INSULIN RESISTANCE, INSULIN-LIKE GROWTH FACTOR 1, AND PEDIATRIC MUSCULOSKELETAL DEVELOPMENT

by

JOSEPH MICHAEL KINDLER

(Under the Direction of RICHARD D. LEWIS)

ABSTRACT

Insulin-like growth factor I (IGF-I) plays a pivotal role in pediatric muscle and bone development. Whereas IGF-I acts directly upon the bone-forming osteoblasts, the tropic effect of IGF-I on skeletal muscle is suspected to, at least in part, facilitate its effect on bone. Insulin and IGF-I act at the level of the muscle and bone through a similar downstream signaling process. Animal and cell culture studies have shown that biological factors that contribute to insulin resistance moderate IGF-I function. Thus, it is plausible that the potentially adverse influence of insulin resistance on pediatric bone health involves IGF-I. The objective of this dissertation is to utilize existing data in order to examine the relationships between insulin resistance and pediatric bone outcomes, in addition to the potential influence of insulin resistance on the IGF-I-bone relationship in children. For all studies, total body bone mass, lean mass, and fat mass were measured via dual-energy X-ray absorptiometry; cortical bone geometry was assessed via peripheral quantitative computed tomography; and insulin resistance was measured via the homeostasis model assessment of insulin resistance. In Manuscript #1, which is a cross-sectional study of black and white girls (N = 147; 50% black, ages 9-11 years), insulin resistance had a significant and negative influence on the lean mass-dependent relationships between IGF-I and

bone mass. In Manuscript #2, which is a cross-sectional study of black and white boys and girls (N = 315; 50% female, 50% black, ages 9-13 years), children with higher insulin resistance had lower cortical bone size and estimated bending strength. In addition, insulin resistance moderated the relationship between IGF-I and lean mass as well as cortical bone size and strength. Our data are the first to implicate a suppression of IGF-I-dependent lean mass accretion as a contributor to the smaller cortical bone in children with insulin resistance. As such, insulin resistance-related deficits in cortical bone size and subsequent bending strength, which might involve IGF-I, could help explain the greater propensity for skeletal fracture in obese youth.

INDEX WORDS: INSULIN-LIKE GROWTH FACTOR 1, INSULIN RESISTANCE, OBESITY, BONE, MUSCLE, CHILDREN, DUAL ENERGY X-RAY ABSORPTIOMETRI, PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

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CHAPTER 1

INTRODUCTION

The adolescent years are characterized by rapid bone mineral accrual, increases in cortical bone size, density and strength, and improvements in trabecular bone microarchitecture.[1-3] Though conflicting data have been reported, there is considerable evidence supporting the position that bone development is compromised in obese youth, potentially contributing to an increased for skeletal fracture.[4] This is of considerable importance given that nearly 20% of US youth are currently considered obese,[5] and upwards to one in four are at risk of developing type 2 diabetes.[6] Various obesity-related factors are suspected to underpin the fat-bone relationship, with insulin resistance receiving considerable recent attention.[7-11]

The majority of the evidence relating to insulin resistance and bone in children has considered two-dimension skeletal outcomes, culminating in the general consensus that insulin resistance is a negative determinant of total body bone mass.[9] Sayers and colleagues were among the first to implicate fasting insulin, an indicator of insulin resistance, as a negative determinant of cortical bone size, volumetric density, and estimated bending strength in English adolescents.[8] However, rather than insulin resistance having a direct adverse influence on cortical bone, insulin resistance may influence bone development through indirect means, specifically by modulating biological factors involved in bone growth.

Of the various hormones involved in pediatric skeletal development, insulin-like growth factor I (IGF-I) plays a pivotal role.[10,12,13] Indeed, IGF-I promotes bone mineral accrual and cortical bone areal expansion by acting directly upon the bone-forming osteoblasts; preferentially

those located toward the periosteum.[14-16] In addition, the trophic effect of IGF-I on lean body mass is suspected to precede skeletal changes.[13,17-19] Therefore, IGF-I orchestrates cortical bone growth through both direct and muscle-dependent processes. Moreover, IGF-I is similar to the pancreatic β-cell-derived insulin in terms of structure, downstream signaling processes, and cellular target tissues (i.e., muscle and bone).[20,21] *In vivo* and *in vitro* studies have shown that various biological components that are suspected to contribute to the progression of insulin resistance might too suppress the effects of IGF-I.[22-24] Therefore, the smaller and weaker cortical bone in children with insulin resistance might involve IGF-I.

The literature review (Chapter 2) provides an overview of the current body of evidence relating to the following topics: 1) bone biology and physiology, 2) pediatric musculoskeletal development, 3) adiposity and pediatric bone development, 4) skeletal muscle and pediatric bone development, 5) insulin resistance and bone, 6) IGF-I and bone, and 7) insulin resistance, IGF-I, and musculoskeletal development. Chapter 3 presents the first manuscript of this dissertation, which is a cross-sectional ancillary study of a previously conducted randomized controlled trial aimed at examining the influence of insulin resistance on the lean mass-dependent link between IGF-I and total body bone mass in girls who were at the early stages of maturation. Finally, Chapter 4 presents a cross-sectional ancillary study of a previously conducted randomized controlled trial aimed at examining the influence of insulin resistance on the relationships between IGF-I and cortical bone outcomes in black and white boys and girls who were at the early stages of sexual maturation.

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CHAPTER 2[1, 2]

REVIEW OF THE LITERATURE

Bone

The human skeleton is comprised to two major types of bone: cortical and trabecular.[1,2] Cortical bone comprises approximately 80% of the total body skeleton and is found primarily within the mid-regions (diaphysis) of the long bones (i.e. the tibia, radius, and femur; **Figure 1**). The cortical compartment is separated into two sub-regions: the endosteum, which is the innermost boarder adjacent to the bone marrow, and the periosteum, which is the outermost boarder adjacent to the skeletal muscle. In contrast, trabecular, or "spongy/cancellous" bone, accounts for approximately 20% of skeletal tissue, is found primarily in the regions of the epiphyses and metaphyses, is directly adjacent to the bone marrow, and is comprised of a matrix of individual "trabeculae." The spine, femoral neck, and ilium are additional skeletal regions of predominantly trabecular bone.

The skeleton is made up of water (10%), an organic compound (70%) consisting predominantly of type-1 collagen, and an inorganic compound (20%) made up predominantly of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$].[1-3] The inorganic component, or hydroxyapatite, is comprised mostly of calcium and phosphorus, but also contains carbonate and magnesium.

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[1] Lewis RD, Kindler JM, Laing EM. 2016. Nutritional Influences on Bone Health: 9th International Symposium. Reprinted here with the permission of the publisher.

[2] Kindler JM, Lewis RD, Hamrick MW. 2015. Current Opinions in Endocrinology, Diabetes and Obesity. Reprinted here with the permission of the publisher.

Osteoblasts, osteoclasts, and osteocytes are the three primary bone cells responsible for the maintenance of skeletal tissue. Osteoblasts, or bone forming cells, are derived from mesenchymal stem cells and are located on the surface of the bone. Osteoclasts, or bone resorbing cells, are derived from hemopoetic stem cells and are responsible for maintaining calcium homeostasis. Osteoclasts resorb bone mineral into the circulation following signaling from, most notably, the osteoblast. Eventually, osteoblasts either become osteocytes, which are bone cells impacted within the bone, or bone lining cells. Through a coordinated effort between these various cell-types, the bone undergoes both "modeling" and "remodeling" processes. Bone modeling is the process through which new bone is added to the bone surface, therefore resulting in a newly shaped bone. In contrast, bone remodeling is the process by which the bone is first resorbed by the osteoclasts, and thereafter, the osteoblasts add new bone upon the previously resorbed area. Whereas bone modeling is the dominant process during growth, bone remodeling, occurs throughout the human lifespan and is responsible for rejuvenating the stored bone mineral.

Pediatric Bone Development

Peak height velocity (PHV) is achieved in girls and boys near the ages of 12 and 13.5 years, respectively.[4-7] Following PHV, peak lean mass velocity is achieved, and is followed months later by peak bone mineral content velocity. Due to a lag between bone elongation and bone mineral accrual, children during this time are at an increased risk for sustaining a skeletal fracture, particularly at the distal radius.[7] Over the 2 years surrounding PHV, nearly one fourth of adult bone mass is accrued.[4] At nearly 18 years of age lean body mass reaches a plateau, which precedes the peak accrual of bone mass (i.e., peak bone mass) by months or even years depending on the skeletal region.[4,5]

Trabecular and cortical bone likewise undergo significant changes during growth. Trabecular bone thickness and separation increase and trabecular number decreases, which ultimately lead to a consistent bone volume to total volume fraction throughout early development. During this time boys have a more optimal trabecular microarchitectural framework; however, in both sexes, cortical bone size, volumetric density, and strength significantly increase and lag behind the growth trajectory of muscle cross-sectional area.[6,8-10] Whereas girls tend to have denser and less porous cortical bones, boys on average have larger, thicker and stronger bones.[8,9] These sex-related differences in cortical bone geometric and material properties are attributed, in part, to differences in skeletal muscle.[6,8] However, sex hormones, particularly estrogen, are believed to contribute to the stronger muscle-bone relationship between girls versus boys despite higher lean body mass in boys.[11]

Adiposity and Pediatric Bone Development

According to Ogden and colleagues,[12] approximately 17% of children between the ages of 2 and 19 years are obese. Whereas conflicting data have been reported, there is considerable evidence supporting the position that bone development is compromised in obese youth.[13-16] This may help explain, at least in part, the greater risk for skeletal fracture in obese children and adolescents.[17,18] In contrast, this elevated fracture risk may also be attributed to deficits in physical function and greater subsequent risk for falling.[19] However, *in vitro* mechanisms implicating adipose tissue as a negative determinant of adolescent bone structure and strength have been supported *in vivo*.[13-15]

Few studies have considered measures of trabecular bone microarchitecture while examining the obesity-bone relationship in children and adolescents. The recent availability of high-resolution imaging techniques, however, has bolstered research endeavors aimed at

examining these skeletal endpoints. Studies in adults support the notion that obesity per se is beneficial to trabecular bone microarchitecture;[20,21] yet, the limited data in younger cohorts have been less clear. For example, in two separate studies, children with greater adiposity had lower trabecular thickness, but greater trabecular number and lower trabecular separation.[22,23] Whereas the findings relating to trabecular thickness can be perceived as detrimental to trabecular bone quality, those pertaining to number and separation are considered advantageous. Data from Hoy et al[16] suggest that these inconsistent relationships in younger children foreshadow suboptimal trabecular bone microarchitectural outcomes in obese individuals during later adolescence and young adulthood (ages 15-21 years).

In contrast to the limited evidence specific to trabecular bone microarchitecture, data on cortical bone outcomes are readily available in the pediatric literature. One study by Pollock et al[13] examined the relationships between fat mass and pQCT-derived measures of cortical and trabecular bone in a cohort of girls at the later stages of adolescence (N = 115, mean age = 18.2 years). Results from this study showed inverse correlations between fat mass and cortical bone geometry and estimated strength (i.e. strength strain index) at the radius, and following statistical adjustment for limb length and muscle cross-sectional area, at the tibia. Lean body mass is one factor explaining why obesity might be advantageous to cortical bone, as fat-free soft tissue (FFST) mass/muscle cross-sectional area tends to be greater in obese youth and is a strong positive predictor of cortical bone.[6,14,24,25] However, despite overt differences in lean body mass, various studies have shown similar cortical bone size and strength between obese versus normal-weight children.[13,14,22] Ducher et al[26] found that overweight children (N = 427, 7-10 years old) had greater measures of bone quality at both the radius and tibia, cortical and trabecular regions, whereas following adjustment for muscle cross-sectional area a number of

these relationships switched direction in favor of the normal weight participants. Sayers et al[27] reported similar findings in boys and girls (mean age = 15.5 years), such that total body fat mass was positively associated with tibia periosteal circumference and negatively with endosteal circumference in girls only. Furthermore, total body FFST positively correlated with cortical BMC in both genders, but more so in girls where positive associations were also found in relation to periosteal circumference. A more recent study by Leonard et al[25] support the notion that obese individuals have more optimal cortical bone due to corresponding differences in skeletal muscle. However, these authors also demonstrate that adiposity-related accelerated maturation additionally contributed to more favorable bone outcomes in obese adolescents. A separate prospective study of adolescents showed similar relationships between adiposity and cortical bone development. Specifically, Glass et al[28] showed that total body fat mass was a positive predictor of changes in cortical bone mass, size, and strength, but that these relationships were nullified or reversed after adjustment for lean body mass. Further, the relationships between fat and bone appeared to be enhanced in the female participants as a result of an earlier onset of maturation.

