THE GUT-BONE AXIS: EFFECTS OF INCRETIN HORMONES ON BONE METABOLISM

by

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(Under the Direction of Connie J. Rogers)

ABSTRACT

Musculoskeletal disorders are a leading cause of years lived with a disability world-wide and represent a major healthcare expenditure in the United States. Nearly 10 million people living in the United States have osteoporosis, a condition associated with an increased risk of fractures. The National Osteoporosis Foundation highlights a myriad of factors that contribute to bone health across the lifespan, including nutrition, physical activity, medications, and hormones. Nutrition plays a pivotal role in maintaining lifelong bone health, and cross-talk between the gut and bone–often referred to as the "gut-bone axis"–is hypothesized to mediate the nutritional effects on bone. Incretin hormones, such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), acutely regulate postprandial bone metabolism. However, the biological mechanisms through which nutrition impacts bone health via the gut-bone axis have not been clearly defined. The objective of this dissertation is to examine the relationship between nutrition, incretin hormones, and bone metabolism in healthy adults and in individuals with endocrine-related conditions such as diabetes and cystic fibrosis-related diabetes (CFRD). In manuscript #1, a cross-sectional study involving 10 healthy

emerging adults (ages 18-25) was conducted to determine the effects of glucose ingestion on C-terminal telopeptide (CTX), a biomarker of bone resorption. Glucose ingestion resulted in an anti-resorptive effect on bone metabolism by minute 30. Manuscript #2 is a systematic review and meta-analysis that summarizes the overall effect of GIP administration on CTX based on previously conducted randomized controlled crossover trials. GIP exerts an anti-resorptive effect on bone, which may be modified in individuals with diabetes. In manuscript #3, a secondary analysis was performed to evaluate the effects of intravenous incretin hormone infusion on bone resorption in adults with cystic fibrosis (CF). CTX decreased during GIP infusion, but not during placebo, indicating that GIP induces a bone anti-resorptive effect in individuals with pancreatic-insufficient CF (PI-CF). Overall, findings suggest that glucose ingestion and incretin hormone infusion have acute anti-resorptive effects on bone. However, metabolic conditions that disrupt the gut-bone axis, such as type 1 and type 2 diabetes and PI-CF, may increase the risk of bone disease in individuals with entero-endocrinopathies.

INDEX WORDS: INCRETINS, GIP, GLP-1, BIOMARKERS, BONE METABOLISM, CTX, P1NP

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DEDICATION

This dissertation is dedicated to my parents, who have been my foundation through this journey. Despite being 9,000 miles away from home, their unwavering support and endless love have been my constant companions. I cherished our weekly conversations during my lunch breaks – these moments of connection were a reminder of the values you instilled in me. You taught me the importance of hard work and putting 100% effort into everything I commit to. Most importantly, you taught me that learning and education are treasures that no one can ever take away from you.

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CHAPTER 1

INTRODUCTION

Musculoskeletal disorders are a leading cause of years lived with a disability world-wide and represent a major healthcare expenditure in the United States [1]. Nearly 10 million people living in the United States have osteoporosis, which is a condition associated with an increased risk for fracture [2]. The National Osteoporosis Foundation's position statement indicate a myriad of factors contributing to bone health across the lifespan, including nutrition [3], physical activity [4], medications [5], and hormones [6].

In recent years, research has explored the potential regulatory role of gut-derived hormones–particularly incretins–in bone metabolism [7]. Enteroendocrine hormones, such as glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1), are released after macronutrient ingestion and play a critical role in regulating satiety, gastric motility, and glucose metabolism [8]. These hormones bind to G-protein coupled receptors on pancreatic beta cells, promoting glucose-dependent insulin secretion, thereby helping maintain glucose homeostasis [9]. Interestingly, these incretin receptors (GIPR and GLP-1R) are also expressed on bone cells, suggesting a possible link between gut-derived incretins and bone metabolism [10].

In-vitro studies have demonstrated that GIP and GLP-1 receptors on bone-resorbing osteoclasts reduce osteoclastogenesis when activated, indicating an anti-

resorptive effect [10]. Studies have also highlighted that glucose ingestion has a more pronounced effect on bone resorption when administered orally compared to intravenous routes, suggesting that the gastrointestinal tract plays a crucial role in this process [11, 12]. This phenomenon has led to the hypothesis of the "gut-bone axis," where gut-derived incretin hormones influence bone metabolism.

Given this potential gut-bone axis, it is plausible that conditions marked by impaired incretin and/or insulin responses–such as diabetes and metabolic disorders–may adversely affect bone health. For example, individuals with hypothyroidism, non-alcoholic fatty liver disease, and type 2 diabetes exhibit significantly blunted bone resorption following macronutrient consumption compared to healthy individuals [12-14]. However, the specific mechanisms through which nutrition impacts bone health remain unclear.

This dissertation aims to investigate these connections. Chapter 2 presents a literature review, focusing on bone biology, insulin's impact on bone health, the gut-bone axis, bone assessment methods, cystic fibrosis (CF) and CF-related diabetes, and anti-diabetes medications. Chapters 3, 4, and 5 contain original research investigating the effects of glucose ingestion and incretin infusion on bone metabolism in both healthy young adults and adults with CF.

Specific Aims and Hypotheses:

- **Aim 1: Determine effects of glucose ingestion on changes in bone resorption in healthy emerging adults.**
    - Hypothesis 1. CTX, a biomarker of bone resorption, decreases significantly following oral glucose tolerance testing (OGTT).

- **Aim 2: Summarize current literature on intravenous infusion of GIP on biomarker of bone resorption.**
    - Hypothesis 2. CTX decreases significantly following GIP infusion compared to placebo infusion in adults.
- **Aim 3: Determine effects of intravenous infusion of incretin hormones on bone metabolism in adults with CF.**
    - Hypothesis 3. Compared to placebo, GIP and GLP-1 infusion significantly decreases CTX in adults with CF.

CHAPTER 2

REVIEW OF THE LITERATURE

Bone Metabolism

Musculoskeletal disorders are a major cause of disability worldwide and contribute significantly to healthcare costs in the United States [1]. In the U.S. alone, nearly 10 million individuals suffer from osteoporosis, a condition linked to an increased risk of fractures [2]. The transition from adolescence to adulthood is crucial for establishing long-term bone health, as peak mass is typically attained around 20 years of age [15]. Because bone health tends to track consistently from childhood into adulthood, disruptions in normal bone accrual during early life can increase the risk of developing osteoporosis and experiencing fractures later in life [16]. Several factors influence bone health across the lifespan, including nutrition [3], physical activity [4], medications [5], and hormonal regulation [6].

The adult skeleton undergoes continuous bone resorption and formation, processes collectively known as bone modeling and remodeling [17]. These activities occur in the periosteum (the outer cellular layer of bone), the endosteum (the internal membrane lining), and the compact cortical bone [18]. Bone remodeling units within these areas comprise bone-forming osteoblasts, bone-resorbing osteoclasts, and osteoprogenitor stem cells [19]. During the developmental years, both the periosteum and endosteum expand to accommodate bone growth. During this critical period, bone modeling occurs independently of remodeling to achieve peak bone mass–the maximum

amount of bone tissue accrued by the end of skeletal development [20]. Once peak bone mass is reached, the integrity of the adult skeleton is maintained through the coordinated activities of osteoclasts and osteoblasts, which are responsible for breaking down and rebuilding bone tissue, respectively [21]. The balance between bone resorption and formation is essential for preserving bone mass and ensuring overall mineral balance [20].

Nutritional Regulation of Bone

The position statement from the National Osteoporosis Foundation highlights the importance of various dietary components in the development of peak bone mass and overall bone health [22]. These modifiable factors include micronutrients, macronutrients, and dietary patterns. However, the biological mechanisms by which nutrition influences bone health throughout the lifespan remain incompletely understood. While the skeleton is regulated by a variety of hormones–such as thyroid hormone, estrogen, testosterone, cortisol, parathyroid hormone, and insulin-like growth factor-1 [23]–it is also influenced by enteroendocrine hormones derived from the intestinal tract.

One proposed mechanism through which nutrition may affect bone health is hormonal regulation, specifically via the activation or deactivation of cells responsible for bone turnover, which in turn influences bone accrual [8]. Following macronutrient consumption, several gut-derived hormones are released to regulate satiety, gastric motility, and metabolism [8]. Among the key hormones are the incretins: glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1). These hormones bind to their respective G-protein coupled receptors on pancreatic beta cells (GIP receptors [GIPR] and GLP-1 receptors [GLP-1R]), triggering the release of insulin

in a glucose-dependent manner to maintain glucose homeostasis [9]. In addition to their roles in glucose regulation, GIP and GLP-1 receptors are also found on bone cells, where they influence bone remodeling processes [10].

For example, consuming a solution containing 75 grams of glucose has been shown to reduce bone resorption by approximately 50%, as indicated by decreases in C-terminal telopeptide of type 1 collagen (CTX) [12, 24-26], whereby these effects are more pronounced when glucose is ingested orally rather than intravenously [11, 12]. This suggests that the suppression of bone resorption is influenced by mechanisms related to the gut, likely involving gut-derived incretin hormones.

The Gut-Bone Axis

Several hormonal mediators of nutrient metabolism are hypothesized to contribute to the "gut-bone axis." Incretin hormones, particularly GIP and GLP-1, are thought to play a key role in this gut-mediated influence on bone health **(Figure 2.1.)**. After a meal, GIP and GLP-1 levels increase approximately 10-fold and 2-fold, respectively [27], together accounting for about 50% of insulin secretion from pancreatic beta cells [28]. These hormones are rapidly broken down in the plasma by the enzyme dipeptidyl peptidase-4 (DPP-4), which is crucial for maintaining glucose homeostasis [29]. In several crossover randomized control trials, GIP infusion consistently decreased bone resorption, as measured by CTX, compared to saline infusion [30, 31]. The rapid suppression of bone resorption following oral glucose ingestion, which is not seen with intravenous glucose administration, suggests that GIP and GLP-1 play a pivotal role in mediating the anti-resorptive effects on bone [30, 32].

Insulin, a peptide hormone produced by pancreatic beta cells, helps regulate blood sugar levels following food ingestion [33]. Under normal physiological conditions, insulin facilitates glucose uptake into peripheral target cells for metabolism. However, when insulin signaling is impaired–commonly referred to as insulin resistance–glucose uptake is diminished, leading to dysglycemia [34]. While the liver, skeletal muscle, and adipose are the primary insulin-dependent tissues managing glucose metabolism, the skeleton is also influenced by insulin [35]. *In vitro* studies have demonstrated that osteoblasts and osteoclasts possess membrane-bound receptors for GIP, GLP-1, and insulin. Experimental studies in humans further indicate that these incretin hormones, along with insulin, can promote bone formation and suppress bone resorption [36-40].

The role of incretins, particularly GIP, in reducing bone resorption has been shown to occur even more prominently in individuals without diabetes compared to those with diabetes [30]. Consequently, medical conditions that alter incretin or insulin responses may negatively impact bone health through disruptions in the gut-bone axis. For instance, in a study of youths with cystic fibrosis-related diabetes (CFRD), we observed a significant reduction in the bone resorption biomarker CTX–approximately 32%–120 minutes after glucose ingestion [41]. This reduction was less than the typical 50% reduction observed in healthy adults during oral glucose tolerance tests [12, 24, 26]. Furthermore, in studies investigating the role of incretins on bone metabolism, GIP receptor antagonism through GIP(3-30)NH2 has been shown to diminish the usual decrease in CTX following oral glucose ingestion [30]. Other studies comparing bone metabolism following macronutrient ingestion have reported similarly diminished effects on bone resorption in adults with hypothyroidism, nonalcoholic fatty liver disease, and

type 2 diabetes compared to healthy controls [12-14]. These findings suggest that the bone's biological responses to food or macronutrient consumption may be altered in individuals with chronic metabolic conditions. Nonetheless, experimental studies indicate that macronutrient consumption generally induces acute anti-resorptive effects on bone, with gut-derived hormones like GIP, GLP-1, and insulin playing a critical role in these processes. In fact, reductions in bone resorption follow mixed meal ingestion have been shown to be rapid and significant, with CTX decreasing by approximately 50% within two hours of ingestion [31]. This decrease in bone resorption is comparable in magnitude to the effect of antiresorptive pharmacological agents; however, it is transient and reverses within 4-5 hours postprandially [42].

Several molecular mechanisms have been proposed to explain the gut-bone axis. Incretin hormones are released by enteroendocrine cells in response to nutrient ingestion and circulate to target tissues such as bone, where they influence osteoblast and osteoclast activity [9, 43] **(Figure 2.2.)**. Upon nutrient ingestion, the K and L cells in the gut sense a variety of nutrients such as glucose, amino acids, long-chain fatty acids (LCFAs), short-chain fatty acids (SCFAs), and peptides [44]. These nutrients interact with specific receptors on the membrane of the enteroendocrine cells, including free fatty acid receptors-1,3,4 (FFAR1, FFAR3, and FFAR4), calcium-sensing receptors (CaSR), amino acid transporters, and taste 1 receptor membrane-1 and 3 (TAS1R1/TAS1R3) [45]. Activation of these receptors triggers intracellular signaling cascades involving the phospholipase C pathway, which leads to calcium release, and the protein kinase C pathway, amplifying calcium signaling [46]. The elevation in intracellular calcium levels results in the secretion of GIP and GLP-1 from K and L cells, respectively, into the

bloodstream. This response is also tightly regulated by nutrient type, such as glucose entering the enteroendocrine cell via transporters like sodium-glucose cotransporter 1 (SGLT1) and glucose transporter 2 (GLUT2), further stimulating incretin release [47].

GIP and GLP-1 bind to their respective receptors on bone mesenchymal stem cells (BMSCs), osteoblasts, and osteoclasts [48]. Binding of GIP and GLP-1 on BMSCs activates G-protein coupled receptor pathways, which in turn increases cyclic AMP levels and activate protein kinase A [49]. These signaling pathways promote differentiation of BMSCs into pre-osteoblasts and ultimately mature osteoblasts. The activation of these pathways inhibits osteoblast apoptosis [50]. At the osteoclasts, GIP and GLP-1 also play an inhibitory role by binding to receptors on osteoclast precursors [51]. This action counteracts the bone resorptive effects mediated by the RANK-RANKL interaction [52]. Specifically, GIP and GLP-1 binding to osteoclast receptors inhibits RANKL-induced osteoclastogenesis, reducing bone resorption [53]. Together, these molecular mechanisms highlight the potential role of incretins in regulating bone turnover in response to nutrient ingestion.

Insulin and Bone Health

Diabetes is among the most common metabolic health conditions world-wide [54]. In the United States alone, over 34 million people have diabetes [55], with approximately 95% of these individuals diagnosed with type 2 diabetes (T2D). Diabetes is associated with several co-morbidities, including increased bone fragility [56]. Despite individuals with T2D often having normal or even elevated bone mineral density (BMD) [57, 58], diabetes is linked to an increased risk of fractures [59] and impaired fracture

healing [60, 61]. Even in the absence of T2D, biological factors involved in diabetes pathogenesis have been implicated in bone health deficits [62].

Insulin, a peptide hormone produced by pancreatic beta cells, is essential for maintaining glycemic control after food consumption [63]. Under normal physiological conditions, insulin facilitates glucose uptake into cells for metabolism. However, in insulin resistance–where insulin signaling is impaired–there is reduced glucose uptake, leading to dysglycemia [34]. While insulin primarily regulates glucose metabolism in the liver, skeletal muscle, and adipose tissue, it also plays a critical role in bone health. Insulin signaling promotes osteoblast differentiation and proliferation, collagen synthesis, and reduces osteoclastogenesis [64]. However, the positive effects of insulin on bone can be disrupted in conditions such hyperglycemia [65], hyperlipidemia [66], and chronic inflammation, all of which suppress insulin signaling [67].

Individuals with insulin resistance often have increased body weight and skeletal muscle mass, subjecting their skeletons to greater mechanical loading, which contributes to increased bone density. However, the relationship between insulin resistance and bone health may be more strongly mediated by body size and glucose dysregulation rather than insulin resistance itself [68].

Bone Assessment – Bone Turnover Markers

Biochemical assays are commonly used to evaluate bone resorption, formation, and the overall regulation of bone turnover [69]. These assays detect enzymes, proteins, and by-products of bone remodeling processes [70], though they are subject to variability. Factors such as age, gender, and ethnicity, along with sample collection variables like fasting state, circadian rhythms, menstrual cycles, and physical activity, can influence

biomarker levels [71]. Nevertheless, bone turnover markers remain valuable for diagnosing and managing various medical conditions like osteoporosis and hyperparathyroidism [72].

Key biomarkers for bone resorption include CTX, tartrate-resistant acid phosphatase 5b (TRAP 5b), and amino-terminal crosslinked telopeptide of type 1 collagen (NTX) [71]. CTX is a particularly sensitive biomarker that can rapidly indicate the effects of treatments like bisphosphonates in postmenopausal women with osteoporosis [70]. However, CTX levels fluctuate with circadian rhythms and are significantly influenced by dietary intake, requiring fasting blood samples for accurate measurement [71].

Biomarkers of bone formation include procollagen type 1 N-terminal propeptide (P1NP) and procollagen type 1 C-terminal propeptide (P1CP), bone-specific alkaline phosphatase (BALP), and osteocalcin (OC) [70]. Additionally, regulators of bone turnover, such as NF-kB ligand (RANKL), osteoprotegerin (OPG), and sclerostin, are critical in assessing bone dynamics [70]. CTX and P1NP are frequently used in clinical studies to assess bone resorption and formation, particularly when evaluating the impact of dietary intake on bone health [11, 12, 24, 26] and at different timepoints [73]. CTX reflects collagen breakdown during bone resorption [74], while P1NP reflects the incorporation of collagen fragments into the bone matrix [70]. These biomarkers are most often detected using enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay techniques and are useful for investigating diet-bone interactions, particularly within the context of the gut-bone axis [75, 76].

Bone Assessment – Imaging

Dual-energy x-ray absorptiometry (DXA) is a well-established, gold-standard imaging method for measuring BMD [77]. The International Society for Clinical Densitometry has provided guidelines for assessing bone health in both adult and pediatric populations [78]. While the lumbar spine and total body are preferred sites for bone health assessment in pediatric populations, the lumbar spine and hip are recommended for adults [79]. However, in individuals with obesity, DXA assessments at these skeletal sites can be challenging due to scanner weigh limits, positioning difficulties, and interference from overlying soft tissue [80, 81]. In such cases, assessing BMD in the forearm may be a more appropriate alternative, particularly in the radius, which is a non-loaded skeletal site vulnerable to fractures and consists of both cortical and trabecular bone regions [82, 83]. In pediatric populations, ultradistal radius BMD is a valuable measure that reflects trabecular bone density without being significantly affected by stature [84].

DXA is widely used in clinical settings to diagnose osteoporosis and evaluate fracture risk, as well as to monitor responses to bone-modulating therapies. However, it is limited by its inability to differentiate between cortical and trabecular bone due to its two-dimensional nature. To address this limitation, the trabecular bone score (TBS) has been developed to estimate trabecular bone microarchitecture at the lumbar spine [85]. TBS is emerging as a tool for diagnosing osteoporosis and predicting fragility fracture risk in at-risk populations [86].

An earlier cross-sectional study of 18- to 19-year-old females found that individuals with obesity had inferior trabecular bone microarchitecture, as assessed by

magnetic resonance imaging, compared to controls with healthy body weight. However, many of these differences were no longer significant after adjusting for insulin resistance [87]. These findings highlight the potential utility of alternative bone imaging technologies that can assess distinct features of cortical and trabecular bone morphology, particularly in the context of insulin resistance and cardiometabolic health conditions. Cross-sectional studies have demonstrated a strong correlation between BMD and TBS [88], suggesting that these imaging techniques could be valuable in guiding clinical management of bone health throughout life. Further research is needed to explore the application of TBS in individuals with metabolic conditions such as diabetes.

Recent advancements in bone densitometry, particularly the development of high-resolution peripheral quantitative computed tomography (HR-pQCT), allow researchers and clinicians to assess nuanced characteristics of cortical and trabecular bone geometry, volumetric density, microstructure, and estimated strength at the appendicular skeleton **(Figure 2.2.)** [89]. HR-pQCT is often referred to as an 'in-vivo bone biopsy' due to its high-resolution capabilities [90]. Although clinical application of HR-pQCT is currently limited by its high cost, required personnel training, and a lack of normative data across different populations (based on medical condition, age, sex, or race/ethnicity) [90], it offers novel insights from a research perspective. HR-pQCT has been identified as a reliable method to detect differences in bone morphology between healthy subjects and individuals with chronic conditions, such as diabetes and osteoporosis [91].

To date, further research is required to fully integrate HR-pQCT into clinical practice for predicting fracture risk and monitoring disease progression in adults. Nevertheless, cortical and trabecular bone respond differently to environmental and

biological factors such as nutrition, physical activity, and disease [92, 93]. Since DXA is unable to distinguish between these bone compartments, HR-pQCT outcomes provide a more accurate reflection of bone strength and are therefore more closely related to fracture risk [89, 94].

Cystic Fibrosis

Cystic fibrosis (CF) is a progressive genetic condition with autosomal recessive inheritance, associated with a defective chloride channel functioning of the cystic fibrosis transmembrane conductance regulator (CFTR) protein [95]. More than 70,000 people globally have CF, with the condition primarily affecting individuals of Caucasian descent [96]. CFTR is a transmembrane protein that regulates ion transport across epithelial cell surfaces, and its dysfunction affects many organs [97]. Mutations in the CFTR gene results in a wide range of clinical manifestations and increased mortality risk. CF is diagnosed when clinical symptoms are present alongside CFTR dysfunction, which is typically characterized by elevated chloride concentrations in sweat (≥60 mmol/L) or confirmed through genetic testing for CFTR mutations [98]. CF remains the most common life-limiting single-gene disorder in Caucasians, affecting about 1 in every 3,500 newborns [99]. However, life expectancy has dramatically improved in recent decades due to advancements in CF-targeted medications and treatments [100]. According to the 2019 Cystic Fibrosis Foundation Registry Report, life expectancy for individuals with CF has increased by approximately 20 years over the last three decades [101].

