum insulin concentrations and lower glucose concentrations than mice receiving a placebo[26]. Mice infused with 30 nanograms of undercarboxylated osteocalcin per hour showed no decrease in blood glucose concentrations[26]. These mice were also tested for insulin sensitivity, where it was found that 0.3 and 3 nanograms of undercarboxylated osteocalcin per hour increased expression of energy expenditure markers such as PGC-1 α and UCP-1, as well as adiponectin, and infusion of 30 nanograms per hour further augmented this effect[26]. Mice receiving all three concentrations of undercarboxylated osteocalcin as well as matched placebo groups were evaluated for energy expenditure markers[26]. It was found that undercarboxylated osteocalcin infusion did not influence food intake or body weight. This study team further investigated exogenous administration of undercarboxylated osteocalcin in mouse models that had either diet induced obesity or hyperphagia induced obesity[26]. Mice fed diets high in fat and time-release undercarboxylated osteocalcin pellets releasing 3 nanograms of undercarboxylated osteocalcin per hour gained less weight, had lower triacylglyceride levels, and were more insulin sensitive than mice not receiving the

undercarboxylated osteocalcin time release pellets[26]. Hyperphagic mice receiving 3 nanograms per hour of undercarboxylated osteocalcin via pump showed similar results to the diet-induced obese mice when compared to hyperphagic mice not receiving undercarboxylated osteocalcin[26]. It is estimated that humans not receiving vitamin K supplementation have circulating undercarboxylated osteocalcin concentrations ranging from 6.3-9.0% of total osteocalcin[27].

The actions, target tissues and feedback mechanism of osteocalcin are summarized in Figure 2. In the osteoblast, osteocalcin is synthesized by the OCN gene. OST-PTP is synthesized by the ESP gene. The presence of OST-PTP up-regulates the carboxylation of osteocalcin. Carboxylated osteocalcin acts in the bone, and potentially increases cross-bridging to increase bone mineral density. Undercarboxylated osteocalcin leaves the bone to act on the pancreas and adipose. The adipose releases the adipocytokines leptin and adiponectin. Adiponectin increases insulin sensitivity in the skeletal muscle and increases energy expenditure. Leptin binds to receptors in ventral medial nucleus of the hypothalamus and causes the sympathetic nervous system to increase β2 adrenergic receptors which decrease osteoblast proliferation and bone formation[8].

CHAPTER 4: BONE

Overview of Bone Turnover

Bone is constantly being remodeled, with bone resorption and bone formation occurring simultaneously but at different rates in different parts of the body. Bone remodeling happens cyclically, starting with the resting phase. In this phase bone lining cells, which are flat, fibrocytic cells derived from osteoblasts, line the surface of the bone. To initiate the resorption phase, bone lining cells retract, exposing the bony surface which acts as chemo-attractant for osteoclast precursors. Osteoclasts are cells that resorb bone. Osteoclasts are derived from a monocyte lineage, and are short-lived, multinucleated cells that undergo apoptosis. During the resorption phase, osteoclast precursors mature to osteoclasts and attach to bone. The breakdown products from osteoclast activity are primarily recycled; the calcium and phosphorus are released into extracellular fluid and used to mineralize other sites of skeletal remodeling. Some of the amino acids that osteoclasts release are reused for protein synthesis, with the exception of amino acids that have undergone post-translational modification, such as the γ-carboxyglutamate in osteocalcin. The resorption phase typically lasts about 40 days. Once an area of bone has been resorbed, bone lining cells again cover the surface. At this point, bone formation begins. This process takes about 145 days. Columnar osteoblasts, bone building cells, migrate to the site and deposit new bone matrix. It is at this point that osteocalcin in secreted from the osteoblast cell. After bone is built, the remaining surface osteoblasts become quiescent, flatten out, turn into bone lining cells, and seal the new bone surface. This turnover process occurs more rapidly in infants and children, and less rapidly in the elderly.

The protein matrix that makes up the bone is primarily collagen, a strong, fibrous protein that gives bone its tensile strength. Non-collagenous calcium binding proteins make up about ten percent of the protein matrix. These include the vitamin K dependent proteins osteocalcin and matrix gla protein as well as the non-vitamin K dependent proteins osteonectin and fibronectin. Also in the bone matrix is a large abundance of minerals including calcium and phosphorus in the form of hydroxyapatite, magnesium, and fluoride, which in addition to being found in the matrix promote new bone formation and mineralization.

Bone formation and resorption is primarily regulated by the interactions of three hormones upon target tissues; parathyroid hormone, calcitriol and calcitonin. Individual nutrients are known to influence these three hormones. Calcitriol is the active form of vitamin D, which has been activated by hydroxylation in both the kidney and the liver.

CHAPTER 5: EFFECT OF NUTRITION, BIOLOGICAL AND LIFESTYLE VARIABLES ON OSTEOCALCIN

The Influence of Macronutrients and Energy on Osteocalcin

Empirical evidence suggests that bone turnover is depressed after feeding[28]. Clinical trials have tested the effect of meals of varying macronutrient compositions on serum osteocalcin concentrations. Because of the designs used, it is unclear whether bone turnover is suppressed by the consumption of energy, glucose, or other nutrients. An acute study by Henricksen et al. found that serum osteocalcin concentration was unaffected by glucose or fructose when ingested orally compared to the fasting state[29]. This study collected blood samples at 30 minutes and hours 1, 2, 3, 6, and 9, and is further detailed in Table 1[29]. An in vivo study using both euglycemic and hypoglycemic-hyperinsulinemic clamps was performed to investigate whether glucose ingestion or the respondent surge in circulating insulin concentration caused suppression of bone turnover[30]. This study showed that the clamp-induced hyperinsulinemia coupled with euglycemia had no significant effect on acute bone formation measured by osteocalcin and serum procollagen type I N-terminal propeptide or acute bone resorption measured by serum C-terminal telopeptide of type I collagen[30]. Hypoglycemia caused by the hyperinsulinemic state resulted in a decrease in both bone resorption and formation[30]. Catecholamines and glucagon are unlikely to play a role in the acute changes in bone turnover as they are released later in the postprandial cycle, compared to observed changes in osteocalcin concentration[30]. The suppression of bone formation in the presence of hypoglycemia may be attributed to the hypoglycemia itself, the release of

counter-regulatory hormones or perhaps the decrease in parathyroid hormone (PTH) which is a known response to hypoglycemia.

Postprandial lipid metabolism is integral to the carboxylation of osteocalcin, as Vitamin K is carried to the osteoblasts via chylomicrons and chylomicron remnants. In bone, in vivo, chylomicron remnant uptake postprandially is equivalent to 20% of liver uptake[31]. In vitro evidence has shown that osteoblasts had a similar chylomicron remnant uptake capacity to liver[31]. An acute study showed that oral ingestion of long chain triacylgycerides had no effect on osteocalcin concentration[29]. Additional studies have investigated the efficacy of conjugated linoleic acids and polyunsaturated fatty acids in the suppression of bone formation markers, with contradictory results[32]. Lipids may also influence bone formation when added to the body's adipose stores through the adiptocytokine leptin. Leptin is an anorexigenic adipocyte-derived hormone found in levels proportionate to fat mass that aids in the control of bone formation. Excess of energy, regardless of nutrient origin adds to fat mass, increasing leptin levels and inhibiting osteoblast activity over the long term. Interestingly, many people who are obese and overweight are leptin resistant[33]. It is suspected that leptin controls bone formation by binding to the β2 adrenergic receptors on ventromedial hypothalamic neurons, which in turn signal the sympathetic nervous system to inhibit osteoblast activity[8].

The effect of dietary protein on bone formation and resorption has yielded controversial results, as non-intervention studies on bone health are often confounded by both low protein and low energy intake. Studies in the elderly have shown that dietary supplementation of an oral beverage containing protein (in the form of an oral

supplement such as Ensure™ or Boost™) improved clinical outcome in patients with femoral neck fractures[34]. Intervention studies conducted in senescent rats have shown that energy restriction as well as low protein diets adequate in energy (diets consisting of 2.5% of energy coming from protein) lead to significantly decreased femoral bone mineral density, a noteworthy marker for osteoporosis[34]. It has also been shown that a supplement of essential amino acids in protein-deficient rats increases trabecular bone strength, bone mineral density, and cortical thickness[34]. Conversely, diets high in protein have been shown to increase renal calcium excretion and decrease calcium absorption, as well as augmenting bone resorption [34]. Few studies have focused solely on the effect of protein on bone formation. One such study was conducted in free-living young women consuming low (0.7 g/kg), medium (1.0 g/kg), or high (2.1 g/kg) levels of dietary protein. This clinical trial found that a high protein diet compared to a low protein diet increased bone resorption but had no significant effect on bone formation[35]. Protein and energy consumption has also been studied in a dietary intervention study using adult Wistar rats. These rats were separated into 6 cohorts, and intervention was separated into control groups, protein and energy restricted groups and energy restricted groups. Each group was then separated into a high protein or a low protein arm. Total osteocalcin was measured after five months. The energy restricted groups as well as the protein-energy restricted groups showed a significant decrease in circulating osteocalcin concentration compared to the control group. Though the higher protein diet in each group was correlated with a higher osteocalcin concentration than the lower protein group, these differences were not significant [34]. In another in vivo cross-over human study, subjects were given protein, carbohydrate or fat beverages. This study is detailed in Table 1.

Orally ingested protein, fat, and glucose led to a lesser amount of circulating osteocalcin compared to the fasting group, with protein having the greatest effect on suppression of osteocalcin excursion[29]. This study collected blood samples at 30 minutes and hours 1, 2, 3, 6, and 9, and used area under the curve statistical analyses to assess the concentration of total osteocalcin over the first three hours of this experiment for each nutrient. Subjects were fed immediately after the fasting blood draw. The differences in area under the curve were not significant for the various macronutrients with a p-value of 0.11[29]. Osteocalcin levels rose above baseline between the 3 and 6 hour blood draws[29]. It is possible if protein had been administered isocalorically to fat and carbohydrate that a more extreme suppression of osteocalcin would be seen. It is important to note that none of these studies measured the carboxylation status of osteocalcin. In short, adequate protein consumption is integral to bone health, yet high protein diets are associated with increased bone loss.

The Influence of Micronutrients on Osteocalcin

Many micronutrients and other non-nutritive, food-based compounds are known to influence bone formation/metabolism. Among the dietary factors thought to have a beneficial effect on bone are copper, zinc, fluoride, magnesium, phosphorus, potassium, manganese, vitamin K, vitamin C, the B vitamins, phtyo-estrogens and non-digestible oligosaccharides (fiber)[36]. Dietary factors that can negatively affect bone health (primarily though increasing osteoclast activity although potentially through inhibiting osteoblast activity) include the excess consumption of alcohol, caffeine, sodium, fluoride, and phosphorus, as well as the excess or insufficiency of vitamin A[36].

The relationship between osteocalcin and vitamin K is well documented, with studies investigating the carboxylation reaction of osteocalcin in the presence of various levels and forms of vitamin K. Vitamin K is integral to the post-translational modification of osteocalcin. This fat soluble vitamin is both consumed exogenously through diet and manufactured in the flora of the colon. Vitamin K is necessary for coagulation in vivo, and acts as a cofactor in the post-translational modification of glutamic acid residues to γ-carboxyglutamic acids. This process occurs in at least 12 proteins in the human body[37]. These modified proteins are implicated in blood coagulation and anticoagulation processes because of the ability of γ-carboxyglutamic acids to bind to membrane surfaces and calcium[38, 39]. Several studies have suggested that vitamin K dependent proteins exhibit additional activities and roles beyond hemostatic functions and bone metabolism, perhaps in vascular calcification and atherosclerotic complications[37]. The ability of γ-carboxyglutamic acids to bind calcium ions explains why carboxylated osteocalcin may play a role in bone mineral density whereas

undercarboxylated osteocalcin has not been shown to do so. A cross sectional study in adult humans found that increased vitamin K intake was significantly inversely correlated with decreased undercarboxylated: intact osteocalcin ratios [4, 40]. The effects of other vitamins on bone health have been investigated with findings that vitamins C, D and E impact bone formation. A classic cell study suggests that vitamin D, in the form of calcitriol ($1,25(OH)_2D_3$) increases the synthesis and secretion of osteocalcin into plasma[3]. Other cell studies also found that osteocalcin regulation and production are dependent on the presence of vitamin D[3, 41]. Another study found a strong inverse correlation between plasma γ-tocopherol (vitamin E) concentrations and undercarboxylated: intact osteocalcin ratio[4]. The role of vitamin C and circulating osteocalcin concentration has also been examined using a porcine model. This study found a significant dose-dependent positive trend between vitamin C intake and osteocalcin concentration, though other measured bone markers and bone strength showed no difference between vitamin C levels[42].

Minerals have also been investigated in relation to bone formation and bone health, with magnesium, copper, zinc, fluoride, phosphorus and potassium being specifically examined. Rats with induced magnesium deficiency showed a 26% decrease in circulating osteocalcin concentration compared to pair-fed magnesium adequate rats[43]. Excess magnesium intake was not investigated. Serum osteocalcin was unaffected by copper supplementation in healthy adult females[44]. A cross sectional study showed that a high calcium diet as measured retrospectively with a food frequency questionnaire had no significant impact on osteocalcin concentrations in free living women in Sweeden[45]. A European observational study found that inadequate zinc status was associated with a

decrease in bone resorption markers but had no significant correlation with bone formation markers[2]. An in vivo rat model study showed a significant positive correlation between fluoride and total serum osteocalcin, while a cell study showed that the effect of fluoride on osteoblast activity was less remarkable[46, 47]. Fluoride, a highly electronegative ion, is typically associated with increasing mineralization within the bone. Excess intake of phosphorus is known to increase parathyroid hormone which in turn increases calcium absorption from the gut and stimulates osteoclastogenesis. Rats fed a high phosphorus diet had significantly higher levels of total osteocalcin compared to rats fed a control or low phosphorus diet, while calcium intake remained the same [48]. A linear relationship was seen between phosphorous intake and circulating osteocalcin concentrations[48]. A trial examining the effect of potassium citrate supplementation on bone formation markers in free living female volunteers showed inconsistent trends between potassium intake and osteocalcin, with half of the women showing an increase in osteocalcin levels and the other half showing a decrease or remaining unchanged[49]. The effect of select micronutrients on total and undercarboxylated osteocalcin is summarized in Table 2.

Hormonal effects on osteocalcin

Various hormones have been studied as effectors of osteocalcin, in hopes of finding biochemical manipulations of osteocalcin. Glucagon like peptide 1 and 2 (GLP 1 and GLP 2) as well as gastric inhibitory protein (GIP) were not found to alter osteocalcin levels. Cortisol resulted in a lower concentration of osteocalcin throughout the day when

administered in the morning[29]. Parathyroid hormone has been shown to increase OST-PTP in vivo, which in turn may up-regulate the carboxylation of osteocalcin [21]. Thus, a high phosphorus or low calcium diet may result in augmented carboxylation of osteocalcin.

Pharmacological agents and osteocalcin

To date, osteocalcin has not been prospectively manipulated through the use of pharmacological agents, but data has been collected about select drugs and their affect on osteocalcin. Warfarin, a known inhibitor of enzymes in the vitamin K cycle, is prescribed for anti-coagulation therapy. Patients on this drug display increased undercarboxylated osteocalcin [50]. Warfarin has been shown to decrease carboxylation of osteocalcin in vivo and in vitro. The administration of heparin in co-culture experiments in adipocytes increased adiponectin concentration[50]. The bisphosphonate class of drugs is commonly prescribed to patients with osteoporosis to inhibit osteoclast resorption causing reduced bone resorption and reduced bone turnover rate. The reduced bone turnover rate is associated with a lower circulating osteocalcin concentration[50]. Glucocorticoid therapy is common in the United States, and is prescribed for its anti-inflammatory and immunosuppressive effects. This class of drugs predisposes patients to both diabetes and osteoporosis[51]. Chronic administration of glucocorticoids suppressed bone formation and osteocalcin expression[50]. It is possible that osteoporosis and diabetes, formerly considered independent manifestations of glucocorticoid therapy may be mechanically linked through the expression of total osteocalcin [50].

Biological and Lifestyle Effects on Osteocalcin

A study investigating lifestyle and osteocalcin found that males had higher circulating concentrations of undercarboxylated osteocalcin and intact osteocalcin compared to females[4]. In females, undercarboxylated osteocalcin concentrations were highest in women under the age of 30, and then steadily declined until the 50-59 age group, which correlates with an increase in incidence of osteoporosis[4]. After a female reached the age of 60, undercarboxylated osteocalcin rose insignificantly[4]. When undercarboxylated osteocalcin was expressed as a ratio of intact osteocalcin, a negative correlation was found with age [4]. A trend was seen in smokers and former smokers having lower concentrations of total osteocalcin, however, diet was not accounted for[4]. This study also showed that menopausal status, alcohol intake, education level and BMI were not associated with the undercarboxylated : intact osteocalcin ratio[4]. A recent in vitro study showed that compression of osteoblasts, simulating physical activity, increased the expression of osteocalcin[52]. Additionally, observational and longitudal studies were conducted in premenopausal women examining the effect of exercise on bone turnover markers[53]. The observational study involved 530 women and found that women who reported participating in an average of at least 30 minutes of physical activity per day had higher total ostecocalcin concentrations, with an average concentration of 0.21 nanograms per milliliter total osteocalcin higher than the non-exercising counterparts after age and BMI were accounted for[53]. The longitudal study investigated healthy, normal weight, premenopausal women who participated in a community exercise program where they exercised 90 minutes 3-4 times a week for 4

weeks[53]. This study found that women participating in the exercise program significantly increased their total serum osteocalcin concentration over 4 weeks[53].

<u>Circadian Rhythm of Osteocalcin</u>

One study group reported that osteocalcin levels vary throughout the day, independent of nutrient consumption[31]. It has been suggested that this variation is controlled by a circadian rhythm[54]. No variation in the rate of carboxylation related to circadian rhythm has been demonstrated[31].

In 1985, the circadian rhythm of osteocalcin in 10 healthy adults was studied. Total osteocalcin, ionized and total calcium, and phosphorus were measured in serum every 30-60 minutes for 24 hours[54]. Three meals, of undescribed composition and energy level were served to the subjects, at 0900, 1200 and 1730. In 9 of the 10 subjects studied, diurnal variations in osteocalcin were seen[54]. Osteocalcin followed a pattern of peaking between 0400 and 0800, falling during the morning after breakfast, and achieving a small rise before lunch. Osteocalcin concentration fell after lunch and then rose slightly before dinner. After dinner the osteocalcin level fell and then gradually rose to peak concentration[54]. It was also found that changing cyclical hormonal status in the 4 women studied did not change the circadian rhythm of total osteocalcin. This research was completed prior to the understanding that feeding acutely affects bone turnover. It is possible that this circadian rhythm is actually due to the administration of meals, and that varying the meals energy, macronutrient and micronutrient content may change the magnitude of nadir and zenith of the graph.