Skeletal Muscle and Pediatric Bone Development

Childhood and adolescence are important life stages characterized by rapid and significant longitudinal bone growth, areal bone expansion and bone mineral accrual.[5,6,9,10] Achieving optimal skeletal gains throughout maturation is of primary importance since the majority of adult bone mass is attained by the twentieth year of life.[5] The link between muscle and bone is complex, and is attributed to various modifiable factors. As an example, skeletal tissue is responsive to the forces exerted upon it, as outlined by the theory set forth by Frost.[29] This theory posits that bone adapts to the mechanical loads that are placed upon it during muscle

contraction. Total body lean mass and muscle cross-sectional area (MCSA) are common surrogates of the muscular contractile forces of which bone is particularly responsive since muscle size and strength are closely related.[30] As a second example, pathogenic fat depots, e.g., adiposity stored within the skeletal muscle, have piqued recent interest, as muscle fat in adults has been shown to be predictive of suboptimal bone outcomes and hip fracture.[31,32] The gaps in our understanding of the complex muscle fat-bone relationships is amplified in children, as these aforementioned relationships have been less studied in pediatric populations. Further, it is important to note that the cross-talk between muscle and bone, which is mediated through a variety of secretory factors, including myostatin, fibroblast growth factor 2, and interleukin-15, is also influenced by insulin-like growth factor I (IGF-I), i.e., one of the major circulatory hormones involved in pubertal growth.[33,34]

For nearly 40 years, dual energy X-ray absorptiometry (DXA) has been the primary imaging technology used for the assessment of the muscle-bone relationship in humans. The majority of pediatric data acquired to date support a positive relationship between total body lean mass and DXA bone outcomes at various skeletal sites.[6,33,35-44] Technological advancements in the past two decades have led to the development of alternative imaging modalities, such as peripheral quantitative computed tomography (pQCT), which allows for the assessment of cortical and trabecular bone geometry and volumetric bone mineral density (vBMD) in addition to muscle size and adipose tissue infiltration at appendicular skeletal regions (**Figure 2**).[13] Whereas positive relationships between trabecular bone outcomes and muscle mass have been reported, the majority of pediatric evidence has focused on cortical bone outcomes.[23,25,45] This review will present the clinical evidence acquired to date concerning the role of skeletal muscle mass, size, and adiposity, and related hormonal factors on pediatric cortical bone outcomes.

Muscle and Cortical Bone Geometry, Volumetric Density and Strength

Collecting data on cross-sectional cortical bone outcomes is important with respect to the muscle-bone relationship because lean body mass exerts its osteogenic effects through differential mechanisms, of which only sophisticated imaging modalities are able to capture. Skeletal muscle mass and size are consistent and positive predictors of cortical bone geometry, predominantly due their role in promoting bone mineral deposition toward the periosteum.[27,38,46-48] This preferential deposition of bone mineral toward the periosteal boarder may help explain the mixed relationships (i.e., positive,[15,23,27,48] null[13,15,25,36,38,47,49,50] and negative[13,27,36,48] associations) between muscle and cortical bone volumetric density. By contrast, muscle mass, size, and strength are consistent and positive determinants of cortical bone size and estimated strength. In a study of boys and girls ranging in age from 8-15 years, those with greater appendicular lean mass (ALM; kg) had a larger periosteal circumference and subsequent cortical bone area at both the ultra-distal radius and tibia.[23] However, ALM was positively associated with vBMD only in boys at the mid-tibia. In a separate cohort of boys and girls (10-15 years), calf muscle cross-sectional area was a strong determinant of section modulus at the tibia 38% site.[25] The authors also noted strong positive associations between upper and lower limb muscle strength with section modulus at the radius and tibia, respectively. Bajaj et al[45] reported similar findings in early pubertal boys and girls via magnetic resonance imaging. This study showed that femoral muscle volume was a positive predictor of femoral cortical bone size and estimated strength, though the strength of the positive relationships were dampened after accounting for the dependence of cortical bone size on bone length. In a prospective study, Moon et al[50] found that total body lean mass was a positive predictor of tibia (38% site) total bone area, cortical bone area, and cortical vBMD in a cohort of young boys and girls tracked from birth to approximately

7 years of age. These findings in younger children were similar to those in early pubertal girls, such that over a period of two years, change in MCSA was a positive predictor of tibia and femur cortical area, cortical thickness, and polar strength strain index (pSSI).[49] Further, in older boys and girls (8 to 18 years), Wey et al[15] showed that over a period of 18-36 months, change in MCSA was a positive determinant of most distal radius (20% site) cortical bone size and strength outcomes. However, in terms of vBMD, MCSA change was a positive predictor in older girls but a negative predictor in younger girls. One explanation for these inconsistencies is that growth trajectories of cortical bone size and density differ at earlier versus later stages of pubertal development. Further, cortical porosity decreases during the latter years of pubertal maturation, which may appear as increases in cortical vBMD.[9]

Muscle Adiposity and Pediatric Bone

Obese children are overrepresented in pediatric skeletal fracture cases; however, the reason for this is unclear.[17] The influence of excess adipose tissue on measures of bone mass, geometry, architecture, and strength are mixed, though studies (from co-author R. Lewis and colleagues) support a negative effect of adiposity on cortical bone outcomes in adolescents.[13,14] The idea that certain adipose depots regulate the fat-bone relationship has been an area of recent interest, specifically with respect to visceral, bone marrow and muscular adiposity. Fat is located between adjacent muscles (i.e., intermuscular fat) as well as within individual muscles (intramuscular fat) and myocytes (intramyocellular lipid).[51] Considering the pQCT-derived measure of muscle density (MD), which account for the fat within and between muscle fibers, Farr et al[52] were the first to show that higher MD (i.e., lower muscle fat) was a significant positive predictor of cortical vBMD (femur), area (femur and tibia), and thickness (tibia), as well as pSSI (femur and tibia) in a cohort of girls (mean age 10.7 years). By contrast, Deere et al[53]

reported negative associations between MD and measures of bone size, volumetric density, and strength in older boys and girls (mean age = 17.8 years). Though the age of the participants differed, it is important to consider that both studies were cross-sectional in nature. Therefore, it is difficult to determine the factors contributing to these conflicting findings despite the authors employing relatively similar statistical analyses.

To date, only two prospective studies have examined the relationships between muscle fat and cortical bone geometry in children.[49,54] Changes in cortical bone outcomes over a 2-year period were compared to MD in a cohort aged 13 years at follow-up.[54] In support of the cross-sectional findings in Deere et al,[53] girls with higher muscle fat at baseline had the lowest gains in trabecular bone outcomes (femur and tibia) and cortical vBMD (tibia). Though the authors did not statistically account for changes in MD across the 2-year period, girls with the lowest MD at baseline had significantly greater positive changes in MD over the duration of this study.[54] Since change in MD was positively associated with change in cortical vBMD at both the femur and tibia, the duration of exposure to muscle adiposity implies an important consideration in these studies and may explain the mixed cross-sectional relationships mentioned previously.[49]

Muscle adipose tissue infiltration is associated with metabolic disturbances, such as insulin resistance, which may adversely influence cortical bone geometry in adolescents.[49,55] Sayers et al[55] suggested that the negative relationship between insulin resistance and cortical bone may be, at least in part, attributed to muscle fat. Though Janz et al[56] showed that the relationships between muscle function and cortical bone outcomes were dependent upon muscle size, it has also been shown in older and younger adults that muscle adiposity may impede muscular function.[57,58] Data in children demonstrate that after controlling for differences in body weight, obese versus normal weight youth have significantly lower peak muscle power and

force production.[59] While the authors did not present data on muscle adiposity, an important consideration is that fat infiltration into the muscle tracks consistently with total body adiposity. Perhaps the greater lower leg fat-to-muscle ratio in the obese children was accompanied by greater levels of muscle fat.[60]

Insulin Resistance and Bone

Of the few studies that have examined the relationships between metabolic health outcomes and bone in children, the vast majority has supported a negative relationship between insulin resistance and bone utilizing 2-dimensional bone imaging techniques (**Table 1**).[55,61-69] Among the first to examine these associations in a younger cohort of overweight American Latino children was Afghani and colleagues.[62] The authors showed that various measures of insulin resistance were inversely associated with total body BMC and aBMD. Lee et al[66] likewise demonstrated that children with higher measures of insulin resistance had lower BMC at various skeletal regions of interest. Other studies in children with prediabetes[67] and who have multiple cardiometabolic risk factors associated with the metabolic syndrome[61] support these findings. Alternatively, Lawlor et al[65] showed in the boys and girls who participated in the ALSPAC study that fasting insulin was a positive predictor of total body BA and BMC, but aBMD in females only. However, after accounting for the effect of fat mass, insulin was a significant negative predictor of aBMD, BA and BMC in all study participants. These latter findings are in agreement with those by Sayers et al[55] who, in this same cohort of ALSPAC children, showed that fasting insulin was negatively associated with periosteal circumference, cortical BMC, cortical vBMD, and polar SSI. Based on the evidence acquired to date, it appears that insulin resistance in children is associated with suboptimal skeletal mass and structure, yet the mediating mechanisms explaining this relationship remain unknown.

Insulin resistance, hyperinsulinemia and hyperglycemia are conditions that typically occur secondary to obesity. Both obesity and type-2 diabetes are pro-inflammatory conditions, and provide one possible explanation for the fat-bone relationship, since exposure to pro-inflammatory factors may modulate bone metabolism. Another possible mechanism by which insulin resistance may impact bone involves the formation of advanced glycation end products. Circulatory glucose concentrations are tightly regulated throughout pubertal growth; however, hyperglycemia may lead to the glycation of bone collagenous proteins and result in compromised bone strength.[69] Pediatric studies relating bone quality with insulin resistance and hyperglycemia-related inflammation and bone glycation are limited, thus future studies should consider these mechanisms.

IGF-I and Bone

Whereas skeletal growth and maturation are under the control of various hormones and sex steroids, IGF-I is considered a major determinant of cortical bone development.[70] Circulatory IGF-I concentrations mirror hepatic origins and both the muscle and bone secrete IGF-I, which acts in an autocrine and/or paracrine fashion.[71-73] Breen et al[33] demonstrated prospectively a strong influence of IGF-I on adolescent bone mass accrual, such that the boys and girls in the 90th percentile of circulating IGF-I concentrations exhibited the greatest gains in BMC at various habitually loaded and unloaded skeletal regions. Though bone outcomes were not reported in the study by Dalskov et al,[74] significant positive associations between IGF-I and fat-free mass index where observed in 8-11 year old girls. Fat-free mass index is a composite measure of muscle mass, bone mass and height, and each is correlated with IGF-I in varying degrees. This may explain why prospective relationships over a period of 3-6 months were marginal in this study. Moreover, the use of two-dimensional DXA-derived measures may also

influence the interpretation of these findings since IGF-I is known to promote periosteal bone mineral accrual, which DXA is not able to detect.[71] Considering that IGF-I has a marked effect on muscle protein synthesis, a muscle-dependent link in the IGF-I-bone relationship in humans has been suggested.[75] Indeed, Xu et al[70] showed that the positive relationships between IGF-I and cortical bone size and mass were attenuated after controlling for muscle size. Further, the majority of circulatory IGF-I is bound to IGF binding proteins that modulate the bioactivity of IGF-I; thus, it is likely that these factors play a major role in regulating the IGF-I-muscle/bone relationships.[76] Additional research is needed to elucidate the mechanisms by which muscle may mediate the IGF-I-cortical bone relationships in children.

Insulin Resistance, IGF-I, and Musculoskeletal Development

Insulin and IGF-I are similar in terms of structure, cellular target tissues (e.g., muscle and bone), and downstream signaling processes, which occur predominantly through the AKT/mTOR pathway.[76-78] Therefore, it is likely that biological factors involved in the progression of insulin resistance may too hinder IGF-I-dependent processes. This has been discussed as a potential explanation for various musculoskeletal phenotypes such as that observed with idiopathic osteoporosis,[79] rheumatoid arthritis,[80] aging,[81] and unloading,[82] to name a few. Sayers and colleagues[55] also discussed the potential role of IGF-I as an explanation for their observed relationships between fasting insulin and cortical bone parameters in the adolescent participants of the ALSPAC.

In vivo and in vitro studies provide insight regarding the manner in which certain biological factors understood to contribute to the progression of insulin resistance and type-2 diabetes may hinder IGF-I-dependent processes. In a rat model of diet-induced type 2 diabetes, Li et al[83] showed significant reductions in total body BMD over a period of 12-weeks. These

changes in bone mass were accompanied by significant modulations to integral components of the IGF-I signaling pathway. For instance, at the muscle and bone, mRNA expression of insulin receptor substrate (IRS), an essential component of the insulin/IGF-I-mediated AKT/mTOR signaling pathway, was significantly reduced. Considering that hyperglycemia and hyperlipidemia are hallmark characteristics of type 2 diabetes, these authors concluded that these associated factors contributed to the changes observed in type 2 diabetes. However, type 2 diabetes is also a pro-inflammatory state, and these inflammatory cytokines are also suspected to contribute to disease progression. In fact, Choucair and colleagues[84] examined the effect of numerous pro-inflammatory cytokines (i.e., TNF-α, IL-6, and IL-1β) on IGF-I-dependent processes is chondrocyte lineage cells. These authors showed that culturing IGF-I with these pro-inflammatory cytokines had a significant attenuating influence on IGF-I-dependent activation of PI3 kinase and map kinase. These changes in cellular signaling were accompanied by corresponding decreases in chondrocyte differentiation, but had no influence on chondrocyte IGF-I receptor expression. Similar results have also been demonstrated in myoblasts in relation to IGF-I signaling components and the pro-inflammatory factors IL-1β and TNF-α.[85,86] Similar to the above-mentioned data in chondrocytes,[84] Broussard et al showed in two separate studies that TNF-α[86] and IL-1β[85] had a significant modulating influence on IGF-I-dependent IRS-1 and IRS-2 phosphorylation, but no effect on IGF-I receptor autophosphorylation. Again, demonstrating that pro-inflammatory cytokines have an adverse influence on IGF-I-dependent processes downstream of the membrane-bound receptor.

Summary

Adolescence is a period of rapid musculoskeletal development, with IGF-I playing a pivotal role in these processes. Various studies have implicated obesity and obesity related

chronic health conditions, specifically insulin resistance, as potential hindrances to optimal bone development during youth. However, the mechanism responsible for these observations has yet to be elucidated. Considering the similarities between the pancreatic β-cell-derived insulin and IGF-I, it is reasonable to suspect that IGF-I function is compromised in individuals with insulin resistance. Therefore, this dissertation seeks to address two specific gaps in the current body of evidence: 1) the relationships between insulin resistance and cortical bone endpoints in children, and 2) the influence of insulin resistance on the relationships between IGF-I and musculoskeletal endpoints.