This increase in survivorship has revealed CF-related complications, which contribute to early morbidity and mortality. Although lung disease remains the primary

concern in CF, many individuals also suffer from endocrine disorder, particularly CF-related diabetes (CFRD) and CF-related bone disease (CFBD), which are among the most common extrapulmonary complications [102].

CFRD is a unique form of diabetes involving impaired incretin and insulin responses to macronutrient intake [103, 104]. Prior to the onset of overt diabetes, approximately 85% of individuals with CF develop pancreatic insufficiency, which impairs nutrient metabolism and nutritional status, increasing the risk of developing diabetes [105]. CFRD is largely attributed to the progressive decline of pancreatic β-cell function, resulting in abnormal insulin secretion, including a diminished early-phase insulin response followed by a pronounced late-phase response [106]. Insulin therapy is the first-line treatment for CFRD [107], though recent data suggest that incretin-based therapies (e.g., GLP-1 receptor agonists) may enhance insulin secretion following meals [108]. Incretin mimetics increase insulin production and reduce glucagon secretion in the pancreas [109], and they are also suspected to benefit bone health [110].

Pancreatic insufficiency and poor incretin response to food intake are implicated in both CFRD [111-113] and CFBD [114, 115]. CFBD has become an increasingly prevalent but complex extrapulmonary complication, particularly in adults with late-stage CF. Currently, CFBD is among the most common complications in CF [116]. Data from the 2020 Cystic Fibrosis Foundation Patient Registry Report indicate that approximately one in five individuals with CF have bone disease, with prevalence increasing with age [117].

Studies in adults with metabolic disorders have demonstrated that impaired glucose tolerance, as seen with pancreatic insufficiency, blunts glucose-mediated changes

in bone metabolism, potentially contributing to low BMD and increased fracture risk in the CF population [65]. Fractures in individuals with CF are concerning, as they can lead to further complications, including reduced lung function, and may be contraindications for lung transplantation [118].

CFBD is primarily diagnosed through DXA screening, following CF Foundation guidelines [114]. Individuals over 18 years old are recommended to undergo DXA screening to assess BMD. If BMD is normal (DXA Z-score ≥-1), screening is repeated every five years. For those with DXA Z-scores between -1 and -2, screening is recommended every 2-4 years, while individuals with Z-scores below -2 should undergo yearly scans. Despite around 20% of adolescents with CF being at risk for suboptimal peak bone mass [119], screening recommendations for those under 18 have not been clearly established. More than 10% of adults with CF are reported to have CFBD, although this figure may be underestimated due to underreporting and insufficient screening [114]. This is concerning, especially since the spin–composed primarily of metabolically active trabecular bone–is a common fracture site in CF patients [120]. The two-dimensional nature of DXA limits its ability to distinguish between trabecular and cortical bone, which may explain why fractures in CF are not fully accounted for by DXA-assessed BMD [121, 122]. Studies utilizing high-resolution imaging methods in people with CF have shown that deficits in bone microstructure and strength are not always reflected in DXA BMD scores [123].

Several studies highlight the potential of HR-pQCT in detecting bone deficits in CFBD, demonstrating more significant findings in bone microstructure and strength compared to DXA [123-125].

While the F508del-CFTR mutation is suspected to contribute to low BMD in patients with CF [126], several other factors–including malnutrition, chronic inflammation, advanced lung disease, hormonal deficiencies (estradiol, testosterone), glucocorticoid use, and vitamin D and insulin deficiencies–are known to increase the risk of low BMD. CFRD, in particular, is believed to contribute to increased bone fragility [57]. Despite people with T2D often having normal BMD, diabetes is associated with increased fracture risk and impaired fracture healing [56]. In CF, impaired insulin signaling may negatively impact bone health, as insulin inhibits osteoclast activity [127], which might contribute to reduced bone resorption during insulin therapy, as observed in a mouse model of type 1 diabetes [128].

Although CF modulator therapies have significantly improved life expectancy in recent years, CFBD contributes to reduce the quality of life for many patients, especially following low-impact fractures, which lead to reduced mobility. Additionally, severe bone disease is a contraindication for lung transplantation, a critical procedure for end-stage CF patients [129]. As such, clinical strategies aimed at preserving bone health, including frequent screening from childhood into adulthood, are crucial in the CF population. Further investigation into the gut-bone axis, particularly in patients with pancreatic insufficiency, is essential for understanding the complex relationships between CFRD and CFBD.

Anti-Diabetes Medication

Several pharmacologic agents are available for treating glucose dysregulation, many of which affect bone density and fracture risk. A recent Bayesian meta-analysis identified several anti-diabetes medications associated with an increased risk of fractures

[5]. Metformin, a frontline therapy for both adult and pediatric T2D, was among the medications linked to a decreased risk of fractures. Preclinical studies suggest that metformin promotes osteoblast differentiation and reduces osteoclastogenesis, resulting in favorable effects on bone [130]. Schwartz et al. [131] utilized data from the Diabetes Prevention Program Outcome Study, which assessed BMD via DXA approximately 16 years after participants were randomized to either intensive lifestyle intervention, metformin, or placebo. Despite significant weight loss in both the metformin and lifestyle intervention groups, neither group experiences bone loss over time, and femoral neck BMD was slightly higher in these groups compared to the placebo group.

However, the effects of incretin-based anti-diabetes therapies on bone health are mixed. Dipeptidyl peptidase 4 (DPP-4) inhibitors have shown inconsistent effects on fracture risk, but GLP-1 receptor agonists are associated with a reduced risk of fractures [132, 133]. In a 26-week randomized placebo-controlled trial evaluating the effects of liraglutide on bone outcomes in adults with T2D, Hygum et al. [134] reported hip bone loss in participants assigned to placebo, but not in those receiving liraglutide. The authors attributed the preservation of bone density in the liraglutide group to its antiresorptive properties. Semaglutide, a recently FDA-approved GLP-1 receptor agonist for weight loss [135], has been shown to improve glucose regulation, , although its effects on bone health have not yet been thoroughly investigated. Since weight loss is typically accompanied by bone loss, it is crucial to assess the impact of Semaglutide on bone health and fracture risk in individuals with obesity and T2D.

Significant advancements have been made in bone health research over the past several decades, which has provided new insights into the subclinical effects of metabolic

conditions on bone. However, substantial knowledge gaps remain. This dissertation aims to address some of these gaps by explored the incretin-mediated effects on bone metabolism in healthy adults, individuals with diabetes, and adults with glucose-intolerance CF.

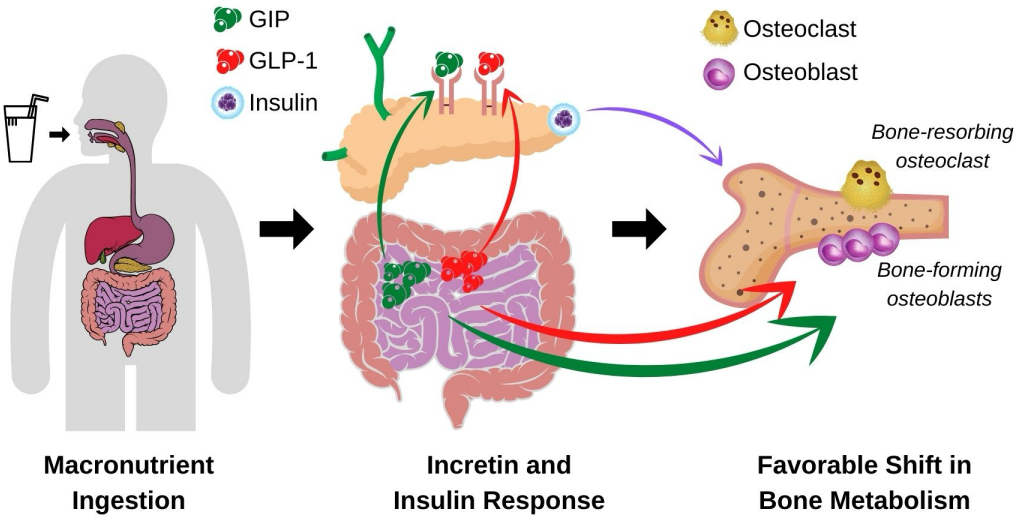

Figure 2.1. Schematic depicting the hypothesized mechanism of the gut-bone axis.

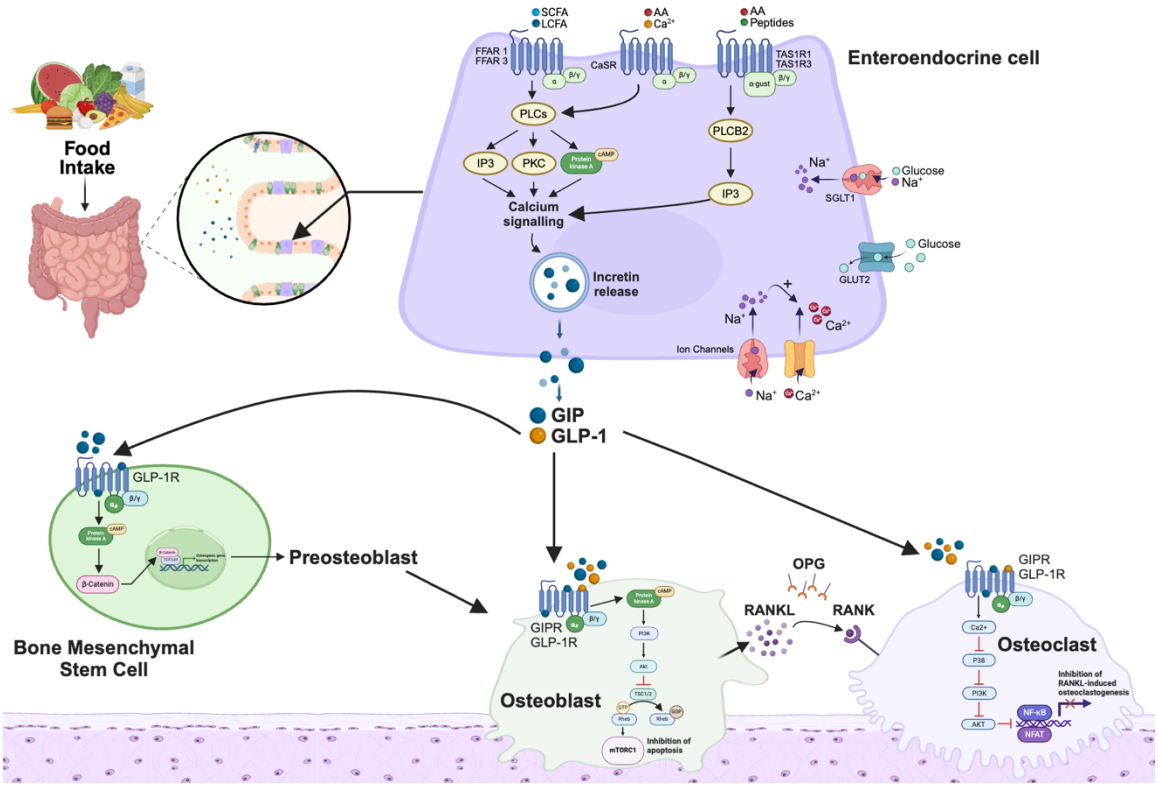

Figure 2.2. Schematic depicting the hypothesized molecular mechanism of incretins on osteoblasts and osteoclasts.

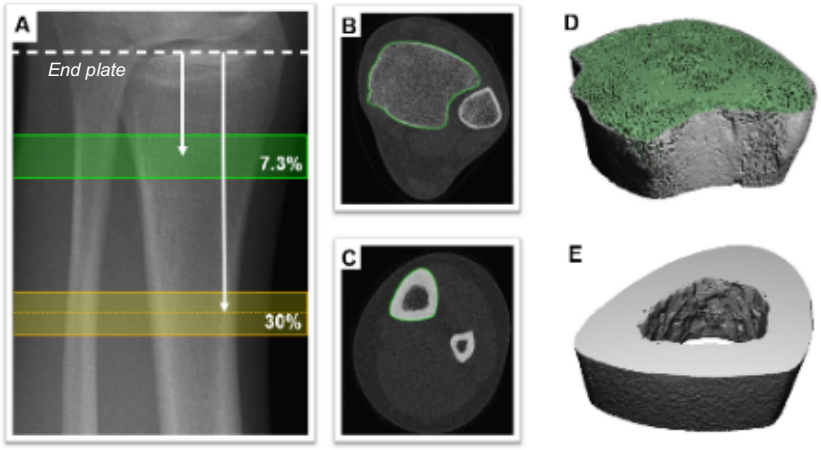

Figure 2.3. Schematic of HR-pQCT "scout view" (A), cross-sectional images of trabecular (B) and cortical (C) sites, and 3-dimensional image of trabecular (D) and cortical (E) sites.

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CHAPTER 3

BONE RESORPTION AND INCRETIN HORMONES FOLLOWING GLUCOSE INGESTION IN HEALTHY EMERGING ADULTS [1]

[1] Lei WS, Rodrick EB, Belcher SL, Kelly A, Kindler JM. 2023. Journal of Clinical and Translational Endocrinology. Reprinted here with permission of the publisher.

Abstract

Background: Studies in adults indicate that macronutrient ingestion yields an acute anti-resorptive effect on bone, reflected by decreases in C-terminal telopeptide (CTX), a biomarker of bone resorption, and that gut-derived incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), facilitate this response. There remain knowledge gaps relating to other biomarkers of bone turnover, and whether gut-bone cross-talk is operative during the years surrounding peak bone strength attainment. This study first, describes changes in bone resorption during oral glucose tolerance testing (OGTT), and second, tests relationships between changes in incretins and bone biomarkers and bone micro-structure. **Methods:** We conducted a cross-sectional study in 10 healthy emerging adults ages 18-25 years. During a multi-sample 2-hour 75g OGTT, glucose, insulin, GIP, GLP-1, CTX, bone-specific alkaline phosphatase (BSAP), osteocalcin, osteoprotegerin (OPG), receptor activator of nuclear factor kappa-β ligand (RANKL), sclerostin, and parathyroid hormone (PTH) were assayed at mins 0, 30, 60, and 120. Incremental areas under the curve (iAUC) were computed from mins 0-30 and mins 0-120. Tibia bone micro-structure was assessed using second generation high resolution peripheral quantitative computed tomography. **Results:** During OGTT, glucose, insulin, GIP, and GLP-1 increased significantly. CTX at min 30, 60, and 120 was significantly lower than min 0, with a maximum decrease of about 53% by min 120. Glucose-iAUC$_{0\text{-}30}$ inversely correlated with CTX-iAUC$_{0\text{-}120}$ (rho=-0.91, P<0.001), and GLP-1-iAUC$_{0\text{-}30}$ positively correlated with BSAP-iAUC$_{0\text{-}120}$ (rho=0.83, P=0.005), RANKL-iAUC$_{0\text{-}120}$ (rho=0.86, P=0.007), and cortical volumetric bone mineral density (rho=0.93, P<0.001). **Conclusions:** Glucose ingestion yields an

anti-resorptive effect on bone metabolism during the years surrounding peak bone strength. Cross-talk between the gut and bone during this pivotal life stage requires further attention.

Introduction

Peak bone mass is achieved around the third decade of life [1], setting the stage for lifelong bone health. Nutrition is a main modifiable factor involved in peak bone mass attainment [2], and endocrine mediators of nutrient metabolism are purported to contribute to these effects [3, 4]. The "entero-insulin axis," for example, involves cross-talk between the gut and the pancreas for regulation of post-prandial macronutrient metabolism [5]. Following food ingestion, K and L cells of the gastrointestinal tract release glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), respectively, which signal the pancreatic beta cells to promote insulin production and pancreatic alpha cells to decrease glucagon production in a glucose-dependent manner [6]. Beyond their well-defined actions in glucose control, these gut-derived hormones, referred to as incretins, also regulate bone turnover [7, 8].

The integral cellular machinery involved in bone metabolism, osteoblasts and osteoclasts, possess membrane-bound receptors for GIP and GLP-1 [9-12]. Clinical studies in adults report greater decreases in bone resorption, measured via CTX, following oral vs. intravenous glucose administration, despite a similar glycemic response [13-15]. Intravenous infusion and subcutaneous injection of incretin hormones results in a bone anti-resorptive effect [15-19]. These collective findings support a gut-mediated mechanism underpinning nutrition effects on bone. Bone biology during the years surrounding peak bone mass attainment is unique to that of the adult skeleton [20]. Bone modeling, a process involving the independent action of the osteoblasts and osteoclasts to enhance bone size, mass, and strength, is dominant in adolescence [21], whereas bone remodeling, a process involving the coordinated action of the osteoblasts

and osteoclasts to maintain mineral homeostasis, is dominant in the ageing skeleton [22]. To this point, clinical studies involving incretin hormones and bone metabolism (i.e., the "gut-bone axis") have primarily focused on adults. As such, studies in individuals experiencing the adolescent-to-adult transition are required to confirm that the gut-bone axis is operative during the important life stage of peak bone mass and peak bone strength attainment.

The Bone Health and Osteoporosis Foundation (BHOF), formerly the National Osteoporosis Foundation, sponsored a 2017 summary statement on lifestyle factors in peak bone mass [23]. This report highlighted the importance of nutrition in peak bone mass attainment and identified critical knowledge gaps in this field of study. Notably, clinical studies defining the intermediary biological mechanisms in nutrition effects on bone and focused on the adolescent to young adult transition were highlighted as critically needed areas of pursuit. To address these needs, we conducted a cross-sectional study in 10 healthy adolescents and young adults ages 18 to 25 years. Our primary aim was to determine normal changes in bone resorption during 2-hour 75g multi-sample oral glucose tolerance testing (OGTT). OGTT was used to streamline comparisons to prior studies that followed similar approaches [13, 14, 16, 17]. The primary outcome of interest was CTX, which is a biomarker of bone resorption that has been reported in numerous adult studies describing bone anti-resorptive effects of glucose ingestion [13-17, 24-27]. Based on these previous studies, our *a priori* hypothesis was that CTX would decrease significantly by min 120 of OGTT. Earlier clinical studies involving the gut-bone axis have mainly focused on CTX and procollagen 1 intact N-terminal propeptide (P1NP) as biomarkers of bone resorption and formation, respectively. For this reason, the extent to

which other biomarkers of bone metabolism and/or bone-derived factors are responsive to OGTT is unclear. [28, 29]. Our secondary aims were to determine relationships between glucose, insulin, and incretin hormones and 1) changes in biomarkers of bone turnover during OGTT and 2) measures of cortical and trabecular bone morphology assessed via second generation high resolution peripheral quantitative computed tomography (HR-pQCT).

Methods

Study design and participants

We enrolled a sample of 10 healthy adolescents and young adults to participate in this cross-sectional study. This desired sample size was based on previously published results from our lab [30] and others [16, 17, 25], indicating that a sample size of n=10 would provide >90% power to observe an approximately 50% decrease in CTX between mins 0 and 120 of OGTT.

Subjects were ages 18 to 25 years, absent of chronic diseases or growth disorders, and had a self-reported body mass index (BMI; kg/m^2) in the 'healthy weight' range. Healthy weight status was based on age-specific cutoffs using BMI-for-age percentile for individuals ages 18 to 19 years [31] and BMI for individuals for individuals ages 20-25 years [32]. Potential subjects were excluded if they recently sustained a fracture or were taking medications known to influence bone metabolism. Subjects participated in two laboratory visits. The OGTT was held at the UGA Clinical and Translational Research Unit, whereas the questionnaires, anthropometric measurements, DXA, and HR-pQCT were completed at the UGA Bone and Body Composition Laboratory. Both laboratory visits were completed within 22 days of one another. Prior to participating in the study,

all subjects provided written informed consent. The Institutional Review Board for Human Subjects at The University of Georgia approved all study protocols and procedures.

Anthropometry

Standing height and weight were measured using a wall-mounted stadiometer and digital scale, respectively. BMI was calculated, and for subjects <20 years of age, BMI-for-age percentile was calculated [33]. All anthropometric measurements were collected in triplicate and averaged by a single trained researcher.

Oral glucose tolerance test

Subjects completed a multi-sample 2-hour OGTT on the morning following an overnight fast. A fasting blood specimen was collected (min 0), at which point subjects were instructed to drink a beverage containing 75g of glucose (Trutol) over a period of 10 minutes. Using an indwelling intravenous catheter, additional blood specimens were collected at mins 30, 60, and 120. Serum samples were collected using tubes pre-treated with EDTA, and plasma samples were collected using tubes pre-treated with protease inhibitors.

Blood biochemistries

Glucose, insulin, total GIP, active GLP-1, CTX, BSAP, osteocalcin, osteoprotegerin (OPG), receptor activator of nuclear factor kappa-β ligand (RANKL), sclerostin, and parathyroid hormone (PTH) were assayed at mins 0, 30, 60, and 120 of OGTT. Glucose, CTX, and BSAP assays were performed at Athens-Piedmont Medical Center, and insulin, GIP, GLP-1, osteocalcin, OPG, RANKL, sclerostin, and PTH were assayed at the University of Georgia College of Veterinary Medicine Cytometry Core.

Serum glucose was measured via spectrophotometry using a Beckman Coulter AU5800 clinical chemistry analyzer (Beckman Coulter, Brea, CA). Serum CTX and BSAP were assayed via immunoassay using the Roche Cobas 602 (Roche Diagnostics, Basel, Switzerland) and Beckman Coulter DxI 800 (Beckman Coulter, Brea, CA), respectively. Insulin, GIP, and GLP-1 were assessed in duplicate via a magnetic bead-based multiplex platform (Millipore, HMHEMAG-34-K). Osteocalcin, OPG, sclerostin, and PTH were assessed in duplicate via a magnetic bead-based multiplex platform (Millipore, HBNMAG-51K), and RANKL was assessed using a single plex assay (Millipore, HRNKLMAG-51K).

Calculations

Data from mins 0, 30, 60, and 120 for each outcome of interest were used to calculate incremental area under the curve (iAUC) for each measure. As an example, iAUC from mins 0 to 120 for CTX is abbreviated as CTX-iAUC$_{0\text{-}120}$. iAUCs capturing the 'early phase' response (OGTT mins 0-30) were also calculated. As an example, iAUC from mins 0 to 30 for GLP-1 is abbreviated as GLP-1-iAUC$_{0\text{-}30}$.