CHAPTER 6: LOW VERSUS HIGH COMPLEX CARBOHYDRATE DIET

DASH diet

The DASH (Dietary Approaches to Stop Hypertension) diet and the Atkin's diet are two diets commonly used by the American public. The DASH diet is often prescribed by clinical practitioners for lowering blood pressure and improving general health. The DASH diet contains reduced amounts of total fat, saturated fat and cholesterol and increased amounts of potassium, calcium, magnesium, dietary fiber and protein compared to the typical American diet. The DASH diet recommends 7-8 servings of grains, 4-5 servings of vegetables, 4-5 servings of fruit, 2-3servings of low fat dairy and 2-3servings of fat or oil per day. This diet limits meat, poultry and fish intake to 2-3servings per day and limits intake of sweets to less than 5 servings per week. Serving sizes for the DASH diet are the same as those recommended by the United States Department of Agriculture food guide pyramid. The DASH diet has been effective in lowering intake of energy, fat, saturated fat and sodium as well as increasing intake of fruits, vegetables and dairy. In a long-term, randomized multicenter study participants consuming the DASH diet consumed at least two thirds of their respective Daily Recommended Intake for most nutrients[55]. The weight loss associated with the DASH diet can be effective in decreasing the morbidities of obesity, chronic hyperglycemia, hypertension, hypercholesterolemia and hyperlipidemia.

A trial performed by Lin et al. studied the effects of the DASH diet versus a control of the typical American diet at various sodium levels on bone turnover markers[55]. This study found that the DASH diet reduced serum total osteocalcin by 8-11% at each

sodium level compared to the control diet[55]. The participants in each of the three subgroups of sodium intake of the control groups showed no significant difference in osteocalcin levels regardless of sodium level. Participants in the DASH group however, showed a decrease in serum osteocalcin by ~3% from the highest to the lowest sodium level[55]. Prominently, this study also showed that when subjects were placed back on the control diet, their osteocalcin levels were increased[55]. Undercarboxylated osteocalcin was not measured in this study. This study suggests that dietary intake of sodium, higher in the Atkin's diet than the DASH diet may positively influence osteocalcin secretion.

Low Carbohydrate Diet

Low Carbohydrate Diets are often used by the American public to initiate and maintain weight loss. Commonly used low carbohydrate diets include The South Beach Diet™ and the Atkin's diet. The Atkins diet consists of foods high in protein and fats, with a limit placed on carbohydrate intake, especially intake coming from refined sugars[56]. No limit is placed on saturated fats. Dr. Atkin, the creator of this diet suggests that obesity is caused by the intake of large amounts of carbohydrates and the associated insulin secretion. Dr. Atkin noted that his diet may be deficient in many vitamins and minerals and suggests that an assortment of vitamins and minerals be supplemented to the diet[57]. A short-term study of obese patients with type II diabetes found that a low carbohydrate diet resulted in decreased energy intake to a level appropriate for height and weight, improved 24 hour blood glucose profiles, insulin sensitivity and HbA1c as well as decreased triacyglyceride and cholesterol levels[58]. This trial, though only enrolling 10

participants shows that the low carbohydrate diets may be equally effective in reducing health risks as other popular prescribed weight loss diets, and may be preferential to patients. This study also showed that subjects on the Atkin's diet had a decreased level of leptin associated with decreased fat mass compared to the control group, suggesting that bone turnover may be decreased.

CHAPTER 7: POTENTIAL IMPACT OF OSTEOCALCIN

Manipulation of undercarboxylated and total osteocalcin excursion may prove therapeutic for glycemic control, obesity, and osteoporosis. Individual macro and micro nutrients have been shown to affect bone turnover and osteocalcin secretion or carboxylation of osteocalcin[2-5].

Osteoporosis

Bone mineral density is a measure of the amount of calcium and other minerals in a specified section of bone. Low bone mineral density is an indicator of future fracture and osteoporosis risk and is routinely measure in females past the age of 50 and other at-risk individuals. In 2005 an estimated 10-20 billion dollars was spent in the United States for osteoporosis treatment[59]. Interestingly, obesity, another major public health concern seems to protect against osteoporosis. Paradoxically, persons with diabetes are prone to fracture, but are not predisposed to osteoporosis because of an increase in bone mineral density[60]. Common theories advocate that adiposity can even be advantageous to bone strength, providing a resistance workout with activities of daily living. Although a correlation has been documented between increased bone mineral density and type II diabetes, a marked decrease in osteocalcin levels was also seen, when compared to a control group[24]. Several studies support the claim that obesity and/or type II diabetes leads to an increase in bone mineral density and that obesity can protect a person from osteoporosis [61-63]. Conflicting studies have reported that excess weight as fat mass does not provide benefit and may actually be a negative factor for bone health[64]. These studies

did not consistently measure osteocalcin levels (total or undercarboxylated osteocalcin), and this conflicting evidence may be clarified by measurements of both forms of this protein.

Obesity

Problems with overweight and obesity are of epidemic proportions in the United States, with only one state in 2007 having a prevalence of under 20% of their population as being overweight[65]. The Centers for Disease Control and Prevention (CDC) have followed the obesity and overweight trends for over 12 years, seeing an increase annually[65]. Body mass index (BMI) is an index of weight in kilograms to height in meters squared. The CDC classifies anyone with a BMI of 25 and above as being overweight and 30 and above as being obese. Weight regulation is a complex puzzle that is still not fully understood. It is the result of genetics and metabolism, environment and life style choices, and has been correlated with select socioeconomic factors. The health consequences of being overweight include a higher risk for hypertension, osteoarthritis, dyslipidemia, Type II diabetes, coronary heart disease, stroke, gallbladder disease, sleep apnea and other respiratory problems and select cancers such as endometrial, breast and colon[65]. Economically, overweight associated health problems are costly. These costs are difficult to estimate, but limited data from 1998 to 2000 estimates that the US spent 75,051 billion dollars on overweight and obesity related health problems [66]. The prevalence of overweight was significantly less from 1998-2000 compared to 2007-2008[65].

Diabetes

The American Diabetes Association estimates that 8 % of the United States population has diabetes, or 23.6 million people[67]. The ADA also estimates that 57 million people are currently "pre-diabetic" or have abnormally high glycemia or impaired glucose tolerance[67]. Diabetes is a disease of chronically elevated blood glucose levels, and in many cases impaired insulin sensitivity and/or production. This disease can be treated pharmacologically, but in many cases is best managed with diet and lifestyle choices. This disease decimates quality of life, and can lead to significant complications and co-morbid conditions such as heart disease, stroke, hypertension, renal failure, neuropathy leading to loss of function and amputation, dental disease, sexual dysfunction and complications of pregnancy. In 2007, the total economic costs attributed both directly and indirectly to diabetes was one hundred and seventy four billion dollars[67]. Ten percent of the health care costs in the United States is attributed to direct diabetes care[67]. The correlation between glycemic control and osteocalcin excursion has been previously demonstrated in an interventional trial. This trial showed the effect of improving glycemic control using diet counseling and medication significantly increased osteocalcin concentration in the body[68]. When HbA1c levels, a marker of three month glycemic control, were higher than 10 (correlating to an average daily glucose level of 279 mg/dL) osteocalcin levels reached a plateau of 2 nanograms per milliliter[68]. When HbA1c levels improved, and were lower than 10, an inverse relationship between osteocalcin concentration and HbA1c can be seen[68]. Empirically, patients with type II diabetes have a lower concentration of serum osteocalcin compared to healthy counterparts[68]. Some trials suggest diabetics to have 71% lower osteocalcin

concentrations than healthy counterparts, while bone resorption markers remained unchanged[68]. Select studies show that having diabetes increases the risk of bone fracture and osteoporosis[69]. As poor glycemic control is associated with a lower concentration of total intact osteocalcin, it is reasonable that regardless of the proportion of osteocalcin being carboxylated, bone mineral density may be compromised. Fracture risk may also be increased in patients with diabetes because of mineral losses related to diabetes-associated diarrhea, polyuria and neuropathy of the feet reducing feeling and normal gait, and increasing fall risk[69].

Type II diabetes is also correlated with decreased levels of insulin like growth factor 1 and insulin. The causation of slow bone turnover and low osteocalcin levels may be attributed to these factors[61]. Conversely, low osteocalcin and/or undercarboxylated osteocalcin levels could contribute to insulin resistance and in turn cause type II diabetes. Semenkovich and Teitelbaum suggest that a cycle exists in which the metabolic events of diabetes down-regulate osteoblast function, which in turn leads to a lesser secretion of osteocalcin and a greater aggravation of insulin resistance[50].

CHAPTER 8: METHODOLOGY

General Design and Purpose:

This was an acute, randomized, within-subject crossover feeding trial of healthy men and women living in the Portland, Oregon area. This substudy of The Energy Balance Study was approved by the Oregon Health & Science University Institutional Review Board. Participation in this study was voluntary, and all subjects provided signed informed consent.

The purpose of this study was to examine the acute effects of high and low carbohydrate meals on circulating postprandial concentrations of undercarboxylated osteocalcin and intact osteocalcin, to provide groundwork for future studies of glycemia, weight management and osteoporosis.

Subject Recruitment and Selection

Four men and six women were recruited from the Portland, Oregon area through recruitment fliers posted throughout Portland, including the Oregon Health & Science University and Portland State University campuses. Inclusion criteria, detailed in Table 3, included a self assessment of general health, normal body mass index and willingness to consume both high and low carbohydrate meals. Exclusion criteria included major debilitating mental or physical illness, weight instability over the past 6 months, and food allergies or restrictions that may interfere with intervention meals. All subjects remained

free-living for the entirety of the study. Subject visits and data collection were completed between April of 2008 and February of 2009.

Screening:

Pre-study screening visits were conducted for each potential participant to determine eligibility for the study. Each subject was read information on the screening consent form (Appendix A) and once the participant fully comprehended the study requirements and gave their written and verbal consent established screening procedures were continued. These procedures included measurement of height, weight, blood pressure, fasting blood glucose and hemoglobin. The Baecke Activity Questionnaire was administered to assess physical activity. Medical history was assessed using the gender specific Cornell Medical Index. Food preferences were discussed to accommodate minor changes and increase compliance to the standardization diets. Subjects judged to be healthy by self-report, review of medical history, medication-use, and lab screening were considered eligible for participation.

Standardization Protocol:

On days 1-3 of the protocol each subject was fed a weight maintenance standard diet (50% carbohydrate, 35% fat, and 15% protein). A table detailing the nutrient composition of the standard diet is presented in Appendix B. Each day, each participant arrived at the Oregon Clinical and Translational Research Institute (OCTRI) Outpatient Unit between 0700 and 1000 am to have their weight measured in the Bionutrition Unit. The participants then ate breakfast in the OCTRI dining room and completed study

related forms. All other meals and snacks for the remainder of the day were prepared for each participant to take home. Participants were asked to consume the food provided to them by the Bionutrition kitchen staff in its entirety, and instructed to abstain from other food intake. Subjects returned empty containers to the Bionutrition Unit every morning from the previous day to check compliance to diet.

Intervention Protocol:

At about 0600 in the morning of the fourth study day, subjects were admitted to the inpatient unit of OCTRI for approximately 12 hours.   For 24 hours prior to the intervention day, subjects were asked to abstain from significant physical activity. On the third day of the standardized diet, subjects were asked to consume all of their meals by 2200 to ensure a ten hour fast before the fasting blood sample was drawn.   Blood pressure and vital signs were measured and an indwelling catheter was placed in a peripheral vein in the subject's arm. Half normal saline was infused throughout the day to preserve patency of the intravenous (IV) line. A fasting blood sample was taken at 0800, after which the study subject consumed either a low carbohydrate or high complex-carbohydrate breakfast meal. The first postprandial blood sample was taken at 0830 and subsequent samples were taken every hour after for nine hours as well as at 1300. The participant consumed a lunch meal between 1300 and 1330.   If the timing of the meals or blood draws for the first visit were postponed for any reason, such as failed IV assess, the second visit was time-matched to eliminate any confounding variables related to circadian rhythms. A detailed table of the blood sample collection schedule is shown in

Appendix C. Blood was collected in pre-chilled heparinized tubes and centrifuged on site at 1500 gravitational fields (g) at four degrees centigrade for 12 minutes. Heparinized plasma was harvested, aliquotted and immediately frozen at negative twenty degrees and then transferred to a negative 80 degrees centigrade freezer within 24 hours. At the end of each intervention day participants were discharged with orders to resume their usual diet and activity. After a washout period of at least three days, participants returned to the research center and completed the same 4 day protocol, but consumed the alternate diet of either low carbohydrate or high complex carbohydrate meals.

Inpatient Intervention Diets:

During the inpatient admissions participants consumed either a low carbohydrate meal, modeled after the Atkins diet induction phase, or a high complex carbohydrate meal, modeled after the DASH diet. Macronutrient composition of the low carbohydrate meal was approximately 66% fat, 30% protein, and 4% carbohydrate. Macronutrient composition of the high complex carbohydrate diet was approximately 28% fat, 58% carbohydrate, and 18% protein. Each meal consumed during the inpatient admission provided 10 kcal/kg body mass. A table detailing the nutrient composition of the two test meals is presented in Appendix D.

Calculations:

The energy intake necessary to maintain the body weight of participants during the standardization phase of the study was calculated using the Harris-Benedict energy prediction equation and multiplied by an activity factor. The activity factor was determined by the average amount of physical activity for each participant as estimated by the Baecke activity questionnaire, and ranged between 1.2 for sedentary persons and 2.0 for highly active persons. The equation accounts for gender, age, height and weight.

Basal Energy Requirements (male) = 66 + 13.7 (weight in kg) + 5 (height in cm) – 6.8 (age in years)

Basal Energy Requirements (female) = 665 + 9.6 (weight in kg) + 1.8 (height in cm) – 4.7 (age in years)

Measurements:

Height was measured without shoes at the screening visit with a wall mounted stadiometer (Holtain Ltd., UK).

Body weight was measured in light clothing each day of the standardized diet and the intervention diets using a stand-on scale with shielded remote digital display (Scale-Tronix, Model 5002, Carol Stream, IL) in the Bionutrition unit or the inpatient unit of the Oregon Clinical and Translational Research Instiute.

Blood pressure was measured at the screening visit and on intervention days using an automatic non-invasive Dinamap XL vital signs monitor (Critkon Corp, Tampa, Fl).

Point of contact *hemoglobin* concentration was determined using a HemoCue B Hemoglobin System (HemoCue, Inc., Lake Forest, CA) at the screening visit.

Point of contact *blood glucose* was determined using a YSI 2300 StatPlus Glucometer (Yellow Springs Instruments, Yellow Springs, OH).

Body composition including total and regional lean, fat and skeletal mass was measured by total body dual energy X-ray absorptiometry (DXA) scan (Discovery Series Densitometer, Hologic Inc., Bedford, MA) on the last day of either the first or second standardized diet period.

Total Intact Osteocalcin was measured in heparinized plasma using a competitive immunosorbant assay (ELISA) [Quidel Metra, San Diego, CA] and analyzed in the OCTRI core lab. To prepare for chemical analysis, samples and reagents were brought to room temperature (20-25 degrees centigrade) at which point samples were mixed to ensure homogenous plasma, and centrifuged for 10 minutes at 15 g and 5 degrees centigrade to precipitate any particulate matter. Samples were diluted 1:2 in wash buffer, and then twenty five microliters (μL) of diluted sample was added to each well along with 125 μL of the anti-osteocalcin antibody. This solution was incubated for 1 hour and 55 minutes at room temperature. This solution was removed and sample wells were washed three times with wash buffer. One hundred and fifty μL reconstituted enzyme conjugate was added to each well, and incubated for 55 minutes at room temperature before being washed three times with wash buffer. One hundred and fifty μL of working

substrate solution was then added to each well and samples were incubated for 35-40 minutes at room temperature. Fifty µL of stop solution was added to each well, and absorbance of each sample was read at 405 nanometers (nm) using a microplate reader (Bio-Rad Benchmark Plus, Hercules, CA). A differential absorbance reading measured at 630 nm was subtracted from the reading measured at 405 nm. A differential reading is used as a reference optical density reading which is subtracted from the primary optical density reading. This adjusts or controls for any optical differences of the individual wells of the plate, such as thickness of the plate, and edge effects at the side of the plate.

Undercarboxylated osteocalcin was measured in heparinized plasma using a sandwich method enzyme linked immunosorbant assays (Takara Bio, Otsu Shiga, Japan) and analyzed in the OCTRI core lab. All reagents were prepared as directed and brought to room temperature prior to analysis. One hundred µl of sample, control or standards were added to the appropriate wells and incubated for 2 hours at room temperature. Sample solution was removed and the wells were washed three times with 400 µl of phosphate buffered saline (PBS). One hundred µl of antibody-peroxidase conjugate solution were added into wells and incubated at room temperature for 1 hour. Solutions were aspirated from wells and washed 4 times with 400 µl of PBS per well, with thorough aspiration between washes. One hundred µl of peroxidase substrate solution was added to each well, and incubated for 15 minutes at room temperature. One hundred µl of stop solution was applied to all wells and mixed gently. Absorbance was read at 450 nm on the microplate reader (Bio-Rad Benchmark Plus, Hercules, CA), and the differential absorbance reading of 630 nm was subtracted.

Data Management:

All data collected as a result of participation in this study was kept confidential. Paper forms, excluding one master copy, identified patients only by a study specific ID. All non-electronic forms were kept in a locked cabinet within a locked office in the OHSU Mark O. Hatfield Research Building. Specific forms were developed for each data set including patient demographics and history, as well as each discrete outcome variable. Password protected computer databases were developed to store information; these were accessible only by select study staff.

Statistical Power Calculations and Analyses:

Power was calculated prior to the start of the study. Power to detect a 1.1 fold difference in area under the curve of circulating intact osteocalcin concentration after the low carbohydrate and high carbohydrate meals is shown in Table 4. Little empirical evidence has been published to suggest a value of change in intact osteocalcin that would represent clinical relevance. A 1.1 fold difference in area under the curve was chosen because this value would show that one meal composition produced at least a 10% greater concentration of intact osteocalcin over time when compared to the other meal composition. A 10% difference in concentration over time of circulating intact osteocalcin attributable to diet, in a controlled setting in the acute time period, may show potential for nutritional intervention in changing intact osteocalcin concentration over the long term. A trial investigating circulating intact osteocalcin concentration after a 6

month intervention by Rosato et al. showed that increasing absolute intact osteocalcin concentration over 2 ng/mL was linearly correlated with improved glycemic control, indicating potential clinical significance for small increases in intact osteocalcin[68]. This power was calculated using hypothesized correlations between response to the high complex carbohydrate and low carbohydrate meals and a within subject coefficient of variation (CV) of 0.2 and 0.3 between the two diets. A significance level of 0.05 and a sample size of 10 subjects is assumed.

Based on the above power calculation, this study would be underpowered (power less than 0.80) if the correlation between meals is low (below 0.75) or if the coefficient of variation of either intact osteocalcin area under the curve or the undercarboxylated : intact osteocalcin ratio area under the curve is high (greater than 0.20). If the coefficient of variation is at most 0.20 and the correlation is at least 0.88, then there will be greater than 80% power to detect a 1.1 fold difference between the diets with 10 subjects.

Means, standard deviations, and standard error of the means were calculated for absolute circulating concentrations of undercarboxylated and intact osteocalcin as well as the undercarboxylated: intact osteocalcin ratio at each time point. Differences in excursion of undercarboxylated osteocalcin and intact osteocalcin concentrations were calculated as symmetric percent change, using a method suggested by Berry and Ayers [72]. This method prevents either of the diets as being treated as a baseline or preferred value and is calculated using the difference of the area under the curve between diets divided by the sum of the area under the curve of both diets per subject. This analysis was performed for intact osteocalcin concentration, undercarboxylated osteocalcin concentration and undercarboxylated: intact osteocalcin ratio. Robust percent change

was calculated from symmetric percent change for better comprehension of results. Robust percent change is calculated using the inverse transformation; robust percent change is equal to 200 * symmetric percent change/ (100 minus symmetric percent change).