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Table 2.1 Insulin resistance and bone

Reference	Population	Bone imaging method and outcomes		Glucose regulation measures and outcomes		Summary of results
Afghani et al[26]	Overweight Latino children with a familial history of type 2 diabetes N (boys/girls) = 106/78	DXA	Whole body BMC and aBMD	Oral glucose tolerance test Frequently sampled intravenous glucose tolerance test	Fasting glucose 2-hour glucose Fasting insulin 2-hour insulin Insulin sensitivity AIR Insulin AUC Incremental insulin AUC	• In all participants, fasting insulin, 2-hour insulin, AIR, AUC, and incremental AUC were negatively correlated with whole body BMC and aBMD. • AIR and AUC were negative predictors of whole body BMC and aBMD, respectively.
Ashraf et al[63]	African and European American female adolescents (n = 29; ages 14-18 years) and premenopausal adults (n = 49; ages 18-55 years)	DXA	Whole body BMC, aBMD, and BMAD	Mixed meal tolerance test	Insulin AUC Whole body insulin sensitivity index Fasting insulin	• In adolescents and adults, insulin AUC was a positive predictor of whole body aBMD but fasting insulin was positively associated with aBMD only in adolescents. • In the adolescent girls, insulin AUC and fasting insulin were positive correlates of whole body BMAD.
do Prado et al[64]	Musculoskeletal responses in response to weekly doses of vitamin D3 (1400 IU and 14,000 IU) over 1 year	DXA	Whole body BMC and aBMD	Fasting blood draw	Fasting insulin and glucose HOMA-IR	• Fasting insulin and HOMA-IR were significant negative predictors of total body aBMD and BMC in boys but not girls.
Lawlor et al[65]	English boys (n = 1100) and girls (n = 1205)	DXA	Whole body BMC, BA	Fasting blood draw	Fasting insulin	• Fasting insulin was positively associated with

	approximately 15.5 years of age who took part in the ALSPAC		and aBMD			aBMD in girls, but not boys. However, fasting insulin correlated positively with BA and BMC in both sexes. • After adjusting for fat mass, insulin was a significant negative predictor of aBMD, BA and BMC in both sexes.
Lee[66]	Korean adolescent boys (n = 315) and girls (n = 303) aged 10-19 years who participated in the 4th Korean National health and Nutrition Examination Survey	DXA	Whole body BMC Proximal femur BMC Lumbar spine BMC	Fasting blood draw	HOMA-IR	• HOMA-IR was a negative predictor of total body, proximal femur and lumbar spine BMC in boys ages 13-16. HOMA-IR also correlated negatively with total body and proximal femur BMC in boys ages 17-19 years. • HOMA-IR negatively correlated with BMC in normal and overweight boys and girls.
Pirgon et al[68]	Obese adolescent boys and girls with (n = 42) and without (n = 40) NAFLD as well as healthy controls (n = 30) approximately 12.3 years of age.	DXA	Lumbar spine BMD	Fasting blood draw	HOMA-IR	• HOMA-IR was negatively associated with lumbar spine aBMD in obese children with and without NAFLD, however, these relationships were substantially stronger in those with NAFLD.
Pollock et al[13]	Prepubertal black and white boys and girls with (n =	DXA	Whole body BMC, BA	Oral glucose tolerance test	Fasting insulin and glucose	• Children with prediabetes had significantly

	41) and without (n = 99) prediabetes		and aBMD	Fasting blood draw	2-hour glucose Glucose AUC 2-hour insulin Insulin AUC HOMA-IR	lower total body BMC and BA than those with normal fasting glucose concentrations. • In the children with prediabetes, 2-hour insulin and insulin AUC were negatively associated with total body BMC.
Pollock et al[14]	Overweight adolescent black and white boys and girls with 0 (n = 55), 1 (n = 46) and >2 (n = 42) cardiometabolic risk factors who were 14-18 years of age	DXA	Whole body BMC, BA and aBMD	Fasting blood draw	HOMA-IR *Cardiometabolic risk factors:* Waist circumference High density lipoproteins Triglyceride level Blood pressure Fasting glucose Fasting insulin	• Children with 1 or ≥ 2 cardiometabolic risk factors had significantly lower total body BMC and aBMD than those with 0 risk factors. • HOMA-IR was a significant and negative determinant of total body BMC in the total sample of overweight children.
Sayers et al[55]	English boys (n = 1344) and girls (n = 1400) approximately 15.5 years of age who took part in the ALSPAC	pQCT (50% tibia)	PC EC Ct.BMC Ct.vBMD pSSI	Fasting blood draw	Fasting insulin	• Fasting insulin was a negative predictor of PC, cortical vBMD, cortical BMC and pSSI after adjusting for confounders. • Through path analysis, insulin was associated with PC via muscle cross-sectional area (positive), muscle density (negative) and subcutaneous fat (positive).

DXA, dual energy X-ray absorptiometry; BMC, bone mineral content; aBMD, areal bone mineral density; AIR, acute insulin response; AUC, area under the curve; BMAD, bone mineral

apparent density; HOMA-IR, homeostasis model assessment of insulin resistance; ALSPAC, Avon Longitudinal Study of Parents and Children; BA, bone area; NAFLD, -alcoholic fatty liver disease; pQCT, peripheral quantitative computed tomography; PC, periosteal circumference; EC, endosteal circumference; Ct.BMC, cortical bone mineral content; Ct.vBMD, cortical volumetric bone mineral density; pSSI, polar strength-strain index

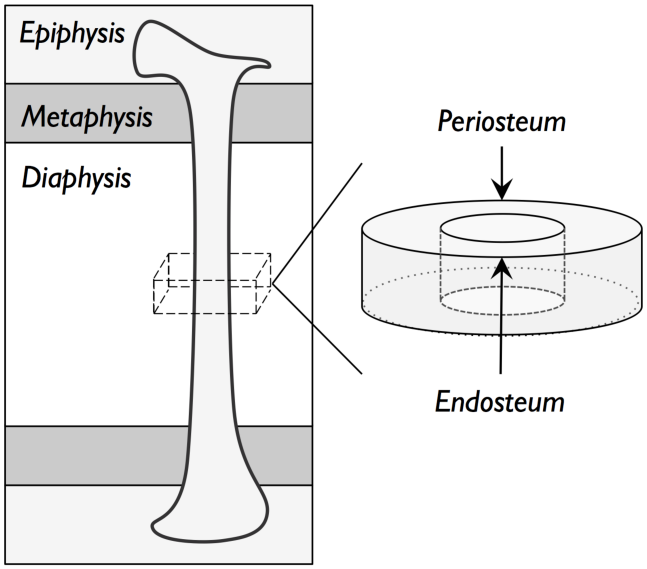

Figure 2.1. Schematic depicting long bone regions.

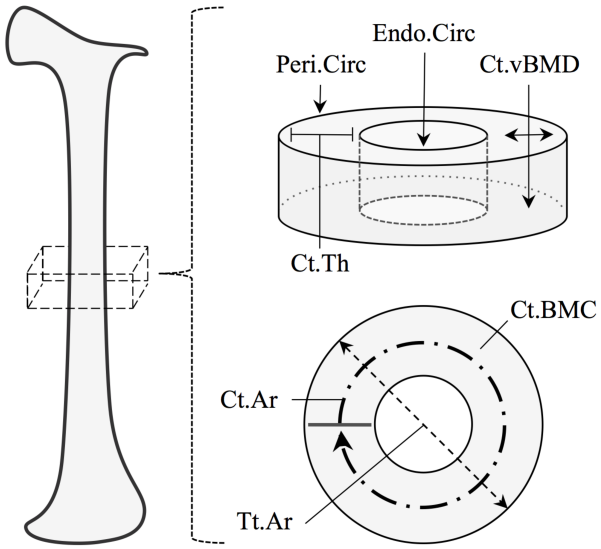

Figure 2.2. Schematic depicting cortical bone endpoints derived via peripheral quantitative computed tomography.

CHAPTER 3[3]

INSULIN RESISTANCE NEGATIVELY INFLUENCES THE MUSCLE-DEPENDENT IGF-I-BONE MASS RELATIONSHIP IN PREMENARCHEAL GIRLS

[3] Kindler JM, Pollock NK, Laing EM, Jenkins NT, Oshri A, Isales C, Hamrick M, Lewis RD. 2016. The Journal of Clinical Endocrinology and Metabolism. Reprinted here with permission of the publisher.

Abstract

Insulin-like growth factor I (IGF-I) promotes bone growth directly and indirectly through its effects on skeletal muscle. Insulin and IGF-I share a common cellular signaling process; thus, insulin resistance may influence the IGF-I-muscle-bone relationship. We sought to determine the effect of insulin resistance on the muscle-dependent relationship between IGF-I and bone mass in pre-menarcheal girls. This was a cross-sectional study conducted at a university research center involving 147 girls ages 9 to 11 years. Glucose, insulin and IGF-I were measured from fasting blood samples. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from glucose and insulin. Fat-free soft tissue (FFST) mass and bone mineral content (BMC) were measured by dual-energy X-ray absorptiometry. Our primary outcome was BMC/height. In our path model, IGF-I predicted FFST mass (b = 0.018; $P = .001$), which in turn, predicted BMC/height (b = 0.960; $P < .001$). IGF-I predicted BMC/height (b = 0.001; $P = .002$), but not after accounting for the mediator of this relationship, FFST mass. The HOMA-IR by IGF-I interaction negatively predicted FFST mass (b = -0.044; $P = .034$). HOMA-IR had a significant and negative effect on the muscle-dependent relationship between IGF-I and BMC/height (b = -0.151; $P = .047$). Lean body mass is an important intermediary factor in the IGF-I-bone relationship. For this reason, bone development may be compromised indirectly via suboptimal IGF-I-dependent muscle development in insulin resistant children.

Introduction

Transient fluctuations in insulin sensitivity are a hallmark of pubertal development. These changes occur irrespective of adiposity, are greatest during early adolescence, and reach a nadir at mid-puberty (1,2). Though girls tend to become more insulin resistant than boys due to greater gains in adiposity, these changes are presumed to accompany normal growth patterns (1). However, there is evidence to suggest that insulin resistance beyond that which occurs normally during the pubertal transition may impede optimal musculoskeletal development. Indeed, several investigators have identified metabolic health outcomes associated with insulin action and glucose homeostasis as negative predictors of bone mass and density in children (3-6). Because the osteoblast is an insulin dependent cell-type, impaired insulin signaling within the bone-forming cells may explain the above associations (7). It is also likely that hyperinsulinemia and/or insulin resistance modulates alternative biological processes involved in musculoskeletal development during youth (5).

Insulin-like growth factor I (IGF-I) is an essential hormone in pediatric muscle and bone development. Whereas IGF-I plays a direct role in bone formation (8,9), it has also been proposed that the effects of IGF-I on skeletal muscle precede its effects on bone (10-14). In a transgenic mouse model, mice overexpressing IGF-I had significantly greater muscle mass and muscle cross-sectional area versus wild type mice (10). These animals also had increases in tibia and femur cortical bone area and thickness, BMC, and periosteal circumference, suggesting a muscle-dependent relationship between IGF-I and cortical bone outcomes. In a cohort of Finnish girls, Xu et al (12) reported positive associations between IGF-I and cortical bone size and mass that were nullified after adjusting for lower leg muscle cross-sectional area. Further, a muscle-

mediated link between IGF-I and bone mass accrual throughout the pubertal years was also proposed by Breen and colleagues (11).

In addition to being an integral link in the IGF-I-bone relationship, skeletal muscle is the primary site of insulin-mediated glucose uptake. Because IGF-I and the pancreatic β cell-derived insulin are structurally similar and share a common downstream cellular signaling process (15-17), it is plausible that insulin resistance has an adverse effect on IGF-I-dependent processes. Some researchers have suggested that insulin resistance during puberty may be accompanied by a resistance to IGF-I (1); however, this was only speculative. The effect of insulin resistance on IGF-I function, in particular with respect to the IGF-I-muscle and bone relationship, has yet to be examined. The aim of this study was to determine the effect of insulin resistance on the muscle-dependent relationship between IGF-I and bone mass in girls at the early stages of pubertal maturation (Figure 1).

Subjects and Methods

Subjects

This cross-sectional study is an ancillary analysis of a double-blinded, randomized, placebo-controlled zinc sulfate supplementation trial, conducted between 2007 and 2010 (18). This study utilized baseline data from black and white girls, ages 9 to 11 years (N = 147), and in the early stages of puberty. In order to determine initial study eligibility, potential participants underwent a telephone pre-screening. Girls were excluded from this study if they had reached menarche, were previously diagnosed with a chronic disease or growth disorder, or reported the use of medications and/or herbal supplements that are known to influence bone metabolism. Sexual maturation was determined by the criteria set forth by Tanner (19), and all participants were required to have a sexual maturation rating stage of 2 or 3 based on breast development.

The parent or guardian of eligible participants were mailed a sexual maturation rating stage self-assessment form, along with photographs and written explanations of each maturation stage, which was completed and mailed back to the laboratory. Once participants met inclusion criteria, an in-lab screening visit was conducted to measure standing height, body weight, sitting height and leg length in order to calculate maturity offset. Maturity offset, or years relative to peak height velocity (PHV), was calculated as: -9.376 + 0.0001882 x interaction of leg length and sitting height + 0.0022 x interaction of age and leg length + 0.005841 x interaction of age and sitting height - 0.002658 x interaction of age and weight + 0.07693 x ratio of weight to height (20). Study protocols and procedures were approved by The University of Georgia Institutional Review Board for Human Subjects, and each participant and their guardian provided written informed assent and consent, respectively.