Dual-energy X-ray absorptiometry

Total body, lumbar spine, and non-dominant forearm dual-energy X-ray absorptiometry (DXA) scans were performed using a Hologic Horizon densitometer (Hologic, Inc.). Scans were performed and analyzed by a single trained research assistant using APEX software version 2.1. In our lab, total body BMD and lumbar spine BMD showed strong reliability in n=32 healthy adults (CVs <1%). BMD Z-scores were computed using published reference ranges from the Bone Mineral Density in Childhood

Study [34]. Since these reference ranges terminate at the age of 20 years, subjects >20 years of age were assigned the age of 20 for Z-score calculations.

High resolution peripheral quantitative computed tomography

The Scanco XtremeCT II HR-pQCT scanner (SCANCO Medical AG) was used for assessment of tibia cortical and trabecular bone characteristics **(Figure 1)**. A single trained research assistant performed and analyzed all scans. First, lower leg length was measured using a sliding caliper from the distance of the medial malleolus to the tibial plateau. Measurements were completed on the non-dominant leg, determined by asking the subject which leg they would use to kick a soccer ball. Next, a scout view scan was completed. The reference line was manually placed at the proximal edge of the distal end plate. Scans were acquired at a fixed offset distance (22.5 mm proximal to the reference line), as previously described by *Whittier* et al [35], and at a relative offset distance (30% relative to the reference line). A series of 168 parallel slices were collected, using a 10.2 mm image stack and 61 mm isotropic voxel size, centered at the fixed and 30% sites proximal to the reference line. At the fixed site, total volumetric BMD (Tt.vBMD), trabecular area (Tb.Ar), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and the bone volume to total volume fraction (BV/TV) were assessed. At the 30% site, cortical volumetric BMD (Ct.vBMD), cortical area (Ct.Ar), cortical thickness (Ct.Th), intra-cortical porosity (Ct.Po), and cortical pore diameter (Ct.Po.Dm) were assessed. Following scan acquisition, the quality of each scan was graded from a scale of 1 (excellent quality) to 5 (poor quality) using the method described by *Whittier* et al [35]. A priori, it was determined that only scans that received a score of 1 to 3 would be included in final analyses.

Statistical analyses

Statistical analyses were performed using STATA version 15. All data were visually inspected for outliers and biologically implausible data points, which were subsequently excluded from the dataset prior to conducting analyses. Descriptive characteristics were summarized using mean/standard deviation for continuous variables, and count (percentage) for categorical variables.

Changes in biomarkers of bone metabolism, incretin hormones, insulin, and glucose during OGTT were evaluated using linear mixed-effects regression ("mixed" command in STATA). Separate analyses were performed for each outcome of interest. For each analysis, min 0 was used as the reference time point against which subsequent time points were compared. Spearman rank order correlation was used to assess associations between iAUCs for biomarkers of bone metabolism, incretins, insulin, and glucose, and bone outcomes from DXA and HR-pQCT. All analyses described above were repeated while excluding the two male subjects to eliminate potential confounding of sex. For all analyses, P-values < 0.05 were considered statistically significant.

Results

Descriptive characteristics

Descriptive statistics are presented in **Table 1**. The study sample was 80% female and 10% Black, with an average age of about 22 years and an average BMI of about 23 kg/m^2. All subjects had a fasting glucose less than 100 mg/dL and a 2-hour glucose less than 140 mg/dL, indicating normal glucose control as defined by the American Diabetes Association [36].

Changes in insulin, incretins, and bone biomarkers during OGTT

Changes in glucose, insulin, and incretins during OGTT are presented in **Figure 2** and changes in bone biomarkers during OGTT are presented in **Figure 3**. Glucose, insulin, GIP, and GLP-1 increased significantly during OGTT and reached a peak at min 30. Whereas glucose and GLP-1 returned to min 0 values by min 120, insulin and GIP at min 120 remained greater than min 0. With respect to biomarkers of bone metabolism, only CTX changed significantly during OGTT. CTX at min 30 (P=0.011), 60 (P<0.001), and 120 (P<0.001) was significantly lower than min 0. By min 30, 60, and 120, CTX decreased by approximately 20%, 30%, and 53% compared to min 0, respectively. Sensitivity analyses excluding the two male subjects revealed similar effects of glucose ingestion on CTX. By mins 30, 60, and 120, CTX decreased by approximately 19% (P=0.030), 36% (P<0.001), and 52% (P<0.001) compared to min 0, respectively, when excluding the two male subjects.

Correlations between incretins and bone biomarkers during OGTT

Bivariate correlations between glucose, insulin, incretins, and bone biomarkers were assessed using Spearman rank correlation (**Table 2**). Glucose-iAUC$_{0\text{-}30}$ was inversely correlated with CTX-iAUC$_{0\text{-}120}$ (**Figure 4**). GLP-1-iAUC$_{0\text{-}30}$ was positively correlated with BSAP-iAUC$_{0\text{-}120}$ and RANKL-iAUC$_{0\text{-}120}$ (**Figure 5**). When excluding the two male subjects, the inverse correlation between glucose-iAUC$_{0\text{-}30}$ and CTX-iAUC$_{0\text{-}120}$ (rho=-0.905, P=0.002) and the positive correlation between GLP-1-iAUC$_{0\text{-}30}$ and RANKL-iAUC$_{0\text{-}120}$ (rho=0.829, P=0.0416) remained significant, but the correlation between GLP-1-iAUC$_{0\text{-}30}$ and BSAP-iAUC$_{0\text{-}120}$ was attenuated (rho=0.643, P=0.119).

Correlations between incretins and HR-pQCT bone outcomes

Overall, HR-pQCT scans were of high quality. For the tibia trabecular bone region (22.5 mm from the distal end plate), five scans received a grade of 1 and three scans received a grade of 2. One scan received a grade of 4 and was excluded from analyses. For the 30% tibia, eight scans received a grade of 1 and two scans received a grade of 2.

Spearman correlations between glucose, insulin, GIP, and GLP-1 iAUCs and bone outcomes from DXA and HR-pQCT are presented in **Supplemental Table 1**. Glucose, insulin, and GIP iAUCs did not correlate with DXA or HR-pQCT bone measures. However, GLP-1-iAUC$_{0\text{-}30}$ was positively correlated with Ct.vBMD (rho=0.93, P>0.001; **Figure 6)**. After excluding the two male subject, the association between GLP-1 iAUC and Ct.vBMD remained significant (rho=0.89, P=0.007).

Discussion

This study fills important knowledge gaps relating to the gut-bone axis during the critical years of peak bone mass and peak bone strength attainment [13-17, 24-27, 37-39]. Our results reveal that glucose ingestion yields a rapid, acute decrease in bone resorption, as indicated by a significant reduction in CTX. Although other biomarkers of bone metabolism did not change significantly during OGTT, GLP-1 response correlated with changes in BSAP and RANKL during OGTT and with tibia Ct.vBMD assessed via HR-pQCT. The current study is the first to support a bone anti-resorptive effect of glucose ingestion and potential involvement of incretin hormones in emerging adults. While these results align closely with prior studies in older adults [13-17, 24-27], they also help to expand our current knowledge on the involvement of incretin hormones in peak bone strength.

The primary aim of this study was to assess changes in bone resorption during OGTT. In agreement with our *a priori* hypothesis, CTX, which is a biomarker of bone resorption [29], decreased significantly by min 120 of OGTT. Whereas a decrease in CTX is consistent with numerous previously published studies in healthy adults [13-17, 24-27], as well as a recently published study in individuals ages 14 to 30 years with pancreatic insufficient cystic fibrosis (CF) [30], the current study is the first to report these effects in healthy adolescents and young adults, which coincides with the typical period of peak bone mass and peak bone strength attainment. We observed a ~53% decrease in CTX by min 120 of OGTT, which is comparable to prior studies in healthy adults that report a relatively consistent ~50% decrease in CTX by min 120 of OGTT [13-17, 25-27]. Since this study was not designed to compare effects of glucose ingestion on bone metabolism at varying stages across the lifespan, future adequately powered studies are warranted to address this knowledge gap.

In contrast to the well-characterized associations between glucose ingestion and bone resorption [13-17, 24-27], effects on bone formation are less clear. While some studies reported that a standard 75g OGTT significantly decreased P1NP [24, 25], a common biomarker of bone formation [40], others reported that bone formation remains unchanged [14, 15, 17, 27]. In our study, we did not assess P1NP since numerous previous studies have consistently reported null associations during 2-hour OGTT [14, 15, 17, 25, 27]. Rather, we evaluated BSAP and total osteocalcin as biomarkers of bone formation, which were unchanged during OGTT. Since CTX was our primary outcome of interest, we might not have had sufficient statistical power to observe effects on other bone outcomes.

Gut-derived incretin hormones, GIP and GLP-1, are proposed regulators of bone metabolism following macronutrient ingestion [15, 37, 41, 42]. Preclinical studies have demonstrated that osteoclasts and osteoblasts express GIP and GLP-1 receptors, and that binding of GIP and GLP-1 to these receptors inhibit bone resorption and promote bone formation [6, 12, 43]. Clinical studies administering exogenous GIP and/or GLP-1 via subcutaneous injection or intravenous infusion consistently report decreases in bone resorption, but mostly null effects on bone formation [15-19]. Observational studies have also reported significant associations between changes in GIP/GLP-1 and CTX during OGTT [15, 16, 27]. In the current study, GIP and GLP-1 response during OGTT did not correlate with CTX, but glucose response was closely related to CTX. Results from a study by Nissen and colleagues help shed light on these findings [44]. These authors compared the independent and combined effects of hyperglycemia (vs. euglycemia) and GIP infusion (vs. saline) on changes in CTX. Hyperglycemia and GIP infusion independently resulted in decreases in CTX, but this effect was more pronounced when GIP infusion was combined with hyperglycemia, suggesting that plasma glucose at least in part influences incretin-mediated bone resorption. In an earlier study from our team [45], increases in GIP correlated with decreases in CTX during OGTT in a sample of young adults with pancreatic insufficient CF, but the majority of participants had either mild glucose dysregulation or diabetes. The participants in the current study were required to have a normal BMI and to be absent of any chronic health conditions. On average, fasting glucose was 81 mg/dL and 2-hour glucose was 93 mg/dL, so subjects have otherwise normal glucose control. Thus, we suspect that the null associations

between incretin hormones and CTX in the current study is partly attributed to the generally normal metabolic health status of our study sample.

In contrast to the null associations between incretins and CTX, we report significant associations between GLP-1 and both RANKL and BSAP. RANKL is an osteoblast-derived cytokine involved in paracrine regulation of bone metabolism [46], and BSAP is a biomarker of bone formation. RANKL promotes osteoclast differentiation, survival, and function, but OPG acts as a decoy receptor for RANKL to limit bone resorption [47]. BSAP, RANKL, OPG, and the RANKL to OPG ratio were unchanged during OGTT, so interpretation of associations with GLP-1 is unclear. Although the RANKL/RANK/OPG pathway is a pivotal mechanism involved in bone modeling and remodeling [48], involvement of this mechanism in the gut-bone axis requires further attention. This study was not originally powered to observe changes in OPG or RANKL during OGTT, or associations with incretin hormones, so these preliminary findings require further confirmation.

The well-defined skeletal sexual dimorphism [49, 50] underscores the need for studies that identify sex differences during macronutrient ingestion and the incretin and bone metabolism responses that follow. Unfortunately, our study was not sufficiently powered to compare males and females. In adults, *Fuglsang-Nielsen* et al reported that women have higher fasting CTX and P1NP, but that men and women experience similar changes in bone metabolism following OGTT and mixed meal tolerance test (MMTT) [24]. In contrast, in individuals with pancreatic insufficient CF, changes in CTX during OGTT were greater in males vs. females. With respect to bone morphology, the differences in bone structure and strength between males and females are substantial.

Males tend to have a more robust trabecular bone network and larger cortex compared to females [51, 52], but cortical bone density tends to be greater in females vs. males [53]. Since 20% of our study sample was male, we performed sensitivity analyses excluding male subjects to minimize potential confounding of sex. Overall, associations between incretins and bone metabolism during OGTT remained significant after excluding the male subjects. In our total sample, GLP-1 correlated positively with tibia Ct.vBMD, and this association was also maintained in sensitivity analyses including female subjects only. GLP-1 was also marginally associated with lower metrics of cortical bone porosity. Overall, the results of these sensitivity analyses suggest that our main findings were likely not attributed to sex confounding. Since distinct differences in bone biology [52], as well as metabolic response to food intake exist between males and females [54, 55], there is a need to understand sex-related differences with respect to gut-bone cross-talk.

Strengths and limitations

A main strength of this study was our focus on adolescents and young adults around the age of peak bone mass attainment. To this point, all prior studies involving effects of macronutrient ingestion on bone metabolism have exclusively included adults [13-17, 24-27, 37-39]. These prior studies mainly focused on CTX as a biomarker of bone resorption, but we also included alternate biomarkers and bone-derived factors involved in bone turnover. For example, this is the first study to assess RANKL and OPG during OGTT and in relation to incretin hormones. Our results highlight the potential involvement of the RANKL/RANK/OPG pathway in the gut-bone axis, but these preliminary findings warrant additional investigation. Further, assessment of cortical and trabecular bone micro-structure and volumetric density via HR-pQCT addresses critical

needs that were described in two separate reports involving determinants of peak bone mass [23, 56]. In sum, studies using high resolution bone imaging modalities during the adolescent-to-adult transition are warranted to help understand the underpinning biological mechanisms and contributors to peak bone strength attainment.

The main limitation of this study was our small sample size and cross-sectional design, which limits inference of causality. We were sufficiently powered for our primary aim, which was to test differences in CTX between mins 0 and 120 of OGTT. However, the small sample size likely limited our ability to observe changes in bone formation, or to detect associations between incretins, bone biomarkers, and bone density and morphology. Our sample size also precluded us from testing for interactions between glucose, insulin, and incretin hormones in relation to CTX, and from comparing effects between males and females. Sensitivity analyses excluding the two male subjects yielded similar results to our main findings, indicating that our results are not attributed to sex confounding. Alternate experimental methods, such as mixed meal tolerance testing, should be considered in future studies to help facilitate translation to free-living conditions. Finally, additional outcomes such as carboxylated and undercarboxylated forms of osteocalcin might provide unique insights into reciprocal actions in gut-bone cross-talk. Total osteocalcin, which was assessed in this study, is considered a biomarker of bone formation, but the undercarboxylated form of osteocalcin is involved in glucose regulation by augmenting insulin production [57]. Thus, potential bi-directional relationships should be explored.

Conclusions

This study addresses an important knowledge gap involving the role of macronutrient ingestion and gut-derived hormones on bone metabolism during the years surrounding peak bone mass attainment. Since younger individuals have otherwise been excluded from studies involving the gut-bone axis, whether findings from adults are translatable to the transitional years from adolescence to young adulthood is unknown. In the current study, glucose ingestion yielded an acute bone anti-resorptive effect that was consistent with findings from prior studies in adults [13-17, 24-27]. Increases in GLP-1, which is an incretin hormone involved in post-prandial insulin production [58], was associated with changes in BSAP and RANKL during OGTT, as well as cortical bone density. These results underscore the need for additional research, specifically involving the gut-bone axis, for the acquisition and maintenance of bone mass across the lifespan. Notably, there is a need for adequately powered studies aimed at comparing effects across race, sex, and age groups, as well as dietary or pharmacologic compounds that might help amplify this process.

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Table 3.1. Participant characteristics.

	Mean ± SD
Age, years	21.8 ± 1.7
Female, n (%)	8 (80)
White, n (%)	8 (80)
Height, cm	165.4 ± 8.0
Weight, lb	140.0 ± 21.0
BMI, kg/m^2	23.2 ± 3.1
Total body BMD, Z-Score	-0.50 ± 1.3
Lumbar spine BMD, Z-Score	-0.29 ± 1.3
1/3 radius BMD, Z-Score	0.02 ± 1.3
Glucose, mg/dL[a]	81.3 ± 6.1
2-hour glucose, mg/dL	92.6 ± 15.7
Insulin, pg/mL[a]	1066.7 ± 1462.5
GIP, pg/mL[a]	84.1 ± 41.2
GLP-1, pg/mL[a]	4.6 ± 3.5
CTX, pg/mL[a]	491.6 ± 130.3
BSAP, mcg/L[a]	8.9 ± 1.8
Osteocalcin, pg/mL[a]	25803.1 ± 17367.9
OPG, pg/mL[a]	394.4 ± 184.1
RANKL, pg/mL[a]	135.6 ± 116.5
Sclerostin, pg/mL[a]	2173.5 ± 824.1
PTH, pg/mL[a]	61.02 ± 24.7

BMI, body mass index; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide 1; CTX, C-terminal telopeptide, BSAP, bone-specific alkaline phosphatase; OPG, osteoprotegerin; RANKL, nuclear factor kappa-β ligand; PTH, parathyroid hormone.
[a]Fasting measure from min 0 of OGTT.

Table 3.2. Spearman correlation between iAUCs for glucose, insulin, GIP, and GLP-1 from minutes 0-30 and biomarkers of bone metabolism from minutes 0-120.

	Glucose		**Insulin**		**GIP**		**GLP-1**	
	Rho	P	Rho	P	Rho	P	Rho	P
CTX	-0.91	<0.001	-0.43	0.244	-0.14	0.701	0.58	0.099
BSAP	-0.41	0.243	0.12	0.765	0.56	0.090	0.83	0.005
OPG	0.21	0.555	0.20	0.606	0.41	0.244	0.35	0.356
Osteocalcin	0.16	0.651	0.10	0.798	0.13	0.726	0.00	1.000
Sclerostin	0.09	0.815	0.40	0.286	0.62	0.054	0.35	0.356
PTH	0.06	0.868	-0.03	0.932	-0.18	0.627	0.13	0.732
RANKL	-0.63	0.070	0.12	0.779	0.17	0.668	0.86	0.007
RANKL to OPG Ratio	-0.12	0.779	0.38	0.352	-0.33	0.420	-0.12	0.779

Supplemental Table 3.1. Spearman correlation between iAUCs for glucose, insulin, GIP, and GLP-1 from minutes 0-30 and DXA and HR-pQCT-derived outcomes.

	Glucose		**Insulin**		**GIP**		**GLP-1**	
	Rho	P	Rho	P	Rho	P	Rho	P
DXA								
Total body BMD	0.04	0.920	-0.17	0.668	-0.44	0.200	0.10	0.798
Lumbar spine BMD	-0.10	0.776	-0.10	0.798	-0.21	0.556	0.35	0.356
1/3 radius BMD	-0.07	0.841	-0.42	0.265	-0.36	0.310	0.08	0.831
HR-pQCT								
Trabecular bone (22.5 mm)								
Tb.vBMD	-0.23	0.544	-0.43	0.289	-0.10	0.798	0.29	0.493
BV/TV	-0.20	0.604	-0.45	0.260	-0.12	0.765	0.29	0.493
Tb.N	0.01	0.983	-0.17	0.693	0.45	0.224	0.48	0.233
Tb.Th	0.23	0.554	-0.19	0.647	-0.51	0.156	-0.46	0.254
Tb.Sp	0.14	0.715	0.36	0.385	-0.37	0.332	-0.48	0.233
Cortical bone (30% tibia)								
Ct.vBMD	-0.60	0.066	-0.03	0.932	0.13	0.726	0.93	<0.001
Tot.Ar	-0.04	0.920	-0.22	0.576	-0.59	0.074	-0.50	0.171
Ct.Ar	-0.04	0.920	-0.37	0.332	-0.39	0.260	-0.42	0.265
Ct.Th	0.10	0.789	0.02	0.966	0.26	0.467	0.08	0.831
Ct.Po	0.16	0.651	-0.37	0.332	-0.52	0.128	-0.63	0.067
Ct.Po.Dm	0.15	0.675	-0.60	0.088	-0.37	0.293	-0.62	0.077

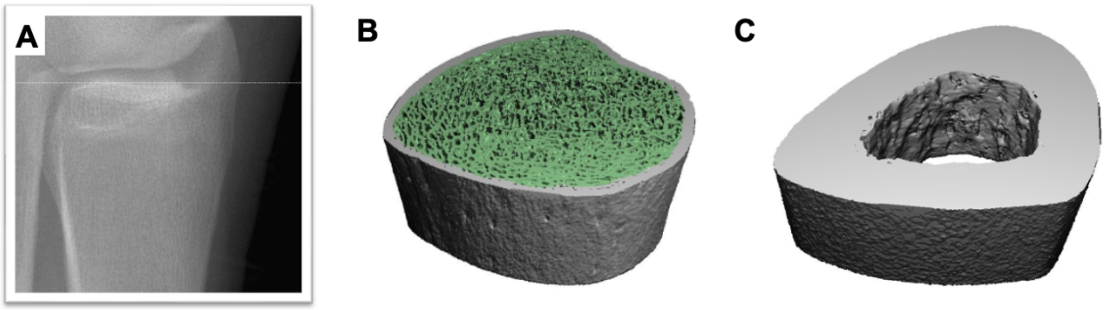

Figure 3.1. "Scout view" scan showing reference line placement (for the 22.5 mm scan region; **A**) and reconstructed 3-dimensional images of trabecular (**B**) and cortical (**C**) bone regions.