The circulating concentrations of intact osteocalcin and undercarboxylated osteocalcin, as well as the undercarboxylated: intact osteocalcin ratio were analyzed using area under the curve analysis calculated by the trapezoidal method[70]. Area under the curve was calculated from baseline throughout the 9.5 hours of intervention as well as for time intervals 0-5 hours and 5-9.5 hours. Hours 0-5 correspond with the time interval from baseline to immediately before the lunch meal. Hours 5-9.5 correspond with the time interval directly before the lunch meal through the duration of the intervention. Incremental area under the curve was also calculated for the duration of sample collection and for hours 0-5 and hours 5-9.5 [71]. Incremental area under the curve was calculated using the trapezoidal method and subtracting for baseline. A negative incremental area under the curve suggests that the concentration of the analyte drops below its baseline value. When considering the second time period from before lunch through the duration of the sample collection (hours 5-9.5), hour 5 was used as the baseline measurement.

Differences in area under the curve of intact osteocalcin, undercarboxylated osteocalcin, and the undercarboxylated: intact osteocalcin ratio after consuming low carbohydrate or high carbohydrate meals were analyzed using one tailed t-tests of symmetric percent change between diets. The area under the curve for the ratio of undercarboxylated: intact osteocalcin was calculated using the undercarboxylated: intact

osteocalcin ratio at each hour, instead of the area under the curve for undercarboxylated osteocalcin divided by the area under the curve for total osteocalcin.

Cumulative area under the curve was calculated for each subject and each intervention using a cubic spline smoothing fit to the 12 time points using the trapezoidal rule. Time in hours to the 25%, 50% 75% and 95% of cumulative area under the curve was calculated. These calculations were performed with the help of Mr. Mike Lasarev, OHSU Research Associate.

A p-value of ≤ 0.05 was considered statistically significant for all calculations. A Bonferroni adjustment was used in all analyses to adjust for the 12 time points being investigated. The resulting p-value of significance is ≤ 0.004. All data analysis was performed using Microsoft Excel 2007 (Seattle, WA).

CHAPTER 9: RESULTS

Descriptive Statistics

Ten subjects, four men and six women, completed both arms of this randomized crossover trial. Participant characteristics are presented in Table 5. All subjects met the inclusion criteria of having a body mass index in the range of 19-25 kg/m^2, an age in the range of 21 - 65 years and were considered to be of good health through self-report using the Cornell Medical Index. All subjects identified themselves as Caucasian. The average age of the participants was 29.7 ± 9.5 years (given as the mean ± standard error of the mean; $\bar{X}$ ± SEM), with a range of 22 to 48 years. The average body mass index was 21.6 ± 1.8 kg/m^2 ($\bar{X}$ ± SEM) with a range of 19.3 – 25.2 kg/m^2.

Missing Data

Five data values were missing from the 480 samples analyzed. Three of these values occurred in the middle of the day, and the values from the time point before and after the missing value were averaged to impute the missing value. None of these values were fasting nor immediately after consumption of a meal. One missing value occurred at the 1130 time point for intact osteocalcin concentration. Concentrations for both intact and undercarboxylated osteocalcin were missing for one subject at the 1430 time point. The final two missing values were from the last time point of the same intervention day for one subject. Analyses for this subject were adjusted to compare the first 11 time points from each visit.

Concentrations of intact osteocalcin and undercarboxylated osteocalcin concentrations and the undercarboxylated: intact osteocalcin ratio

The means, standard deviation and range of circulating intact osteocalcin concentration, circulating undercarboxylated osteocalcin concentration and the undercarboxylated: intact osteocalcin ratio at each time point are presented in Table 6.

Intact Osteocalcin

The excursion of intact osteocalcin concentration over time is shown in Figure 3 and Table 6. There was no difference in the mean fasting intact osteocalcin concentrations between the high (10.4 ± 4.0 ng/mL, as $\bar{X}$ ± SD) and low carbohydrate meals (12.6 ± 4.6 ng/mL, as $\bar{X}$ ± SD; two tailed paired t-test, p = 0.09). From this point on, all p-values will refer to a one tailed t- test unless otherwise noted.

Response of Intact Osteocalcin to High Carbohydrate Meals

There was no change in intact osteocalcin concentration between the 0800 (10.7 ± 4.0 as $\bar{X}$ ± SD) and 0830 (10.7 ± 4.2 as $\bar{X}$ ± SD) time point. Intact osteocalcin concentration dropped 21% from 0830 to 1030 (8.4 ± 3.6 ng/mL, as $\bar{X}$ ± SD) and then gradually increased 24% until 1300 (10.4 ± 5.0 ng/mL, as $\bar{X}$ ± SD). From 1300, immediately before lunch, until 1530 (9.4 ± 4.7 ng/mL, as $\bar{X}$± SD), the concentration of intact osteocalcin decreased by 10%. From 1530 until 1730 (9.8 ± 5.0 ng/mL, as $\bar{X}$± SD) the concentration of intact osteocalcin increased by 4%. Overall, intact osteocalcin

concentration dropped 8% from 0800 (10.7 ± 4.0 ng/mL, as $\bar{X}$ ± SD) to 1730 (9.8 ± 5.0 ng/mL, as $\bar{X}$ ± SD) after consumption of high carbohydrate meals.

Figure 4 illustrates the percent change from baseline of intact osteocalcin in response to the high and low carbohydrate meals. At 0830, after consumption of the high carbohydrate meal the percent change from baseline in intact osteocalcin concentration was a small positive change. From 0930 until 1030 the change in intact osteocalcin from baseline was negative and increasing in magnitude. After 1030 the percent change from baseline was negative and decreasing in magnitude, i.e., was more similar to baseline, until 1300. After 1300 the change in intact osteocalcin from baseline was negative and increasing in magnitude until 1530. From 1530 until 1630 the change from baseline in intact osteocalcin concentration was negative and decreasing in magnitude. From 1630 until 1730 the change in intact osteocalcin concentration from baseline was negative and increasing in magnitude. Percent change from baseline was lowest immediately after consumption of the breakfast meal (1.2 ± 16.0%, as $\bar{X}$ ± SD) and immediately before the lunch meal (-3.5 ± 38.8, as $\bar{X}$ ± SD). Percent change from baseline was highest at 1030 (-20.5 ± 24.8%, as $\bar{X}$ ± SD).

Differences in intact osteocalcin concentration were measured fasting, before the lunch meal and at the end of the collection period by post hoc analyses. No significant difference was observed between the concentration of intact osteocalcin obtained at baseline (10.7 ± 4.0 ng/mL, as $\bar{X}$ ± SD) compared to the concentration found before lunch at 1300 (10.4 ± 5.0 ng/mL, as $\bar{X}$ ± SD; two tailed paired t-test, p = 0.75) or at the end of the collection period at 1730 (9.8 ± 5.0 ng/mL, as $\bar{X}$ ± SD; two tailed paired t-test, p= 0.39). These time points were chosen because they represented fasting (0800), the last

postprandial measurement before lunch (1300) and the end of the sample collection period (1730). These analyses examined if the concentration of intact osteocalcin was increasing or decreasing over time.

Response of Intact Osteocalcin to the Low Carbohydrate Meals

The concentration of intact osteocalcin decreased 13% following consumption of the low carbohydrate breakfast meal from 0800 (12.6 ± 4.6 ng/mL, as $\bar{X}$ ± SD) to 0830 (10.9 ± 4.5 ng/mL, as $\bar{X}$ ± SD), then rose 4% in the next hour (0930; 11.3 ± 4.1 ng/mL, as $\bar{X}$ ± SD), before decreasing 7% from 0930 to 1030 (10.5 ± 3.6 ng/mL, as $\bar{X}$ ± SD). The concentration of intact osteocalcin remained stable over the next seven hours, fluctuating at most by 0.04 ng/mL (~3%) between time points. Over the entire study period, intact osteocalcin concentration dropped 18% from 0800 (12.6 ± 4.6 ng/mL, as $\bar{X}$ ± SD) until 1730 (10.3 ± 4.0 ng/mL, as $\bar{X}$ ± SD).

The percent change in intact osteocalcin concentration from baseline is shown in Figure 4. Each postprandial time point showed a negative change from baseline in response to the low carbohydrate meals. There was less variation in the change from baseline after the low carbohydrate meals (range of mean % change from baseline: -16.6 to -7.5 %) compared to the high carbohydrate meals (range of mean % change from baseline -20.5 to 1.2 %). The range of the standard deviation for percent change from baseline was smaller following low carbohydrate meals (range of SD: 14.25 – 27.94) compared to high carbohydrate meals (range of SD: 16.00 – 40.79, data not shown). This

suggests a lower inter-individual variation in response to the low carbohydrate meals compared to the high carbohydrate meals.

Area Under the Curve for Intact Osteocalcin

Total and incremental area under the curve is presented in Table 8. There was no difference in the mean incremental area under the curve for intact osteocalcin concentration after consumption of high (11.66 ± 7.42 ng*$hr_{0800-1730}$/mL, as $\bar{X}$ ± SEM) or low carbohydrate meals (-18.04 ± 5.74 ng*$hr_{0800-1730}$/mL, as $\bar{X}$ ± SEM) between 0800 and 1730 (p=0.19). Following both high and low carbohydrate meals, there was a reduction in postprandial intact osteocalcin concentration over time, with the response to both meals being less than, on average, the fasting value. No difference in area under the curve in circulating intact osteocalcin was seen when baseline was accounted for after the breakfast or lunch meals ($iAUC_{0800-1300}$: -6.78 ± 3.15 ng*$hr_{0800-1300}$/mL vs. -8.65 ± 2.68 ng*$hr_{0800-1300}$/mL , high vs. low carbohydrate meals, respectively, p= 0.25; $iAUC_{1300-1730}$: 4.92 ± 2.91 ng*$hr_{0300-1730}$/mL vs. -3.06 ± 2.52 ng*$hr_{0300-1730}$/mL, high vs. low carbohydrate meals, respectively, p= 0.13; All iAUC measurements given as $\bar{X}$ ± SEM).

When total area under the curve was calculated, not accounting for differences in baseline values, the area under the curve after breakfast and throughout the day was lower after consumption of high carbohydrate meals compared to low carbohydrate meals ($AUC_{0800-1300}$: 46.75 ± 5.45 ng*$hr_{0800-1300}$/mL vs. 54.11 ± 5.92 ng*$hr_{0800-1300}$/mL, respectively, p=0.01; $AUC_{0800-1730}$: 88.56 ± 10.80 ng*$hr_{0800-1730}$/mL vs. 99.35 ± 10.23 ng*$hr_{0800-1730}$/mL, respectively, p= 0.01. All AUC measurements given as $\bar{X}$ ± SEM).

There was a trend of lower area under the curve after consumption of the high carbohydrate meal between 1300 and 1730 compared to consumption of the low carbohydrate meal ($AUC_{1300\text{-}1730}$: 41.78 ± 5.77 ng*$hr_{1300\text{-}1730}$/mL vs. 54.24 ± 4.78 ng*$hr_{1300\text{-}1730}$/mL, respectively, as $\bar{X}$ ± SEM, p= 0.06). The differences in significance between incremental and total area under the curve analyses may be explained in part by the dissimilarity between the fasting concentrations of the two meal compositions, though this dissimilarity is not statistically significant. The fasting concentrations were 10.7 ± 4.0 ng/mL vs. 12.6 ± 4.6 ng/mL following high vs. low carbohydrate meals, respectively, as $\bar{X}$ ± SEM at 0800, and 10.4 ± 5.0 ng/mL and 10.7 ± 3.9 ng/mL, respectively, as $\bar{X}$ ± SEM, at 1300.

Undercarboxylated Osteocalcin

The excursion of undercarboxylated osteocalcin concentration over time as shown in Figure 5 and Table 6. There was no difference in the mean fasting undercarboxylated osteocalcin concentrations before the high and low carbohydrate meals (3.0 ± 0.3, 1.5 ± 0.5, respectively as $\bar{X}$ ± SD; two tailed paired t-test, p = 0.19).

Response of Undercarboxylated Osteocalcin to High Carbohydrate Meals

The concentration of undercarboxylated osteocalcin rose 7% from 0800 (3.0 ± 1.0 ng/mL, as $\bar{X}$ ± SD) to 0830 (3.2 ± 1.2 ng/mL, as $\bar{X}$ ± SD). In the hour from 0830 to 0930 circulating undercarboxylated osteocalcin concentration increased 22% from 3.2 ±

1.2 ng/mL to 3.9 ± 2.0 ng/mL, both values given as $\bar{X}$ ± SD. This was followed by a 10% decrease in undercarboxylated osteocalcin concentration over the next hour (3.5 ± 1.8 ng/mL at 1030, as $\bar{X}$ ± SD). There was no change in circulating undercarboxylated osteocalcin concentration from 1030 until 1130 (3.5 ± 1.6 ng/mL, as $\bar{X}$ ± SD). From 1130 to 1230 (4.5 ± 2.2 ng/mL, as $\bar{X}$ ± SD) circulating undercarboxylated osteocalcin concentration increased 29% and then decreased 7% in the half hour between 1230 and 1300 (4.2 ± 2.1 ng/mL, as $\bar{X}$± SD). From 0800 (3.0 ± 1.0 ng/mL, as $\bar{X}$ ± SD) to 1300 (4.2 ± 2.1 ng/mL, as $\bar{X}$± SD) there was a net increase of 40% in circulating undercarboxylated osteocalcin concentration. Circulating undercarboxylated osteocalcin concentration increased by 2% after the lunch meal (4.3 ± 2.6 ng/mL at 1330 as $\bar{X}$ ± SD). From 1330 to 1430 (3.6 ± 1.5 ng/mL, as $\bar{X}$ ± SD), circulating undercarboxylated osteocalcin concentration decreased 16% and then rose until 1730 (4.3 ± 2.6 ng/mL, as $\bar{X}$ ± SD) increasing 19%. There was a 2% net increase in circulating undercarboxylated osteocalcin concentration from immediately before lunch to the end of the study collection (4.2 ± 2.1 ng/mL at 1300 compared to 4.3 ± 2.6 ng/mL at 1730, values given as $\bar{X}$ ± SD). Throughout the nine and a half hours studied, circulating undercarboxylated osteocalcin concentration increased 43% (3.0 ± 1.0 ng/mL at 0800 compared to 4.3 ± 2.6 ng/mL at 1730, values given as $\bar{X}$ ± SD).

The percent change of circulating undercarboxylated osteocalcin concentration compared to baseline is shown in Figure 6. There was a positive change at each time point from baseline, with the highest percentage of change (54%) occurring at 1230 and 1730. The least amount of change from baseline, 8%, occurred at the first postprandial

time point of 0830. The average amount of change from baseline was 33% over the nine and a half hour sample collection period.

Post-hoc analyses showed no significant difference in absolute circulating concentration of undercarboxylated osteocalcin between 0800 (3.0 ± 1.0, as $\bar{X}$ ± SD) and 1300 (4.2 ± 2.1, as $\bar{X}$ ± SD, two-tailed paired t-test, p=0.07) or between 0800 and 1730 (4.3 ± 2.6, as $\bar{X}$ ± SD two-tailed paired t-test, p= 0.14).

The inter-individual variation in undercarboxylated osteocalcin concentration was similar after the high and low carbohydrate diets. There appears to be a larger standard deviation in percent change from baseline following the high carbohydrate meals (range of SD: 26.27 – 87.01) compared to the low carbohydrate meals (range of SD: 31.73 – 63.96, data not shown), suggesting more variance in inter-individual response after the high carbohydrate meals.

Response of Undercarboxylated Osteocalcin to Low Carbohydrate Meals

The response of undercarboxylated osteocalcin to the low carbohydrate meals showed less magnitude of change in comparison to the response to high carbohydrate meals, fluctuating within a 0.8 ng/mL range (range of mean undercarboxylated osteocalcin concentration of 2.9 - 3.7 ng/mL) throughout the day. Immediately following consumption of the breakfast meal there was a decrease of 0.1 ng/mL, (3%) in circulating undercarboxylated osteocalcin concentrations (0800; 3.4 ± 1.5 ng/mL, 0830; 3.3 ± 1.6 ng/mL, values given as $\bar{X}$ ± SD). There was a net decrease of 12% in undercarboxylated osteocalcin from 0830 (3.3 ± 1.6 ng/mL, as $\bar{X}$ ± SD) to 1130 (2.9 ± 1.4 ng/mL, as $\bar{X}$ ±

SD), followed by a 17% increase from 1130 to 1230 (3.4 ± 1.5 ng/mL, as $\bar{X}$ ± SD). From 0800 (3.4 ± 1.5 ng/mL, as $\bar{X}$ ± SD) to 1300 (3.3 ± 1.6 ng/mL, as $\bar{X}$ ± SD) there was a 3% decrease in undercarboxylated osteocalcin concentration. From 1330 (3.7 ± 2.1 ng/mL, as $\bar{X}$ ± SD) to 1530 (3.0 ± 1.2 ng/mL, as $\bar{X}$ ± SD) there was a decrease of 19% in undercarboxylated osteocalcin concentration, followed by an increase of 15% until 1730 (3.4 ± 1.5 ng/mL, as $\bar{X}$ ± SD). The net reduction in undercarboxylated osteocalcin concentration from 1330 (3.7 ± 2.1 ng/mL, as $\bar{X}$ ± SD) to 1730 (3.4 ± 1.5 ng/mL, as $\bar{X}$ ± SD) was 12%.

Change from baseline to each time point is shown in Figure 6, with the highest percentage of change from baseline, 20%, occurring at 1330. Average circulating undercarboxylated osteocalcin concentrations for the first two and a half hours following the breakfast meal were lower than baseline, with the greatest negative changes (10%) from baseline occurring at 0930 and 1130. All changes from baseline from 1230 on were positive. The average percent change in circulating undercarboxylated osteocalcin concentration following the low carbohydrate meals from baseline was 3.9%.

Post-hoc analyses showed no significant difference between values at 0800 (3.4 ± 1.5 as $\bar{X}$ ± SD) and 1300 (4.3 ± 1.6 as $\bar{X}$ ± SD, two-tailed paired t-test, p= 0.99) or between 0800 and 1730 (3.4 ± 1.5 as $\bar{X}$ ± SD, two-tailed paired t-test, p= 0.89), signifying that the circulating undercarboxylated osteocalcin concentration was not different from fasting to four hours postprandially after each meal.

Area Under the Curve for Undercarboxylated Osteocalcin

The incremental and total area under the curve values for each time interval are shown in Table 8. When accounting for baseline, there is a higher incremental area under the curve after consumption of the high carbohydrate vs. low carbohydrate meals after the breakfast meal and through the duration of sample collection ($iAUC_{0800\text{-}1300}$: 3.24 ± 2.25 ng*$hr_{0800\text{-}1300}$/mL vs. -1.55 ± 215 ng*$hr_{0800\text{-}1300}$/mL, respectively, p= 0.02; $iAUC_{0800\text{-}1730}$: 7.01 ± 4.51 ng*$hr_{0800\text{-}1730}$/mL vs. -1.77 ± 4.51 ng*$hr_{0800\text{-}1730}$/mL, respectively, p= 0.03. All iAUC measurements given as $\bar{X}$ ± SEM). After consumption of high carbohydrate meals, absolute undercarboxylated osteocalcin concentrations were higher postprandially compared to the lower concentrations in undercarboxylated osteocalcin seen after consumption of low carbohydrate meals. After the lunch meal, there was no difference between meals as measured by incremental area under the curve ($iAUC_{1300\text{-}1730}$: -1.64 ± 2.10 ng*$hr_{1300\text{-}1730}$/mL vs. -0.19 ± 1.79 ng*$hr_{1300\text{-}1730}$/mL, respectively, measurements given as $\bar{X}$ ± SEM, p= 0.26).