Anthropometric measurements

One trained researcher performed all anthropometric measures of participants who wore light indoor clothing. Weight was measured to the nearest 0.1 kg using an electronic scale (Seca Bella 840). Standing height, sitting height and leg length were measured to the nearest 0.1 cm using a wall-mounted stadiometer (Novel Products Inc.). Each measure was performed twice and then averaged. In our lab, ten girls ages 6-10 years were measured twice over a 2-week period to determine measurement reliability, and intraclass correlation coefficients (ICC) and coefficients of variation (CVs) were computed for standing height (0.99 and 0.4%), body weight (0.99 and 1.4%) and sitting height (0.97 and 0.9%).

Dual energy X-ray absorptiometry

Total body fat-free soft tissue (FFST) mass (kg), fat mass (kg), percent body fat (%BF), and bone mineral content (BMC; g) were assessed via dual-energy X-ray absorptiometry (DXA;

Delphi A, Hologic Inc.). In order to ensure quality assurance, the DXA machine was calibrated against a three-step soft tissue wedge (Hologic, Inc.), which was composed of different thickness levels of aluminum and Lucite. The same researcher performed and analyzed all DXA scans through instrument-specific software and procedures (Whole Body Analysis software, Hologic Inc., version 11.2). In our lab, ten girls ages 5 to 8 years were scanned twice over a 7-day period in order to determine measurement reliability, all ICCs for bone and body composition outcomes were ≥ 0.98. For all analyses, total body BMC was normalized for height (i.e., BMC/height).

Serum biochemistries

Blood samples were collected by a trained phlebotomist between 0700 and 1030 on the morning following an overnight fast. Samples were placed on ice immediately after collection, centrifuged, and stored in a -80 °C freezer until analysis for glucose, insulin and IGF-I. From sera, glucose was measured in triplicate using a microtiter modification of the enzymatic Autokit Glucose method (Wako Chemicals), which has a detection limit of 0-500 mg/dL. The mean intra- and inter-assay CVs for this analysis was 1.8% and 2.2%, respectively. Insulin was assayed in duplicate from sera using the Human Insulin Specific radioimmunoassay (HI-14K), which has a detection limit of 3.125-100 uU/mL. The mean intra- and inter-assay CV for this analysis was 3.5% and 5.3, respectively%. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as: fasting insulin (uU/mL) x fasting glucose (mg/dL) / 405 (21). From blood collection tubes that were pretreated with EDTA, plasma IGF-I (ng/mL) concentrations were measured in duplicate using a quantitative sandwich immunoassay technique with recombinant human IGF-I (R&D Systems). Each plasma sample was pretreated with an acidic solution for the purpose of dissociation prior to treatment with a buffered protein that contained blue dye and

preservatives, then lyophilized to release IGF-I that was bound to IGF binding proteins (IGFBPs). The inter- and intra-assay CVs were 7.5-8.3% and 3.5-4.3%, respectively.

Statistical analyses

Histograms for all variables were visually inspected for outliers and normal distribution. Skewed or kurtotic distributions were confirmed if greater than 2.0. All variables of interest followed an approximately normal distribution. Pearson's bivariate and partial correlations were performed. Linear regression was performed to determine the relationship between IGF-I and BMC/height while controlling for sexual maturation rating stage. These analyses were performed using SPSS (version 21; Chicago, IL).

To test the moderating effect of HOMA-IR on the FFST mass-mediated relationship between IGF-I and BMC/height, a Model 2 moderated mediation analysis was performed as described by Preacher et al (22). Given model convergence issues pertaining to excessive variances in certain model parameters, FFST mass (log), IGF-I (square root), BMC/height (square root), and the interaction between HOMA-IR and IGF-I (i.e., HOMA-IR*IGF-I; square root) were transformed. Both HOMA-IR and IGF-I were centered on their respective grand means and the maximum likelihood estimation was used. We regressed BMC/height on FFST mass (path B1), IGF-I, HOMA-IR and HOMA-IR*IGF-I. FFST mass was regressed on IGF-I (path A1), sexual maturation rating stage, HOMA-IR and HOMA-IR*IGF-I (path A3). IGF-I covaried with sexual maturation rating stage and HOMA-IR. HOMA-IR*IGF-I covaried with HOMA-IR, IGF-I and sexual maturation rating stage. Sexual maturation covaried with HOMA-IR. The pathway from sexual maturation rating stage to BMC/height was removed from our final model due to the non-significant relationship, and therefore generated an over-identified model. Overall model goodness of fit was determined through previously set criteria on multiple fit

indices (23). Accordingly, the following absolute and relative model fit indices were considered for our model: Root Mean Square Error of Approximation (RMSEA) < .08, Comparative Fit Index (CFI) > .90, Tucker-Lewis Index (TLI) > .90, and Standardized Root Mean Square Residual (SRMR) < .07. We tested our moderation at a HOMA-IR value of 4.0 through the following equation: (path A1 + path A3 x 4.0) x path B (24). Mplus software (version 7.31) was used for our moderated mediation analysis. The statistical significance level for all analyses was set at $P < .050$.

Results

Descriptive participant characteristics are presented in Table 1. IGF-I was positively associated with FFST mass and BMC/height in both unadjusted and sexual maturation-adjusted analyses (all $P < .010$; Table 2). After additional adjustment for FFST mass, the relationship between IGF-I and BCM/height was nullified. HOMA-IR was positively associated with both FFST mass and BMC/height in unadjusted and sexual maturation-adjusted analyses (all $P < .050$). After additional adjustment for FFST mass, HOMA-IR was no longer associated with BMC/height. FFST mass and BMC/height were strongly correlated with one another in unadjusted ($r = 0.790$, $P < .001$) and sexual maturation-adjusted ($r = 0.758$, $P < .001$) analyses. IGF-I and HOMA-IR did not correlate with one another in either unadjusted ($r = 0.085$, $P = .305$) or sexual maturation-adjusted ($r = 0.063$, $P = .453$) analyses.

The path model examining the effect of HOMA-IR in the FFST mass-dependent relationship between IGF-I and BMC/height is depicted in Figure 2. In this model, the paths from IGF-I to FFST mass (positive), FFST mass to BMC/height (positive), and HOMA-IR*IGF-I to FFST mass (negative) were statistically significant (all $P < .050$). Testing the moderating effect of HOMA-IR at a cut-point of HOMA-IR = 4.0, the test for an indirect effect was

statistically significant and negative ($P < .050$), meaning that the FFST mass-mediated relationship between IGF-I and BMC/height was stronger in participants with HOMA-IR < 4.0 versus those with HOMA-IR > 4.0.

Discussion

We examined the effect of insulin resistance on the muscle-dependent relationship between IGF-I and bone mass in children. The rationale for this study is based on the understanding that IGF-I promotes muscle and bone growth (11,12,14), the effect of IGF-I on bone is presumed to be mediated through skeletal muscle (10-12), and that insulin and IGF-I share a common downstream cellular signaling process (15,17). Thus, it is plausible that insulin resistance may impair IGF-I action. The main finding from this study was that insulin resistance had a significant negative effect on the FFST mass-dependent relationship between IGF-I and bone mass in pre-menarcheal girls. Our data provide a possible explanation of why insulin resistant children may be at risk for suboptimal bone mass accrual (3,6,25) and cortical bone development (5).

Through path analysis, we showed that the muscle-dependent link between IGF-I and bone mass was attenuated in our insulin resistant girls. These findings suggest that despite having similar IGF-I concentrations, the myotrophic effects of IGF-I may differ between insulin resistant versus insulin sensitive children. Based on the hypothesis that IGF-I cellular signaling processes are compromised secondary to insulin resistance, particularly within muscle tissue, this explanation is plausible (1). Indeed, attenuated IGF-I cellular signaling in the muscle and/or bone has been shown in animal models of ageing (8) and physical unloading (9). Additionally, in a rat model of diet-induced type-2 diabetes, Li et al (26) showed significant reductions in mRNA and protein expression of muscle and bone cell insulin receptor substrate, an essential component of

insulin/IGF-I-mediated AKT/mTOR signaling processes (17). The concept of "IGF-I resistance" has been proposed as one explanation for the skeletal phenotype that is characteristic of idiopathic osteoporosis; however, to our knowledge, this has not yet been studied in the context of insulin resistance in humans (27).

It is important to consider that insulin resistance may negatively influence IGF-I-dependent muscle growth since lean body mass is a strong determinant of pediatric bone development and is presumed to facilitate the IGF-I-bone relationship (12,28). As an example, Xu et al (12) showed that the positive relationship between IGF-I and mid-tibia cortical BMC was nullified after controlling for muscle cross-sectional area in a prospective study of girls in the early to later stages of maturation. This relationship was also evident in mice overexpressing IGF-I, demonstrating a muscle-dependent effect of IGF-I on cortical bone mass and size (10). In the current study, the significant and positive correlation between IGF-I and bone mass was attenuated after adjusting for FFST mass. This finding was replicated in our path model, i.e., the non-significant direct pathway from IGF-I to bone mass represents the relationship between IGF-I and BMC/height while controlling for the mediator (i.e., FFST mass). The rationale for these findings is that IGF-I promotes muscle growth, which in turn, leads to bone mass accrual and cortical bone areal expansion (11,12,14). Though these data provide valuable insight regarding the contribution of skeletal muscle in the IGF-I-bone relationship, stating that the link between IGF-I and bone is mediated by muscle mass is premature. In our causal path model, however, we show for the first time that FFST mass is a true mediator in the pathway from IGF-I to bone mass in our sample of early pubertal girls. We do not dispute that IGF-I is involved in bone development through direct processes. Circulatory IGF-I concentrations most closely mirror hepatic production. Moreover, both the muscle and bone produce IGF-I, acting in an autocrine

and/or paracrine fashion (29,30), and may further explain why the direct path from IGF-I to BMC/height was not significant in the current study.

Because insulin promotes hepatic IGF-I production, it would be expected that the insulin resistant girls in the current study with higher fasting insulin concentrations would also have higher IGF-I concentrations (31). However, we showed only marginal associations between HOMA-IR and IGF-I. A number of explanations may account for this null finding, including a non-linear relationship between insulin resistance and circulatory IGF-I (32) as well as the relatively high mean HOMA-IR (approximately 5.5) in our sample. We used a conservative cut-point of 4.0 to test the interaction between HOMA-IR and IGF-I in our causal model (24), however, more liberal values of HOMA-IR to denote "insulin resistance," e.g., 3.16, have been published previously (33). Despite having similar total IGF-I concentrations, insulin resistant and hyperinsulinemic children may have higher biologically active IGF-I than their healthier counterparts due to modulations in IGFBPs (34). These differences in IGF-I availability in favor of the insulin resistant children further support the hypothesized modulation of IGF-I action secondary to insulin resistance.

The majority of the girls in the current study were proximal to the estimated age of PHV, which precedes peak lean mass velocity (PLMV) and peak bone mass velocity (PBMV). We speculate that the 12% of study participants who had surpassed the estimated age of PHV had not yet reached PLMV nor PBMV, since these pubertal milestones are not typically achieved until approximately 13 years of age (35). Further, plateaus in peak lean mass precede plateaus in peak bone mass, neither of which are typically achieved until young adulthood (36). Because impaired IGF-I-dependent muscle accretion may result in discrepancies in the attainment of peak bone mass in insulin resistant children, this should be of particular concern since nearly one fourth of

adult bone mass is accrued during the two years surrounding PHV (37). Our participants were on average 10.5 years of age and nearly one year away from estimated PHV. Thus, one could argue that there is substantial time to mitigate the trajectory of insulin resistance-related muscle and bone developmental inadequacies by optimizing insulin sensitivity during youth. Prospective studies are needed to confirm whether the effect of insulin resistance on IGF-I-dependent muscle development, and subsequently bone development, is hindered throughout maturation.

The major strength of this study was our utilization of path analysis statistical techniques while exploring a novel mechanism through which insulin resistance may negatively influence pediatric muscle and bone development. From our path analysis, the criterion for each of our model fit indices was met, highlighting the merit of our hypothesized model despite our relatively small sample size. Given that our study used cross-sectional data, we cannot be certain that insulin resistance has a direct effect on the IGF-I-muscle-bone relationship. Our inclusion of only two-dimensional (i.e., DXA-derived) musculoskeletal outcomes limits the interpretation of our findings since we are unable to ascertain whether these relationships apply to cortical and/or trabecular bone structural indices. Future studies should prospectively examine cortical and trabecular bone at appendicular skeletal sites since IGF-I promotes cortical bone areal expansion (38) and insulin resistance is a negative determinant of cortical bone size in adolescents (5). Because others have shown agreement between total body BMC with tibia diaphyseal cortical bone area in children (39), our findings may be attributed to cortical bone characteristics that we did not measure. The current study included only female participants, thus we cannot draw conclusions on whether these relationships are consistent across sexes. We suspect that these relationships would differ in boys versus girls given the sex-related differences in the strength of the muscle-bone relationship (40).