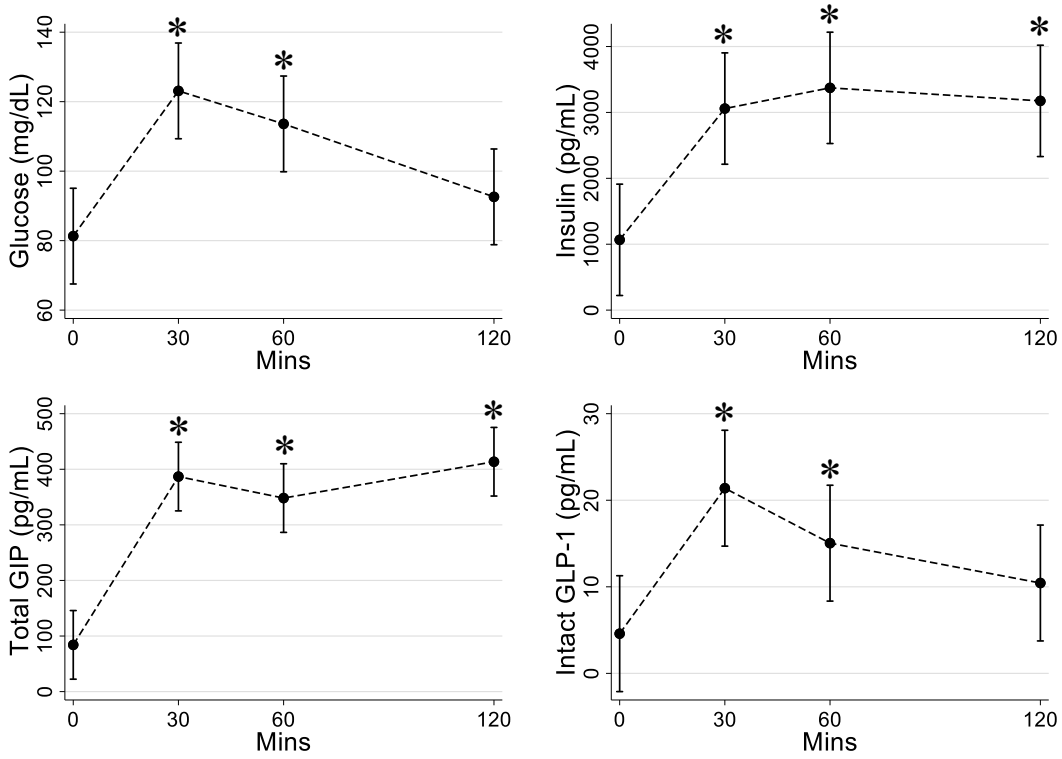

Figure 3.2. Changes in glucose, insulin, total GIP, and intact GLP-1 during OGTT in healthy emerging adults. Error bars indicate standard error. *Significantly different than min 0 ($P<0.05$).

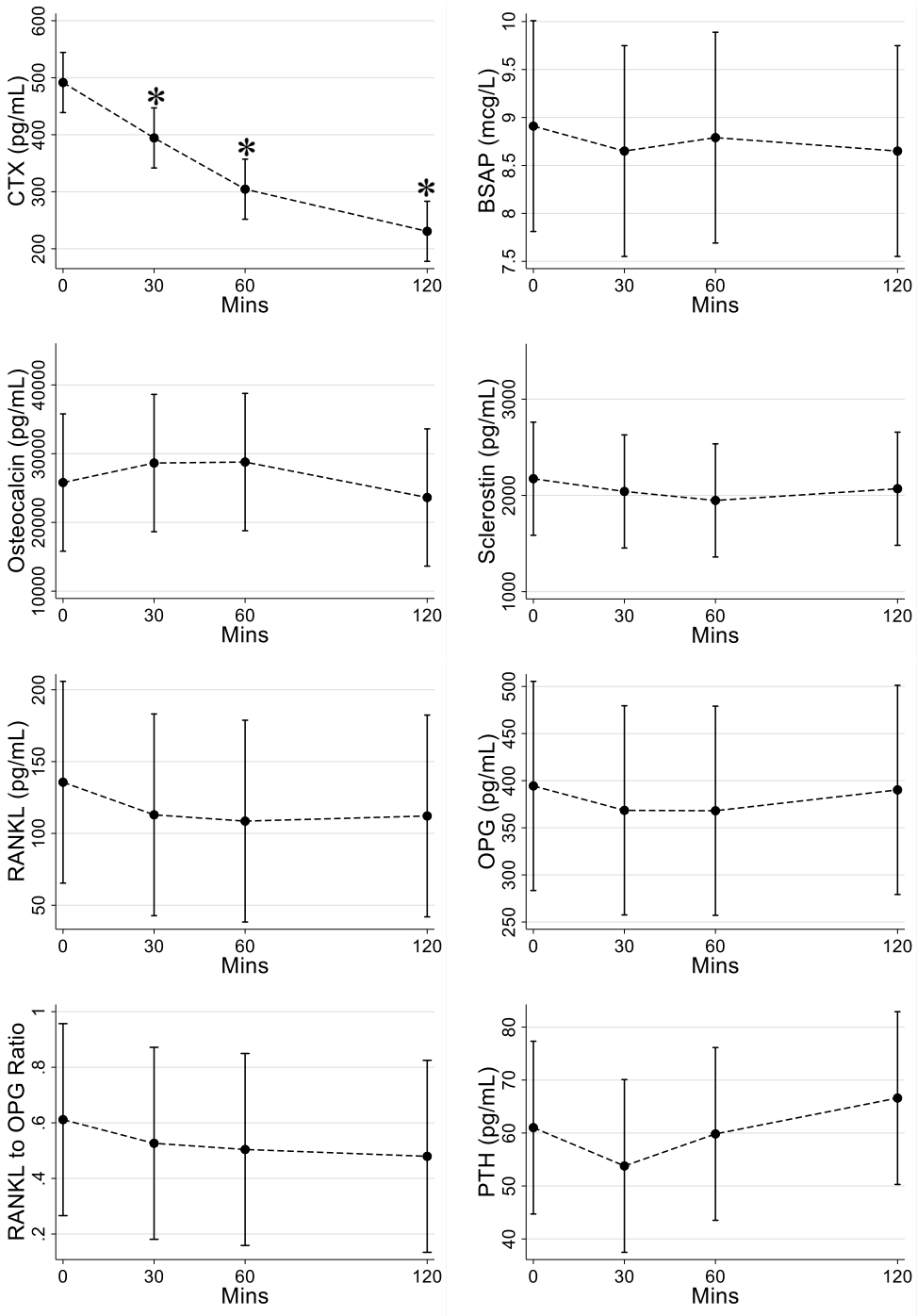

Figure 3.3. Changes in biomarkers of bone metabolism during OGTT in healthy emerging adults. *Significantly different than min 0 (P<0.05).

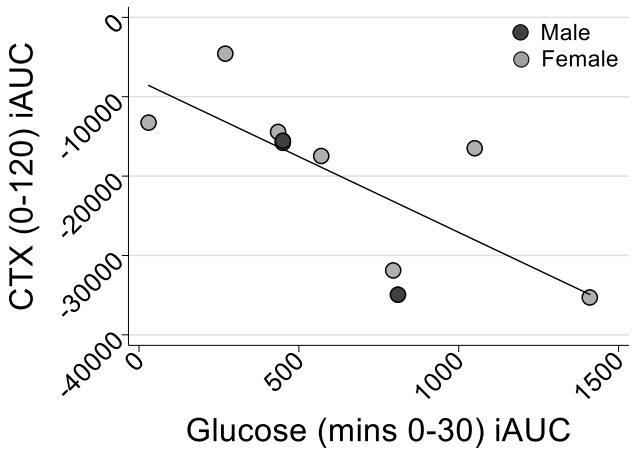

Figure 3.4. Association between glucose-iAUC$_{0\text{-}30}$ and CTX-iAUC$_{0\text{-}120}$ in healthy emerging adults. Black dots are for males and gray dots are for females.

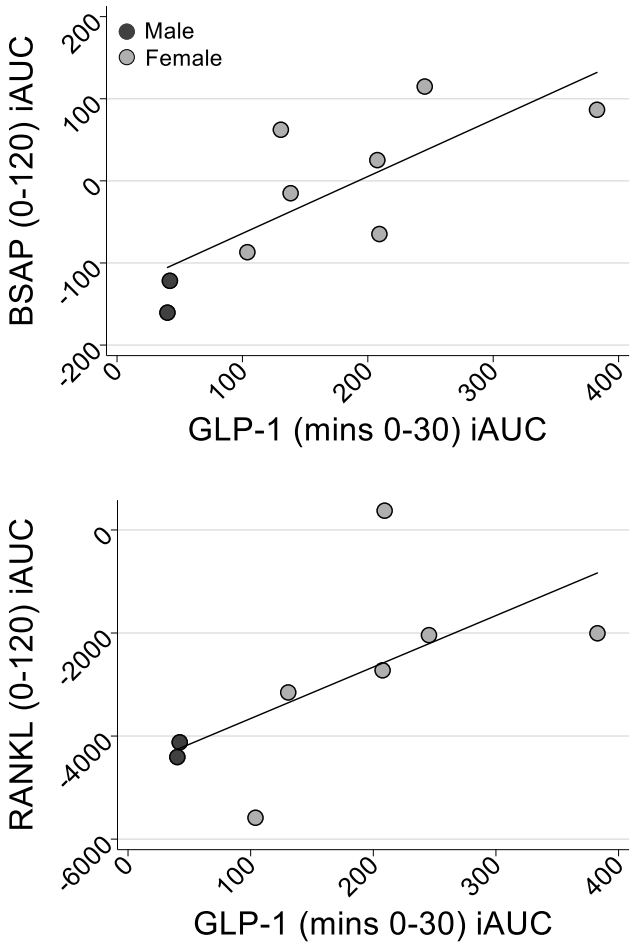

Figure 3.5. Association between GLP-1-iAUC$_{0\text{-}30}$ and BSAP-iAUC$_{0\text{-}120}$ (top) and RANKL-iAUC$_{0\text{-}120}$ (bottom) in healthy emerging adults. Black dots are for males and gray dots are for females.

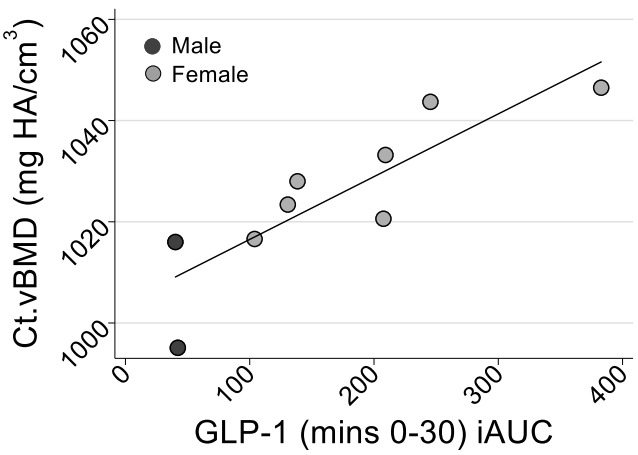

Figure 3.6. Association between GLP-1-iAUC$_{0\text{-}30}$ and Ct.vBMD in healthy emerging adults

CHAPTER 4

GLUCOSE-DEPENDENT INSULINOTROPIC PEPTIDE AND BONE RESORPTION: A SYSTEMATIC REVIEW AND META-ANALYSIS OF RANDOMIZED CONTROL TRIALS[2]

[2] Lei WS, Chen XY, Kelly A, Isales C, Kindler JM. 2024.
To be submitted to *Journal of Clinical and Translational Endocrinology*.

Abstract

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are gut-derived hormones that augment post-prandial insulin production in a glucose-dependent manner but also regulate bone metabolism. Bone-resorbing osteoclasts possess receptors for GIP and GLP-1, referred to as "incretin" hormones and clinical trials generally support a bone anti-resorptive effect of acute incretin administration. To this point, results from these trials have not been systematically evaluated and summarized. We performed a systematic review and meta-analysis summarizing randomized controlled crossover trials testing the effect of GIP/GLP-1 administration (via intravenous infusion or subcutaneous injection) on bone resorption in adults. A systematic search of PubMed was performed for studies published between January 2000-2024 that reported baseline carboxy-terminal collagen crosslinks (CTX) and percent change in CTX relative to baseline following placebo and incretin administration. Among the 168 records that were identified, 24 underwent full-text review, and 7 met the criteria for inclusion in this analysis. Two studies performed GLP-1 administration (n=27) and seven studies performed GIP administration (n=77, 100% male), so a meta-analysis was only performed for GIP. Among the GIP studies, most included healthy subjects, except two that included 22 people with diabetes (n=12 with type 1, n=10 with type 2). Five studies performed intravenous infusion (n=59) and two performed subcutaneous injection (n=18). For each study, baseline CTX and percent change in CTX following GIP and placebo administration were extracted, and a mean difference (MD) between the two treatments was computed. A random-effects meta-analyses were performed using the R package meta. For studies that reported PTH (n=3 studies) and P1NP (n=4 studies)

during GIP administration, additional meta-analyses these measures were also considered as outcomes. GIP administration was associated with a greater decrease in CTX compared to placebo ($P<0.01$), but there was relatively high heterogeneity ($I^2=87.2\%$, $P<0.01$). Comparing the MD between studies of people with diabetes (MD: 0.12, 95% CI: 0.08-0.16) vs. those without diabetes (MD: 0.19, 95% CI: 0.15-0.23), the decrease in CTX following GIP administration was greater in those without diabetes ($P=0.03$). Egger's test revealed an absence of publication bias ($P=0.014$). GIP had a null effect on PTH ($n=30$, $P=0.84$) and P1NP ($n=47$, $P=0.95$). In summary, GIP has a bone anti-resorptive effect, which may be modified in people with diabetes. The involvement of GIP in diabetes-related bone disease requires further attention.

Introduction

Osteoporosis is a condition associated with skeletal fragility and increased risk for fracture [1]. In the United States alone, it is estimated that over 10 million individuals aged 50 and older are living with osteoporosis [2]. Globally, osteoporosis contributes to approximately 9 million fractures annually [3]. The adult skeleton relies on the balanced and coordinated actions of osteoclasts and osteoblasts to resorb and deposit bone mineral [4]. However, this bone remodeling cycle can be disrupted by various factors, including metabolic disturbances such as diabetes [5]. Over 38 million people in the United States have diabetes, and epidemiological studies suggest that people with both type 1 diabetes and type 2 diabetes are at increased risk for fracture [6, 7]. Biological mechanisms underpinning the diabetes-bone connection have yet to be elucidated.

Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1), referred to as "incretins," are peptides that are secreted by enteroendocrine cells to regulate satiety, gastric motility, and metabolism [8]. GIP and GLP-1 bind to G-protein coupled receptors (GIP receptors [GIPR] and GLP-1 receptors [GLP-1R]) on pancreatic beta cells to exert a glucose-dependent release of insulin to facilitate glucose homeostasis [9]. A perturbed entero-insular response has been purported to contribute to diabetes progression [10], and the incretin effect is often absent in people with type 2 diabetes [11]. Beyond the role of GIP and GLP-1 in the regulation of glucose metabolism, the presence of GIPR and GLP-1R on bone-resorbing osteoclasts and bone-forming osteoblasts support their involvement in bone metabolism by augmenting insulin production [12]. Preclinical studies show that bone-resorbing osteoclasts possess GIP receptors, and in-vitro models show that short-term treatment of GIP inhibits

osteoclastogenesis and bone resorption [13]. Additionally, murine studies show that GIP receptor knockout mice results in decreased bone size, lower bone mass, and altered bone microarchitecture [14]. Moreover, GLP-1 activation of GLP-1R, primarily on mesenchymal stem cells, promotes osteoblast differentiation [15]. Recent clinical studies have similarly highlighted the importance of incretin hormones in regulating bone metabolism [12, 16].

Following glucose ingestion, C-terminal telopeptide (CTX) – a biomarker of bone resorption [17], decreases acutely and rapidly [18], suggesting an anti-resorptive effect of glucose on bone turnover. These glucose-mediated effects on bone resorption are more pronounced following oral glucose administration compared to routes of glucose administration that bypass the gastrointestinal tract (e.g., intravenous/injection administration), suggesting that gut-mediated mechanisms are likely involved [19, 20]. In clinical studies, oral glucose tolerance testing (OGTT) in adults is associated with a consistent decrease in CTX by around 50% for one to two hours following ingestion of the glucose solution [19-27]. Additionally, infusion of GIP(3-30)NH2, a selective GIPR antagonist, attenuates post-prandial decreases in bone resorption, demonstrating a key role of GIP in regulating bone-augmenting effects of macronutrient ingestion [28]. These findings highlight the critical role that incretins, namely GIP, exert on bone metabolism, which might have important implications in people with entero-endocrinopathies.

Diabetes, a condition characterized by perturbed incretin and/or insulin responses to nutrient ingestion, may threaten bone health through chronic modification of the gut-bone axis [29]. In both type 2 diabetes and cystic fibrosis-related diabetes (CFRD), the incretin response is notably altered [30]. While CFRD is a unique form of diabetes, it

shared similarities with type 2 diabetes, including incretin dysregulation [31]. In these conditions, the impaired incretin response, especially the diminished secretion of GIP and GLP-1, affects postprandial insulin secretion, thereby impacting bone metabolism [9]. People with diabetes have decreased bone density, altered bone quality, and a heighted risk for fractures [32]. While the link between diabetes and bone health is complex and influenced by various factors such as insulin resistance and inflammation [33], recent studies have reported that people with diabetes have suppressed bone metabolism, where CTX only decreases by about 30% following glucose ingestion compared to the 50% reduction observed in healthy people [34]. That the incretin response is perturbed in diabetes, thus the biological responses of bone to macronutrient and/or food consumption as mediated through incretins might be modified in people with diabetes.

This systematic review and meta-analysis summarizes the effect of GIP infusion on bone resorption, and identifies potential metabolic conditions and experimental protocols that potentially contribute to variations in the pronounced decrease in CTX following incretin administration.

Methods

Design overview

We followed a standardized protocol for meta-analyses according to the Transparent Reporting of Systematic Reviews and Meta-analyses (PRISMA) and reviewed published literature of crossover randomized controlled trials (RCTs). Articles that evaluated for changes in CTX during exogenous administration of GIP were considered for inclusion in the meta-analysis. Additionally, we extracted data for procollagen propeptide 1 (P1NP), a bone formation marker, and parathyroid hormone

(PTH) from studies reporting on these biomarkers. To determine our effect size, we extracted data for CTX (ng/mL), P1NP, and PTH at baseline and percent change from baseline at CTX, P1NP, and PTH nadir during placebo and intervention arms to calculate a mean difference (MD) between groups and compared the MD between groups to derive an effect estimate. The Cochrane risk-of-bias tool for randomized trials (RoB 2, Version 2) was used to assess RCT quality in this meta-analysis.

Inclusion and Exclusion Criteria

To meet our criteria for review, studies had to 1) be crossover, double-blinded, RCTs involving incretin (GIP and/or GLP-1) infusion or injection; 2) include a placebo control arm; 3) report baseline data in ng/mL or equivalent units; and 4) include percent change data for CTX.

Search strategy

We used the following strategy to search the PubMed database: (("parenteral administration" [TIAB] OR "subcutaneous injection*" [TIAB] OR "insulin tolerance test" [TIAB] OR "glucose tolerance test" [TIAB] OR "insulin stimulation test*" [TIAB] OR infusion* [TIAB] OR saline [TIAB] OR "glucose infusion*" [TIAB] OR "s.c. injection" [TIAB] OR "glucose clamp" [TIAB] OR "insulin clamp" [TIAB] OR "intravenous" [TIAB] OR "GIPR" [TIAB] OR "co-agonist*" [TIAB]) AND ("Gastrointestinal Hormone*" [MH] OR "Gastric Inhibitory Polypeptide" [MH] OR "Glucagon-Like Peptide 1" [MH] OR "Glucagon-Like Peptide 2" [MH] OR "incretins" [MH] OR "incretin*" [TIAB] OR "gut hormone*" [TIAB] OR "enteric hormone" [TIAB] OR "gut-bone axis" [TIAB] OR insulin [MH] OR GIP [TIAB] OR GLP-1 [TIAB] OR GLP-2 [TIAB]) AND ("Bone remodeling" [TIAB] OR "Bone turnover" [TIAB] OR

"Biomarker*" [TIAB] OR "Collagen Type I" [MH] OR "procollagen" [MH] or "bone resorption" [MH] OR "bone homeostasis" [TIAB]" OR "Osteoclasts" [MH] or "bone turnover" [TIAB] or "bone resorption" [TIAB] or collagen [TIAB] OR CTX [TIAB] OR P1NP [TIAB] or PTH [TIAB]). We limited the search to studies conducted in humans with an abstract and/or full text that was available in English. Two independent reviewers scanned titles and available abstracts to identify potentially relevant articles. Full-text review and data abstraction were performed by two researchers in parallel, and disagreements were resolved via consensus. For studies that were determined to be eligible for inclusion in this meta-analysis but did not report the necessary data in the publication, corresponding authors were contacted via email.

Effect Size Calculation

Our primary outcome of interest was MD in CTX between GIP and placebo administration. For both the GIP and placebo arms of each study, we extracted baseline values of CTX ± SEM (ng/mL) and percent change ± %CV from baseline CTX. Effect sizes were calculated by converting percent change from baseline into ng/mL units of CTX to represent 'final' value. Next, baseline value of CTX in ng/mL was multiplied by %CV/100 from the percent change value to obtain a standard deviation for the baseline CTX value. For each study, the overall MD in change in CTX, expressed in units of ng/mL, was calculated by subtracting the MD between the GIP infusion and placebo group. To help facilitate interpretation, a MD of zero is indicative of no difference in the change in CTX during GIP vs. placebo administration, and a positive effect size estimate indicates that GIP infusion resulted in a greater decrease in CTX (relative to baseline)

compared to placebo. We also computed MDs utilizing the same approach for our secondary outcomes of interest, P1NP and PTH.

Analytic Plan

We included studies that reported differences in diabetes status, route of GIP administration (i.e., subcutaneous injection or intravenous infusion), duration of infusion, and low and/or high glycemic status, which was achieved via clamp technique. For diabetes status, both type 1 and type 2 diabetes were included. To determine if there was heterogeneity between these studies, we performed a random-effects meta-analysis of MD. We identified subgroups for diabetes status, administration route, glycemic status, and timepoint of CTX nadir (e.g., mins 60 or 90), and treated these groups as binary predictors in our analysis. Interaction tests were performed to test for potential differences between subgroups.