When total area under the curve is considered, the difference between high and low carbohydrate diets disappears. ($AUC_{0800\text{-}1300}$:18.44 ± 2.32 ng*$hr_{0800\text{-}1300}$/mL following high carbohydrate meals vs. 15.65 ± 2.29 ng*$hr_{0800\text{-}1300}$/mL following low carbohydrate meals, p= 0.11; $AUC_{1300\text{-}1730}$ 17.11 ± 2.44 ng*$hr_{1300\text{-}1730}$/mL following high carbohydrate meals vs. 14.69 ± 1.95 ng*$hr_{1300\text{-}1730}$/mL following low carbohydrate meals, p= 0.15; $AUC_{0800\text{-}1730:}$ 35.55 ± 4.56 ng*$hr_{0800\text{-}1730}$/mL following high carbohydrate meals vs. 30.34 ± 3.95 ng*$hr_{0800\text{-}1730}$/mL following low carbohydrate meals, p= 0.12. All AUC measurements given as $\bar{X}$ ± SEM). Though not significant,

there is a pattern of a larger area under the curve following the high carbohydrate meals, in concurrence with the incremental area under the curve findings.

The Undercarboxylated: Intact Osteocalcin Ratio

Fasting analysis

The excursion of undercarboxylated osteocalcin indexed to intact osteocalcin, or the undercarboxylated: intact osteocalcin ratio over time is shown in Figure 7 and Table 6. There was no difference in the mean fasting ratio at the start of each day (high carbohydrate 0.31 ± 0.12 ng/mL, low carbohydrate; 0.29 ± 0.13 ng/mL, values given as $\bar{X}$ ± SD; two tailed paired t-test, p = 0.64).

Response of the Undercarboxylated: Intact Osteocalcin Ratio Following High Carbohydrate Meals

Undercarboxylated osteocalcin concentration was indexed to intact osteocalcin concentration for each time point. There was a 183% increase in the undercarboxylated: intact osteocalcin ratio between 0800 (0.31 ± 0.125, as $\bar{X}$ ± SD) and 1730 (0.88 ± 0.1.52, as $\bar{X}$ ± SD). A 10% decrease in the undercarboxylated: intact osteocalcin ratio occurred between 0800 (0.31 ± 0.12, as $\bar{X}$ ± SD) and 0830 (0.28 ± 0.13, as $\bar{X}$ ± SD). There was a 186% increase in the proportion of undercarboxylated osteocalcin from 0830 (0.28 ± 0.13, as $\bar{X}$± SD) to 0930 (0.80 ± 1.45, as $\bar{X}$ ± SD), followed by a 18% decrease until 1030 (0.66 ± 0.97) and a 14% increase until 1230 (0.75 ± 1.08, as $\bar{X}$ ± SD). There was a

21% decrease in this ratio in the half hour from 1230 (0.75 ± 1.08, as $\bar{X}$ ± SD) until 1300 (0.59 ± 0.72 as $\bar{X}$± SD), then the ratio was stable for an hour before increasing 49% from 1430 (0.59 ± 0.72) until 1730 (0.88 ± 1.52, as $\bar{X}$± SD). From 0800 (0.31 ± 0.12, as $\bar{X}$ ± SD) through 1300 (0.59 ± 0.70, as $\bar{X}$ ± SD) there was a 90% increase in the undercarboxylated: intact osteocalcin ratio. In the time interval from 1300 (0.59 ± 0.70 as $\bar{X}$ ± SD) through 1730 (0.88 ± 1.52 as $\bar{X}$ ± SD) there was a 49% increase in this ratio.

There were large standard deviations for the undercarboxylated: intact osteocalcin ratio after consumption of the high carbohydrate meals (range of standard deviation 0.12 – 0.86), suggesting a wide inter-individual variance in response.

Post-hoc analyses showed no significant difference in the undercarboxylated: intact osteocalcin ratio between 0800 (0.31 ± 0.12 as $\bar{X}$ ± SD) and 1300 (0.59 ± 0.70 as $\bar{X}$ ± SD, two-tailed paired t-test, p= 0.09) or between 0800 (0.31 ± 0.12 as $\bar{X}$ ± SD) and 1730 (0.88 ± 1.52 as $\bar{X}$ ± SD, two-tailed paired t-test, p= 0.11).

Percent change in the undercarboxylated: intact osteocalcin ratio between adjacent time points was calculated (data not shown). A large standard error of the mean was seen at 0930 following the high carbohydrate breakfast. The median percent change at the 0930 time point was -23.63, with a range of -1093 – 38.62 %. One subject exhibited a -1093% change in undercarboxylated: intact osteocalcin ratio. Percent change from 0830 to 0930 for this subject is shown in Table 7. This value, though considered an outlier when drawn on a box plot, was not removed because there is a lack of empirical evidence to suggest that this change is not physiologically normal. The subject exhibiting this drastic change was a 48 year old female, subject 10, dissimilar in age from the other subjects. Subject 10 displayed a 70% increase in intact osteocalcin

concentration and a 251% decrease in undercarboxylated osteocalcin concentration at 0930. These changes, coupled with the small absolute concentrations of intact and undercarboxylated osteocalcin resulted in the large variation seen at 0930.

Response of the Undercarboxylated: Intact Osteocalcin Ratio Following Low Carbohydrate Meals

The undercarboxylated: intact osteocalcin ratio remained stable in response to the low carbohydrate meals. Little inter-individual variation was seen at each time point as demonstrated through small standard deviation (range of SD: 0.082 – 0.160). Throughout the nine and a half hour collection period, the undercarboxylated: intact osteocalcin ratio stayed within a 0.14 unit range (0.26 ± 0.08 – 0.40 ± 0.35, values given as $\bar{X}$ ± SD). Because this number is minute, percent change from between time points may be artificially inflated. From 0800 (0.29 ± 0.13 as $\bar{X}$± SD) to 1300 (0.32 ± 0.12 as $\bar{X}$ ± SD) the undercarboxylated: intact osteocalcin ratio fell 8%. The ratio rose 11% from 1330 (0.36 ± 0.18 as $\bar{X}$ ± SD) to 1730 (0.35 ± 0.14 as $\bar{X}$ ± SD). There was a net increase of 3% from 0800 (0.29 ± 0.13 as $\bar{X}$ ± SD) to 1730 (0.35 ± 0.14 as $\bar{X}$ ± SD).

The change from each time point compared to baseline is shown in Figure 8. When each time point was compared to the baseline value, all values showed a mean increase of less than 50%. The highest percent change from baseline occurred at1430 (45%) and the lowest percent change from baseline occurred at 0930 (-1.1%).

Area Under the Curve for Undercarboxylated: Intact Osteocalcin

The incremental and total area under the curve values for each time interval are shown in Table 8. There was no difference in the proportion of undercarboxylated: intact osteocalcin ratio at any of the three time intervals using incremental area under the curve analyses ($iAUC_{0800-1300}$: 1.64 ± 1.31 $ng*hr_{0800-1300}$ /mL vs. 0.04 ± 0.16 $ng*hr_{0800-1300}$ /mL following high vs. low carbohydrate meals, respectively, p= 0.12; $iAUC_{1300-1730}$: 0.50 ± 0.47 $ng*hr_{1300-1730}$ /mL vs. 0.14 ± 0.20 $ng*hr_{1300-1730}$ /mL following high vs. low carbohydrate meals, respectively, p= 0.27; $iAUC_{0800-1730}$: 3.41 ± 2.64 $ng*hr_{0800-1730}$ /mL following vs. 0.32 ± 0.38 $ng*hr_{0800-1730}$ /mL following high vs. low carbohydrate meals, respectively, p= 0.14. All iAUC measurements given as $\bar{X}$ ± SEM). Following both high and low carbohydrate meals, there was an augmentation in the proportion of undercarboxylated osteocalcin to intact osteocalcin concentration postprandially, when baseline was accounted for.

When total area under the curve is considered, not accounting for differences in baseline, there is no significant difference in the undercarboxylated: intact osteocalcin ratio between high and low carbohydrate meals ($AUC_{0800-1300}$: 3.19 ± 1.44 $ng*hr_{0800-1300}$ /mL following high carbohydrate meals vs. 1.50 ± 0.17 $ng*hr_{0800-1300}$ /mL following low carbohydrate meals, p= 0.13; $AUC_{1300-1730}$: 3.15 ± 1.45 $ng*hr_{1300-1730}$ /mL following high carbohydrate meals vs. 1.56 ± 0.17 $ng*hr_{1300-1730}$ /mL following low carbohydrate meals, p= 0.15; $AUC_{0800-1730}$: 6.33 ± 2.88 $ng*hr_{0800-1730}$ /mL following high carbohydrate meals vs. 3.06 ± 0.39 $ng*hr_{0800-1730}$ /mL following low carbohydrate meals, p= 0.14. All AUC measurements given as $\bar{X}$ ± SEM). The lack of significance between interventions may be attributed to the large standard error of the mean observed at each

time point after consumption of the high carbohydrate meals Though not significant, there is a pattern of a greater proportion of undercarboxylated osteocalcin as measured by total area under the curve following the high carbohydrate meals compared to the low carbohydrate meals.

Symmetric and Robust Percent Change

Individual and overall symmetric and robust percent change in area under the curve for circulating intact osteocalcin concentration, circulating undercarboxylated osteocalcin concentration and the undercarboxylated: intact osteocalcin ratios are shown in Table 9. The difference between the area under the curves was first calculated as a simple difference between high and low carbohydrate meals. Symmetric percent change and robust percent change were calculated with respect to the change in area under the curve from the high carbohydrate diet compared to the low carbohydrate diet. Robust percent change can be intuitively understood on the percent change scale. For example, the area under the curve for intact osteocalcin was 32% lower following the high carbohydrate diet versus the low carbohydrate diet. The area under the curve of absolute undercarboxylated osteocalcin throughout the day was 13% lower after the high carbohydrate diet versus low carbohydrate diet, and the area under the curve of undercarboxylated: intact osteocalcin ratio was 106% higher following consumption of high versus low carbohydrate meals. Subject 10, previously discussed, demonstrates a large robust percent change in AUC for both intact osteocalcin and the undercarboxylated: intact osteocalcin ratio (high carbohydrate AUC; -18.20 ng*hr$_{0800-}$

$_{1730}$/mL, low carbohydrate AUC; 18.54 ng*hr$_{0800-1730}$/mL, robust percent change in area under the curve of intact osteocalcin between diets; 198%, undercarboxylated osteocalcin: intact osteocalcin AUC; 15.98 ng*hr$_{0800-1730}$/mL, low carbohydrate AUC; 1.55 ng*hr$_{0800-1730}$/mL, robust percent change in the undercarboxylated: intact osteocalcin ratio 929%). These values are considerably higher than values calculated for any other study participant.

Cumulative Area Under the Curve

Cumulative area under the curve is shown in Table 10, and investigates at which time point 25%, 50%, 75% and 95% of the cumulative area under the curve was amassed for each diet intervention. Time in hours to percent of accumulation is listed per subject, per analyte. For example, as seen in Table 10, Subject 5 accumulated 50% of the total area under the curve of intact osteocalcin at 4.58 hours following high carbohydrate meals and at 4.58 hours following low carbohydrate meals. A one tailed paired t-test was used to investigate differences in time to accumulation for area under the curve for intact osteocalcin concentration, absolute undercarboxylated osteocalcin concentration and the undercarboxylated: intact osteocalcin ratio. Differences were not found to be significant for any analyte. There were no significant differences in the time needed for intact osteocalcin to reach the 25th, 50th, 75th and 95th percent of accumulation (the average time to 25 percent of accumulation of the AUC after the high carbohydrate meal was 2.26 hours vs. 2.26 hours after low carbohydrate meals; $p= 0.50$. Average time to reach the 50th percent of accumulation following high carbohydrate meals was 4.68 hours vs. 4.61 hours following low carbohydrate meals, $p= 0.34$. Average time to reach the 75th

percent of accumulation following high carbohydrate meals was 7.05 hours vs. 7.00 hours following low carbohydrate meals p= 0.36. The average time to reach the 95th percent of accumulation following high carbohydrate meals was 9.03 hours vs. 8.93 hours following low carbohydrate meals p= 0.26). There were no significant differences in the time needed for undercarboxylated osteocalcin to reach the 25th, 50th, 75th and 95th percent of accumulation. (The average time to reach 25 percent of accumulation following high carbohydrate meals was 2.55 hours vs. 2.51 hours following low carbohydrate meals; p= 0.40. The average time to accumulate 50 percent of the area under the curve on high carbohydrate meal was 4.86 hours vs. 4.97 hours following low carbohydrate meals; p= 0.34. The average time to accumulate 75 percent of accumulation following high carbohydrate meals was 7.05 hours vs. 7.00 hours following low carbohydrate meals; p= 0.36. Average time to reach the 95th percent of cumulative area under the curve following high carbohydrate meals was 9.03 hours vs. 8.93 hours following low carbohydrate meals; p= 0.26). There were no significant differences in the time needed for undercarboxylated: intact osteocalcin ratio to reach the 25th, 50th, 75th and 95th percent of accumulation. (The average time to reach the 25th percent of cumulative area under the curve following high carbohydrate meals was 2.62 hours vs. 2.68 hours following low carbohydrate meals; p= 0.36. The average time to reach the 50th percent of accumulation on high carbohydrate meal 4.90 hours vs. 5.13 hours following low carbohydrate meals; p= 0.13. The average time to reach the 75th percent of accumulation following high carbohydrate meals was 7.19 hours vs. 7.13 hours following low carbohydrate meals; p= 0.38. The average time to reach the 95th

percent of accumulation following high carbohydrate meals was 9.04 hours vs. 8.94 hours following low carbohydrate meals; p= 0.16).

CHAPTER 10: DISCUSSION

This preliminary study was novel in several ways. This is the first acute study, in our knowledge, to investigate the effects of whole meals on intact and undercarboxylated osteocalcin, and is one of few feeding trials examining the effects of nutrients on intact and undercarboxylated osteocalcin. Empirically most studies examining the effect of diet on osteocalcin concentration are observational or long term because osteocalcin has traditionally been a long term marker of bone turnover. The study design used in this trial is unique to previous studies, examining the acute effects of controlled whole meals on circulating undercarboxylated and intact osteocalcin concentrations. Additionally, to our knowledge this is the first feeding trial to examine the effects of high complex vs. low carbohydrate diets on circulating concentrations of undercaboxylated and intact osteocalcin. Because so little was known about the effects of high and low carbohydrate meals on osteocalcin concentration apriori, this trial serves as a hypothesis generating study providing steering for future research.

The fasting values for both undercarboxylated and intact osteocalcin concentration measured from this study confirms those reported in a cross-sectional study investigating undercarboxylated and intact osteocalcin, and a variety of lifestyle factors. This cross-sectional trial by Nimptsch et al. reports an average absolute undercarboxylated osteocalcin concentration of ~3.5 ng/mL, an intact osteocalcin concentration of ~10 ng/mL and an undercarboxylated: intact osteocalcin ratio of ~0.35 in men and women between the ages of 18 and 30[4]. However, the fed or fasting state at time of blood draw was not reported. Undercarboxylated osteocalcin and intact

osteocalcin were measured using the same enzyme linked immunosorbant assays in the Nimpstch et al. study as our feeding trial. When values from our feeding trial were pooled regardless of diet and fed or fasting state, our values were similar to Nimpsch et al., with an average intact osteocalcin concentration of 10.2 ± 4.1 ng/mL ($\bar{X}$ ± SD), an average undercarboxylated osteocalcin concentration of 3.5 ± 1.7 ng/mL ($\bar{X}$ ± SD) and an average undercarboxylated osteocalcin ratio of 0.34 ± 0.72 ($\bar{X}$ ± SD).

Our feeding trial also supports the work of Clowes et al., who first reported that energy intake results in an acute postprandial reduction in circulating intact osteocalcin concentration over time[28]. In our trial, circulating intact osteocalcin concentration was acutely reduced following both high and low carbohydrate meals, though the postprandial pattern of change in intact and undercarboxylated osteocalcin concentrations generated by these meals was different.

There was no significant difference between diets in intact osteocalcin using incremental area under the curve analyses, resulting in the rejection of the first hypothesis that consumption of high carbohydrate meals will result in greater concentrations of intact osteocalcin postprandially compared to consumption of low carbohydrate meals. Although the fasting values of intact osteocalcin were not statistically different ($p = 0.86$) between the two intervention days, the fasting value before the high carbohydrate meals was 1.7 ng/mL lower than the fasting value before the low carbohydrate meals. This difference, multiplied over the nine and a half hour study period, may contribute to the concentrations over time, and may result in the significant difference between diets as measured by total area under the curve as well as the lack of significance measured by

incremental area under the curve. However, the pattern of excursions appeared different between diets, suggesting that a difference may exist if this study had more power. The incremental area under the curve following the lunch meal was calculated using the intact osteocalcin concentration obtained immediately before lunch. Excursion of circulating intact osteocalcin concentration over time as measured by area under the curve shows less change from baseline after the lunch meal on both diets compared to the change observed after the breakfast meal.

The area under the curve for intact: undercarboxylated osteocalcin ratio was not significantly lower after the high carbohydrate meals than the low carbohydrate meals, leading to the rejection of the second hypothesis that consumption of high carbohydrate meals will result in a lower undercarboxylated: intact osteocalcin ratio compared to the low carbohydrate meals. Following the high carbohydrate breakfast meal, absolute undercarboxylated osteocalcin concentrations were increased, compared to a reduction in absolute undercarboxylated osteocalcin concentration following the low carbohydrate breakfast meal. Following both lunch meals absolute undercarboxylated osteocalcin concentrations were reduced; a greater, albeit non-significant reduction from baseline was seen following the high carbohydrate diet. This is consistent with the hypothesis that high carbohydrate meals result in a lower area under the curve of undercarboxylated osteocalcin concentration for the after lunch time interval. The response of absolute undercarboxylated osteocalcin to the high carbohydrate meals was more dynamic than the stable response displayed following consumption of low carbohydrate meals.