Conclusions

We are the first to report that insulin resistance is an important consideration in the context of the IGF-I-muscle and bone relationships. Through path analysis we showed that the muscle-dependent relationship between IGF-I and bone mass was compromised in insulin resistant children. This is of particular importance during the years surrounding PHV, given the rapid muscle and bone mass accrual that occurs during this specific developmental period (37,41). Based on our results, it is premature to state that excessively insulin resistant children are also resistant to IGF-I; however, as discussed previously, this is one possible explanation for our results. Though *in vivo* and *in vitro* data are lacking, the concept of IGF-I resistance has been proposed in pubertal children (1) and is suspected to contribute to the skeletal inadequacies that are characteristic of idiopathic osteoporosis (27). Nearly 25% of US youth are currently considered prediabetic (42). These children may be prone to suboptimal IGF-I-dependent muscle and bone mass accrual since insulin resistance is a seminal characteristic of type-2 diabetes progression. Prospective studies including both boys and girls, along with measures of appendicular cortical and trabecular bone structure and IGFBPs are warranted to enhance our understanding of IGF-I, insulin resistance, and muscle and bone development during youth.

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Table 3.1. Descriptive participant characteristics

	Mean	SD
Age (years)	10.52	0.72
Race (n; black/white)	67/80	
Sexual maturation stage (n; 2/3)	103/43	
Maturation offset (years to PHV)	-0.84	0.67
Weight (kg)	47.03	11.27
Height (cm)	148.35	6.68
BMI (kg/m^2)	21.26	4.4
BMI-for-age percentile (%)	75.07	26.06
FFST mass (kg)	31.11	5.08
Fat mass (kg)	15.39	7.27
Percent body fat (%)	30.72	8.09
BMC/height (g/cm)	8.86	1.2
Fasting insulin (uU/mL)	25.71	10.79
Fasting glucose (mg/dL)	86.05	7.58
HOMA-IR	5.51	2.47
Fasting IGF-I (ng/mL)	407.65	244.7

PHV, peak height velocity; BMI, body mass index, FFST, fat-free soft tissue; BMC, bone mineral content; HOMA-IR, homeostasis model assessment of insulin resistance; IGF-I, insulin-like growth factor I

Table 3.2. Bivariate and partial correlations between IGF-I and HOMA-IR with total body FFST mass and BMC/height in pre-menarcheal girls

	Unadjusted		Sexual maturation-adjusted		Sexual maturation + FFST mass-adjusted	
	r	*P*	r	*P*	r	*P*
IGF-I						
FFST mass	**0.294**	**< 0.001**	**0.262**	**0.002**		
BMC/height	**0.279**	**0.001**	**0.253**	**0.002**	0.087	0.300
HOMA-IR						
FFST mass	**0.381**	**< 0.001**	**0.188**	**0.024**		
BMC/height	**0.240**	**0.004**	**0.342**	**< 0.001**	-0.115	0.170

Values in bold are statistically significant at the level of P < .050 IGF-I, insulin-like growth factor I; HOMA-IR, homeostasis model assessment of insulin resistance; FFST, fat-free soft tissue; BMC, bone mineral content

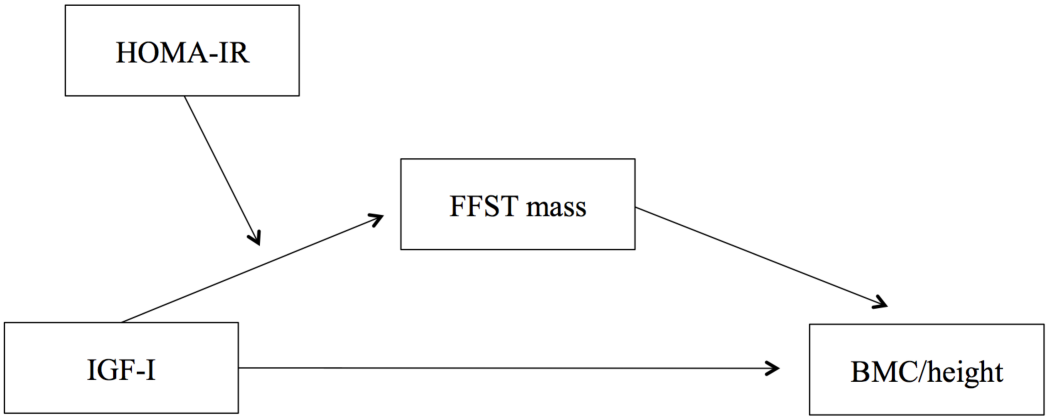

Figure 3.1. Hypothesized effect of insulin resistance (i.e., HOMA-IR) on the FFST mass-dependent relationship between IGF-I and BMC/height. HOMA-IR, homeostasis model assessment of insulin resistance; IGF-I, insulin-like growth factor I; FFST, fat-free soft tissue; BMC, bone mineral content

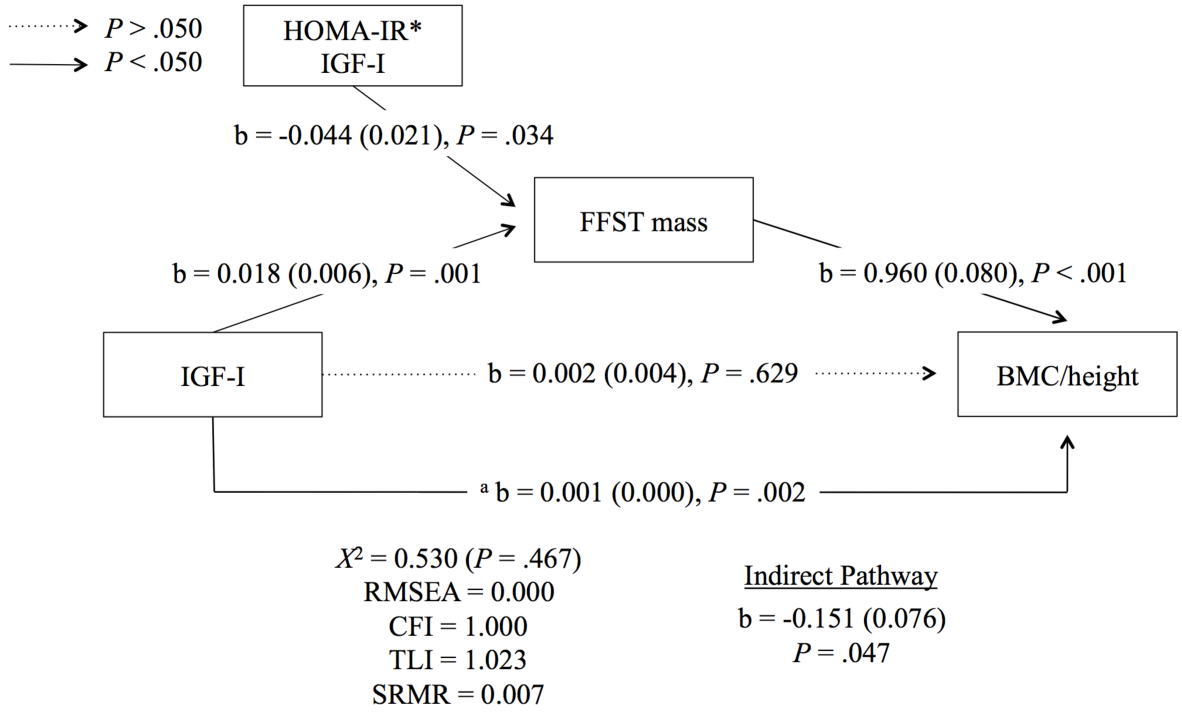

Figure 3.2. Insulin resistance has a negative effect on the FFST mass-dependent relationship between IGF-I and bone mass. The interaction between HOMA-IR and IGF-I was tested at a cut-point of HOMA-IR = 4.0. Unstandardized regression coefficients are presented as b (SE). Sexual maturation and HOMA-IR were included as covariates in this model but are not displayed. [a] Indicates the relationship between IGF-I and BMC/height after adjusting for sexual maturation. Broken lines represent non-significant relationships. HOMA-IR, homeostasis model assessment of insulin resistance; IGF-I, insulin-like growth factor I; FFST, fat-free soft tissue; BMC, bone mineral content; RMSEA, Root Mean Square Error of Approximation; CFI, Comparative Fit Index; TLI, Tucker-Lewis Index; SRMR, Standardized Root Mean Square Residual

CHAPTER 4[4]

INSULIN RESISTANCE AND THE IGF-1-CORTICAL BONE RELATIONSHIP IN CHILDREN AGES 9-13 YEARS

[4] Kindler JM, Pollock NK, Laing EM, Oshri A, Jenkins NT, Isales CM, Hamrick MW, Ding KH, Hausman DB, McCabe GP, Martin B, Hill Gallant KM, Warden SJ, Weaver CM, Peacock M, Lewis RD. 2017. Submitted to The Journal of Bone and Mineral Research.

Abstract

IGF-I is a pivotal hormone in pediatric musculoskeletal development. Though recent data suggest that the role of IGF-I in total body lean mass and total body bone mass accrual may be compromised in children with insulin resistance, cortical bone geometric outcomes have not been studied in this context. Therefore, we explored the influence of insulin resistance on the relationship between IGF-I and cortical bone in children. A secondary aim was to examine the influence of insulin resistance on the lean mass-dependent relationship between IGF-I and cortical bone. Children were otherwise healthy, early adolescent black and white boys and girls (ages 9-13 years) and were classified as having high (n=147) or low (n=168) insulin resistance based on the homeostasis model assessment of insulin resistance (HOMA-IR). Cortical bone at the tibia diaphysis (66% site) and total body fat-free soft tissue mass (FFST) were measured by pQCT and DXA, respectively. IGF-I, insulin and glucose were measured in fasting sera and HOMA-IR was calculated. Children with high HOMA-IR had greater unadjusted IGF-I ($p<0.001$). HOMA-IR was a negative predictor of cortical bone mineral content, cortical bone area (Ct.Ar) and polar strength strain index (pSSI; all $p<0.05$) after adjusting for race, sex, age, maturation, fat mass, and FFST. IGF-I was a positive predictor of most musculoskeletal endpoints (all $p<0.05$) after adjusting for race, sex, age, and maturation. However, these relationships were moderated by HOMA-IR (all $p_{Interaction}<0.05$). FFST positively correlated with most cortical bone outcomes (all $p<0.05$). Path analyses demonstrated a positive relationship between IGF-I and Ct.Ar via FFST in the total cohort ($\beta_{Indirect\ Effect}=0.321$, $p<0.001$). However, this relationship was moderated in the children with high ($\beta_{Indirect\ Effect}=0.200$, $p<0.001$) versus low ($\beta_{Indirect\ Effect}=0.408$, $p<0.001$) HOMA-IR. These data implicate insulin resistance as a

potential suppressor of IGF-I-dependent cortical bone development, although prospective studies are needed.

Introduction

Nearly one third of US children have a body mass index (BMI) ≥ 85th percentile (for sex and age), and are thus considered overweight or obese.[1] Of the various adverse health characteristics that have been linked to childhood overweight and obesity, musculoskeletal health has received relatively little attention. Some studies have demonstrated lower cortical bone mass, size, volumetric density, and estimated bending strength in obese compared with normal-weight adolescents,[2,3] lending a reasonable explanation for the greater propensity for skeletal fractures in overweight and obese youth.[4,5] One obesity-related condition that may underpin the adiposity-bone connection is insulin resistance.[6,7] For instance, in the English boys and girls who participated in the Avon Longitudinal Study of Parents and Children, fasting insulin, an indicator of insulin resistance, was a negative predictor of mid-tibia cortical bone volumetric density, size, and estimated bending strength. Therefore, these data suggest that processes involved in cortical bone areal expansion may be affected.[7]

Of the various hormones involved in pediatric skeletal development, insulin-like growth factor I (IGF-I) plays a pivotal role.[8-10] Indeed, IGF-I promotes bone mineral accrual and cortical bone areal expansion by acting directly upon the bone-forming osteoblasts; preferentially those located toward the periosteum.[11-13] In addition, the trophic effect of IGF-I on lean body mass is suspected to precede skeletal changes.[10,14-16] Therefore, IGF-I promotes cortical bone growth through both direct and lean mass-dependent processes. Moreover, IGF-I is similar to the pancreatic β-cell-derived insulin in terms of structure, downstream signaling processes, and cellular target tissues (e.g., muscle and bone).[17,18] Skeletal muscle is most prone to developing

insulin resistance and, as noted above, is an integral link between IGF-I and bone. Therefore, recent cross-sectional data showing a suppressed total body lean mass-dependent relationship between IGF-I and total body bone mass in girls with high insulin resistance may be attributed to suboptimal IGF-I action.[9] Cortical bone outcomes have yet to be studied in the context of insulin resistance, IGF-I, and pediatric bone; thus representing a key gap in the current body of evidence. In this study, we explored the influence of insulin resistance on the relationship between IGF-I and cortical bone in children. Considering the role of IGF-I in promoting cortical bone areal growth, we hypothesized that insulin resistance would moderate the relationship between IGF-I and cortical bone size, and consequently estimated bending strength. As a secondary aim, we examined the influence of insulin resistance on the lean mass-dependent relationship between IGF-I and cortical bone.

Subjects and Methods

Subjects

This is a cross-sectional, ancillary study using baseline data from children who participated in the GAPI study (The University of Georgia (UGA), Purdue University (PU), and Indiana University (IU) multi-site, double blinded, randomized placebo-controlled vitamin D supplementation trial).[19,20] This secondary data analysis considers all participants with available data on the homeostasis model assessment of insulin resistance (HOMA-IR), and includes black and white males and females, ages 9 to 13 years, who were in the early stages of pubertal development (N=315). All children were recruited at sexual maturation rating stage 2 or 3 based on self-reported breast or genital development.[21-23] Potential participants were excluded from this study if they already commenced menarche (females), had a prior diagnosis of any chronic disease or growth disorder, or were using any medications and/or dietary/herbal supplements

known to influence musculoskeletal metabolism. “High” and “low” HOMA-IR groups were determined using a HOMA-IR cutoff of 4.0.[24] Those designated as having low HOMA-IR (i.e., HOMA-IR < 4.0) represent the group with “normal” insulin sensitivity, and those designated as high HOMA-IR (i.e., HOMA-IR ≥ 4.0) represent the group with the greatest insulin resistance. The Institutional Review Board for Human Subjects at UGA, PU, and IU approved all study protocols and procedures. All participants and parents/guardians provided written informed assent and permission, respectively.