For analyses involving CTX, sensitivity analyses were performed using the leave-one-out method[35] to test whether individual studies contributed to the effect size and subsequent overall heterogeneity. Forest plots for overall and subgroup MD were generated accordingly. Heterogeneity was assessed using the I^2 statistic, and publication bias was assessed by funnel plot and Egger's test. Egger's linear regression test is presented to evaluate asymmetry. For all analyses, $P < 0.05$ was considered statistically significant. All analyses were performed using R version 4.1.1.1.

Results

Search results

Our initial search yielded 168 studies. Title and abstracts were reviewed for relevance by two independent reviewers. The majority of RCTs did not include CTX as

an outcome. Following title and abstract review, 24 articles were included in full-text review. Following full-text review, 24 reports were assessed for eligibility. Of the 24 studies, seven studies were included in our meta-analysis [26, 28, 34, 36-39] . A summary of our search is shown in **Figure 4.1.**

Study characteristics

A summary of the characteristics of the studies that were included in our final analyses is displayed in **Table 4.1.** Overall, a total of 60 subjects were included in the meta-analysis. Per our inclusion criteria, all studies were crossover, double-blinded, RCTs. All studies were conducted in Denmark, and for the placebo arm of the trial, used saline. Studies were published between 2014-2022. Subjects were adult males above 18 years of age. Five RCTs were conducted in healthy males, while two RCTs were conducted in males with type 1 (n=10) or type 2 (n=12) diabetes. Of the seven studies, two administered GIP/saline via subcutaneous injection and five administered GIP/saline via intravenous infusion. For studies that performed GIP/saline administration via intravenous infusion, the infusion rate was relatively consistent across studies. Two studies used a 4 pmol/kg/min dose of GIP for 15 minutes that was followed by 2 pmol/kg/min of GIP for the remaining time, two studies used a consistent 4 pmol/kg/min dose of GIP, and one study used a continuous 1.5 pmol/kg/min dose of GIP. For the two studies [37, 39] that employed subcutaneous injection, GIP was injected at 100 ug/mL. Although the duration of the intervention experiments ranged from 90 to 240 minutes, reported timepoint for CTX nadir was relatively consistent. Relative to the start of the infusion/injection, four studies reported CTX nadir at 90 minutes, two studies [28, 36] reported CTX nadir at 120 minutes, and one study reported CTX nadir at 90 during high

glycemia protocol and 120 minutes during low glycemia protocol. In addition to CTX, studies reported data on other bone-related markers, including P1NP (n=4 studies), PTH (n=3 studies), alkaline phosphatase (n=1 study), osteocalcin (n=1 study), and IGF-1 (n=1 study). Three studies [34, 36, 39] provided descriptive data on PTH and P1NP, but without extractable values and/or corresponding authors could not be reached to provide data, and were therefore excluded from final analyses [34, 36, 39].

Quality assessment

A risk-of-bias diagram is presented in **Figure 4.2.** Overall, studies presented with good quality. All studies demonstrated low risk of bias arising from the randomization process, deviations from intended interventions, missing outcome data, or measurement of outcomes of interest.

Total MD and subgroup analyses for CTX

A summary forest plot for MD between intervention and placebo groups is presented in **Figure 4.3A.** All studies reported a significant MD between treatment and placebo arms. The summary effect estimate indicated that compared with placebo, GIP infusion was associated with a greater decrease in CTX (MD: 0.16, 95% CI: 0.12 to 0.19, $P < 0.01$).

Subgroup analyses were performed based on study characteristics such as diabetes status, glycemic status, administration route, and timepoint of CTX nadir. A subgroup forest plot for MD between intervention and placebo group is presented in **Figure 4.4.** Results showed that diabetes status was a significant predictor of the effect size. In studies that included people without diabetes, the MD between GIP and placebo group was significantly greater (MD: 0.19, CI: 0.15 to 0.23, $P = 0.03$) than the studies that

included people with diabetes (MD: 0.12, CI: 0.08 to 0.16). As such, this indicates that GIP had a greater effect on CTX in people without diabetes vs. people with diabetes. Glycemic status, administration route, and timepoint of CTX nadir were not significant predictors of effect size.

Total MD for P1NP and PTH

A summary forest plot for P1NP and PTH MD between intervention and placebo groups is presented in **Figure 4.3B** and **Figure 4.3C,** respectively**.** All studies reported a non-significant MD between treatment and placebo arms. The summary effect estimate indicated that GIP infusion was not associated with differences in either P1NP (MD: 2.62, 95% CI: -2.60 to 7.84, P = 0.95) or PTH (MD: 0.01, 95% CI: -0.60 to 0.63, P = 0.84).

Homogeneity of results

The significant MD between GIP intervention and placebo for CTX presented with high heterogeneity (Q = 78.30, I^2 = 87.2%, 95%CI: 79.1 to 92.2%, P < 0.01). The between-study heterogeneity variance was estimated at τ^2 = 0.003 (95%CI: 0.001 to 0.009). The prediction interval ranged from *g* = 0.03 to 0.28, indicating a likely significant difference between GIP and placebo intervention. Since results are presented with substantial heterogeneity, sensitivity analysis using the leave-one-out method was performed (**Figure 4.5.)**, which revealed that overall MD between intervention and placebo groups were not affected by the sequential exclusion of individual studies. For P1NP and PTH, the absence of heterogeneity (I^2 = 0% and τ^2 = 0) is likely influenced by the small number of studies that reported on these biomarkers.

Assessment of bias

Based on visual interpretation of the funnel plot presented in **Figure 4.6.**, publication bias for effect sizes could not be ruled out. However, we had a limited number of studies available, with all studies reporting similar sample sizes. As such, results from our funnel plot should be interpreted with caution. As an alternative approach, the Egger's test revealed an absence of publication bias (P = 0.14).

Discussion

People with diabetes are at a up to an increased 3-fold risk of falls and fracture [40]. Recent clinical studies report that GLP-1 receptor agonists may improve bone quality and prevent fractures in patients with diabetes [41], but conflicting findings from others [42, 43] highlight the need for further work in understanding the effects of incretin-based medications on fracture. The primary objective of this systematic review and meta-analysis was to summarize the effect of GIP administration on bone resorption in previously completed RCTs. Our results suggest that CTX decreases significantly following GIP administration compared to saline, but that this effect may be less pronounced in people with diabetes. Since GIP is hypothesized to play a role in the gut-bone axis, a perturbed gut-bone cross-talk may contribute to deranged bone resorption.

Based on seven cross-over RCTs [26, 28, 34, 36-39], we found that GIP infusion/injection consistently decreased CTX compared to saline infusion/injection, indicating that GIP has a bone anti-resorptive effect. This study's results align with other studies that suggest GIP might directly affect bone [13, 44]. Gasbjerg et al demonstrated that co-infusing GIP with the GIP selective receptor antagonist, GIP(3-30)NH_2, diminished the CTX response compared to infusing GIP alone [28]. Moreover, studies in murine models revealed that GIP administration in rat models with osteoporosis

prevented bone loss [45]. In-vitro studies also detected the presence of GIP receptors on osteoclasts [13], and binding of GIP inhibits osteoclast differentiation and function, which could contribute to its anti-resorptive effects. In light of these findings, GIP plays a pivotal role in modulating bone resorption and presents a potential avenue for vulnerable population at heighted risk for poor bone health.

Type 1 diabetes (T1D) and T2D are metabolic conditions associated with pancreatic beta cell derangements and impaired insulin secretion [46]. In people with T2D, glucose-mediated suppression of bone resorption is significantly dampened compared to healthy individuals with normal glucose tolerance [22], and impaired incretin and insulin secretion is hypothesized to contribute to these effects. In this meta-analysis, we stratified our analyses by diabetes status and found that diabetes is associated with a significantly lower effect size compared with healthy people. Since diabetes is also associated with dysregulated insulin and glucose regulation during the post-prandial state [47], suppressive effects of GIP on CTX may be glucose and/or insulin dependent. While our sub-group and analyses show that glycemic status and glucose concentration was not associated with MD in CTX, we did not perform analyses based on insulin concentrations due to unavailable data. Additionally, insulin responses differ during type 1 and type 2 diabetes, with type 1 diabetes typically showing little to no insulin response, especially during advanced stages, while type 2 diabetes may exhibit individually varied responses, which can be present in some individuals and absent in others [48]. This discrepancy in insulin response presents a limitation in our study, since our “diabetes” subgroup included both type 1 and type 2 diabetes. In a clinical trial, Christensen et al reported that a double dose of GIP infusion at 130 pmol/L reduced CTX

by only about 40% in patients with T1D and infusion of GIP led to a reduction in CTX that was significantly less than in healthy people [36]. In-vivo and in-vitro studies also show that diabetes may be linked to osteoclast resistance to GIP [13, 49], and clinical observational studies show that people with diabetes have a less pronounced reduction in CTX following an OGTT compared to healthy individuals [22]. While we had a limited number of studies available for subgroup analysis, as well as a lack of a healthy control group in the RCTs performed in people with diabetes, these studies highlight the potential effects of incretins to modify bone metabolism.

We did not study the dose-dependent effects of GIP on bone, but studies included in this meta-analysis reported GIP concentration at 70-155 pmol/L, which similarly reflects the maximal concentration of plasma GIP released (~125 pmol/L) following meal consumption in healthy adults [50]. In patients with diabetes, the incretin response is dampened following nutrient ingestion, and clinical studies using OGTT methods have only reported about a 30% decrease in CTX patients with T2D, which is considerably less than the decrease previously reported in healthy adults.[19] This study examined a relatively homogenous range of GIP infusion (1.5-4 pmol/L) and injection (100 ug/mL). Dose-dependent effects of GIP on bone resorption warrant investigation, notably in people with medical conditions associated with a diminished incretin effect, such as T2D.

Several other hormones may also drive the antiresorptive effect on bone. Notably, GLP-1 is an incretin hormone secreted from enteroendocrine L cells following nutrient ingestion [51], and membrane-bound receptors for GLP-1 on bone cells may play a role in acute decreases in CTX following macronutrient ingestion [52]. Infusion of GLP-1 also leads to significant decreases in CTX, but a GIP/GLP-1 co-infusion led to an 84%

reduction in CTX compared to a 60-70% reduction when infusion either GIP and GLP-1 alone, suggesting that GIP and GLP-1 may have partially additive bone anti-resorptive effect [26]. Similarly, subcutaneous injection of glucagon-like peptide 2 (GLP-2), while having no effect on glucose-dependent insulin production, has been shown to suppress bone resorption for a prolonged period when co-injected with GIP [37]. In patients with ileostomy, CTX decreased significantly following subcutaneous injection of 1600 ug of GLP-2 [53], also suggesting its antiresorptive effect. However, given the limited number of studies available, we were unable to conduct a meta-analysis investigating the effects of GLP-1 and GLP-2 on biomarkers of bone metabolism.

These findings have potential clinical translation due to the advent of incretin-based therapeutics. While GLP-1 receptor agonists are clinically used for glucose control in individuals with type 2 diabetes [54], as well as chronic weight management [55], the recent FDA approval of Tirzepatide, a dual GIP/GLP-1 receptor agonist, have also shown promising outcomes in treating people with diabetes and/or obesity [56]. Since GIP yields reductions in bone resorption, dual incretin-mimetics may be potential routes for improving skeletal health in patients with diabetes and increased risk of fracture. Clinical studies investigating relationships between incretin-based therapies and bone outcomes have reported an association between GLP-1 receptor agonists and decreased risk for fracture [57]. For example, a 26-week RCT evaluating effects of liraglutide on changes in bone mineral density in adults show that liraglutide, but not placebo, was associated with reduced bone loss which is likely attributed to the incretin-mediated antiresorptive effects [58]. However, others have shown null effects of GLP-1 receptor agonists on falls and fractures [59]. Gut-derived hormones play an important anti-resorptive role and may act

simultaneously to inhibit osteoclast activity potentially by acting on their respective receptors [23], but whether these effects translate towards a therapeutic aspect on bone will require further investigation.

This study has several strengths and limitations. Despite choosing a random effects model to address the heterogeneity in our studies, the variability in study protocols and methodologies between studies limited our ability to interpret findings. For example, duration of the infusion protocols ranged from 90 to 240 minutes, and while some studies used a higher starting GIP dose (4 pmol/kg/min) for the first 15 minutes of infusion, others used a consistent dose (2 pmol/kg/min) throughout the infusion period. Additionally, we were limited by the number of articles available to run additional analyses based on glucoregulatory variables. For example, insulin likely plays a role in the incretin-mediated antiresorptive process, but the lack of studies limited our ability to interpret these results. Additionally, we performed subgroup analyses to test the differences in effect size between studies performing subcutaneous injection of GIP versus intravenous infusion of GIP, but our small study sample likely limited our ability to detect differences between groups. Subject characteristics across studies were also relatively homogenous, where RCTs were performed in mostly healthy, Caucasian males in Denmark. As such, interpretation of our findings to other populations is limited, and we were unable to draw conclusions based on sex, race, age, and/or other medical conditions. Since bone modeling is dominant during the years preceding peak bone mass, and bone remodeling is dominant during adulthood [60], the effect of incretins on bone metabolism is likely dependent on age. Further, other endocrine-related complications beyond type 1 diabetes and type 2 diabetes such as cystic fibrosis-related diabetes and

non-alcoholic fatty liver diseases are likely associated with diminished gut-bone pathway due to a perturbed incretin response [19, 61].

Finally, since CTX is a preferred biomarker of bone resorption [17], all studies reviewed herein included CTX as a the primary outcome of interest. Only a limited number of studies have investigated biomarkers of bone formation in the setting of GIP infusion/injection [19, 20, 25]. Among them, the three studies [34, 36] that assessed P1NP following GIP and placebo administration reported that P1NP levels were not significantly affected by GIP administration. This is notable despite preclinical evidence suggesting that GIP receptor activation is associated with increased P1NP secretion from osteoblastic cells [36]. Furthermore, PTH, which plays a role in regulating bone turnover and calcium homeostasis, was found to be modestly suppressed by GIP in prior research involving patients with T2D [34]. However, the three studies included in our meta-analysis [28, 34, 39], of which had extractable data, showed that PTH levels remained relatively unchanged following GIP infusion. Given the role of PTH in promoting calcium release from bone, a suppressive effect of GIP on PTH could imply a bone-forming effect [36, 62]. Nonetheless, with few studies included in this meta-analysis, further research is needed to detect any significant differences in P1NP and PTH between interventions. Future studies should consider other biomarkers of bone turnover. For example, osteoprotegerin (OPG) and receptor activator of nuclear factor-kB ligand (RANKL) have both been indicated in animal models, showing that GLP-1 is associated with upregulation of OPG mRNA expression and bone formation [63], and that liraglutide, a GLP-1 receptor agonist, decreased the RANKL/OPG ratio in diabetic rats [64], but experimental studies in humans have yet to be conducted.

Conclusion

In summary, GIP has an antiresorptive role on bone in adults. Nutrition is an important modifier in bone health, and the gut-bone axis is hypothesized to play a role in modifying bone resorption. Metabolic conditions such as diabetes are associated with an increased risk of fragility and fracture, and incretin regulation of bone may be involved in these processes. Individuals with type 2 diabetes experience altered responses to GIP and GLP-1, which may impair normal incretin-mediated modulation of bone metabolism, further increasing the risk of skeletal fragility. Data from this systematic review and meta-analysis highlight the need for additional research that continue to study the potential involvement of incretins in defining skeletal integrity.

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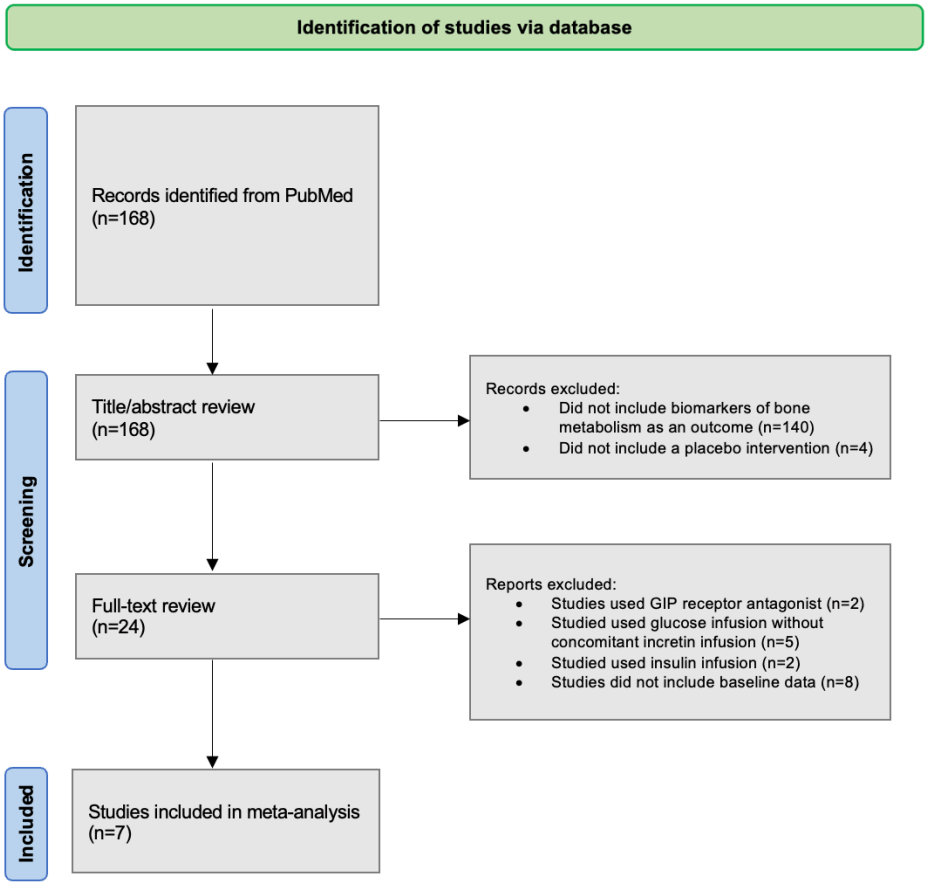