The primary aim of this study investigated the effect of macronutrient composition, particularly carbohydrate content, on circulating intact and

undercarboxylated osteocalcin concentrations. When examining total area under the curve, significant differences can be seen after the breakfast meal and throughout the day, with high carbohydrate meals having a lower concentration of circulating intact osteocalcin concentration over time. As previously discussed this may be in part, attributable to the difference in baseline values. This study did not detect differences in circulating intact osteocalcin concentration over time, as measured by incremental area under the curve, perhaps due to lack of power. However, though not significant, the incremental area under the curve analyses suggest that circulating intact osteocalcin concentration may be higher following the high carbohydrate breakfast and throughout the day and lower following the lunch meal when compared to the low carbohydrate meals. Though macronutrient composition cannot be discarded as a major effector of circulating intact osteocalcin concentration, the incremental area under the curve analyses suggest that with the same macronutrient composition within a set of meals the response in circulating intact osteocalcin concentration was potentially different after lunch compared to breakfast. This limited data suggests that the relative content of protein, carbohydrate or fat were not the primary effectors of circulating intact osteocalcin concentration. Other factors such as circadian rhythm or micronutrients may be effectors of excursion

Though macronutrient composition was standardized between breakfast and lunch meals of the same diet, micronutrient composition was not consistent, which may account in part for the lack of difference seen in circulating intact osteocalcin concentration response to the breakfast versus lunch meals. Calcium, vital to all cells in the body, is stored in bone. A common theory is that calcium intake does not increase bone

formation, but inadequate calcium intake increases bone resorption, through the parathyroid hormone. A meta-analysis of calcium intake and bone demonstrated that many research studies have shown that adequate or high intakes of calcium resulted in reduced age-related bone loss, reduced osteoporotic fractures, and augmented bone gain during growth, supporting the idea that adequate to high calcium intake will slow bone loss and remodeling[78]. These findings are consistent with the theory that calcium intake does not acutely increase bone formation. Bone formation follows bone resorption in healthy adults, and as such we would not expect calcium content to be an acute effector of circulating intact osteocalcin concentrations. Empirical cohort studies have not found a correlation between dietary calcium intake and osteocalcin concentration[45]. This feeding trial, however, may suggest a correlation between dietary calcium intake and acute bone formation, using osteocalcin as a bone formation marker. The ten subjects in this trial consumed an average of 683 calories at each meal. The high carbohydrate meals provided 645 mg and 151 mg of calcium at the breakfast and lunch meals, respectively, per 683 calories. The low carbohydrate meals provided 276 mg and 445 mg of calcium at the breakfast and lunch meals, respectively, per 683 calories. The higher calcium intakes correlated with the greater amount of circulating osteocalcin for each time interval. Though the differences in circulating intact osteocalcin concentration were not significant, and the difference in calcium concentration was not a consistent amount between lunch and breakfast meals regardless of macronutrient composition, this correlation requires further investigation in future research. The amount of micronutrients supplied at each meal is detailed in Appendix E

Parathyroid hormone is positively modulated by decreased serum calcium levels, and is inhibited by high serum calcium levels. For this reason we would expect lower undercarboxylated osteocalcin concentrations after a high calcium meal. The breakfast meal in this trial was served after a ten hour fast. Because of this we would expect parathyroid hormone concentrations to be higher while fasting, and lower postprandially. Parathyroid hormone positively modulates the gene ESP which synthesizes the protein OST-PTP, positively influencing osteocalcin carboxylation[21]. A higher concentration of parathyroid hormone may result in a greater degree of carboxylation of osteocalcin. This effect was seen following the high carbohydrate breakfast in our study. This meal delivered 65% of the recommended dietary intake of calcium, and resulted in a significantly higher absolute undercarboxylated osteocalcin area under the curve (p= 0.02). No difference in area under the curve of absolute undercarboxylated osteocalcin was seen following the lunch meal, though the low carbohydrate meal higher in calcium correlated with the higher value of undercarboxylated osteocalcin.

Vitamin D, like calcium, was found in higher concentrations in the high carbohydrate breakfast meal (121 mcg) and the low carbohydrate lunch meal (18 mcg), compared to the low carbohydrate breakfast meal (83 mcg) and high carbohydrate lunch meal (8 mcg), correlating with the greater concentrations of intact osteocalcin. Vitamin D is needed for transcription of osteocalcin in the osteoblast[3, 41]. It is possible that if one mechanism is causing a decrease in circulating intact osteocalcin concentration, that vitamin D is counteracting this effect, potentially through increasing transcription of the protein. The majority of the vitamin D and calcium in high and low carbohydrate meals came from dairy products; skim milk in the high carbohydrate breakfast meal and cheese

in the low carbohydrate lunch meal. It is also possible that an unknown compound in dairy is an effector of circulating intact osteocalcin concentration.

The post-translational carboxylation of osteocalcin is known to be affected by the presence or absence of vitamin K, as well as the presence or absence of the protein OST-PTP. An acute study by Sokoll et al. administered 420 mg vitamin K (phyloquinones) divided between breakfast and lunch[75]. This study found that supplementation of vitamin K at this amount reduced the variation in postprandial undercarboxylated osteocalcin concentrations. The high carbohydrate meals of this study supplied a greater amount of vitamin K compared to the low carbohydrate meals. If dietary content of vitamin K acutely affects osteocalcin carboxylation then a greater concentration of vitamin K should result in a greater concentration of carboxylated osteocalcin and thus a lower proportion of circulating undercarboxylated osteocalcin following the high carbohydrate meals. Though a significant difference was seen in the area under the curve of undercarboxylated osteocalcin, the difference in the vitamin K content of the breakfast meals (1 vs. 3 mcg per 683 kcal meal, high carbohydrate versus low carbohydrate, respectively) does not seem to be significant enough to explain differences in carboxylation. This suggests that acute differences in dietary vitamin K content may not contribute to the acute differences in osteocalcin carboxylation seen in this trial. There was no significant difference in incremental area under the curve for absolute undercaboxylated osteocalcin after the lunch meals though different amounts of vitamin K were provided (87 mcg vs. 55 mcg per 683 kcal, high vs. low carbohydrate meals, respectively).

The differences in intact osteocalcin concentration between fed and fasting values, as well as between diets suggest that when osteocalcin is being measured clinically as a marker of bone health or vitamin K status, precautions should be taken to standardize this assessment by analyzing the concentration in fasting blood samples. This precaution will allow more consistent tracking of bone health and vitamin K status between multiple visits.

The response of intact osteocalcin concentration to consumption of high carbohydrate meals appears to be is bimodal, decreasing within 2 hours of meal consumption, and then increasing towards fasting values within 3-4 hours. This pattern suggests that hormones or compounds that have similar or inverse responses to meals may influence the excursion of intact osteocalcin concentration. Hormones or circulating compounds known to respond to high carbohydrate meals in an inverse fashion, such as insulin or glucose should be investigated to assess their influence on, or relationship with, changes in intact osteocalcin concentration. The postprandial pattern of change of intact osteocalcin generated by the high carbohydrate meals may be similar to the response this meal pattern has on ghrelin, which increases before meals and decreases after meals. Both insulin and glucose have inverse curves to the pattern of circulating intact osteocalcin concentration seen. If high insulin concentrations or hyperglycemia suppress intact osteocalcin concentration, it would support Semenkovich and Teitelbaum's theory that a metabolic sequelae exists between glycemic control and osteocalcin[50]. Numerous studies have reported that diabetics with chronic hyperglycemia have significantly lower total osteocalcin concentrations compared to healthy cohorts [7, 8,37,68]. In vivo clamp studies suggest that hyperinsulinemia has no significant effect on intact osteocalcin

concentration, but hypoglycemia results in a decreased intact osteocalcin concentration[30]. Perhaps a change in glycemia, either an increase or a decrease from the normal range, can cause a decrease in intact osteocalcin concentrations. These findings support the theory that glycemic control and intact osteocalcin concentration may be related.

High protein meals (20-30% protein) comparable to the low carbohydrate intervention meals served in this study (consisting of 30% protein) have been shown to increase the concentration of anorexigenic hormones in vivo[76]. Anorexigenic hormones like leptin, α-melanocyte stimulating hormone, cholecystokinin, and glucagon-like-peptide were considered as potential effectors of the excursion of intact osteocalcin seen in this study. Leptin was considered as it is known to increase adrenergic beta receptor synthesis and decrease osteoblast proliferation. However, leptin was not considered to be a major affecter of the osteocalcin pattern seen in this acute study because leptin exhibits a steady decrease throughout the day in response to low carbohydrate meals, and a steady increase throughout the day in response to high carbohydrate meals. This pattern was demonstrated in the work of Melissa Kumagai, an OHSU nutrition graduate student who analyzed fasting and postprandial serum samples from 6 subjects in this study for leptin concentration (data not shown). No association was identified between circulating leptin concentration and intact osteocalcin concentration. Alpha-melanocyte stimulating hormone was considered unlikely as a major affecter of intact osteocalcin concentration because it is primarily stimulated by leptin, and thus a curve similar to that of leptin would be expected. Glucagon-like peptide, is stimulated by the presence of all macronutrients in the gut, and thus if it was a major effector of intact osteocalcin concentration, similar patterns between intervention meals would be expected.

Cholecystokinin is stimulated primarily by the presence of fat and protein in the gut and has a half life of 2.5 minutes[77]. Because of these factors it is not likely that cholecystokinin is responsible for the bimodal pattern seen in intact osteocalcin concentration following the high carbohydrate meals, nor the stable intact osteocalcin concentration seen in response to the low carbohydrate meals. Although not tested, it seems unlikely that α-melanocyte stimulating hormone, cholecystokinin, and glucagon-like-peptide were related to change in osteocalcin.

The effect of growth hormone and somatomedins (insulin–like-growth factor) were considered in relation to the patterns exhibited by intact osteocalcin in response to the intervention meals. Insulin-like growth factors, which increase bone remodeling, are affected by nutrition only when protein or energy is deficient, not in the fed state [73]. Obesity is also correlated with decreased concentrations of growth hormone and insulin-like-growth factors [74]. Although growth hormone and insulin-like growth factor were not measured in this study, the subjects in this study were all of healthy weight with presumably normal concentrations of these two factors.

Unique to this study design was the use of a pre-intervention standardization diet. The purpose of the standardization diet was to minimize differences in outcome variables that could be magnified or masked by differences in individual diets. In addition to standardizing the diet, subjects were asked not to participate in strenuous exercise for 24 hours before each intervention, to minimize any differential impact that strenuous activity may have on stress and tension related osteoblast activity, a confounding variable for different intact osteocalcin excursions. Because the pattern of excursion of intact

osteocalcin between these two types of diets appear to be different, it is unlikely that this difference was due to differences in diurnal or circadian rhythmicity alone.

The time required for each subject to accumulate 25%, 50% 75% and 95% of the cumulative area under the curve for intact osteocalcin, undercarboxylated osteocalcin and the undercarboxylated: intact osteocalcin ratio was not significantly different between diets, and seemed to follow similar trends, suggesting that both diets accumulated both undercarboxylated and intact osteocalcin at similar rates.

Limitations

This study was underpowered; post-hoc power analyses show a high coefficient of variation of intact osteocalcin area under the curve between diets (0.04). Because of this, this study had 41% power to detect differences of 1.1 magnitudes in intact osteocalcin concentration between diets. If this study or a similar study is to be repeated, and similar within-subject coefficients of variation and correlations between diets are assumed, then at least 25 subjects would need to complete the study to using a cross-over achieve 80% power.

Ascertainment bias may be present in this study. The majority of subjects in this study were medical or health science professionals or students. Many of the subjects were interested in nutrition. The majority of subjects reported frequent weight bearing exercise. One of the ten subjects recruited was almost 20 years older than the other subjects, which may contribute in part to the variation seen in response to the meals.

The acute 9.5 hour duration during which these subjects were studied may not accurately capture the effects of these two dissimilar diets on either intact or undercarboxylated osteocalcin excursion. Once fat soluble nutrients are digested, absorbed, and transported to the bone, a 9.5 hour time period may just start to capture the differential changes in osteocalcin concentration and carboxylation status. Additionally, if diet composition affects gene transcription, which is one of the roles that vitamin D may play, more time may be needed before a measureable change is seen.

The disparity in micronutrient content between meals of the same diet may act as confounding variables in this trial. Future studies should examine the effects of these diets with similar levels of vitamins D, K and E, calcium, phosphorus, sodium, and magnesium to eliminate confounding variables. Furthermore, the standardization diet used in this substudy had varying amounts of micronutrients from day to day, notably of vitamin K. Though subjects consumed the same standardized meals in the same order, a more stable vitamin K intake over three days would be preferable.

The whole meals used in this study are both a strength and a limitation. Use of whole meals is a strength because it increases the generalizability and real-life effectiveness of the results. Use of whole meals is a limitation, because it is difficult to draw conclusions with respect to mechanism when so many variables are different between the two diets.

Chemical analyses were performed on heparinized plasma. The enzyme linked immunosorbant assay that was used to measure intact osteocalcin may be more accurately performed on serum. Some subjects had higher absolute values for undercarboxylated

osteocalcin concentration than they did for intact osteocalcin concentration. As undercarboxylated osteocalcin is a component of intact osteocalcin, this finding is physiologically impossible, and speaks to the limitations of the combination of assays used here and the blood product they were performed on. Additionally, the ELISA (Takara) used to measure undercarboxylated osteocalcin has not been used in any published studies in the United States, and it is possible that this is the first time this kit has been used in this country. Finally, research associates in the Oregon Clinical and Translational Research Institute Core Lab had not worked with the intact osteocalcin ELISA (Quidel-Metra) prior to this study. Likewise, this author had minimal laboratory experience prior to completing these assays, increasing the probability of technician error. All three of these factors may have impacted the accuracy and precision of primary outcome measurements.

Strengths

The design and execution of this trial offers many strengths. It is among the first trials to prospectively examine intake of whole meals of disparate macronutrient composition on postprandial intact and undercarboxylated osteocalcin excursion.

Prior to this study, only two published studies could be found that examined osteocalcin concentration over an acute period. The first trial measured only intact osteocalcin and may have fed subjects meals of differing compositions[54]. The second trial focused on the effects of differing sources of vitamin K on intact and

undercarboxylated osteocalcin concentrations, and may have offered differing amounts of other nutrients[75]. These studies were both published more than 10 years ago, and used different techniques than the current trial to quantify osteocalcin concentration. This trial examined postprandial changes in both intact and undercarboxylated osteocalcin after consuming meals of known nutrient content.

The cross-over design of this study allowed responses to each intervention diet to be compared within each subject. Because of this, each subject acted as their own control minimizing confounding variables found between cohorts, such as age, race, BMI, and activity level.

As previously mentioned, the three day standardization diet was a strength for this study, as it minimized differences in nutrient intake within and between subjects prior to intervention, eliminating confounding variables of previous vitamin and mineral intake.

The population of young, healthy subjects offers a model that has fewer confounding variables compared to an older or diseased population.

Future Research

To expand on the results of this preliminary study, future research should examine the long term effects of these two disparate diets on fasting and postprandial intact and undercarboxylated osteocalcin concentrations. The nutrients which are suspected to play an integral role in intact and undercarboxylated osteocalcin excursion are the fat soluble

vitamins D and K. In future studies, these two vitamins should be controlled for to minimize any confounding effect that differences in these nutrients would create.

Individual nutrients should also be examined in future research. Though previous studies have investigated the effects of individual nutrients on intact osteocalcin, little is known about the effects of nutrients other than vitamin K on postprandial undercarboxylated osteocalcin excursion. The results of this study suggest that vitamin K is not the only factor affecting carboxylation of osteocalcin. The effects of nutrition related-hormones such as parathyroid hormone also need to be investigated. The acute effect of calcium on circulating undercarboxylated and intact osteocalcin concentration should be examined, to further elucidate the correlation of high calcium and high intact osteocalcin concentration seen in this trial.

The efficacy of these two diets to change intact and undercarboxylated osteocalcin concentration should be investigated in different populations. An older population at risk for both osteoporosis and diabetes may respond differently than this young, healthy normal weight population. Populations of subjects with osteoporosis and subjects with diabetes should be investigated; as there is potential for these two diets to change intact and undercarboxylated osteocalcin excursion in a clinically relevant way.

CHAPTER 11: SUMMARY AND CONCLUSIONS

This prospective randomized crossover clinical trial characterized the postprandial excursion of undercarboxylated and intact osteocalcin in ten healthy, normal weight adults following consumption of low versus high complex carbohydrate meals. If total concentration and carboxylation status of this protein can be manipulated by diet, novel medical nutrition therapies may be developed to aid in the treatment of osteoporosis, diabetes mellitus and obesity.

The first hypothesis was rejected, high complex carbohydrate meals containing high amounts of fruits, vegetables and select vitamins and minerals failed to significantly increase circulating concentrations of intact osteocalcin postprandially compared to the low carbohydrate meals. Both types of meals resulted in decreased concentrations of intact osteocalcin over time. Significant differences in incremental area under the curve were not seen between diets.

The second hypothesis, that high complex carbohydrate meals would result in a lower undercarboxylated: intact osteocalcin ratio was also rejected. There was a pattern of higher undercarboxylated: intact osteocalcin ratio following the high carbohydrate meals than the low carbohydrate meals, however differences were not significant.

Both high and low complex carbohydrate meals resulted in a postprandial reduction of intact osteocalcin concentration over time. A significantly lower total area under the curve of intact osteocalcin was found after the high carbohydrate breakfast meal as well as throughout the day. Though incremental area under the curve of intact osteocalcin was not significant between diets, significant differences may be found using

the incremental area under the curve measurement if more subjects were studied over a greater period of time.

The mechanism responsible for the differences in concentration and carboxylation status of osteocalcin excursion are unknown. Possible mechanisms include the actions of nutrients such as vitamin D or K, or calcium, as well as certain hormones and includes both nutritional and non- nutritional related factors. Other potential mechanisms have not yet been elucidated, and may be better investigated using different study designs.

The results of this preliminary study suggest that it is possible that diet can manipulate both undercarboxylated and intact osteocalcin concentrations. Longer sample collection period may be needed to capture acute effects, and a longer intervention period may be needed to elucidate the effects of diet on the concentrations and carboxylation status of osteocalcin. Diet remains a potential therapy for manipulating undercarboxylated and intact osteocalcin, as trends in patterns of excursion and carboxylation status were observed in this acute cross-over study.

TABLES

Table 1: Summary of Henriksen et al. trial[29]

Nutrient	Grams	Energy Content (Kilocalories)	Incremental Area Under the Curve, % of baseline per hour for 0-3 hours*
Fat	70 mL emulsion of long chain triacylglycerides equivalent to 35 grams vegetable fat, originating from peanuts	315	~(-30%)
Glucose	75 grams of glucose and juice of ½ of a lemon dissolved in 300 mL of water	300	~(-25%)
Fructose	75 grams of fructose and juice of ½ of a lemon dissolved in 300 mL of water	300	Data not available
Protein	40 grams of protein powder corresponding to 35 grams pure protein originating from milk	140	~ (-75%)
Fasting	No energy consumed	000	~ (-12%)

* Percent of total osteocalcin from baseline was calculated each hour for the three hours. Values are approximated. This chart shows that consumption of protein at about half the energy content of other macronutrients resulted in approximately a 75% lower total osteocalcin concentration than baseline over the three hours studied.

Table 2: The Effect of Select Micronutrients on Total and Undercarboxylated Osteocalcin

Total Osteocalcin	**Micronutrient**	**Undercarboxylated Osteocalcin**
?	↑ Vitamin K	↓
↑	↑ Vitamin D	?
↑	↑ Vitamin C	?
↓	↑ Vitamin E	?
↑	↑ Fluoride	?
↑	↑ Phosphorus	?
↓	↓Magnesium	?

Table 3: Inclusion and Exclusion Criteria	
Inclusion Criteria - BMI 19-25.5 kg/m^2 - Age: 21-65 - Good health - Willingness to eat both a high and low carbohydrate diet - Willingness to stop taking multivitamins or any other dietary supplements for the duration of the study	Exclusion Criteria - Major debilitating mental or physical illness that would interfere with participation. - Pregnancy or lactation within the last 12 months - Weight instability (loss or gain of more than ± 5% within last 6 months) - Current participation in a self-directed or commercial weight loss plan - Any self imposed food restrictions (eg: kosher, vegetarian diet) that the participant would not be willing to stop for the duration of the study. - Any food allergies or food preferences that are not consistent with the research diets - Prescription medication use, with the exception of birth control and intermittent over the counter analgesics.