Anthropometric measurements

Weight was measured using an electronic scale, height was measured using a wall-mounted stadiometer, and BMI percentiles (for sex and age) were calculated.[27] Single-measure intraclass correlation coefficients (ICCs) and test-retest coefficients of variation (CV) for height (0.99% and 0.4%) and weight (0.99% and 1.4%) were determined previously in our lab in 6 to 10-year-old girls (N=10) who were measured by the same researcher twice over a 2-week period.[19]

Dual energy X-ray absorptiometry

Fat mass (kg), fat-free soft tissue mass (FFST; kg) and percent body fat (%) were measured via DXA at each study site (Delphi-A, Hologic Inc (UGA); Lunar iDXA, GE Medical Instruments (PU); and Discovery-W, Hologic Inc (IU)). The same researcher at each site performed and analyzed all DXA scans through instrument-specific software and procedures. At the UGA study site, ICCs were calculated from ten females ages 5-8 years who were scanned twice over a 7-day period (all ≥ 0.98). As reported previously,[19,20,25] DXA scanners at each testing site were cross-calibrated and regression formulae were derived and used to adjust data from UGA and IU to PU values.

Peripheral quantitative computed tomography

As reported previously,[20] peripheral quantitative computed tomography (pQCT) scans were performed using Stratec XCT 2000 scanners (Stratec Medizintechnik GmbH, Pforzheim, Germany). To ensure comparability of machines between each testing site, a cortical bone phantom with known properties was scanned a minimum of 20 times on each scanner. The variation in phantom measures differed by < 1%. Scans were performed on the non-dominant lower leg, as determined by self-report. Tibia length (cm) was measured using the medial tibial plateau and the distal edge of the medial malleolus as points of reference. Relative to the total leg length and measured from the distal region, a pen mark was placed upon the 66% site of the tibia diaphysis. The lower leg was centered within the gantry while the subject was sitting upright and facing the instrument. The scan beam was placed upon the pen mark and a single tomographic slice was taken using a slice thickness of 2.3 mm, voxel size of 400 μm and a scan speed of 20 mm/s.

Using a threshold of 710 mg/cm^3, cort mode 1 was used to determine cortical volumetric bone mineral density (Ct.vBMD, mg/cm^3), cortical bone mineral content (Ct.BMC, mg/mm) and cortical bone area (Ct.Ar, cm^2). Using this same threshold, contour mode 1 was used to define the outermost edge of the bone and peel mode 2, using a threshold of 400 mg/cm^3, was used to separate the cancellous and cortical bone compartments. Total bone area (Tt.Ar, mm^2), cortical thickness (Ct.Th, mm), periosteal circumference (Peri.Circ, mm) and endosteal circumference (Endo.Circ, mm) were measured. Cort mode 2 (threshold of 400 mg/cm^3) was used to determine polar strength strain index (pSSI), which uses Ct.vBMD, section modulus, and normal physiological bone density that is estimated at 12,000 mg/mm^3.[20,28,29] Muscle cross-sectional area (MCSA) was measured using a F03F05 filer (contour mode 3 [threshold of -100 mg/cm^3]

and peel mode 2). At the UGA study site, test-retest reliability was performed by scanning five healthy females (ages 18 to 24 years).[2] One-way random effects model, single measure ICCs for all pQCT measurements were $R \geq 0.97$.

Serum biochemistries

Blood samples were collected in the morning following an overnight fast and were stored in a -80 °C freezer until the time of analyses. Serum glucose was measured in triplicate using a microtiter modification of the enzymatic Autokit Glucose method (Wako Chemicals). The mean intra-assay CV for this analysis was 1.8% and the mean inter-assay CV was 2.2%. Serum insulin was assayed in duplicate using the Human Insulin Specific RIA (HI-14K, Millipore). The mean intra-assay CV for this analysis was 3.5% and the mean inter-assay CV was 5.3%. The homoeostasis model assessment of insulin resistance (HOMA-IR) was calculated (fasting insulin [uU/mL] x fasting glucose [mg/dL]/405).[30] As described previously,[9] serum IGF-1 (ng/mL) was measured in duplicate using a quantitative sandwich immunoassay technique with recombinant human IGF-1 (R&D Systems). Mean interassay CVs ranged from 5.6 to 8.7%.

Statistical analyses

Histograms of all variables were inspected for outliers and non-normal distributions. Non-normal distributions were corrected by performing log (insulin, HOMA-IR, FFST, fat mass, Tt.Ar, Ct.Th and pSSI) or square root (tibia length) transformations. The results of the descriptive comparisons using the transformed and untransformed values were similar. Thus, the untransformed data are presented in **Table 4.1** for ease of interpretation. Unadjusted, between-group differences in participant characteristics were determined using independent samples *t*-tests and *X*-square tests.

The relationship between HOMA-IR and musculoskeletal endpoints was examined using liner regression while including race, sex, age, sexual maturation rating stage, and total body fat mass as covariates. Analyses involving cortical bone measures included FFST as an additional covariate. Including tibia length as a covariate did not influence these relationships, and was therefore not include in the final analyses.

Linear regression analyses predicting musculoskeletal outcomes from IGF-I and FFST were performed. All analyses included race, sex, age, and sexual maturation rating stage as covariates. A two-step linear regression procedure was used to assess whether HOMA-IR moderated the relationship between IGF-I/FFST and musculoskeletal endpoints. In the first step of this procedure, the covariates, moderator variable (i.e., HOMA-IR), and independent variable were entered into the regression model. Second, the HOMA-IR by independent variable interaction was entered into the model, and the F change statistic was evaluated (i.e., $p_{Interaction}$). This procedure was also performed while using group and sex as moderator variables. The above-mentioned statistical analyses were performed using SPSS version 23.

The SPSS PROCESS program was used to perform a Model 58 moderated mediation to determine whether the indirect relation between IGF-I and Ct.Ar via FFST differed between HOMA-IR groups.[31] The index of moderated mediation, standard error, and the bias corrected 95% confidence interval (10,000 bootstrap samples) were calculated. The index of moderated mediation was statistically significant (**Figure 4.1**), indicating that 1) the IGF-I-FFST-Ct.Ar relationship was moderated in those with high HOMA-IR and 2) that this difference was attributed to the suppressed path from IGF-I to FFST. Therefore, justifying the comparison of path analyses between the two groups. Using Mplus software (version 7.31), path analysis was performed to examine the FFST-mediated relationship between IGF-I and Ct.Ar. Indirect effects

tests were conducted using the product coefficient method.[32] Each of the above-mentioned path models were just-identified and included race, sex, and age as covariates. IGF-I was log-transformed for each analysis. All significant p-values within each path analysis remained significant after adjusting for multiple comparisons through the Holm-Bonferroni technique. A p-value < 0.05 was considered statistically significant for all analyses.

Results

Descriptive participant characteristics are presented in **Table 4.1**. The high versus low HOMA-IR group had a greater number of black and female participants, were on average more sexually mature, heavier, had a higher BMI-for-age percentile, and greater tibia length, FFST, fat mass, percent body fat, MCSA, insulin, glucose, HOMA-IR, and IGF-I (all $p<0.05$). With the exception of Ct.vBMD and Ct.Th, the unadjusted cortical bone outcomes were higher in the children with high HOMA-IR versus low HOMA-IR (all $p<0.01$).

After controlling for race, sex, age, sexual maturation rating stage, and fat mass, HOMA-IR was a positive predictor of FFST and MCSA (both $p<0.01$; **Table 4.2**). However, HOMA-IR was a negative predictor of Ct.BMC, Ct.Ar, and pSSI after adjustment for race, sex, age, sexual maturation rating stage, fat mass, and FFST (all $p\leq0.01$).

IGF-I was a significant positive predictor of FFST, MCSA, Ct.BMC, Tt.Ar, Ct.Ar, Ct.Th, Peri.Circ, and pSSI in each of our analyses after adjusting for race, sex, age, and sexual maturation rating stage (**Table 4.3**). However, IGF-I was a negative predictor of Ct.vBMD and a positive predictor of Endo.Circ in our total cohort and low HOMA-IR group only (all $p<0.05$). The relationship between IGF-I and FFST, MCSA, Ct.vBMD, Tt.Ar, Ct.Ar, Ct.Th, Peri.Circ, Endo.Circ, and pSSI was moderated by HOMA-IR (all $p_{Interaction}<0.05$). After additional

adjustment for FFST, IGF-I did not correlate with any of the cortical bone outcomes in the total cohort, the high HOMA-IR group, or the low HOMA-IR group (data not shown).

After adjusting for race, sex, age, and sexual maturation rating stage, FFST was a positive predictor of Ct.BMC, Tt.Ar, Ct.Ar, Ct.Th, Peri.Circ, Endo.Circ, and pSSI in each of our analyses (all $p \leq 0.001$, **Table 4.4**), but a negative predictor of Ct.vBMD in our total cohort and low HOMA-IR group only (both $p < 0.005$; $p_{Interaction} < 0.005$).

The path models presented in **Figure 4.2** represent the FFST-dependent relationship between IGF-I and Ct.Ar while controlling for race, sex, and age. In each of our analyses, IGF-I was a positive predictor of FFST and FFST was a positive predictor of Ct.Ar (all $p < 0.001$). IGF-I did not predict Ct.Ar in any of the path models after controlling for the mediator, FFST. The test for an indirect effect was significant in the total cohort, high HOMA-IR group, and low HOMA-IR group (all $p < 0.001$). However, this relationship was moderated in the high HOMA-IR group. The explained variability of Ct.Ar was 7% greater in those with low versus high HOMA-IR.

Relationships between HOMA-IR, IGF-I, and FFST with musculoskeletal outcomes in males versus females are presented in **Table 4.5**. HOMA-IR was positively associated with FFST (females and males) and MCSA (males), but negatively associated with Ct.BMC (females and males), Ct.Ar (males), and pSSI (males; all $p < 0.05$). In females and males, IGF-I was positively associated with FFST, MCSA, Ct.BMC, Tt.Ar, Ct.Ar, Ct.Th, Peri.Circ, and pSSI (all $p < 0.05$). However, IGF-I correlated negatively with Ct.vBMD ($p < 0.05$) and positively with Endo.Circ ($p < 0.005$) in males only. The relationship between IGF-I and musculoskeletal endpoints, with the exception of Endo.Circ, was moderated in females (all $p_{Sex\ diff.} < 0.05$). FFST was positively correlated with most cortical bone outcomes in females and males (all $p < 0.05$). However, FFST negatively correlated with Ct.vBMD in males only ($p < 0.05$; $p_{Sex\ diff.} < 0.05$).

Discussion

The primary aim of this study was to examine the influence of insulin resistance, as measured by HOMA-IR, on the relationship between IGF-I and cortical bone in children. These data show that the relationship between IGF-I and cortical bone are moderated in children with higher insulin resistance. Insulin resistance also suppressed the prediction of FFST and MCSA from IGF-I. Consequently, the lean body mass-dependent relationship between IGF-I and cortical bone was moderated in the children with high versus low HOMA-IR. Considering the role of IGF-I in promoting cortical bone areal expansion, the smaller and consequently weaker cortical bone of children with higher insulin resistance may be attributed to mechanisms involving IGF-I.

To date, this is the first study to examine the IGF-I-cortical bone relationship within the context of insulin resistance. The role of IGF-I in musculoskeletal development during adolescence has been well characterized.[8,10,33] With respect to cortical bone, Xu and colleagues[10] showed in a cohort of Finnish girls that IGF-I was an important determinant of skeletal development, specifically in relation to periosteal expansion and cortical bone mass accrual, over a period of seven years. Likewise, in the current study IGF-I was a positive predictor of various cortical bone size and strength outcomes. However, we also identified inconsistencies in these relationships between the children with high versus low HOMA-IR. Specifically, insulin resistance blunted the strength of the relationship between IGF-I and Tt.Ar, Peri.Circ, Ct.Th, and Ct.Ar. In a liver-specific IGF-I-deficient mouse model (i.e., the LID mouse), Yakar et al[13] showed reductions in femoral cortical bone area and strength compared to wild-type controls, yet tissue mineral density did not differ between the two. Indirectly, these data in the murine model help clarify the negative relationship between IGF-I and Ct.vBMD.

One explanation is that these inverse associations are attributed to the IGF-I-related preferential deposition of bone mineral toward the periosteum, therefore occurring at the expense of the inner-cortex. Taken together, the suppressed relationship between IGF-I and pSSI, as well as Ct.BMC, in the children with higher insulin resistance was attributed to IGF-I-related deviations in cortical bone size, rather than volumetric density (**Figure 4.3)**. Moreover, it is plausible that the lower Ct.BMC, Ct.Ar, and pSSI in those with higher HOMA-IR involve IGF-I-related mechanisms. These results are of concern given that cortical bone bending strength is highly dependent upon areal dimensions[34] and that the majority of skeletal fractures sustained by children and adolescents,[35-37] particularly those with excess adiposity,[4,38] occur at long-bone sites of predominantly cortical bone.