Figure 4.1. PRISMA Diagram

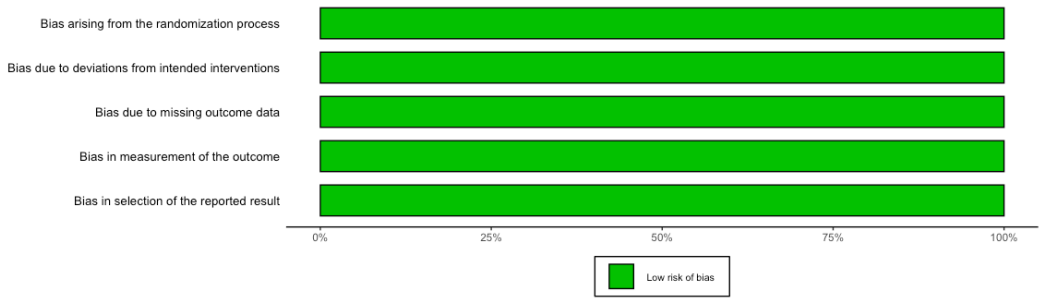


Figure 4.2. Risk-of-bias plot

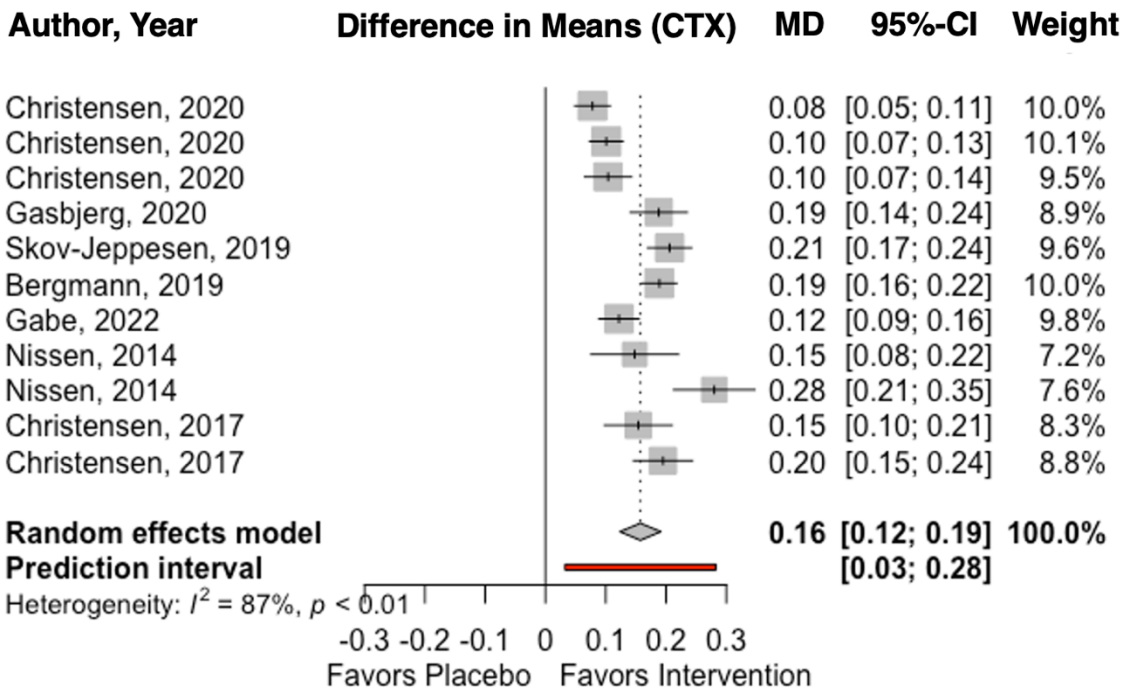


Figure 4.3A. Forest plot of total mean difference in CTX

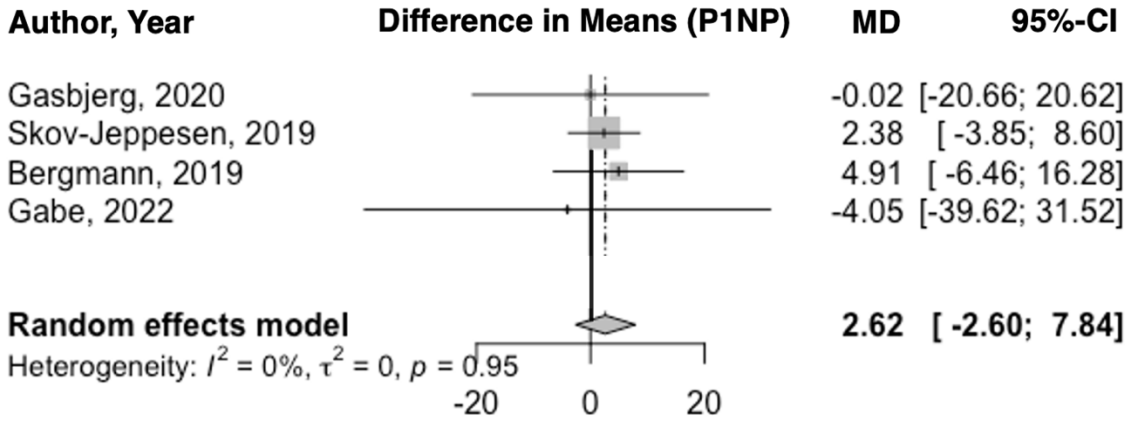


Figure 4.3B. Forest plot of total mean difference in P1NP

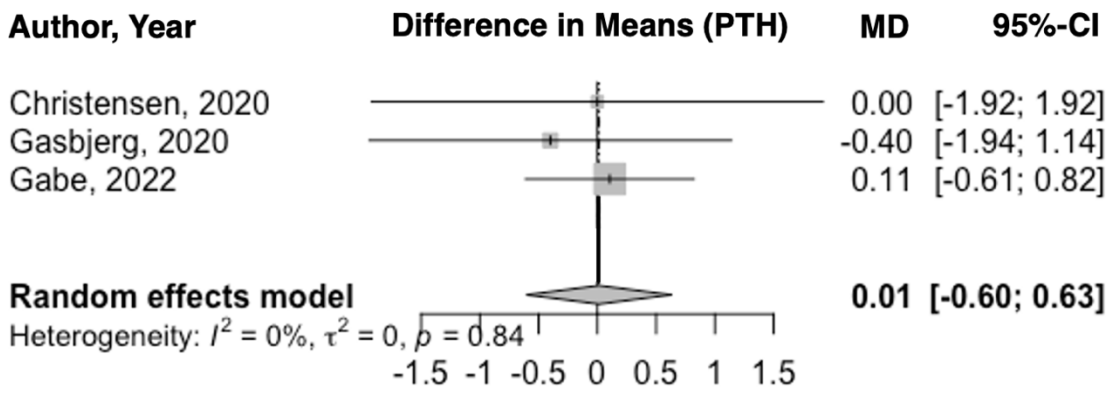


Figure 4.3C. Forest plot of total mean difference in PTH

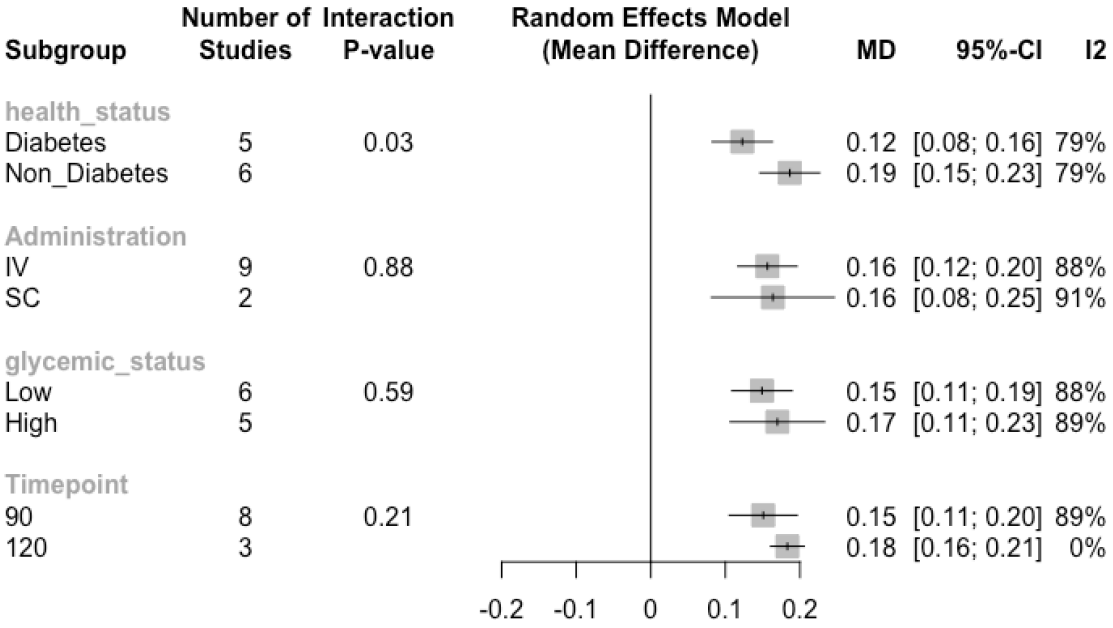


Figure 4.4. Forest plot of subgroup analysis

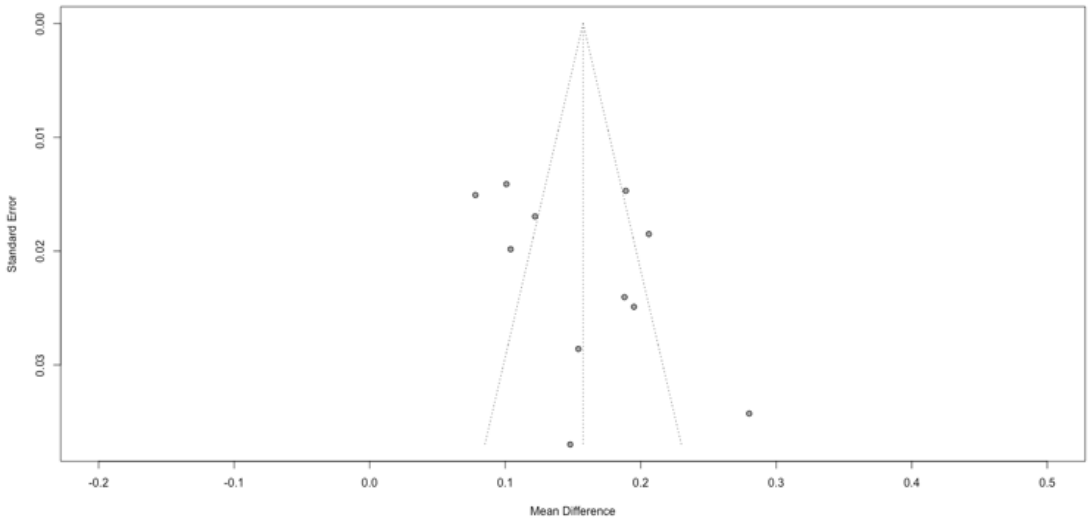


Figure 4.5. Leave-one-out analysis of overall mean difference in CTX

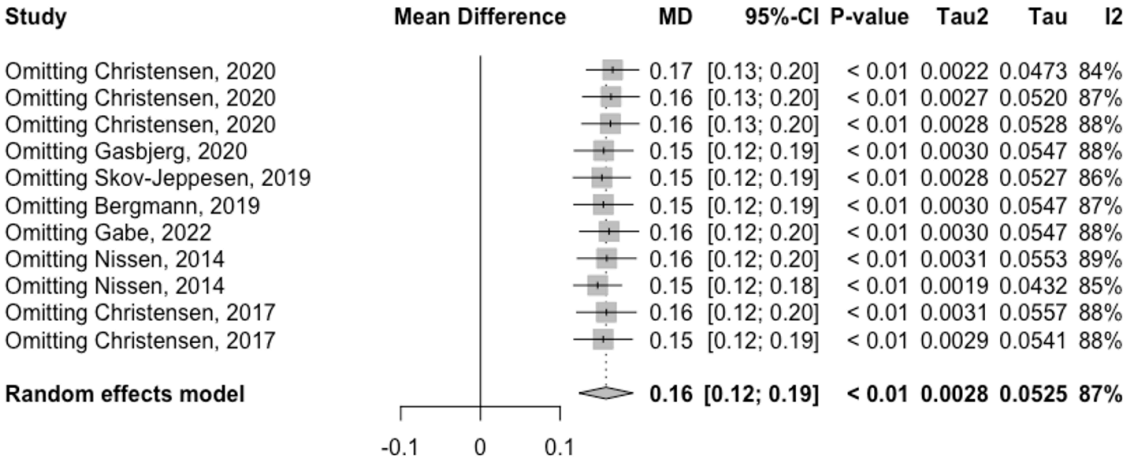


Figure 4.6. Funnel plot of publication bias

Table 4.1. Summary of RCTs included in the Meta-Analysis

								Control			Treatment		
Author, Year	**Country**	**Study Design**	**N**	**Population**	**Female, %**	**Age, Mean (Range), Years**	**Administration**	**Regimen**	**Duration, min**	**% from baseline +/- SD**	**Regimen**	**Duration, min**	**% from/of baseline +/- SD**
Christensen, 2020	Denmark	RCT, crossover	12	T2D	0	Mean +/- SD: 62 +/- 5	IV	saline during insulin-induced hypoglycemia (PG: 3 mmol/L)	90 (nadir: 90)	CTX: Reduced by 12 ± 11% P1NP: Reduced by -5 ± 15% PTH: Reduced by -25 ± 11%	GIP (4 pmol/kg/min for 15 minutes, 2 pmol/kg/min for 75 minutes) during insulin-induced hypoglycemia (PG: 3 mmol/L)	90 (nadir: 90)	CTX: Reduced by 40 ±15% P1NP: Reduced by -9 ± 10% PTH: Reduced by -19 ± 12%
								saline during fasting hyperglycemia (PG: 8 mmol/L)		CTX: Reduced by 0 ± 9% P1NP: Reduced by 2 ± 8% PTH: Reduced by -3 ± 11%	GIP (4 pmol/kg/min for 15 minutes, 2 pmol/kg/min for 75 minutes) during fasting hyperglycemia (PG: 8 mmol/L)		CTX: Reduced by 36 ± 15% P1NP: Reduced by -3 ± 5% PTH: Reduced by 8 ± 14%
								saline during aggravated hyperglycemia (PG: 12 mmol/L)		CTX: Reduced by 10 ± 9% P1NP: Reduced by 1 ± 11% PTH: Reduced by -8 ± 9%	GIP (4 pmol/kg/min for 15 minutes, 2 pmol/kg/min for 75 minutes) during aggravated hyperglycemia (PG: 12 mmol/L)		CTX: Reduced by 47 ± 23% P1NP: Reduced by -3 ± 5% PTH: Reduced by 3 ± 13%
Gasbjerg, 2020	Denmark	RCT, crossover	10	Healthy	0	Mean (range): 22.5 (21-25)	IV	saline during hyperglycemia (12 mmol/L)	CTX: 120 (nadir: 120) P1NP: 40 PTH: N/A	CTX: 81 ± 10% of baseline P1NP: 104 ± 5.4% of baseline PTH: 115 ± 20% of baseline	GIP (1.5 pmol/L) during hyperglycemia (12 mmol/L)	120 (nadir: 120) P1NP: 104 ± 5.4% of baseline PTH: 115 ± 20% of baseline	CTX: 53 ± 6.9% of baseline P1NP: 109 ± 6.7% of baseline PTH: 114 ± 47% of baseline
Skov-Jeppesen, 2019	Denmark	RCT, crossover	8	Healthy	0	Median (range): 27 (20-34)	SubQ	Saline	CTX: 240 (nadir: 90) P1NP: 30 PTH: 15	CTX: 82.3 ± 3.2% of baseline P1NP: 103.1 ± 1.5% of baseline PTH: 71.2 ± 4.0% of baseline	GIP (100 ug/mL)	CTX: 240 (nadir: 90) P1NP: 30 PTH: 15	CTX: 55.3 ± 6.3% of baseline P1NP: 115.1 ± 2.2% of baseline PTH: 103 ± 4.8% of baseline

Bergmann, 2019	Denmark	RCT, crossover	17	Overweight, obese	0	Mean +/- SD: 42 +/- 16	IV	IIGI + Saline	240 (nadir: 120)	CTX: Reduced by 27.8 ± 11.7% P1NP: Reduced by 104 ± 3.39%	IIGI + GIP (4 pmol/kg/min)	240 (nadir: 120)	CTX: Reduced by 74.5 ± 9.2% P1NP: Reduced by 108 ± 5.06%
Gabe, 2022	Denmark	RCT, crossover	10	Healthy	0	Mean (range): NA (20-40	SubQ	Saline	240 (nadir: 90)	CTX: 71 ± 4.1% of baseline	GIP (100 ug/mL)	240 (nadir: 90)	CTX: 57 ± 5.3% of baseline
Nissen, 2014	Denmark	RCT, crossover	10	Healthy	0	Median (range): 22 (19-30)	IV	Saline during euglycemic clamp (PG: 5 mmol/L)	90 (nadir: 90)	CTX: 86.9 ± 6.8% of baseline	Euglycemic clamp (5 mmol/L) + GIP (4 pmol/kg/min for 15 minutes, 2 pmol/kg/min for 45 mins)	90 (nadir: 90)	CTX: 67.3 ± 12.6% of baseline
								Saline during hyperglycemic clamp (PG: 12 mmol/L)		74.1 ± 8.6% of baseline	Hyperglycemic clamp (12 mmol/L) + GIP (4 pmol/kg/min for 15 minutes, 2 pmol/kg/min for 45 mins)		49.2 ± 8.3% of baseline
Christensen, 2017	Denmark	RCT, crossover	10	T1D	0	Mean +/- SD: 26 +/- 4	IV	Saline during low glycemia (PG: 3-7 mmol/L)	120 (nadir: 120) P1NP: 60	CTX: Reduced by 24 ± 10% P1NP: Reduced by 3 ± 8%	Low glycemia (3-7 mmol/L) + GIP (4 pmol/kg/min)	CTX: 120 (nadir: 120) P1NP: 60	CTX: Reduced by 59 ± 18% P1NP: Reduced by +6 ± 10%
								Saline during high glycemia (PG: 12 mmol/L)	90 (nadir: 90)	CTX: Reduced by 7 +/- 90%	High glycemia (12 mmol/L) + GIP (4 pmol/kg/min)	90 (nadir: 90)	CTX: Reduced by 59 ± 19%

CHAPTER 5

EFFECT OF GIP AND GLP-1 INFUSION ON BONE RESORPTION IN GLUCOSE INTOLERANT, PANCREATIC INSUFFICIENT CYSTIC FIBROSIS[3]

[3] Lei WS, Chen XY, Zhao LY, Daley T, Phillips B, Rickels MR, Kelly A, Kindler JM. 2024. Submitted to *Journal of Clinical Endocrinology and Metabolism,* 09/07/2024.

Abstract

Context. Diabetes and bone disease are common in cystic fibrosis (CF) and primarily occur alongside exocrine pancreatic insufficiency (PI). “Incretins,” glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), augment insulin secretion and regulate bone metabolism. In CF, PI dampens the incretin response. Loss of the insulinotropic effect of GIP in CF was recently identified, but effects on bone are unknown. **Objective.** Determine effects of incretins on bone resorption markers in adults with PI-CF. **Design.** Secondary analysis of a mechanistic double-blinded randomized placebo-controlled crossover trial including adults ages 18-40 years with PI-CF (n=25) and non-CF healthy controls (n=3). **Intervention.** Adults with PI-CF received either GIP (4 pmol/kg/min) or GLP-1 (1.5 pmol/kg/min) infusion, followed by double-blind randomization to either incretin or placebo infusion. Non-CF healthy controls received double-blind GIP (4 pmol/kg/min) or placebo. Serum C-terminal telopeptide (CTX), a bone resorption marker, was assessed during the infusion over 80 (GIP) or 60 (GLP-1) minutes. **Main Outcome Measures.** CTX (mg/dL) concentrations. **Results.** In PI-CF, CTX decreased during GIP infusion, but not during placebo (time-by-treatment interaction $P<0.01$). GLP-1 did not affect CTX. In non-CF healthy controls, time-by-treatment interaction was not significant ($P=0.23$), but CTX decreased during GIP ($P=0.02$) but not placebo ($P=0.47$). Over 80 minutes, change in CTX during GIP infusion did not differ between the PI-CF and non-CF healthy controls ($P=0.68$). **Conclusions.** GIP evokes a bone anti-resorptive effect in people with PI-CF. Since the incretin response is perturbed in PI-CF, involvement of the “gut-bone axis” in CF-related bone disease requires attention.

Introduction

Cystic fibrosis (CF) is a genetic disorder arising from recessive mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) [1]. Recurrent pulmonary infections and compromised lung function characterize CF, but non-pulmonary complications, including CF-related diabetes (CFRD) and CF-related bone disease (CFBD) are also common, particularly with increasing age [2]. According to the 2022 CF Foundation Registry [3], >40% of adults have CFRD and >30% have CFBD. An extensive body of evidence links both type 1 diabetes and type 2 diabetes to bone health deficits and increased risk for fracture in non-CF populations [4-6], suggesting that CFRD might contribute to CFBD. Limited studies in people with CF have found that diabetes is associated with worse bone health [7, 8], but biological mechanisms linking CFRD and CFBD have received limited attention.

The adult skeleton relies on a finely orchestrated interplay between osteoclast-mediated bone resorption and osteoblast-mediated bone formation for bone remodeling [9]. Disruptions in this process can lead to the development of osteoporosis, characterized by low bone density and vulnerability to fracture [10]. Many modifiable factors, including nutritional status and food intake, influence bone metabolism [11]. Following ingestion of a meal or isolated macronutrients, as in an oral glucose tolerance test (OGTT), a rapid and acute reduction in C-terminal telopeptide (CTX), a biomarker of bone resorption, occurs and signifies a decrease in bone resorption [12-15]. Providing potential mechanistic insights, the effects of glucose on bone resorption are more pronounced with enteral administration vs intravenous infusion or injection [15-17]. The dampened effect that occurs with bypassing of the gastrointestinal tract suggests that

mechanisms mediated through the gut likely play a significant role in post-prandial bone metabolism.

In response to food ingestion, enteroendocrine K- and L-cells secrete glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), respectively [18]. These hormones are involved in regulating satiety, gastric motility, and insulin secretion to regulate glucose [19]. Both GIP and GLP-1 bind to specific G-protein coupled receptors to stimulate glucose-dependent pancreatic β-cell insulin secretion [20]. In addition to their well-established roles in insulin production for glucose control, incretins are also involved in regulating bone metabolism [21, 22]. In clinical studies, short-term intravenous infusion of GIP reduces bone resorption in people with type 2 diabetes. GLP-1 receptors have been identified in primary mouse osteoblasts [23, 24] and osteoclasts [23, 24]. The impact of GLP-1 on human bone metabolism remains unclear. Insulin, a well-known anabolic hormone, also plays a role in bone turnover [25]. Accordingly, medical conditions like CF, characterized by disrupted incretin and insulin responses [26, 27] and a diminished insulinotropic effect of GIP [28] may jeopardize bone health by altering the gut-bone axis.

The primary goal of this study was to determine the effect of incretins on a biomarker of bone resorption in adults with CF. Using data and blood specimens from a previously completed mechanistic double-blinded randomized placebo-controlled crossover study in adults with CF, we compared changes in CTX during GIP or GLP-1 infusion vs. placebo [28]. Based on prior research conducted in healthy adults [17, 29-31], we hypothesized that GIP, but not GLP-1, infusion would result in a decrease in CTX compared to placebo.

Methods

Study design and participants

We performed a secondary analysis of data and blood specimens from a double-blinded randomized placebo-controlled crossover trial in adults with CF that was conducted at the Hospital of the University of Pennsylvania. The study design and participants are described in greater detail elsewhere [28]. Briefly, the goal of the original trial was to determine the effects of intravenous infusion of GIP and GLP-1 on pancreatic islet function in people with pancreatic insufficient CF (PI-CF). The current study includes 25 adults with PI-CF and 3 non-CF healthy controls that participated in the original trial and had sufficient stored blood specimens for the assessment of our primary outcome of interest.

To be eligible for inclusion into the original study, all subjects were required to be ≥18 years of age at the time of enrollment. Confirmation of CF diagnosis was established through *CFTR* mutation analysis and/or a positive sweat test. These diagnostic criteria adhered to the requirements set by the CF Foundation [32]. Exocrine PI was confirmed by the need for pancreatic enzyme replacement therapy. For individuals with PI-CF, subjects were required to have undergone a 75g OGTT within six months of enrollment to determine their glucose tolerance status. All subjects were required to have abnormal glucose tolerance defined as follows: early glucose intolerance (1-hour glucose ≥155 mg/dL, 2-hour glucose <140 mg/dL [33], impaired glucose tolerance (2-hour glucose 140-199 mg/dL), or CFRD without fasting hyperglycemia (2-hour glucose ≥200 mg/dL or previously confirmed CFRD diagnosis with fasting glucose <126 mg/dL).

The randomization scheme is illustrated in **Fig. 5.1**. People with PI-CF were randomly assigned to either the GIP or GLP-1 infusion group. In addition to receiving the active incretin to which they were assigned (either GIP or GLP-1), participants underwent double-blind randomized administration of a placebo infusion on a different day. Healthy controls without CF were all assigned to receive GIP infusion. On a separate day, they also completed a double-blind randomized placebo infusion. The active and placebo experiments were performed in random order within 1-4 weeks of each other.

All study protocols and procedures were approved by the University of Pennsylvania and the Children's Hospital of Philadelphia Institutional Review Board for Human Subjects. The study was conducted under an Investigational New Drug application with the US Food and Drug Administration (IND 117381), and the protocol was registered with ClinicalTrials.gov (NCT01851694). Prior to beginning study procedures, all participants provided written informed consent.