Table 4: Power Analysis Assuming Significance Level if 0.05 and Sample Size of 10 Subjects

Coefficient of Variation	Correlation between high complex and low complex meals within subjects	Power to detect a 1.1 fold difference in area under the curve
0.2	0.9	0.87
	0.88	0.81
	0.75	0.49
0.3	0.5	0.27
	0.9	0.53
	0.75	0.24
	0.5	0.14

Table 5. Subject Demographics

Sample Characteristics	Women	Men	Total
	6	4	10
Age (years)	29.7 ± 9.5 [22 – 48]	24.8 ± 0.5 [24 – 25]	27.7 ± 7.5 [22 – 48]
Height (centimeters)	168.0 ± 5.32 [162.3 – 174.4]	182.7 ± 6.9 [175.8 – 190.5]	173.88 ± 9.4 [162.3 – 190.2]
Weight (kilograms)	61.4 ± 5.8 [53.3 – 67.7]	78.7 ± 10.2 [64.5 – 87.3]	68.3 ± 11.5 [53.3 – 87.3]
BMI (kg/m^2)	21.6 ± 1.8 [19.3 – 24.0]	23.4 ± 1.7 [20.9 – 25.2]	22.31 ± 1.9 [19.3 – 25.2]
Body Fat (Percent)	25.8 ± 5.7 [17.0 – 31.2]	19.0 ± 3.4 [14.8 – 22.2]	23.1 ± 5.8 [14.8 – 31.2]

Mean ± standard deviation, [range]

Table 6. Osteocalcin	Descriptive Statistics	0800	0830	0930	1030	1130	1230	1300	1330	1430	1530	1630	1730
Intact Osteocalcin	High Carbohydrate												
	Average (ng/mL)	10.7	10.7	9.0	8.4	8.9	9.5	10.4	9.7	9.6	9.4	9.7	9.8
	St Dev	4.0	4.2	3.2	3.6	3.6	4.3	5.0	4.2	5.1	4.7	5.3	5.0
	Minimum ng/mL)	4.4	5.8	1.7	2.0	1.7	2.0	1.9	2.1	2.6	1.2	1.7	1.2
	Maximum (ng/mL)	17.2	17.5	13.1	15.5	14.0	16.8	17.8	15.5	16.7	16.1	18.4	18.0
	Low Carbohydrate												
	Average (ng/mL)	12.6	10.9	11.3	10.5	10.5	10.4	10.7	10.5	10.1	10.5	10.4	10.3
	St Dev	4.6	4.5	4.1	3.6	4.3	3.1	3.9	3.8	3.9	4.5	4.2	4.0
	Minimum ng/mL)	6.6	5.4	5.6	6.2	3.6	6.7	5.5	6.0	5.3	4.9	5.4	5.6
	Maximum (ng/mL)	18.7	19.2	17.7	16.7	17.2	16.8	17.3	18.4	16.3	18.6	16.9	19.0
Undercarboxylated Osteocalcin	High Carbohydrate												
	Average (ng/mL)	3.0	3.2	3.9	3.5	3.5	4.5	4.2	4.3	3.6	3.6	3.8	4.3
	St Dev	1.0	1.2	2.0	1.8	1.6	2.2	2.1	2.6	1.5	1.5	1.8	2.6
	Minimum ng/mL)	1.8	2.0	2.0	2.0	1.1	2.0	1.8	1.7	1.5	1.7	1.9	1.6
	Maximum (ng/mL)	4.6	5.7	8.4	6.7	6.4	7.9	8.8	10.0	6.5	6.6	6.7	9.8
	Low Carbohydrate												
	Average (ng/mL)	3.4	3.3	3.1	3.0	2.9	3.4	3.3	3.7	3.4	3.0	3.3	3.4
	St Dev	1.5	1.6	1.9	1.4	1.4	1.6	1.6	2.1	2.0	1.2	1.4	1.5
	Minimum ng/mL)	1.5	1.0	1.5	1.6	1.4	1.9	1.4	1.7	1.4	1.2	1.3	1.9
	Maximum (ng/mL)	6.1	6.0	7.4	5.7	6.2	6.5	5.7	8.8	8.0	4.5	6.2	6.3
Undercarboxylated: intact osteocalcin	High Carbohydrate												
	Average	0.31	0.34	0.80	0.66	0.69	0.75	0.59	0.63	0.59	0.78	0.74	0.88
	St Dev	0.12	0.16	1.45	0.97	1.10	1.08	0.70	0.78	0.72	1.31	1.07	1.52
	Minimum	0.12	0.13	0.20	0.23	0.11	0.21	0.21	0.15	0.14	0.18	0.16	0.15
	Maximum	0.52	0.64	4.92	3.40	3.79	3.79	2.54	2.80	2.54	4.45	3.64	5.16
	Low Carbohydrate												
	Average	0.29	0.31	0.26	0.29	0.32	0.33	0.32	0.36	0.40	0.31	0.36	0.35
	St Dev	0.13	0.14	0.08	0.12	0.17	0.12	0.12	0.18	0.35	0.14	0.22	0.14
	Minimum	0.13	0.16	0.17	0.13	0.12	0.15	0.16	0.17	0.18	0.19	0.18	0.16
	Maximum	0.60	0.52	0.45	0.57	0.59	0.55	0.49	0.78	1.30	0.61	0.91	0.52

Table 7. Change in Intact Osteocalcin, Undercarboxylated Osteocalcin and Undercarboxylated Osteocalcin Ratio from 0830 to 0930 for Subject 10.

Time	0830	0930	% change
Intact osteocalcin (ng/mL)	5.822	1.714	70.56
Undercarboxylated osteocalcin (ng/mL)	2.401	8.431	-251.20
Undercarboxylated: intact osteocalcin	0.412	4.919	-1092.92

Table 8. Area Under the Curve of Intact Osteocalcin, Undercarboxylated Osteocalcin and Undercarboxylated: Intact Osteocalcin

	Intact Osteocalcin			Undercarboxylated Osteocalcin			Undercarboxylated Ratio		
	Incremental AUC 0800-1300	Incremental AUC 1300-1730	Incremental AUC 0800-1730	Incremental AUC 0800-1300	Incremental AUC 1300-1730	Incremental AUC 0800-1730	Incremental AUC 0800-1300	Incremental AUC 1300-1730	Incremental AUC 0800-1730
High carbohydrate (ng*h/mL)	-6.78 ± 3.15	-4.92 ± 2.91	-11.66 ± 7.42	3.24 ± 2.25	-1.64 ± 2 .10	7.01 ± 4.51	1.64 ± 1.31	0.50 ± 0.47	3.41 ± 2.64
Low carbohydrate (ng*h/mL)	-8.65 ± 2.68	-3.06 ± 2.52	-18.04 ± 5.74	-1.55 ± 2.15	-0.19 ± 1.79	-1.77 ± 4.51	0.04 ± 0.16	0.14 ± 0.20	0.32 ± 0.38
One tailed paired t-test; p-value	0.25	0.13	0.19	0.02	0.26	0.03	0.12	0.27	0.14
	Total AUC 0800-1300	Total AUC 1300-1730	Total AUC 0800-1730	Total AUC 0800-1300	Total AUC 1300-1730	Total AUC 0800-1730	Total AUC 0800-1300	Total AUC 1300-1730	Total AUC 0800-1730
High carbohydrate (ng*h/mL)	46.78 ± 5.45	41.78 ± 5.77	88.56 ± 10.80	18.44 ± 2.32	17.11 ± 2.44	35.55 ± 4.56	3.19 ± 1.44	3.15 ± 1.45	6.33 ± 2.88
Low carbohydrate (ng*h/mL)	54.11 ± 5.92	54.24 ± 4.78	99.35 ± 10.23	15.65 ± 2.29	14.69 ± 1.95	30.34 ± 3.95	1.50 ± 0.17	1.56 ± 0.24	3.06 ± 0.39
One tailed paired t-test; p-value	0.01	0.06	0.01	0.11	0.15	0.12	0.13	0.15	0.14

AUC; area under the curve. Shown as mean ± SEM.

Table 9. Symmetric and Robust Percent change in Area Under the Curve

	Intact Osteocalcin					Undercarboxylated Osteocalcin					Undercarboxylated: Intact Osteocalcin				
Subject	AUC High CHO	AUC Low CHO	Δ (High CHO – Low CHO)	Symmetric % Δ	Robust % Δ	AUC High CHO	AUC Low CHO	Δ (High CHO – Low CHO)	Symmetric % Δ	Robust % Δ	AUC High CHO	AUC Low CHO	Δ (High CHO – Low CHO)	Symmetric % Δ	Robust % Δ
1	21	26.28	-5.28	-11.17	-20.1	29.78	36.19	-6.42	-9.73	-17.73	1.43	1.3	0.13	4.78	10.03
2	55.89	58.11	-2.22	-1.94	-3.81	74.17	78.21	-4.03	-2.65	-5.16	1.19	1.23	-0.04	-1.84	-3.62
3	16.39	23.73	-7.34	-18.3	-30.94	46.38	52.32	-5.94	-6.02	-11.35	3.26	2.87	0.39	6.28	13.41
4	19.48	19.27	0.21	0.54	1.08	35.15	36.93	-1.78	-2.47	-4.82	2.18	2.35	-0.17	-3.75	-7.23
5	38.34	38	0.34	0.44	0.89	51.39	49.7	1.69	1.68	3.41	1.27	1.12	0.15	6.43	13.75
6	21.13	31.59	-10.46	-19.84	-33.1	35.6	43.64	-8.04	-10.14	-18.42	2.1	1.39	0.71	20.34	51.06
7	28.12	29.01	-0.89	-1.55	-3.06	45.86	40.58	5.28	6.11	13.02	1.86	1.39	0.47	14.52	33.98
8	48.51	50.76	-2.25	-2.27	-4.43	58.53	62.83	-4.3	-3.54	-6.84	0.83	0.9	-0.07	-3.85	-7.41
9	41.88	56.71	-14.83	-15.04	-26.15	64.35	77.82	-13.47	-9.47	-17.3	1.64	1.29	0.35	11.83	26.84
10	-18.2	18.54	-36.74	-10864.3	-198.18	10.41	26.98	-16.57	-44.32	-61.42	15.98	1.55	14.43	82.29	929.13
Average	27.25	35.2	-7.95	-1093.34	-31.78	45.16	50.52	-5.36	-8.05	-12.66	3.17	1.54	1.63	13.7	105.99
SEM	6.61	4.75	3.56	1085.66	18.96	5.84	5.54	2.05	4.35	6.26	1.44	0.19	1.42	8.02	91.65

*AUC high CHO and AUC low CHO measured as ng*h/mL

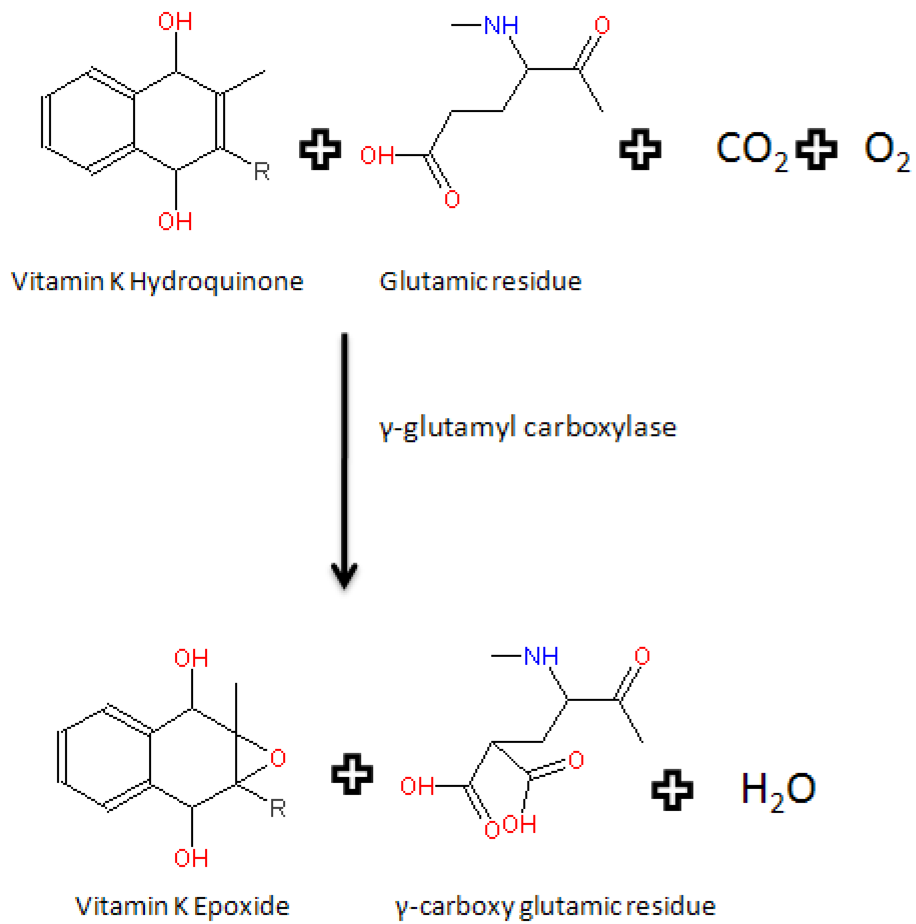
Table 10. Time in hours to Cumulative Area Under the Curve

		Intact Osteocalcin				Undercarboxylated Osteocalcin				The Undercarboxylated: Intact Osteocalcin Ratio			
	Subject	25% *	50% *	75% *	95% *	25% *	50% *	75% *	95% *	25% *	50% *	75% *	95% *
High Carbohydrate	1	2.29	4.49	7.16	9.02	2.10	4.49	7.07	9.02	2.20	4.73	7.02	9.02
	2	2.48	4.87	7.26	9.12	2.34	4.54	6.40	8.83	2.24	4.39	6.30	8.74
	3	2.05	4.30	6.54	8.97	2.48	4.92	6.83	9.17	3.06	5.54	8.07	9.21
	4	2.48	5.01	7.30	9.07	2.20	4.49	6.87	9.02	2.15	4.11	6.68	8.97
	5	1.96	4.58	7.11	9.02	2.34	4.54	6.83	8.93	2.48	4.58	6.73	8.93
	6	2.15	4.30	6.78	9.02	2.67	5.39	7.69	9.17	3.25	6.21	7.69	9.17
	7	1.05	3.77	6.35	8.78	2.53	4.68	7.11	9.07	2.77	4.96	7.59	9.17
	8	2.34	4.68	6.97	9.07	2.72	4.87	7.26	9.07	2.82	4.96	7.35	9.02
	9	2.39	5.20	7.35	9.12	2.29	4.92	7.30	9.12	2.29	4.49	7.02	9.02
	10	3.39	5.63	7.64	9.07	3.87	5.78	7.88	9.21	2.96	5.01	7.49	9.17
	Average	2.26	4.68	7.05	9.03	2.55	4.86	7.12	9.06	2.62	4.90	7.19	9.04
	Subject	25% *	50% *	75% *	95% *	25% *	50% *	75% *	95% *	25% *	50% *	75% *	95% *
Low Carbohydrate	1	2.39	4.87	7.26	9.07	3.10	5.54	7.49	9.07	3.15	5.49	7.45	9.07
	2	1.92	4.19	6.54	8.12	1.67	3.93	5.85	7.99	1.88	4.06	5.77	7.90
	3	1.77	3.82	6.40	9.02	2.29	4.82	6.64	9.07	3.34	5.82	7.16	9.07
	4	2.29	4.82	7.45	9.17	2.58	4.96	7.11	8.97	2.63	4.96	6.92	8.83
	5	2.01	4.58	7.11	9.07	2.15	4.63	7.07	9.02	2.53	4.77	7.07	9.02
	6	2.29	4.49	6.64	9.02	2.82	5.44	8.07	9.26	3.15	6.16	8.16	9.26
	7	2.29	4.73	7.11	9.02	2.01	4.25	6.44	9.07	2.15	4.06	6.35	9.02
	8	2.58	4.77	6.97	8.93	2.63	5.06	7.40	9.17	2.48	5.16	7.69	9.26
	9	2.53	5.06	7.40	9.02	2.96	5.78	7.59	8.97	2.72	5.59	7.45	8.97
	10	2.53	4.82	7.11	9.12	2.86	5.25	7.30	9.07	2.72	5.20	7.30	9.02
	Average	2.26	4.61	7.00	8.96	2.51	4.97	7.10	8.97	2.68	5.13	7.13	8.94
	One Tailed Paired T Test, p-value	0.50	0.34	0.36	0.26	0.40	0.28	0.43	0.16	0.36	0.13	0.38	0.16

* Time in hours to accumulate% of total area under the curve

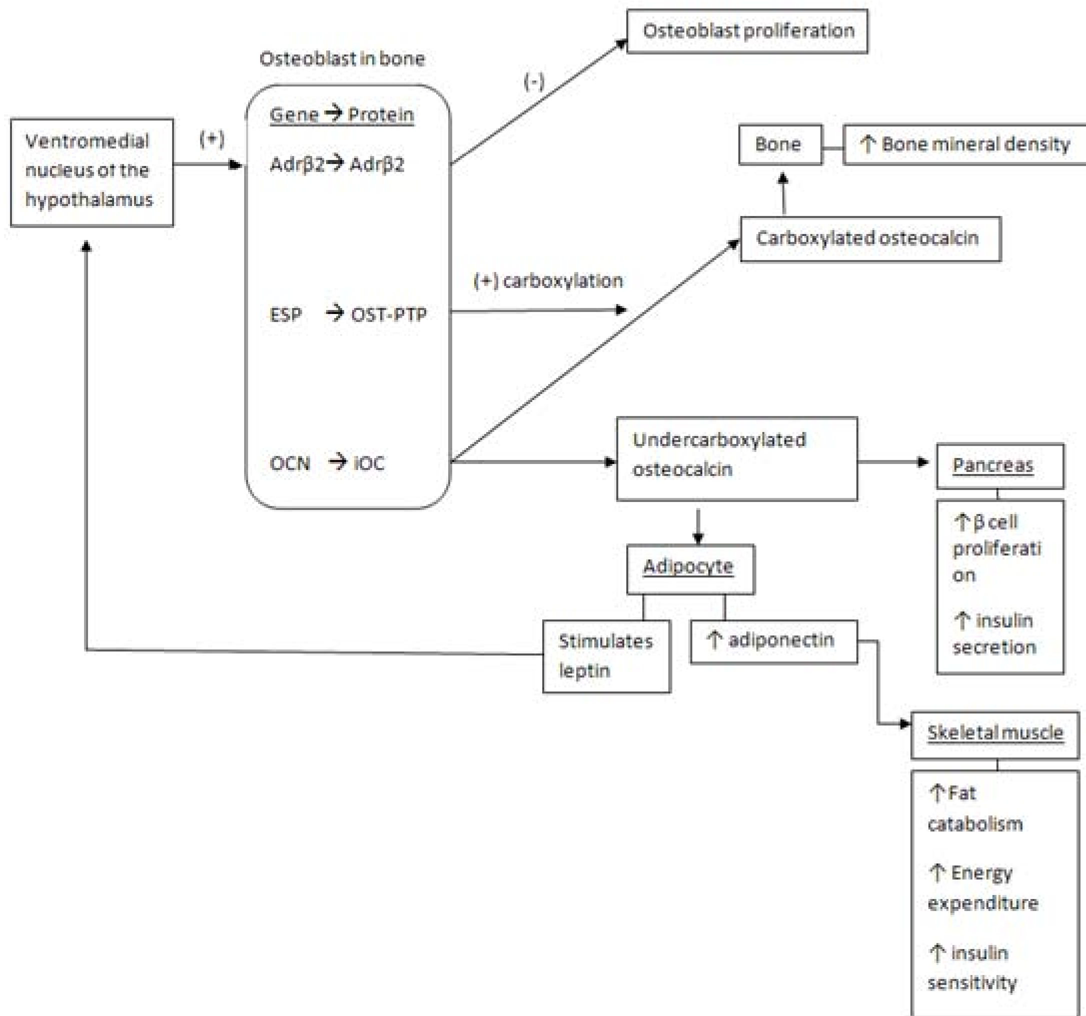
FIGURES

Figure 1. The Carboxylation of Osteocalcin



This figure drawn using Java Molecular Editor [13].