Accompanying the moderated IGF-I-cortical bone relationship, the children with high HOMA-IR also had lower FFST and MCSA relative to IGF-I. Lean body mass and MCSA are strong predictors of cortical bone areal measures[39-42] and are an integral link between IGF-I and bone.[9,10,41] Mouse[41] and human[9,10] studies have provided evidence supporting the facilitative role of lean body mass in the link between IGF-I and bone. In a previous cross-sectional study of premenarcheal girls,[9] our group showed an indirect relationship between IGF-I and total body bone mass via lean body mass. However, we also demonstrated that the IGF-I-lean body mass relationship was attenuated in the girls with higher insulin resistance (i.e., HOMA-IR > 4.0).[24] Likewise, in the current study, the relationship between IGF-I and Ct.Ar was FFST-dependent and was suppressed in the children with high versus low HOMA-IR due to differences in the path from IGF-I to FFST. Between the two groups, we found an approximate 9% difference in explained variability of FFST in favor of those with low HOMA-IR. If in fact IGF-I-dependent lean mass and skeletal muscle accrual is hampered in children with insulin resistance, this may,

in turn, have a downstream influence on skeletal development considering that muscle growth precedes and contributes to, bone accretion.[39,43] We have speculated previously that the insulin resistance-related suppression of the IGF-I-FFST-total body bone mass relationship was accompanied by corresponding deficits in cortical bone geometry.[9] The results of the current study are in support of this hypothesis.

Whereas the inevitable question, "*Are children who are insulin resistant also IGF-I resistant?*" remains unanswered, previous studies provide indirect evidence in support of this position. Insulin and IGF-I are similar in terms of structure, cellular target tissues (e.g., muscle and bone), and downstream signaling processes, specifically through the AKT/mTOR pathway.[17,18,44] As implied in the current study, lean body mass is a facilitator of the relationship between IGF-I and bone and is the primary site of insulin-mediated glucose uptake,[45] thus being most prone to fluctuations in insulin sensitivity. The bone-forming osteoblasts are also insulin-dependent and susceptible to impaired downstream signaling.[46] Factors that contribute to insulin resistance, such as chronic low-grade inflammation, compromise the myogenic and osteogenic effect of IGF-I.[47,48] Therefore, it is reasonable to suspect that the role of IGF-I in pediatric musculoskeletal development is altered in individuals with impaired glucose handling. Despite being tightly regulated throughout maturation, fasting serum glucose was higher in those with higher HOMA-IR. Hyperglycemia may lead to the non-enzymatic glycation of bone collagenous proteins and consequently the accumulation of advanced glycation end products.[49] In addition to being directly implicated in skeletal fragility,[50-52] advanced glycation end products may modulate osteoblast IGF-I function.[53,54] Further, insulin promotes hepatic IGF-I production,[55] likely contributing to the ~20% greater total IGF-I in the high HOMA-IR group. However, the majority of systemic IGF-I is bound to a

variety of regulatory binding proteins. Due to alterations in IGF binding proteins, obese and/or hyperinsulinemic individuals may have a greater proportion of bioavailable relative to total IGF-I versus their healthier counterparts.[56] Therefore, we do not suspect that the insulin resistance-related musculoskeletal inadequacies reported in the current study were attributed to differences in total and/or bioavailable IGF-I.

When interpreting our findings, certain aspects of this study warrant consideration. First, making causal inferences based on our data would be inappropriate given the cross-sectional design. Second, we measured only total circulatory IGF-I concentrations and did not have data available on IGF binding proteins, so we can only speculate on differences in IGF-I bioavailability. Third, although HOMA-IR performs well against the oral glucose tolerance test in children,[58] including more dynamic measures of glucose metabolism would strengthen our methodological approach. Finally, whereas our sample size was sufficient to explore the intended research question, we were unable to perform analyses in groups stratified by race and sex. In accordance with one previous study,[7] relationships between insulin resistance and cortical bone did not differ between sexes. However, with respect to IGF-I, relationships with most musculoskeletal outcomes were stronger in the males versus females while adjusting for covariates including race, age, and maturation. Data pertaining to the sex-dependency of the IGF-I-bone relationship in humans are scarce, yet animal studies indicate that the growth hormone/IGF-I axis, along with sex steroids, contributes to the cortical bone sexual dimorphism.[14] Given that insulin resistance is greater in females versus males during maturation,[59] it is plausible that the influence of insulin resistance on IGF-I-dependent musculoskeletal development differs by sex. Data from our previous study indicate that insulin resistance moderates the relationship between IGF-I and musculoskeletal outcomes in females

who were at the early stages of sexual maturation.[9] However, additional work is needed to corroborate these findings in males. Furthermore, we were not adequately powered to include additional control variables into our path models. The influence of insulin resistance on the relationships between IGF-I and musculoskeletal endpoints was evident whether or not sexual maturation was included as a covariate. Therefore, we do not suspect this omission to be problematic.

The unique strengths of this study include our utilization of path analysis statistical techniques for the testing of FFST as a mediator in the IGF-I-cortical bone relationship. Additionally, we included pQCT-derived measures of appendicular cortical bone geometry and strength, which addresses the most evident limitation of our previous work.[9]

Conclusions

This cross-sectional study corroborates the positive relationship between IGF-I and cortical bone size and strength outcomes in children, and we show for the first time that insulin resistance moderated these relationships. Given that lean body mass is an integral intermediary in the IGF-I-bone relationship and is prone to fluctuations in insulin sensitivity, our results may have been attributed to the suppressed lean body mass-dependent link between IGF-I and cortical bone. Future studies examining the role of IGF-I in pediatric musculoskeletal development within the context of insulin resistance should include measures of IGF-I bioavailability and prospective data collected throughout the adolescent years, specifically in children with obesity-related chronic health conditions. IGF-I is suspected to contribute to the sexual dimorphism observed in skeletal development.[14] Thus, whether insulin resistance influences the IGF-I-bone relationship differently in boys versus girls warrants exploration. Furthermore, biological factors associated with insulin resistance and hyperglycemia, for instance, advanced glycation end

products and biomarkers of inflammation, also warrant consideration in subsequent studies. Since nearly one in four US children and adolescents is at risk of developing type-2 diabetes, a condition characterized by insulin resistance, it is a viable concern that IGF-I-dependent skeletal development is hampered in a relatively large subset of American youth.[60]

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Table 4.1. Participant characteristics

	Total Cohort N=315	High HOMA-IR n=147	Low HOMA-IR n=168	p[a]
Demographics				
Race (n; black)[b]	159	95	64	<0.01
Sex (n, female)[b]	154	86	68	<0.01
Age (years)	11.3 (1.2)	11.2 (1.3)	11.5 (1.2)	0.07
Sexual maturation stage	2.4 (0.5)	2.4 (0.5)	2.3 (0.5)	0.02
Anthropometrics				
Height (cm)	150.7 (9.3)	151.5 (9.3)	150.1 (9.3)	0.18
Weight (kg)	47.4 (12.1)	52.1 (12.4)	43.3 (10.3)	<0.01
BMI-for-age (percentile)	68.2 (29.3)	81.0 (21.5)	57.0 (30.7)	<0.01
Tibia length (cm)	35 (3.5)	35.7 (3.2)	34.3 (3.7)	<0.01
Body Composition				
FFST (kg)	30.5 (6.9)	31.7 (6.4)	29.3 (7.1)	<0.01
Fat mass (kg)	14.7 (7.3)	17.9 (8.0)	11.9 (5.3)	<0.01
Percent body fat (%)	31 (9.4)	34.6 (9.5)	27.9 (8.1)	<0.01
MCSA (mm^2)	4848.8 (1050.0)	5071.4 (998.6)	4655.7 (1058.1)	<0.01
Serum Biochemistries				
Insulin (uU/mL)	19.9 (10.1)	27.3 (10.2)	13.4 (3.3)	<0.01
Glucose (mg/dL)	89 (7.1)	91.3 (7.2)	87.0 (6.5)	<0.01
HOMA-IR	4.4 (2.4)	6.2 (2.4)	2.9 (0.7)	<0.01
IGF-I (ng/mL)	232.4 (97.9)	256.3 (107.3)	211.5 (83.7)	<0.01
Cortical Bone				
Ct.vBMD (mg/cm^3)	1062.6 (35.0)	1066.7 (35.0)	1059.1 (34.8)	0.06
Ct.BMC (mg/mm)	252.2 (47.8)	262.0 (48.0)	243.7 (46.1)	<0.01
Tt.Ar (mm^2)	446.6 (86.5)	464.8 (87.2)	430.8 (83.0)	<0.01
Ct.Ar (mm^2)	237.4 (44.5)	245.4 (43.1)	230.4 (44.5)	<0.01
Ct.Th (mm)	3.8 (0.5)	3.8 (0.5)	3.7 (0.5)	0.12
Peri.Circ (mm)	74.6 (7.2)	76.1 (7.2)	73.3 (6.9)	<0.01
Endo.Circ (mm)	50.9 (6.7)	52.1 (6.9)	49.8 (6.3)	<0.01
pSSI (mm^3)	1684.2 (467.0)	1786.7 (475.9)	1594.9 (441.5)	<0.01

Data are presented as mean (standard deviation) unless otherwise indicated

[a]Test of between-group significance based on independent samples *t*-test

[b]Test of between-group significance based on *X*-square test

Table 4.2. Relationships between HOMA-IR and musculoskeletal outcomes while adjusting for covariates

	β	p
FFST	0.185	<0.001
MCSA	0.149	0.007
Ct.vBMD	-0.094	0.133
Ct.BMC	-0.128	<0.001
Tt.Ar	-0.065	0.111
Ct.Ar	-0.116	0.001
Ct.Th	-0.103	0.075
Peri.Circ	-0.074	0.068
Endo.Circ	-0.033	0.562
pSSI	-0.090	0.010

Each analysis includes, race, sex, age, sexual maturation rating stage and total body fat mass as covariates.
Analyses involving cortical bone outcomes also include FFST as an

Table 4.3. Relationships between IGF-I and musculoskeletal outcomes while adjusting for covariates

	Total Cohort		High HOMA-IR		Low HOMA-IR		$p_{Interaction}$ [a]
	β	p	β	p	β	p	
FFST	0.402	<0.001	0.260	0.001	0.501	<0.001	<0.001[b]
MCSA	0.329	<0.001	0.190	0.042	0.408	<0.001	<0.001[b]
Ct.vBMD	-0.136	0.024	-0.059	0.486	-0.264	0.001	0.001[b]
Ct.BMC	0.278	<0.001	0.216	0.005	0.322	<0.001	0.002
Tt.Ar	0.266	<0.001	0.188	0.035	0.310	<0.001	<0.001
Ct.Ar	0.309	<0.001	0.240	0.003	0.367	<0.001	<0.001[b]
Ct.Th	0.254	<0.001	0.215	0.016	0.308	<0.001	0.035
Peri.Circ	0.264	<0.001	0.191	0.032	0.307	<0.001	<0.001
Endo.Circ	0.163	0.009	0.107	0.275	0.180	0.026	0.012
pSSI	0.292	<0.001	0.216	0.008	0.337	<0.001	<0.001

Race, sex, age, and sexual maturation rating stage were included as covariates for all analyses
[a]Represents the IGF-I x HOMA-IR interaction
[b]IGF-I x group interaction p-value < 0.05

Table 4.4. Relationships between FFST and cortical bone outcomes while adjusting for covariates

	Total Cohort		High HOMA-IR		Low HOMA-IR		$p_{Interaction}$[a]
	β	p	β	p	β	p	
Ct.vBMD	-0.195	0.002	-0.061	0.496	-0.280	0.002	0.001[b]
Ct.BMC	0.690	<0.001	0.639	<0.001	0.771	<0.001	0.838
Tt.Ar	0.747	<0.001	0.783	<0.001	0.747	<0.001	0.727
Ct.Ar	0.736	<0.001	0.675	<0.001	0.811	<0.001	0.219
Ct.Th	0.444	<0.001	0.300	0.001	0.569	<0.001	0.102
Peri.Circ	0.748	<0.001	0.786	<0.001	0.750	<0.001	0.841
Endo.Circ	0.586	<0.001	0.686	<0.001	0.521	<0.001	0.267[b]
pSSI	0.750	<0.001	0.758	<0.001	0.785	<0.001	0.552

Race, sex, age, and sexual maturation rating stage were included as covariates for all analyses
[a]Represents the IGF-I x HOMA-IR interaction
[b]IGF-I x group interaction p-value < 0.05

Table 4.5. Relationships between HOMA-IR, IGF-I, and FFST with musculoskeletal endpoints in males versus females while adjusting for covariates

	Female		Male		$p_{Sex\ diff.}$
	β	p	β	p	
HOMA-IR[a]					
FFST	0.185	0.017	0.207	0.004	0.337
MCSA	0.190	0.014	0.143	0.072	0.753
Ct.vBMD	-0.090	0.315	-0.106	0.233	0.696
Ct.BMC	-0.109	0.045	-0.148	0.003	0.945
Tt.Ar	-0.079	0.215	-0.063	0.266	0.576
Ct.Ar	-0.097	0.072	-0.135	0.006	0.872
Ct.Th	-0.068	0.436	-0.128	0.108	0.646
Peri.Circ	-0.083	0.191	-0.074	0.185	0.550
Endo.Circ	-0.067	0.439	-0.006	0.935	0.329
pSSI	-0.095	0.070	-0.099	0.049	0.796
IGF-I[b]					
FFST	0.342	<0.001	0.505	<0.001	<0.001
MCSA	0.306	<0.001	0.393	<0.001	0.043
Ct.vBMD	-0.073	0.366	-0.216	0.013	0.018
Ct.BMC	0.215	0.003	0.378	<0.001	0.003
Tt.Ar	0.178	0.027	0.389	<0.001	0.017
Ct.Ar	0.243	0.001	0.422	<0.001	<0.001
Ct.Th	0.213	0.012	0.326	<0.001	0.043
Peri.Circ	0.172	0.032	0.394	<0.001	0.007
Endo.Circ	0.075	0.388	0.280	0.002	0.095
pSSI	0.210	0.005	0.410	<0.001	0.011
FFST[b]					
Ct.vBMD	-0.077	0.349	-0.271	0.002	0.021
Ct.BMC	0.670	<0.001	0.673	<0.001	0.880
Tt.Ar	0.698	<0.001	0.765	<0.001	0.493
Ct.Ar	0.725	<0.001	0.723	<0.001	0.453
Ct.Th	0.430	<0.001	0.417	<0.001	0.780
Peri.Circ	0.696	<0.001	0.768	<0.001	0.936
Endo.Circ	0.497	<0.001	0.638	<0.001	0.937
pSSI	0.714	<0.001	0.754	<0.001	0.156