Incretin administration

The experimental protocol has been reported previously [28]. The current study includes only measurements from the early-phase period of the experiment due to limited availability of stored blood specimens during the later time points. The experimental protocol is illustrated in **Fig. 5.2.**

Briefly, subjects underwent an infusion protocol, either involving incretin or placebo, on two separate days in a randomized and double-blinded cross-over fashion. Their visit to the clinical research center occurred in the morning following an overnight fast. The evening before each study visit, lyophilized GIP (1-42 amide) or GLP-1 (7-36 amide) was reconstituted in a solution consisting of 0.9% saline and 0.25% human serum

albumin, resulting in a solution with a concentration of 1 ug/mL, or placebo was made as a solution consisting of 0.9% saline and 0.25% human serum albumin in order to mask appearance. Baseline fasting blood samples were collected at minutes -5 and 0 using an indwelling catheter inserted into a forearm vein. Following the minute 0 blood draw, the infusion of either incretin or saline commenced. Over the course of the infusion period from minutes 0 to 90, GIP was administered at a rate of 4 pmol/kg/min, whereas GLP-1 was administrated at a rate of 1.5 pmol/kg/min. The infusion rate for each incretin over the first 10 minutes of infusion was doubled to rapidly achieve steady-state concentrations. These rates and resulting concentrations of GIP and GLP-1 are considered supraphysiologic, and have been previously demonstrated to enhance insulin response in individuals with impaired glucose tolerance and type 2 diabetes [28, 34, 35].

Following the minute 30 specimen collection, 5g of 10% arginine was infused over a 1-minute period as part of the glucose-potentiated arginine (GPA) test. Subsequently, at minute 40, a hyperglycemic clamp was initiated using a variable-rate infusion of a 20% glucose solution to achieve and maintain a plasma glucose concentration of approximately 230 mg/dL. In people with CF, those that were assigned to the GIP group had stored blood specimens available at minute 80, and those that were assigned to the GLP-1 group had stored blood specimens available at minute 60. In non-CF healthy controls, stored blood specimens were available at minute 60.

Blood biochemistries

Blood samples were collected in EDTA tubes that had protease inhibitors, including dipeptidyl peptidase-4 inhibitor, added immediately following collection. Samples were then centrifuged at 4°C, and plasma separated and frozen at -80°C for

future analyses. Total GIP and active GLP-1 were assayed and measured in duplicate by ELISA (Millipore, Billerica, Massachusetts). CTX was evaluated using the Cobas e411 automated analyzer (Roche Diagnostics International ltd., Basel, Switzerland). Percent change relative to baseline from minutes -5 to 30 was computed and is abbreviated as $CTX\%\Delta_{-5\text{-}30}$.

Anthropometry

Standing height and weight were assessed using a wall-mounted stadiometer and electronic scale, respectively. Body mass index (kg/m^2) was calculated.

Pulmonary function

Forced expiratory volume, the gold standard for evaluating pulmonary disease in CF, was collected for all subjects from a recent clinic appointment.

Statistical analysis

Data were visually inspected for outliers, non-normal distributions, and influential data points before conducting statistical analyses. All statistical analyses were performed with R version 4.2.2. Descriptive characteristics were summarized using mean (standard deviation) for continuous variables and count (percentage) for categorical variables.

Linear mixed effects models were used to compare CTX during incretin and placebo infusion in the GIP and GLP-1 groups separately. The models treat time, condition (incretin or placebo), and their interaction term as fixed effects, and subject, condition nested under subject, and individual slopes in condition nested under subject, as random effects. Post-hoc analyses were performed to compare the changes in CTX over time within each condition, using the minute -5 timepoint as the reference. Dunnett's method was used to adjust the P-value for multiple comparisons. Additional post-hoc

analyses with Bonferroni adjustment were conducted to compare the changes of CTX between incretin and saline condition. A two-sample t-test was performed to compare changes in CTX during GIP infusion, expressed as CTX%$\Delta_{-5\text{-}30}$, between the CF and non-CF healthy control groups. For all analyses, significance was defined as P-values <0.05 (two-tailed).

Results

Descriptive Characteristics

Descriptive statistics are presented in **Table 5.1**. Sixty percent of the study sample was female (n=15). The average age was 27.1 years and the average BMI was 23.3 kg/m^2. Forty-eight percent of participants had early glucose intolerance (n=12), 36% had impaired glucose tolerance (n=9), and 16% had CFRD without fasting hyperglycemia (n=4).

Changes in CTX during incretin infusion

The effects of GIP and GLP-1 infusion on CTX in adults with PI-CF are illustrated in **Fig. 3**. GIP infusion had a significant effect on CTX compared to the placebo infusion, as supported by a significant time by treatment interaction (P=0.013). During GIP infusion, CTX at minute 30 (P=0.0002) and 80 (P=0.012) were significantly lower than minute -5. When comparing the two conditions, the change in CTX from minute -5 to 30 was significantly greater under the GIP vs. placebo condition (P=0.005), and this difference remained but was not statistically significant from minute -5 to 80 (P=0.13). No differences in CTX under the GLP-1 vs. placebo condition were found.

The effects of GIP infusion on CTX in the three non-CF healthy controls are illustrated in **Supplemental Fig. 5.1.** Briefly, the time by treatment interaction was not significant (P=0.23). However, CTX decreased significantly during GIP infusion (P=0.02) but not during placebo infusion (P=0.47).

Comparisons of changes in CTX during GIP infusion between CF and non-CF healthy control groups are shown in **Supplemental Fig. 5.2.** No significant differences were found when comparing CTX%$\Delta_{-5\text{-}30}$ between the two groups (P=0.68). By minute 30, CTX changed by a mean of -14 ± 11% and -17 ± 11% in the CF and non-CF healthy controls, respectively.

Discussion

Incretins augment post-prandial insulin secretion to regulate glucose and also play a key role in bone metabolism [36]. Post-prandial incretin secretion and the insulinotropic effect of GIP are impaired in people with PI-CF [28, 37], but the extent to which the bone anti-resorptive effect of GIP is preserved is unknown. This study experimentally tests the effect of incretin hormones on bone metabolism in adults with CF. Our main finding was that intravenous infusion of GIP yielded a significant decrease in bone resorption, as indicated by decreases in CTX, compared to placebo. Change in CTX during GIP infusion was similar between CF and non-CF healthy controls, but it is important to note that our small sample of non-CF healthy control subjects likely limited our statistical power to observe between-group differences. In contrast, GLP-1 did not affect CTX compared to placebo.

The primary aim of this study was to determine the effects of intravenous infusion of incretin hormones on bone resorption in adults with PI-CF. Pre-clinical studies

indicate that osteoclasts possess receptors for GIP and that GIP treatment in osteoclast-osteoblast co-cultures inhibits osteoclast activity and delays bone resorption [38]. Clinical studies have further demonstrated that GIP administration via intravenous infusion or subcutaneous injection results in a significant decrease in CTX, a biomarker of bone resorption, thereby indicating a bone anti-resorptive effect [17, 29, 39-41]. Whereas the majority of these studies were conducted in otherwise healthy adults, few studies included people with type 1 diabetes [39], type 2 diabetes [40], and hypoparathyroidism [42]. In the current study of adults with PI-CF, we further report a significant decrease in CTX during intravenous GIP administration. This finding suggests that, despite the abnormal glucose tolerance status and impaired beta cell function observed in PI-CF, the bone anti-resorptive effect of GIP remains intact. Nevertheless, the absence of an adequately sized non-CF healthy control group limits the interpretation of these findings.

The original trial from which blood specimens for the current study were derived included only a small number of non-CF healthy controls that underwent an identical GIP infusion protocol as the PI-CF group. While CTX was significantly reduced from minutes 0 to 30 in non-CF healthy controls during GIP infusion, the time by treatment interaction was not significant. Rather than GIP having a null biological effect on bone metabolism in people without CF, we suspect that our small sample size limited our power to detect significant differences between the GIP and placebo experiments. Sufficiently powered studies that include an appropriate control group are required to determine whether the bone anti-resorptive effect of GIP is modified in people with CF.

In contrast to the findings relating to GIP, GLP-1 had a null effect on CTX. Osteoclasts possess membrane-bound GLP-1 receptors, and whole-body GLP-1-receptor

knockout mice exhibit reduced bone mass and a significantly increased number of osteoclasts [24, 43]. However, clinical studies have reported inconsistent effects of GLP-1 infusion or injection on biomarkers of bone resorption in humans [17, 44]. In a rodent model of streptozotocin-induced type 1 diabetes, GLP-1 receptor agonism inhibited osteoclastogenesis by modifying the RANKL to OPG ratio [45]. A prior study from our lab involving healthy young adults found a positive association between GLP-1 and RANKL following a 75g OGTT [13]. CF is associated with high bone turnover [46], and a greater RANKL/OPG ratio has been identified in people with CFBD compared to those without CFBD [47]. While we did not find significant associations between GLP-1 and CTX, previous clinical studies indicate that GLP-1 receptor agonists may influence biomarkers of bone turnover and bone density in people with type 2 diabetes [48, 49], which highlights the need for studies investigating biological mechanisms linking GLP-1 to bone health.

Incretins play a key role in regulating insulin production for glucose control following a meal [50]. Beyond its known glucoregulatory effects, insulin is also suspected to impact bone metabolism by having a bone-augmenting effect [51, 52]. Insulin deficiency, as in type 1 diabetes, is associated with bone deficits and increased risk for fracture [53, 54]. While studies that have experimentally tested the effects of insulin on bone resorption are limited, recent studies in people with type 1 diabetes lacking endogenous insulin production have reported that insulin infusion did not significantly alter CTX [55], but GIP infusion led to a substantial decrease in CTX [39]. Similarly, a recent study in individuals with type 1 diabetes found that an OGTT and a subsequent isoglycemic intravenous glucose infusion significantly reduced CTX

independent of plasma glucose excursion and insulin secretion [56]. In the present study that previously reported on the loss of GIP's insulinotropic action in PI-CF, the effect of GIP to suppress CTX in PI-CF occurred in the presence of similar concentrations of insulin that were not different under conditions of GIP or placebo infusion [28]. Results from these studies suggest that the effect of incretins on bone resorption may be independent of insulin

As with other studies [17, 29, 39-41], we used supraphysiological doses of GIP and GLP-1 for our experimental protocol. Although this approach might limit the direct translation of our results to the clinical settings, our findings support the potential for emerging incretin-based therapies, which have gained attention for treating obesity and type 2 diabetes [57], to impact bone health in patient populations that are vulnerable to musculoskeletal complications. GLP-1 receptor agonists (e.g., liraglutide, semaglutide) and dual GIP/GLP-1 receptor agonists (e.g., tirzepatide) have demonstrated promising effects on weight loss and glycemic control in patients with and without diabetes [58-61]. Emerging evidence suggests that some of these incretin mimetic therapies influence bone metabolism by reducing bone resorption and promoting bone formation [49, 62]. Although there has been conflicting evidence relating to fracture [63], in a 52-week clinical trial, the GLP-1 receptor agonist exenatide increased bone mineral density in patients with type 2 diabetes despite significant weight loss [48]. Given that weight loss is typically accompanied by bone demineralization [64], clinical studies examining incretin-based medications and fracture are needed.

Strengths and limitations of this study that should be taken into consideration when interpreting our results. We used previously acquired data and blood specimens

from a double-blinded randomized placebo-controlled crossover trial that was aimed at investigating the β-cell response to GIP and GLP-1 infusion in adults with PI-CF. Leveraging existing data and blood specimens to investigate the causal link between incretins and bone metabolism in people with CF is a resourceful approach. Although the crossover design helps minimize potential confounding across subjects because each individual served as their own control, some subjects from the original study were not included in the current analyses due to insufficiently stored specimens for CTX assays. Thus, our statistical power to observe significant associations was limited. As described in greater detail in the parent study by Nyirjesy et al [28], the entire experimental protocol was about 260 minutes in duration. In addition to infusing GIP/GLP-1 or placebo, there were also components of the protocol that included arginine infusion for GPA stimulation testing during hyperglycemic clamping. Due to our small sample size, limited number of blood specimens, and potential confounding of glycemic status, we focused solely on the immediate period following incretin infusion. Arginine infusion was initiated after the minute 30 timepoint, so the initial decrease in CTX during the first 30 minutes of GIP infusion suggests that the reported effect of GIP on bone resorption is not confounded by arginine. Additionally, since the hyperglycemic clamp was initiated after the minute 40 timepoint, we performed analyses using data from minutes -5 to 30. $CTX\%\Delta_{-5\text{-}30}$ did not differ significantly between the PI-CF and non-CF healthy controls. This suggests that these null findings were not confounded by hyperglycemia. Our study's observation period is shorter than other investigations that extend up to 240 minutes following infusion [17], and limits our ability to capture long-term antiresorptive effects of incretin hormones. The lack of additional bone biomarkers, such as procollagen

1 intact N-terminal propeptide (P1NP), osteocalcin, RANKL, and OPG, due to limited sample availability, underscores the need for further investigation. RANKL and OPG regulate osteoblast differentiation and activation and are likely involved in incretin regulation of bone [65]. Additionally, GIP and GLP-1 have also been shown to upregulate bone formation by inhibiting osteocalcin synthesis in in-vitro models [66]. As such, assessing other biomarkers of bone turnover beyond CTX would provide important additional insight into mechanisms concerning the gut-bone axis in CF.

Conclusions

In conclusion, the results from this study suggest that GIP has a bone anti-resorptive effect in people with PI-CF. Maldigestion resulting from exocrine PI hinders the post-prandial GIP and GLP-1 response in people with CF [28], and the insulinotropic effect of GIP is suppressed in CF [28]. Since diabetes and bone disease are among the most common complications of CF, entero-endocrinopathies warrant further investigation with respect to CFBD. Even more, with the emergence of incretin-based therapies for the treatment of type 2 diabetes and weight loss (e.g., GIP and/or GLP-1 receptor agonists) [67], their impact on fracture and osteoporosis risk requires attention.

Table 5.1. Participant characteristics.

	Mean ± SD
Age, years	27.1 ± 7.4
Female, n (%)	15 (60)
White, n (%)	25 (100)
Height, cm	166.7 ± 11.0
Weight, kg	64.9 ± 13.6
BMI, kg/m^2	23.3 ± 3.8
Fasting glucose, mg/dL[a]	90.9 ± 9.4
1-hour glucose, mg/dL	210.8 ± 28.7
2-hour glucose, mg/dL	144.2 ± 67.7
HbA1c[a]	5.6 ± 0.4
Fasting GIP, pg/mL[a]	56.4 ± 40.3
Fasting GLP-1, pmol/L[a]	3.8 ± 2.1
Fasting CTX, ng/mL[a]	0.67 ± 0.3
FEV1 (% Predicted)	85.7 ± 21.2

BMI, body mass index; HbA1c, hemoglobin A1c; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide 1; CTX, C-terminal telopeptide; FEV1, forced expiratory volume; FVC, forced vital capacity.
[a]Fasting measure from minute -5 of infusion.

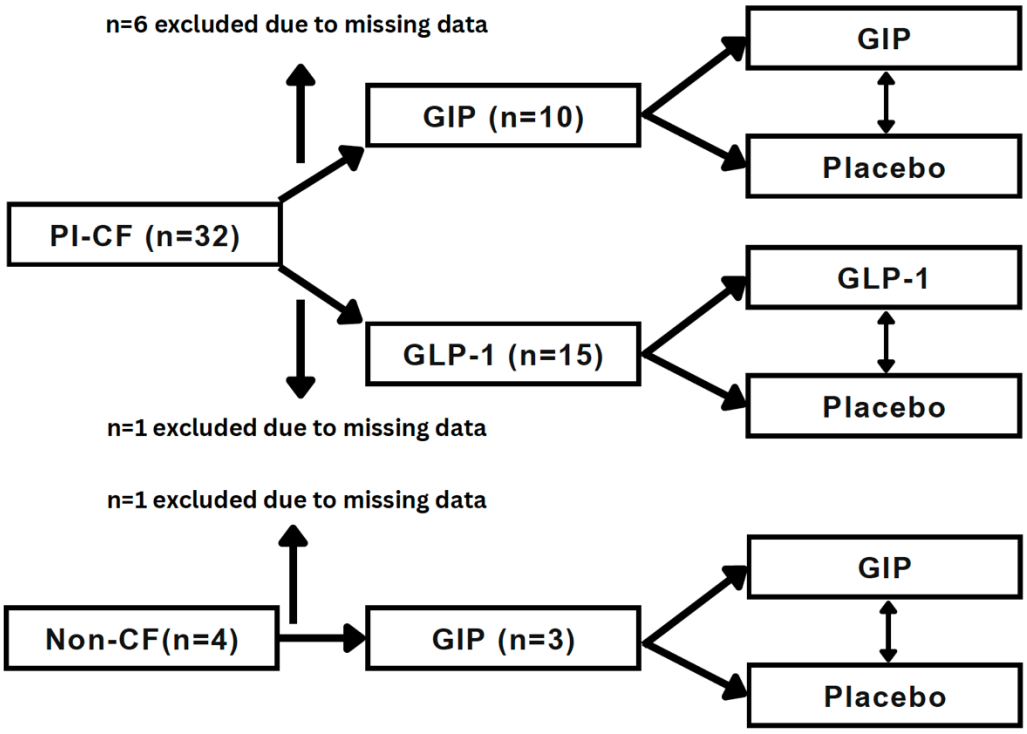


Figure 5.1. Schematic depicting the design and flow of participants throughout the study.

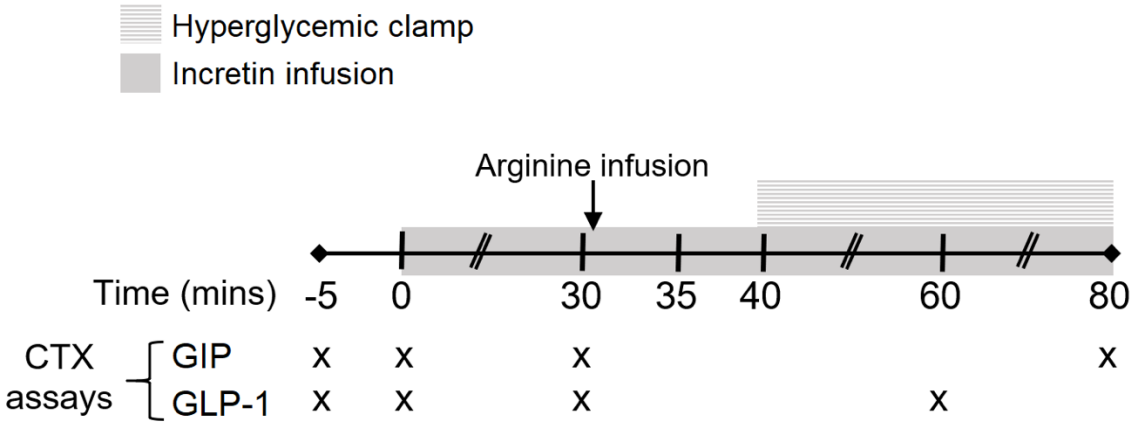


Figure 5.2. Schematic depicting the experimental protocol.

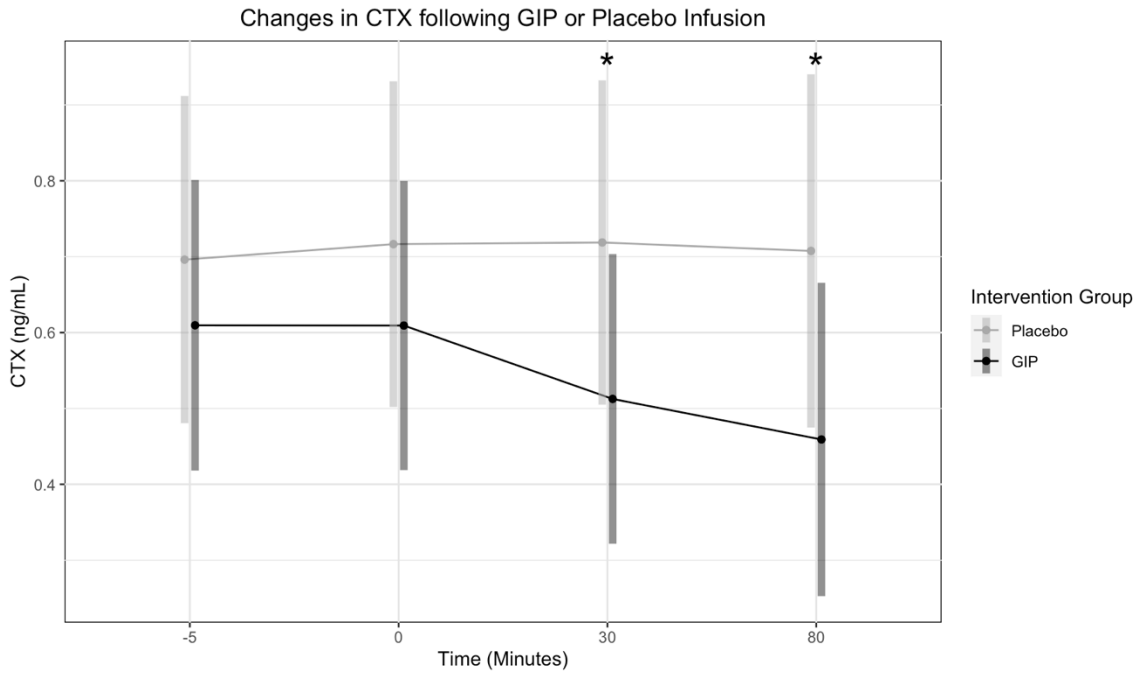


Figure 5.3A. CTX response to GIP infusion in adults with PI-CF. Data presented as means and vertical bands represent 95% confidence interval. *Corresponding timepoint during GIP infusion differs significantly from minute -5 (P<0.01). CTX, C-terminal telopeptide; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1.