Figure 2. The Actions, Target Tissues and Feedback Mechanisms of Osteocalcin in the Mouse Model



Abbreviations and Legend

(+) = positively modulates

(-) = negatively modulates

Adrβ2 = Beta 2 adrenoreceptor (gene and protein)

OST-PTP = osteotesticular protein tyrosine phosphatase

iOC = intact osteocalcin

ESP = gene that synthesizes OST-PTP

OCN = gene that synthesizes iOC

Figure 3. The Excursion of Intact Osteocalcin Concentration over Time*

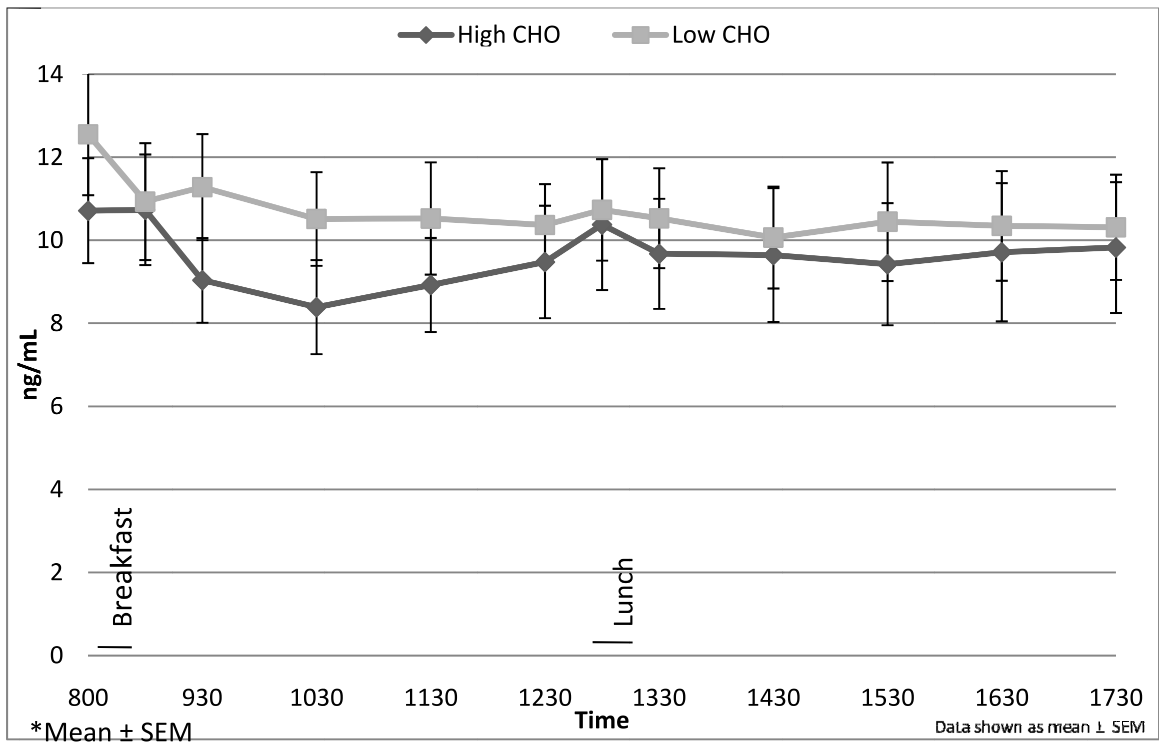


Figure 4. Percent Change in Intact Osteocalcin Concentration from Baseline*

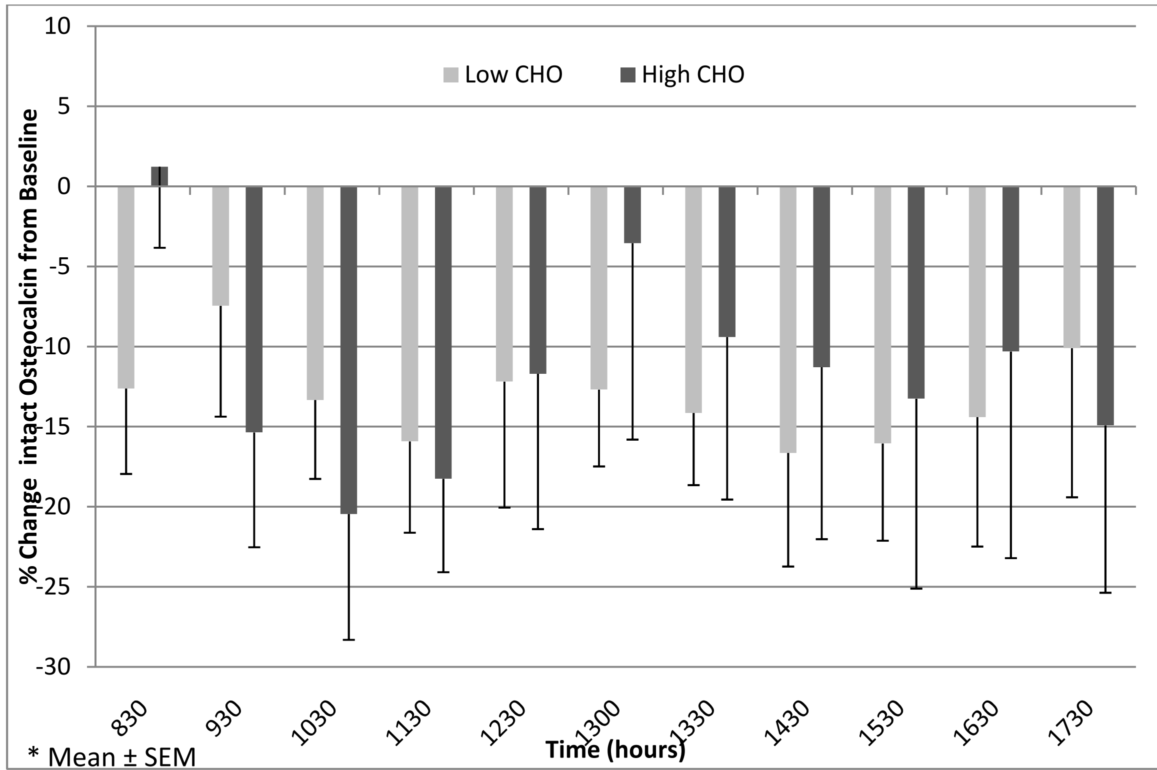


Figure 5. The Excursion of Undercarboxylated Osteocalcin Concentration over Time*

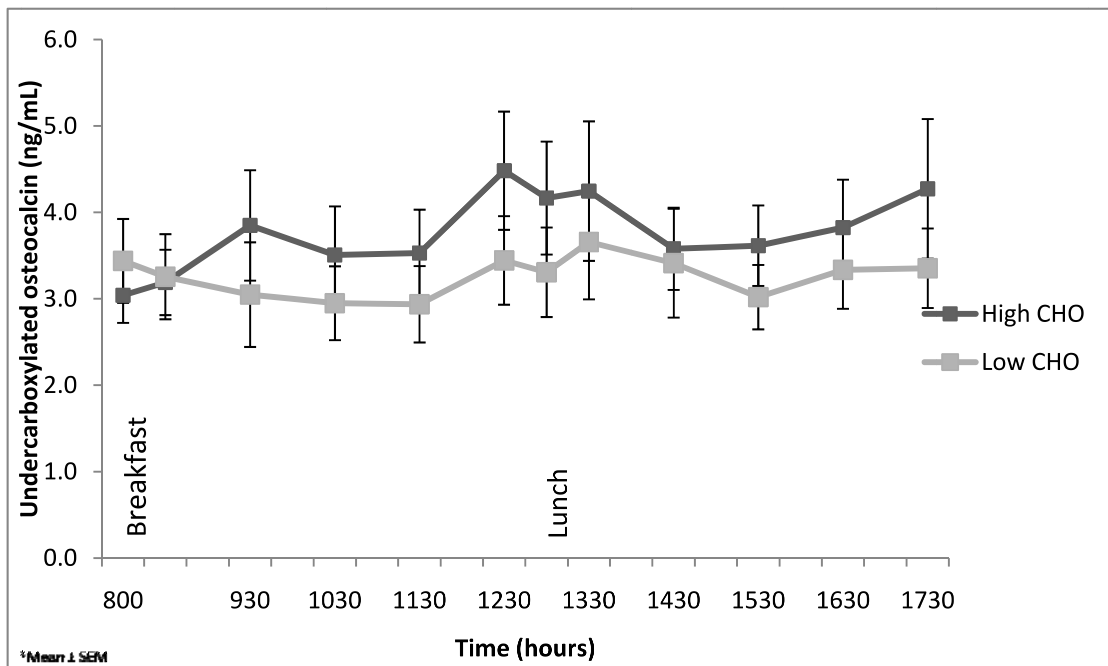


Figure 6. Percent Change in Undercarboxylated Osteocalcin Concentration from Baseline *

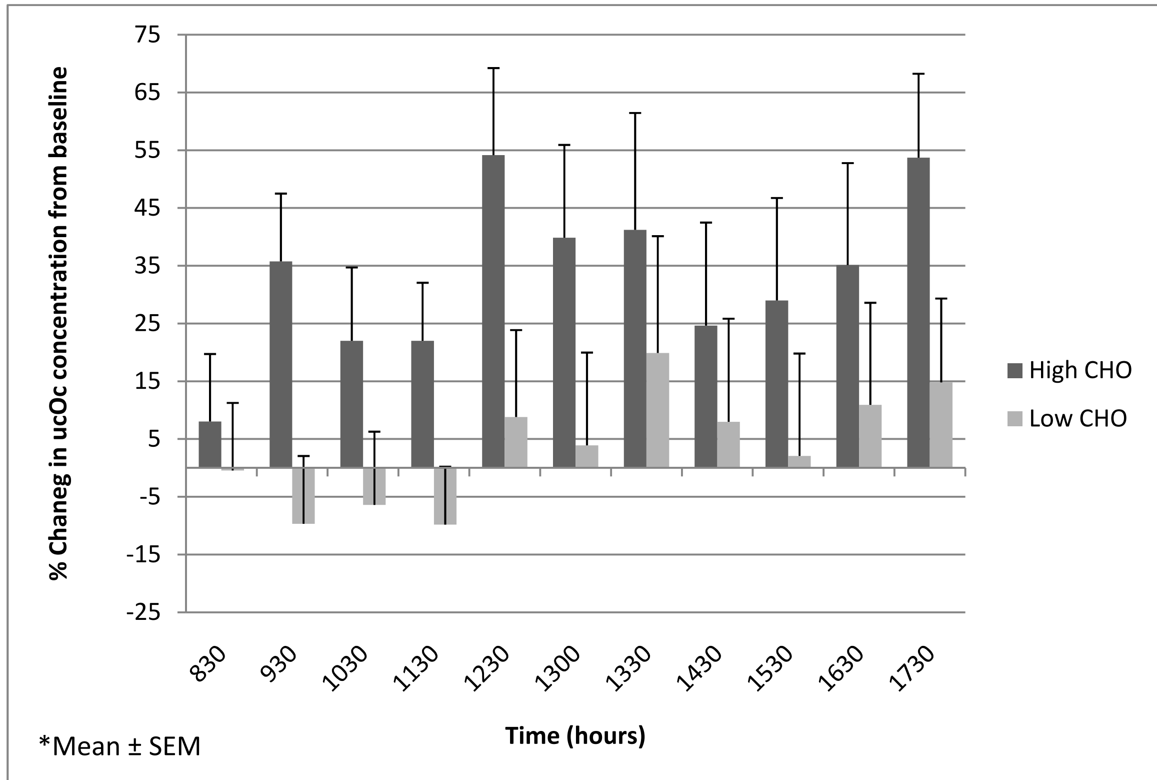


Figure 7. The Postprandial Excursion of the Undercarboxylated: Intact Osteocalcin Ratio Over Time *

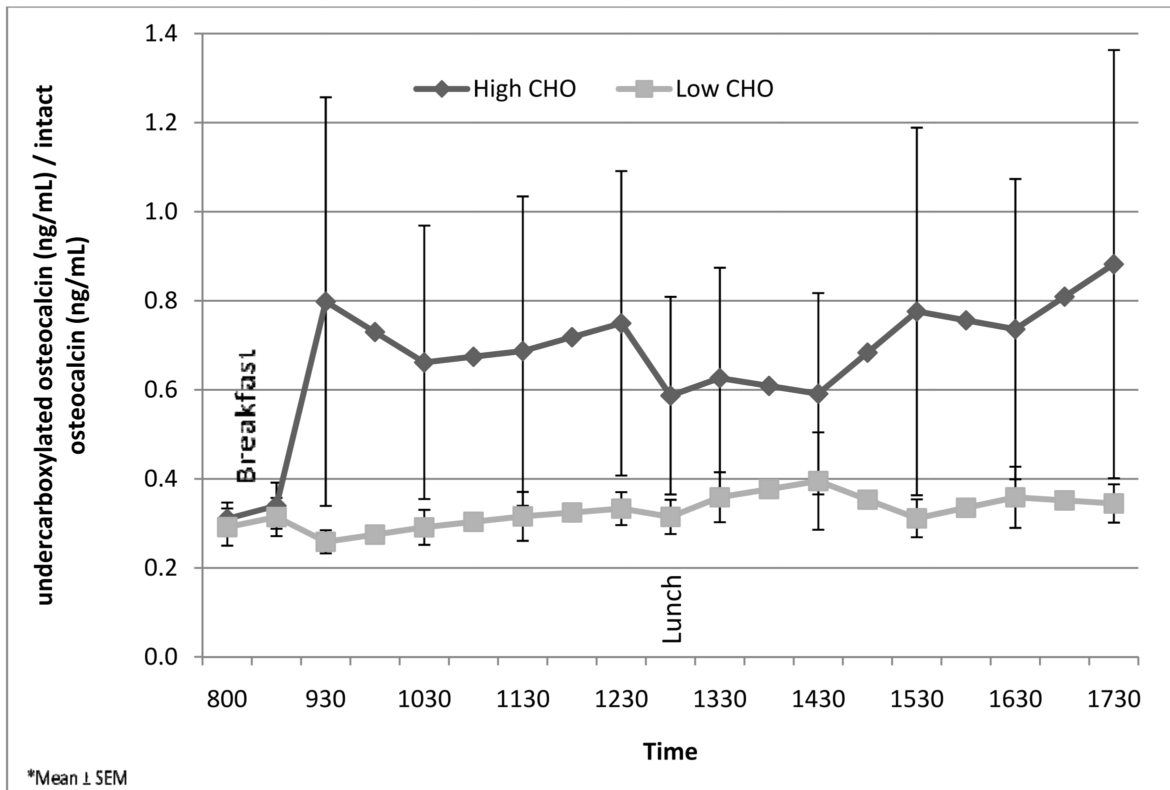
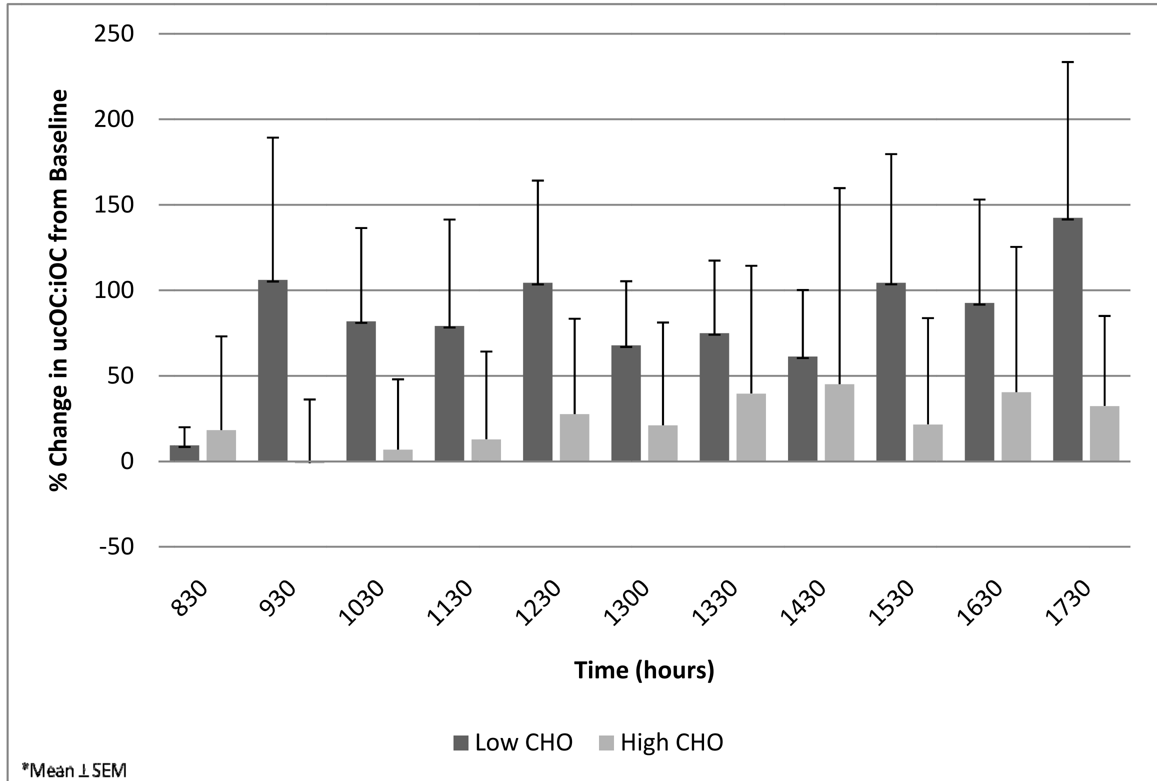


Figure 8. Percent Change in the Undercarboxylated: Intact Osteocalcin Ratio from Baseline*



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APPENDIX A: Informed Consent

IRB# e1867

Protocol Approval Date: Pending

OREGON HEALTH & SCIENCE UNIVERSITY

Consent Form and HIPPA Authorization- Student Research Project-2006

Study Title: Energy and Appetite Regulation by High and Low CHO Diets (a.k.a., The Energy Balance Study) Graduate Student Research Project sub-study

Principal Investigator:	Diane Stadler, PhD, RD	(503) 494-0168
Co-Investigators:	Melissa Kumagai, RD	(503) 494-0156
	Julia Jordan, MS, RD	(503) 494-6234
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	Paul Duell, MD	(503) 494-8986

Sponsor: National Institutes of Health, National Center for Complimentary and Alternative Medicine

PURPOSE:

Very low carbohydrate diets and high-complex carbohydrate, low-fat diets are popular weight-loss methods in the United States. The purpose of this study is to compare the impact of these diets on factors that influence energy balance. You are invited to participate in this research because you are healthy and between 21 and 65 years of age and have met the screening criteria established for this study. We plan to enroll up to 10 subjects into this study over the next six months. All study related procedures will take place at the Oregon Clinical and Translational Research Institute (OCTRI) at OHSU.