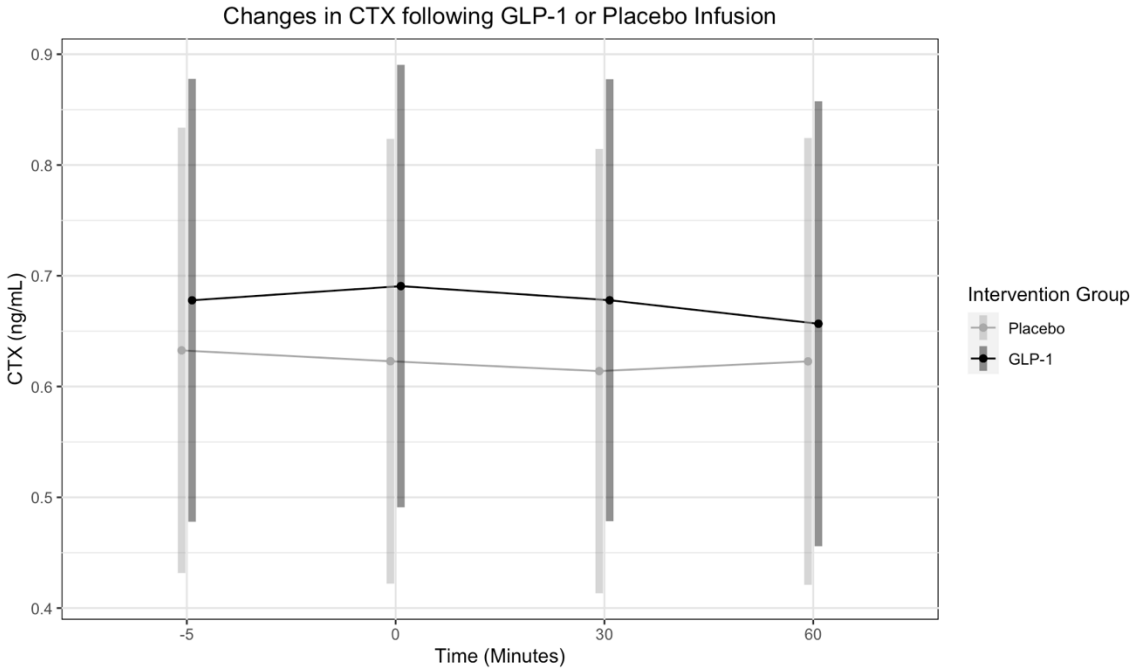
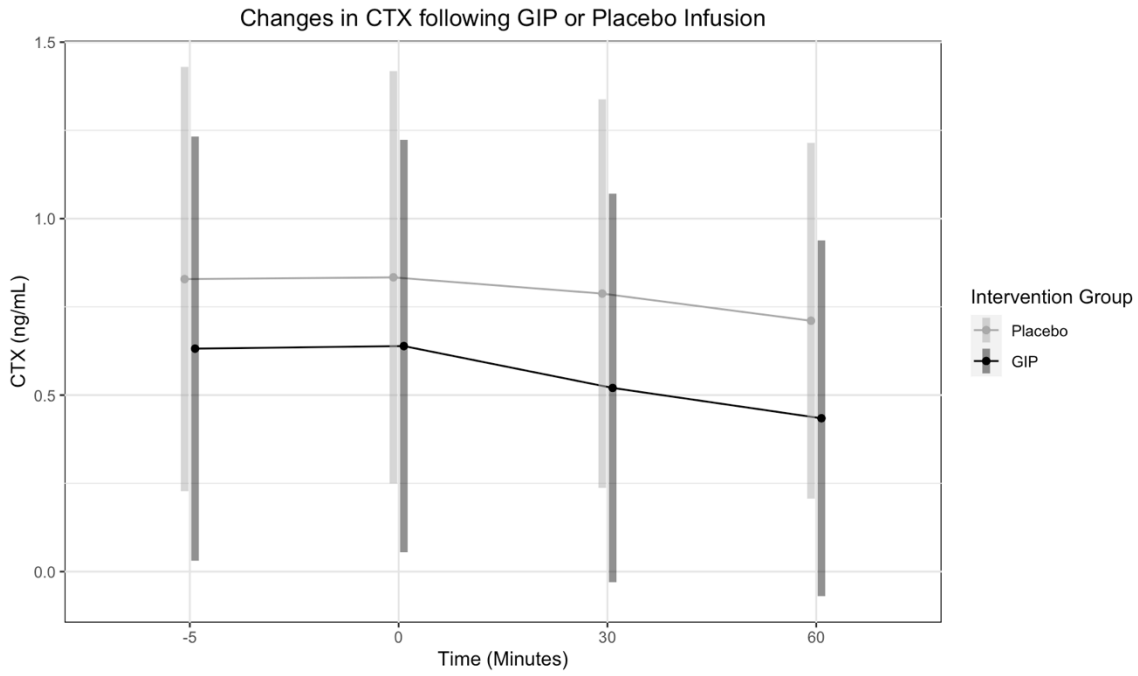
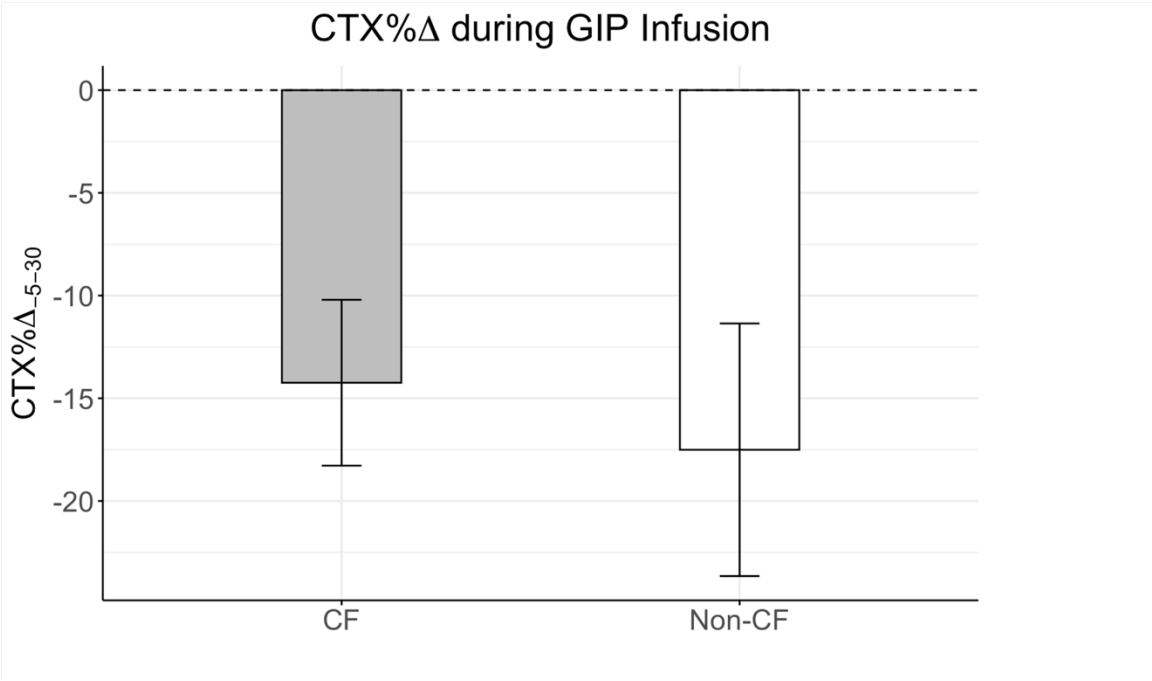


Figure 5.3B. CTX response to GLP-1 infusion in adults with PI-CF. Data presented as means and vertical bands represent 95% confidence interval. *Corresponding timepoint during GIP infusion differs significantly from minute -5 ($P<0.01$). CTX, C-terminal telopeptide; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1.



Supplemental Figure 5.1. CTX response to GIP infusion in non-CF healthy controls. Data presented as means and vertical bands represent 95% confidence interval. CTX, C-terminal telopeptide; GIP, gastric inhibitory polypeptide.



Supplemental Figure 5.2. CTX%Δ from mins -5 to 30 during GIP infusion in CF (gray bar) and non-CF healthy controls (white bar). Vertical bands represent standard error of the mean.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The objective of this dissertation was to explore the relationship between incretin hormones and bone metabolism. To address this, we conducted a cross-sectional study (Chapter 3) with 10 healthy emerging adults (ages 18-25), where we monitored changes in biomarkers of bone metabolism during a 2-hour oral glucose tolerance test (OGTT). Previous research has shown that glucose ingestion has an anti-resorptive effect on bone, as indicated by a ~50% reduction in CTX, a biomarker of bone resorption [1-3]. Our findings corroborated this, showing a significant 52% decrease in CTX at 120 minutes following OGTT ($P < 0.05$). Additionally, glucose was inversely correlated with CTX, while GLP-1 was positively correlated with BSAP, a biomarker of bone formation, and RANKL, a biomarker of bone turnover. GLP-1 was also positively correlated with cortical volumetric bone density.

These findings are clinically relevant, as they highlight the gut-bone interaction during the period of peak bone mass attainment–a previously understudied area. However, like other OGTT studies, we could not conclusively determine whether glucose or incretins were responsible for the observed anti-resorptive effect. Notably, glucose response correlated with CTX, whereas GIP and GLP-1 responses did not.

To further explore this, we conducted a systematic review and meta-analysis (Chapter 4) on the effects of GIP infusion on bone resorption biomarkers. Our review revealed that GIP infusion significantly reduces CTX in healthy adults, but this effect is

diminished in individuals with diabetes (P=0.03), suggesting that metabolic dysfunction may impair the gut-bone axis.

Additionally we explored bone metabolism in adults with CF (Chapter 5), a population characterized by pancreatic insufficiency (PI) and metabolic dysfunction [4]. In a secondary analysis of a randomized, double-blind crossover trial, we found that GIP infusion, but not GLP-1, significantly reduced CTX in adults with CF ($P < 0.01$), suggest that the bone anti-resorptive effect of GIP is preserved despite PI. However, further studies are needed to understand the connection between glucose dysregulation, CF-related diabetes, and bone health in this population.

In summary, this dissertation provides new insights in the gut-bone axis in both healthy young adults and adults with CF. Our findings show that incretin hormones, particularly GIP, have a bone anti-resorptive effect in healthy adults, though this effect may be altered in individuals with metabolic conditions like diabetes or CF. With the increasing use of incretin-based therapies (e.g., GIP and GLP-1 receptor agonists) for type 2 diabetes and weight loss, further research is needed to assess their impact on bone health, particularly in relation to fracture and osteoporosis risk.

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APPENDIX A

CONSENT FORM

Approved by University of Georgia
Institutional Review Board
Project ID VERSION00001314
Approved on: 1/27/2022

Behavioral Threats to Peak Bone Strength

UNIVERSITY OF GEORGIA
CONSENT FORM

Researcher's Statement: You are being asked to take part in a research study. Before you decide if you may participate in this study, it is important that you understand why the research is being done and what it will involve. This form is designed to give you the information about the study so you can decide whether you wish to be in the study or not. Please take the time to read the following information carefully. Please ask the researcher if there is anything that is not clear or if you need more information. When all of your questions have been answered, you can decide if you want to be in the study or not. This process is called "informed consent." A copy of this form will be given to you.

If you are interested in participating in the study, please read the additional information on the following pages, and feel free to ask questions at any point.

Principal Investigator:	*Dr. Joseph M. Kindler, PhD, CTR* *Nutritional Sciences* *kindlerj@uga.edu* *717-798-0776*	**Study Coordinator:**	*Ms. Staci Belcher, MS, RDN, LDN* *Nutritional Sciences* *stacibelcher@uga.edu* *706-542-7466*

- The purpose of this study is to identify the impact of health behaviors on bone health in healthy young adults.
- You are being asked to participate in this research study because you are between the ages of 18 and 25 years and are generally healthy.
- Your involvement in the study is voluntary, and you may choose not to participate or to stop at any time without penalty or loss of benefits to which you are otherwise entitled.
- This research study involves two appointments: one at the Nutrition and Skeletal Health Lab (approximately 2 hours), which is located in room 275 Dawson Hall on the main UGA campus (305 Sanford Drive, Athens GA) and the other at the Clinical and Translational Research Unit (approximately 2.5 hours) on the Health Sciences Campus (109 Bowstrom Road)
- During the visits, you will be asked to complete anthropometrics (height, weight), health-related questionnaires, a diet recall, bone assessment measures, and an Oral Glucose Tolerance Test, which involves drinking a glucose -containing solution and have a blood draw performed.
- Risks include exposure to a small amount of radiation, possible psychological discomfort while answering questionnaires, and possible physical discomfort after consuming the glucose solution and when having your blood drawn.

If you are interested in participating in the study, please read the additional information on the following pages, and feel free to ask questions at any point.

Study Procedures and Time Commitment: As a participant in this study, you will be asked to complete the following procedures at two separate study visits. Visits can occur in any order, but must be completed within 3 months of one another

Nutrition and Skeletal Health Laboratory Visit (approximately 2 hours):

Health history questionnaire: Brief questionnaire involving overall health status, bone fractures/injuries, medication use, and chronic health conditions. This questionnaire will be conducted at the first visit – either at the NASH lab or CTRU.

Body size: Height and weight will be assessed.

Dual-energy X-ray absorptiometry (DXA): The DXA machine is a specific X-ray machine used for bone density assessment. Several DXA scans will be completed to assess bone density and body

composition. DXA scans of your whole body, lumbar spine (lower back), forearm, and hip will be completed. Scans will be completed in a laying or seated position.

High-resolution peripheral quantitative computed tomography (HR-pQCT): The HR-pQCT is a device similar to a CT scanner used in healthcare settings, but smaller so only your arm or leg can be scanned. HR-pQCT scans will be used to assess the strength of your leg and arm. A total of six HR-pQCT scans will be completed. Two scans will be completed at your wrist, one scan around your mid-arm, two scans around your ankle, and one scan around your calf muscle. Scans will be completed in a seated position.

24-hour diet recall: This dietary recall is collected through an interview with a trained study team member. This interview includes questions regarding what foods and how much of each foods/beverages you consumed in the previous 24 hours.

Physical activity questionnaire: This questionnaire includes questions regarding your typical activity levels and specific activities you commonly participate in.

Sleep questionnaire: This questionnaire includes questions regarding your perceived sleep quality, amount of time spent sleeping, and how tired/refreshed you feel in the morning.

Demographics Questionnaire: This questionnaire includes questions regarding your race/ethnicity, sex, socioeconomic status, and education history.

Clinical and Translational Research Unit Visit (approximately 2.5 hours):

Body size: Height and weight will be assessed.

Health history questionnaire: If the CTRU is the first visit, this questionnaire will be collected.

Demographics questionnaire: If the CTRU is the first visit, this questionnaire will be collected.

Oral Glucose Tolerance Test: This test includes drinking a sweet drink and having blood drawn four times over two hours. While fasting, you will be asked to have blood drawn by a qualified and experienced phlebotomist. After one blood draw measurement, you will be asked to drink ~10 ounces of Trutol, a commercially available sweetened beverage containing 75 grams of sugar in less than ten minutes. After finishing the beverage, blood draws (20 mLs each) will take place after 30 minutes, 60 minutes, and 120 minutes. In total, four blood draws will collect approximately five tablespoons (~80 mLs total) of blood. You will be given a snack after the measurement is complete. Your blood will be analyzed for blood glucose levels, hormones, and measures of bone metabolism for this study. Stored samples may also be analyzed at a future date in conjunction with research also related to factors affecting bone strength and growth. Any unused portions of the blood that is collected will be discarded after 10 years post-collection.

Potential Risks and Discomforts: There are some potential risks and/or discomforts associated with the procedures outlined above.

Radiation exposure: You will be exposed to a small amount of radiation during the DXA and HR-pQCT scans. The total radiation from both the DXA scans and HR-pQCT scans result in about 46 µSV of radiation. For comparison, natural and man-made background exposure is approximately 8 µSV per day, and a round-trip airline flight from Athens, GA to Athens, Greece would be approximately 46 µSV of radiation exposure. In the event that information from any scan is lost or unusable, additional scans might be performed.

Embarrassment/discomfort from questionnaires: You may experience some psychological discomfort from the disclosure of information relating to health history, demographics, 24- hour recall, physical

activity, and sleep quality. You may skip any question that may be distressing. If undue discomfort or stress occurs, you have the right to discontinue participation in the study at any time.

Physical Discomfort from oral glucose tolerance test:

You may experience some unpleasant side effects after drinking the Trotol solution. The package label for Trutol lists the following rare but known side effects: nausea, vomiting, abdominal bloating, and/or headache.

You may experience some discomfort or stress when your blood is drawn. The risks of drawing blood from your arm include the unlikely possibilities of a small bruise or localized infection, bleeding and fainting. These risks will be reduced in the following ways: your blood will be drawn only by a qualified and experienced person who will follow standard sterile techniques, who will observe you after the needle is withdrawn, and who will apply pressure to the blood draw-site.

Additional risk for pregnant females: Being a part of this study while pregnant may expose the unborn child to a yet undiscovered risk. Therefore, pregnant females or those who suspect they could be pregnant will be excluded from this study.

Benefits of Participation: There are no potential benefits of participating in this study.

Incentives for participation: You will receive a $50 gift card after completing each study visit, for a total of $100. If you elect to withdraw from the study in the middle of a study visit, you will still receive compensation for that study visit. Payment will be awarded in the form of a gift card at each study visit (NASH Lab, CTRU visit). Your signature will be required to confirm you received the gift card. Records will be retained in a locked cabinet in the Nutrition and Skeletal Health Lab.

Privacy and Confidentiality: Though some individually-identifiable information will be collected from you for contact purposes, all data and specimens collected as part of the study procedures will be coded using a subject ID, which consists of letters and numbers. The key to the above mentioned code will be kept in a password protected computer file. Only the researcher and members of this research team will have access to identifiable data. The project's research records may be reviewed by departments at UGA responsible for regulatory and research oversight. The key to the code matching your name with your ID number will be destroyed following a ten-year retention period. Researchers will not release identifiable results of the study to anyone other than individuals working on the project without your written consent unless required by law.

Voluntary Consent: Your participation in this study is voluntary. You can refuse to participate or stop taking part at any time without giving any reason, and without penalty or loss of benefits to which you are otherwise entitled. If you decide to withdraw from the study, the information that was previously collected will be kept as part of the study and may continue to be analyzed, unless you make a written request to remove, return, or destroy the information. If you are a student, your academic standing or grades will not be impacted in any way by participating in this study, or choosing to withdraw from this study.

Use of Data and Specimens in Future Studies: If you consent to your data and/or blood specimen being used in future studies, use of this data will be limited to ten years. All of your data will be deidentified. These data include: biospecimen, bone and body composition measurements, and/or questionnaires. You will not be informed of the details of any scientific research studies that might be conducted using your private information or biospecimens; this research would relate to factors affecting bone strength and growth and you may not have chosen to consent to some of those specific research studies. We may collaborate with other academic institutions in these future studies and share the deidentified data with these institutions.

If you are injured by this research: The researchers will exercise all reasonable care to protect you from harm during your participation in this study. In the event of an injury as an immediate and direct result of participation,

the researchers' sole responsibility is to arrange for transportation to an appropriate facility if additional care is needed. If you think that you have suffered a research-related injury, you should seek immediate medical attention and then contact Dr. Joseph Kindler right away at 717-798-0776. In the event that you suffer a research-related injury, the medical expenses will be your responsibility or that of your third-party payer, although you are not precluded from seeking to collect compensation for injury related to malpractice, fault, or blame on the part of those involved in the research.

If you have questions: The main researcher conducting this study is Dr. Joseph Kindler, an Assistant Professor at UGA. Please ask any questions you have now. If you have questions later, you may contact Dr. Kindler at kindlerj@uga.edu or 717-798-0776. If you have any questions or concerns regarding your rights as a research participant in this study, you may contact the Institutional Review Board (IRB) Chairperson at UGA at 706-542-3199 or irb@uga.edu.

Questions to be answered by the study participant:

Pregnancy: I certify that I am not currently pregnant.

Yes: _____ No: _____

**if no, this individual is not eligible to participate in this study*

Use of Research Data/Blood Specimens for Future Study: I give the researchers of this study permission to use my data/blood specimens in future studies.

Yes: _____ No: _____

Research subject's consent to participate in research
To voluntarily agree to take part in this study, you must sign on the line below. Your signature below indicates that you have read or had read to you this entire consent form, and have had all of your questions answered.

_____	_____	_____
Name of Researcher	Signature	Date
_____	_____	_____
Name of Participant	Signature	Date

Continued on next page…

Future Contact: I give the researchers of this study permission to contact me regarding participation in future research studies.

Yes: _____ No: _____

**if yes, please provide the following information*

Name: _____

Address: _____

Telephone: _____

Email (work/school): _____

Email (person): _____

Please sign both copies, keep one and return one to the researcher.

APPENDIX B

HEALTH HISTORY QUESTIONNAIRE

Study ID#:_____

Behavioral Threats to Peak Bone Strength

Health History Questionnaire

1. Are you currently taking any medications?
   ☐ Yes
   ☐ No

   If 'yes,' please list the medications, dose, and reason for use

Medication	Dose	Reason for Use

2. Are you currently taking any dietary/herbal supplements?
   ☐ Yes
   ☐ No

   If 'yes,' please list the dietary/herbal supplements, dose, and reason for use

Supplement	Dose	Reason for Use

3. Have you ever broken a bone before?
   ☐ Yes
   ☐ No

If 'yes,' please provide information regarding each time that you have broken a bone:

	Which specific bone did you break?	**At what age did the fracture occur?**	**How did the fracture occur?**
Broken bone 1			
Broken bone 2			
Broken bone 3			

4. Have you taken a medication or hormonal supplement (excluding hormonal birth control) prescribed by a doctor in the last year?

☐ Yes ☐ No

If yes, specify: _____

_____

_____

5. If female, at what age did you have your first menstrual period? _____

6. If female, do you currently have a 'regular' menstrual period?

☐ Yes
☐ No

If "No," please describe why you don't consider your menstrual period to be 'regular."

7. If female, have you ever taken any form of birth control?
   - ☐ Yes
   - ☐ No

8. If female, are you currently using any form of birth control?
   - ☐ Yes
   - ☐ No

If 'yes' to either of the two previous questions, please provide information regarding each type of birth control you have ever used

Mode (patch, implant, pill, injection, etc.)	Name of birth control	Dose	Duration of use

9. What do you consider your race? You can indicate more than one.
   - ☐ American Indian or Alaskan Native
   - ☐ Native Hawaiian or Pacific Islander
   - ☐ Asian
   - ☐ White
   - ☐ Black or African American

10. Do you consider yourself to be Hispanic or Latino?
   - ☐ Yes
   - ☐ No