PROCEDURES:

Summary of Procedures:

One or two pre-study screening visits are required to determine eligibility for this research project.

The first screening visit will take up to 60 minutes to complete and will involve:

- Having your weight, height, and blood pressure measured.
- Completing a medical history questionnaire and other study related forms.
- Providing a fasting blood sample of less than 2 tsp. [Note: You will need to stop eating or drinking any food or beverages (except for water) after 7 p.m. the night before the blood sample is drawn.]
- Reviewing your medical history and having a brief physical examination with the study physician.
- Meeting with the study dietitian to review food preference and activity patterns. This part of the screening may be performed at a separate visit if you prefer.

You was offered a complimentary breakfast in the OCTRI after all the procedures for the screening visit are complete. The main study will involve two 4-day controlled dietary phases separated by at least three days.

During days 1-3 of the first controlled dietary phase you was asked to:

- Have your weight measured, complete study related forms, and eat breakfast in the OCTRI outpatient unit between 7 and 10 a.m.
- Take prepackaged meals and snack foods prepared for you by the OCTRI kitchen staff to eat during the rest of the day (arrangements can be made to eat other meals at the OCTRI if preferred).
- Eat all of the food provided and nothing else so that you do not gain or lose weight.
- Return all of your food containers (and any uneaten food) to the OCTRI the day after the food was to be eaten.
- Wear an activity monitor for up to 7 days.

On Day 3 of either the first or the second controlled dietary phase you will also be asked to:.

- Have your body composition (the amount of fat and muscle tissue you have) measured by:
    - Bioelectrical Impedance Analysis (BIA): The BIA procedure passes a very small, unnoticeable electrical current between electrode pads attached to your hands and feet.

- DEXA scan: A DEXA scan passes a very small amount of X-rays through your body while you are lying on your back on a scanning bed. You was asked to take off any jewelry or metal items that are part of your clothing during this measurement; hospital gowns was available for you to use. Because of the exposure to X-rays all women was asked to provide a urine sample for a pregnancy test on the day of the measurement.

A subgroup of participants will have internal body temperature measured:

On day 3 of both controlled dietary phases you will swallow a single-use, disposable, sensor capsule (about the size of a vitamin tablet). The capsule moves through your stomach and intestines in about 1 to 3 days. Every 15 seconds, the capsule sends information to a "pager-sized" monitor that you wear at your waist. You will need to wear the monitor for about 3 days until the capsule passes through your intestinal tract. The monitor can be placed within 3 feet of your body while you sleep. You will need to wear a wrist-band that states "MRI Risk: DO NOT Perform MRI; a metal-containing thermometer was swallowed on Day-MO-YR for research purposes. Contact Dr. Diane Stadler at 503-706-2074 in an emergency" until the capsule has passed from your intestinal tract.)

Early in the morning of Day 4 (by 6 am) you was admitted to the OCTRI Inpatient Unit. When you arrive in the morning it should be before eating or drinking foods (except water) or performing any significant exercise.

During your visit to the Inpatient Unit you will:

- Be asked to start a 24-hour urine collection.
- Have your blood pressure, pulse, heart rate, and temperature measured.
- Have a blood sampling tube placed in your arm vein so that blood samples can be drawn 12 times (each sample will contain about 1 1/3 TB of blood for a total of about 1 cup of blood) over a period of about 10 hours.
- Have your "resting energy (calorie) use" measured. This process involves placing a lightweight, clear, Plexiglas canopy, with an adjustable air flow rate, over your head and chest to collect samples of the air that you breathe out while you rest on a hospital bed for 45 minutes.
- Eat very-low carbohydrate breakfast and lunch meals or high complex carbohydrate breakfast and lunch meals. The very low carbohydrate meals will include foods like meat, poultry, fish, eggs, cheese, small amounts of vegetables but no fruits, cereals or bread products. The high carbohydrate/low fat meals will include foods like fruits, vegetables, cereal, bread, and low-fat meat and dairy products.
- Have your calorie use associated with meals measured for 45 minutes each hour for 10 hours. This process involves placing the same breath-collection canopy over your head and chest after you eat the research meals and while you rest on a hospital bed. You will need to remain awake during this process but you will not be allowed to engage in any activities other than quiet pursuits such as listening

to music or watching TV. You will only be allowed to get up for very light activity (stretch, walk to the bathroom, etc) for 15 minutes each hour.

- Select a dinner meal from the OHSU hospital menu to eat in the OCTRI or to take with you.

You will then be discharged from the OCTRI Inpatient Unit to follow your typical diet and activities.

After the activity monitoring period is finished, you will return the physical activity monitor and core body temperature monitor.

At least three days later you will repeat the study procedures described for days 1-3 except that you will not have your body composition measured by DEXA if you did so during the first controlled dietary phase.

The following day you was readmitted to the OCTRI Inpatient Unit and you will repeat the procedures described for day 4 except that you will eat the other combination of breakfast and lunch meals (very low carbohydrate or high complex carbohydrate meals).

<u>Sample Storage</u>:

- Blood and urine was stored for other analyses if additional funds become available.
- Potential measurements will include heart, kidney, bone, and indicators of weight regulation.
- No samples was used for genetic testing.

<u>If you have any questions about this study, now or in the future, please contact Dr. Stadler at (503) 494-0168.</u>

<u>RISKS AND DISCOMFORTS</u>:

<u>Very low carbohydrate meals</u>: Risks associated with consuming a very low carbohydrate diet for a day are very low. Because the very low carbohydrate diet is low in fiber, you may experience transient changes in your bowel movement frequency and/or consistency that may result in constipation or diarrhea. Because this diet has a very low water content you was provided with non-carbohydrate containing beverages and reminded to consume adequate fluid during the inpatient admissions.

High complex carbohydrate/low fat diet: Risks associated with consuming a high carbohydrate/low fat diet for a day are very low. This diet may have a higher fiber content than your typical diet and you may notice changes in your bowel movement pattern and/or consistency. You was encouraged to consume water with and between meals throughout each inpatient admission.

Whole Body DEXA Measurement: The procedure takes about 5 minutes to complete. You was exposed to a small amount of radiation (x-rays) from the two whole body DEXA scans. While no amount of radiation has been proven safe, there is no direct evidence that small doses of radiation, similar to those used in the body scan, cause harmful effects in the persons who are exposed. Before each whole body DEXA scan, every female subject must have a urine pregnancy test because of the exposure to x-rays. The reason we do this is to be as careful as possible to not scan a woman who is pregnant. The results of the urine pregnancy test will remain private. We will inform you of the results and, if positive, refer you to your regular doctor or health care provider for ongoing care.

Internal Body Temperature Measurement: There are minimal risks associated with measuring internal body temperature. Internal temperature information was transferred by radio frequency transmission from the capsule to the external monitor. The capsule was administered by the OCTRI nursing staff. The capsule may be swallowed with water or other beverages. The capsule must be swallowed without chewing. There is a small chance that choking may occur when the capsule is swallowed. Ingestion of the capsule may result in gastrointestinal discomfort including nausea, vomiting, or pain. To minimize these risks, you was screened for abnormalities in swallowing, esophageal or bowel strictures, fistulas, or gastrointestinal obstructions. If you have any one or combinations of these conditions, you will not be allowed to participate in this procedure. If medically necessary for non-study related purposes, an MRI should not be conducted until the capsule has passed from the digestive system. Study participants was asked to wear a "MRI Warning" wristband until the capsule has passed through the digestive system. The study physician will provide on-going oversight and follow-up throughout this procedure.

Repeated Blood Samples: You will have 13 blood samples of about 1 tablespoon each drawn from a catheter (tube) placed in an arm vein two times during the study. Approximately 7/8th cup of blood was collected during each inpatient admission. If the catheter stops working at any time during the inpatient admission, you may need to have a new catheter placed in your other arm. You may get an infection where the tube is placed. This would cause swelling, redness, and pain. You may bleed or get a bruise. There is a small chance your blood stream or heart valves might get a serious infection. You may get a blood clot that could go to your lungs. These problems are very rare. If you have these problems, you will need hospital care. Your blood-drawing catheter was in place in your arm for about 11 hours.

Single Fasting Blood Samples: You will have a single fasting blood sample of about 2 teaspoons drawn from an arm vein or a few drops drawn by fingerstick once during the screening phase of the study. You may feel some pain when your blood is drawn. There is a small chance the needle will cause bleeding, a bruise, or an infection. This process will take about 5 minutes to complete.

Estimation of Resting and Meal-Related Energy (Calorie) Use: There are no risks associated with having resting or meal-related calorie use measured by the proposed methods. Some people may feel "closed-in" while lying under the plastic canopy or the air may feel "stuffy". This procedure takes about an hour to complete. These measurements will be performed over about 11 hours during each inpatient admission.

Bioelectrical Impedance Measurement: The electrical conductivity tests are painless to the extent that you will not feel any procedure taking place other than having the electrode pads placed on and removed from your ankles and wrists. The electrical conductivity test takes less than 1 minute to complete.

BENEFITS:

You may or may not notice any health or personal benefits from your participation in this study. However, by serving as a subject in this study, you may contribute new information that may benefit other patients in the future. You was informed of any clinically significant abnormalities and the safety monitoring blood test results was provided to your physician upon request and discussed with you at the conclusion of the study.

ALTERNATIVES:

You may choose not to be in this study.

CONFIDENTIALITY:

We will not use your name or your identity for publication or publicity purposes. Research records may be reviewed and/or copied by all investigators listed on page one of this consent form, others at OHSU who are participating in the conduct of this research protocol, the OHSU Institutional Review Board, and the Oregon Clinical and Translational Research Institute.

COSTS:

There was no cost to you for participating in this study. The study will pay for all study-related examinations and laboratory procedures. In addition, the study will pay for the costs of your food and its preparation.

LIABILITY:

It is not the policy of the U.S. Department of Health and Human Services, or any federal agency funding the research project in which you are participating to compensate or provide medical treatment for human subjects in the event the research results in physical injury.

The Oregon Health & Science University is subject to the Oregon Tort Claims Act (ORS 30.260 through 30.300). If you suffer any injury and damage from this research project through the fault of the University, its officers or employees, you have the right to bring legal action against the University to recover the damage done to you subject to the limitations and conditions of the Oregon Tort Claims Act. You have not waived your legal rights by signing this form. For clarification on this subject, or if you have further questions, please call the OHSU Research Integrity Office at (503) 494-7887.

PARTICIPATION:

Dr. Diane Stadler (503) 494-0168) has offered to answer any questions you may have about this study. If you have any questions regarding your rights as a research subject, you may contact the OHSU Research Integrity Office at (503) 494-7887.You do not have to join this or any research study. If you do join, and later change your mind, you may quit at any time. If you refuse to join or withdraw early from the study, there was no penalty or loss of any benefits to which you are otherwise entitled.

The investigators may withdraw you from this research study at any time if they believe it is in your best interest. You may be asked to withdraw from the study at the investigator's discretion, sponsor's discontinuation, or because of pregnancy or serious side effects, or because of your failure to comply with instructions or unwillingness to participate in study procedures. If you decide to withdraw from this study, we will ask you to complete one final follow-up and discharge visit. We will inform you of any new findings that may affect your willingness to continue or to withdraw from this research study. We will give you a copy of this consent form.

The participation of OHSU students or employees in OHSU research is completely voluntary and you are free to choose not to serve as a research subject in this protocol for any reason. If you do elect to participate in this study, you may withdraw from the study at any time

without affecting your relationship with OHSU, the investigator, the investigator's department, or your grade in any course.

SIGNATURES: Your signature below indicates that you have read this entire form and that you agree to be in this study.

____

Signature of Subject Date

____

Signature of Person Obtaining Consent Date

____

Signature of Investigator Date

APPENDIX B:Nutrient Composition of Standard Diet*

Dietary Component	Days 1-3
Carbohydrate	
g/1000 kcal	130
% of energy	51
mean ± SD (grams)	360 ± 57
Protein	
g/1000 kcal	36
% of energy	14
mean ± SD (grams)	101 ± 16
Fat	
g/1000 kcal	40
% of energy	35
mean ± SD (grams)	111 ± 18
Saturated Fatty Acids	
g/1000 kcal	13
% of energy	11
mean ± SD (grams)	35 ± 9
Monounsaturated Fatty Acids	
g/1000 kcal	14
% of energy	13
mean ± SD (grams)	44 ± 9
Polyunsaturated Fatty Acids	
g/1000 kcal	9
% of energy	8
mean ± SD (grams)	28 ± 14

Cholesterol (mg/1000 kcal)	104
mean ± SD (mg)	228 ± 51
Total Dietary Fiber (g/1000 kcal)	9
mean ± SD (grams)	32 ± 0.43
Sodium (mg/1000 kcal)	1454
mean ± SD (mg)	4035 ± 709
Potassium (mg/1000 kcal)	1089
mean ± SD (mg)	3022 ± 646
Calcium (mg/1000 kcal)	577
mean ± SD (mg)	1876 ± 86
Phosphorus (mg/1000 kcal)	577
mean ± SD (mg)	1601 ± 399

* The amount per 1000 kcal and percent of total energy for each nutrient was calculated from the average amount consumed during the standardization phase.

APPENDIX C: Blood Sampling and Processing Schedule

Table 4. Plasma biomarker analysis														
Collection Tube	**Analyte**	**Aliquot Vol (µl)**	**Time Points (hr)**											
In order of priority	**In order of priority**	**2 sets/ analyte if possible**	**0800**	**0830**	**0930**	**1030**	**1130**	**1230**	**1300**	**1330**	**1430**	**1530**	**1630**	**1730**
10 mL red top, deliver 6.0 mL whole blood	TSH*	500	X											
	hsCRP	100	X			X		X			X		X	
	C-peptide	500	X	X	X	X	X	X	X	X	X	X	X	X
	Insulin	500	X	X	X	X	X	X	X	X	X	X	X	X
	Leptin	500	X	X	X	X	X	X	X	X	X	X	X	X
2 mL green top (heparin)	Osteocalcin	500	X	X	X	X	X	X	X	X	X	X	X	X
	Carboxylated osteocalcin	500	X	X	X	X	X	X	X	X	X	X	X	X
2-mL grey top (NaFl/K-oxalate)	Glucose	500	X	X	X	X	X	X	X	X	X	X	X	X
6-mL purple top (K3-	Total Triglyceride	500	X	X	X	X	X	X	X	X	X	X	X	X

	Total ghrelin	500	X	X	X	X	X	X	X	X	X	X	X	X
	Active ghrelin	500	X	X	X	X	X	X	X	X	X	X	X	X
	TNF-α	500	X			X		X			X		X	
	IL-6	500	X			X		X			X		X	
	Fatty acid profile	100	X			X		X			X		X	
3-mL purple top (K3-EDTA) no vacuum, pretreated with 15 µl DPP-IV and 90 µl aprotinin deliver 1.5 mL whole blood	Active PYY (3-36)	300	X	X	X	X	X	X	X	X	X	X	X	X
3-mL purple top (K3-EDTA) no vacuum, pretreated with 15 µl DPP-IV, deliver 1.5 mL	Active GLP-1	300	X	X	X	X	X	X	X	X	X	X	X	X

whole blood														
3-mL purple top (K3-EDTA) no vacuum, pretreated with 20 μl THL, deliver 2.0 mL whole blood	Non-esterified Free Fatty Acid (NEFA)	400	X	X	X	X	X	X	X	X	X	X	X	X
Total volume drawn per time point (mL)			19	19	19	19	19	19	19	19	19	19	19	19

NOTE: All vacutainers, except for red top, should be pre-chilled on ice before collecting blood samples. Once blood is collected, all tubes (except red top) should be returned to ice and spun in a refrigerated centrifuge within 15 minutes. Red top tube should be allowed to sit at RT for 15-20 minutes before spinning. Make second set of aliquots if additional serum/plasma is available. Aliquot tubes for total and active ghrelin should be treated with HCl and PMSF. Aliquots should be frozen immediately at -20 C for up to 72 hours and then transferred to a -80 C freezer. *TSH is drawn with fasting sample during first inpatient admission, only. All samples were stored at -80 C at the GCRC Core Lab for EOS analysis. Total blood Volume = 228 mL (~1 cup total).

APPENDIX D: Nutrient Composition of High- and Low-Carbohydrate Test Meals*

Dietary Component	High Carbohydrate Meals	Low Carbohydrate Meals
Carbohydrate		
g/1000 kcal	142	9
% of energy	55	4
Protein		
g/1000 kcal	46	75
% of energy	18	30
Fat		
g/1000 kcal	30	73
% of energy	27	66
Saturated Fatty Acids		
g/1000 kcal	6	32
% of energy	6	29
Monounsaturated Fatty Acids		
g/1000 kcal	10	24
% of energy	9	21
Polyunsaturated Fatty Acids		
g/1000 kcal	11	5
% of energy	10	4
Cholesterol (mg/1000 kcal)	137	653
Total Dietary Fiber (g/1000 kcal)	12	2
Sodium (mg/1000 kcal)	1255	2044
Potassium (mg/1000 kcal)	1313	1077
Calcium (mg/1000 kcal)	480	533
Phosphorus (mg/1000 kcal)	808	952

* The energy content of each individual's research meal was indexed to baseline body weight and provided 10 kcal/kg.

APPENDIX E: Select Micronutrient Composition of Intervention Meals

The following micronutrients were provided at each test meal, based on the average 683 kcal intake.

	Micronutrient	Ca	Fe	Mg	P	K	Na
	Amount /683 kcal meal	mg	mg	mg	mg	mg	mg
high carbohydrate	Breakfast	653	2	119	658	1001	437
high carbohydrate	Lunch	217	8	169	647	1280	1303
Low Carbohydrate	Breakfast	276	5	56	679	678	1765
Low Carbohydrate	Lunch	445	4	104	618	777	1026

	Micronutrient	Ribo-flavin	Niacin	Panto-thenic acid	B-6	Folate	B-12
	Amount/ 683 kcal meal	mg	mg	mg	mg	mcg	mcg
high carbohydrate	Breakfast	1	4	2	1	287	6
high carbohydrate	Lunch	1	18	2	1	241	0
Low Carbohydrate	Breakfast	2	6	4	1	110	3
Low Carbohydrate	Lunch	1	8	5	2	58	5

	Micronutrient	Zn	Cu	Mn	Se	Vit C	Thia-min
	Amount / 683 kcal meal	mg	mg	mg	mcg	mg	mg
high carbohydrate	Breakfast	5	1	1	22	6	0
high carbohydrate	Lunch	4	1	3	67	32	1
Low Carbohydrate	Breakfast	5	0	0	92	19	0
Low Carbohydrate	Lunch	10	0	0	38	12	1

	Micronutrient	Vit A*	Vit E*	Vit D*	Vit K	Fluoride*
	Amount / 683 kcal meal	mg RE	mg tocopherol	mcg	mcg	Mg
high carbohydrate	Breakfast	275	3	121	1	21
high carbohydrate	Lunch	404	4	8	87	0
Low Carbohydrate	Breakfast	64	1	83	3	0
Low Carbohydrate	Lunch	268	1	18	55	0