[a]Race, age, sexual maturation rating stage, and total body fat mass were included as covariates. For analyses involving cortical bone endpoints, FFST was also included as a covariate

[b]Race, age, and sexual maturation rating stage were included as covariates

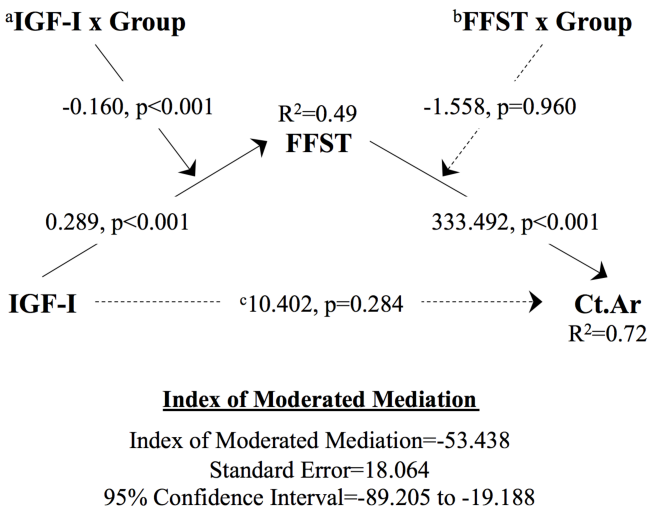

Figure 4.1. The attenuated IGF-I-FFST-Ct.Ar relationship in the children with high HOMA-IR was attributed to the moderated path from IGF-I to FFST. Low HOMA-IR and high HOMA-IR groups were coded as 0 and 1, respectively. [a]Prediction of FFST from the IGF-I by group interaction. [b]Prediction of Ct.Ar from the FFST by group interaction. [c]Relationship between IGF-I and Ct.Ar while controlling for the mediator (i.e., FFST). Broken lines represent nonsignificant relationships. Values are path coefficient, p-value.

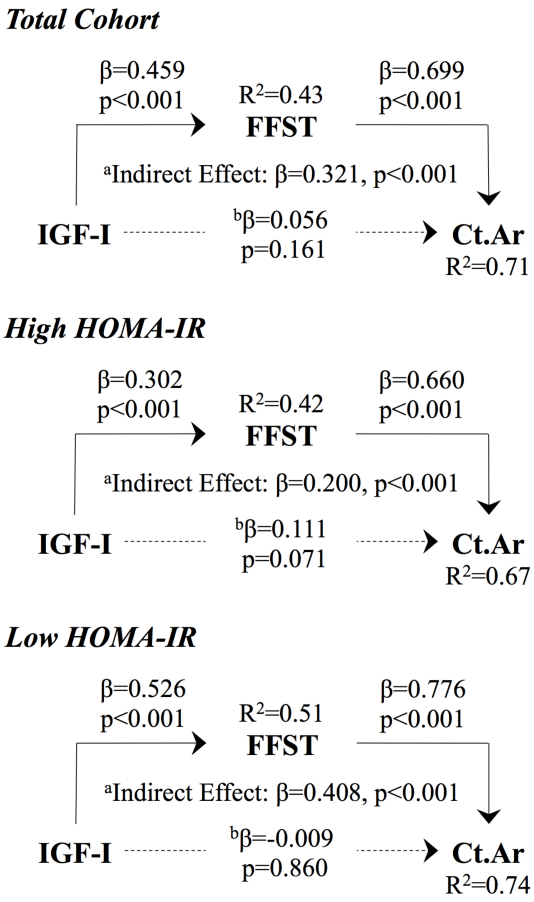

Figure 4.2. IGF-I predicts Ct.Ar via FFST in the total cohort, low HOMA-IR group, and high HOMA-IR group. However, this FFST-dependent relationship is moderated in the children with high HOMA-IR. [a]Relationship between IGF-I and Ct.Ar through FFST. [b]Relationship between IGF-I and Ct.Ar while controlling for the mediator (i.e., FFST). Broken lines represent nonsignificant relationships.

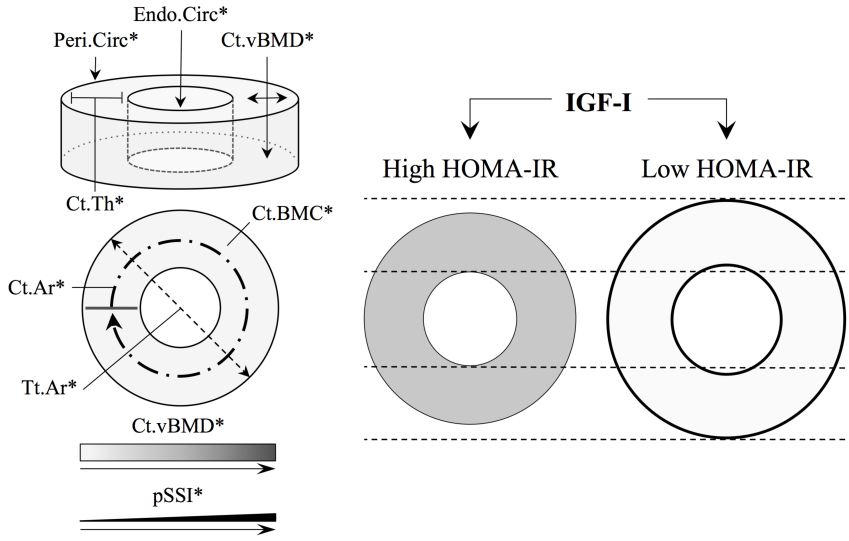

Figure 4.3. Schematic depicting the differences in the IGF-I-cortical bone relationship in the children with high versus low HOMA-IR. The strength of the relationship between IGF-I and Ct.BMC, Tt.Ar, Ct.Ar, Ct.Th, and Peri.Circ, was suppressed in the children with high versus low HOMA-IR. However, IGF-I was a negative predictor of Ct.vBMD (depicted by shading of the cortical compartment) and a positive predictor of Endo.Circ in the children with low but not high HOMA-IR. Consequently, IGF-I was a stronger positive predictor of pSSI in those with low versus high HOMA-IR (depicted by line thickness). *Significant IGF-I by HOMA-IR interaction ($p_{Interaction}<0.05$) for the corresponding cortical bone outcome.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The objective of this dissertation was twofold: first, to determine the relationships between insulin resistance and musculoskeletal endpoints in children, and second, to explore the influence of insulin resistance on the relationships between IGF-I and musculoskeletal outcomes. The study presented in Chapter 3 was conducted with the primary objective of determining the influence of insulin resistance on the lean mass-dependent relationships between IGF-I and total body bone mass in premenarcheal girls. Previous studies have shown both positive and negative relationships between measures of glucose metabolism and bone mass in children.[1] In our minimally adjusted models, the homeostasis model assessment of insulin resistance (HOMA-IR) was positively associated with total body lean and bone mass (all $P < 0.05$). However, after additional adjustment for total body lean mass, the relationship between HOMA-IR and bone mass was attenuated ($P > 0.05$). Similar relationships between IGF-I and musculoskeletal endpoints were identified. Specifically, IGF-I was positively associated with total body lean mass and bone mass in minimally adjusted models (all $P < 0.05$). However, the relationship between IGF-I and bone mass was attenuated after additional adjustment for total body lean mass ($P > 0.05$). Finally, we tested the moderating influence of insulin resistance on the lean body mass-dependent relationship between IGF-I and bone mass. In our path model, IGF-I predicted fat-free soft tissue mass (FFST; b = 0.018, $P = 0.001$), which in turn, predicted bone mass (b = 0.960, $P < 0.001$). IGF-I predicted bone mass (b = 0.001, $P = 0.002$), but not after accounting for the mediator of this relationship, FFST. The HOMA-IR by IGF-I interaction negatively predicted

FFST (b = -0.044, $P = 0.034$). Finally, HOMA-IR had a significant and negative influence on the lean mass-dependent relationship between IGF-I and bone mass (b = -0.151, $P = 0.047$).

The major limitation of the study presented in Chapter 3 was our lack of data on appendicular cortical bone geometric endpoints. This is of particular importance considering that insulin resistance has been associated with cortical bone deficits in adolescents,[2] and that IGF-I is most known for its role in promoting periosteal bone mineral apposition.[3,4] Therefore, the study presented in Chapter 4 was performed to build upon these earlier results. HOMA-IR was a negative predictor of cortical bone mineral content, cortical bone area (Ct.Ar) and polar strength strain index (pSSI; all $p<0.05$) after adjusting for race, sex, age, maturation, fat mass, and FFST. IGF-I was a positive predictor of most musculoskeletal endpoints (all $p<0.05$) after adjusting for race, sex, age, and maturation. However, these relationships were moderated by HOMA-IR (all $p_{Interaction}<0.05$). FFST positively correlated with most cortical bone outcomes (all $p<0.05$). Path analyses demonstrated a positive relationship between IGF-I and Ct.Ar via FFST in the total cohort ($\beta_{Indirect\ Effect}=0.321$, $p<0.001$). However, this relationship was moderated in the children with high ($\beta_{Indirect\ Effect}=0.200$, $p<0.001$) versus low ($\beta_{Indirect\ Effect}=0.408$, $p<0.001$) HOMA-IR. These data implicate insulin resistance as a potential suppressor of IGF-I-dependent cortical bone development, although prospective studies are needed.

These are the first data to identify a blunting influence of insulin resistance on the relationships between IGF-I and musculoskeletal endpoints. Since IGF-I is suspected to contribute to cortical bone areal expansion, the pQCT-derived data presented in the manuscript #2 (Chapter 4) build upon the results presented in manuscript #1 (Chapter 3), which utilized only DXA-derived skeletal outcomes. One limitation of this work that requires consideration is the cross-sectional design of these studies. Further, additional components of the growth

hormone/IGF-I axis such as IGF binding proteins should be taken into account in subsequent investigations, as should sensitive measures of insulin resistance and glucose control.

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APPENDICES I

Supplemental vitamin D in early adolescence

ASSENT FORM (CHILD)

PARENTAL PERMISSION FORM

HEALTH HISTORY QUESTIONNAIRE

SEXUAL MATURATION QUESTIONNAIRES

APPENDIX A

ASSENT FORM (CHILD)

Assent Form (Child)

I, _____, agree to take part in a research study about bone health and growth.

I do not have to be in the study if I do not want to be. I have the right to leave the study at any time without giving any reason, and without penalty. I may have any of my information returned to me, removed from the laboratory, or destroyed. By participating in this study, I will learn about my diet, physical activity and growth. I will learn about vitamin D and if it can help me to be a healthy child, and grow to be a healthy teen and adult.

I will take my vitamin D supplements every day according to the directions. I will not take any other vitamin, mineral or herbal supplements during the study. I will follow my normal dietary habits and will not be asked to avoid certain foods. I will also follow my normal physical activity patterns during the study. I will bring my unused vitamin D supplements to the researcher after 3 weeks so that he or she may count how many I missed. Too much Vitamin D in the diet can cause stomachaches, dizziness, and/or nausea. If I feel any of these side effects, I will report them to the researcher. I will also be asked to answer questions about how the supplements are affecting me.

Before entering the study:

- I will receive a sexual maturation self-assessment form in the mail that I will complete in private at home. I will compare my own appearance to pictures/drawings of growth stages (pictures/drawings of genital areas) and circle the drawing that looks most like me.
- If this procedure causes me to be uncomfortable, I may skip this portion and any information about me will not be shared with anyone else.

At the beginning of the study and at 3, 6, 9, and/or 12 weeks later:

- A trained nurse will take a blood sample from my arm.
- I will provide a urine sample in a private bathroom.
- I will have my height measured against a wall and my weight measured on a scale.
- My parent and I will write down what I eat during two weekdays and one weekend day.
- I will answer questions about my physical activity.
- If I complete these measures listed above, I will receive $50 for the beginning of the study, $50 for 3 weeks, $20 for 6 weeks, $20 for 9 weeks, and $60 for 12 weeks (for a potential total of $200).
- I may experience hunger before the blood and urine collection, but I will receive a snack after these tests.
- I may experience a bruise under my skin after the blood draw, which should disappear within a few days.
- If any of these procedures or questions asked of me cause me to be uncomfortable, I may skip those procedures/ questions and any information about me will not be shared with anyone else.

At the beginning of the study and 12 weeks later, I will have my muscle strength tested by squeezing a handgrip machine, and have pictures taken of my bones and muscles. During these sets of pictures I will lie on a table for approximately 5-10 minutes, and will sit up in a chair for approximately 20-30 minutes. These pictures provide a small amount of radiation, similar to the

X-ray pictures taken at the dentist's office. If any of these procedures or questions cause me to be uncomfortable, I may skip those procedures/ questions and any information about me will not be shared with anyone else.

Before I have the pictures of my bones and muscles taken, I will be asked if I am pregnant. If I am not sure, I will be given a pregnancy test. If I am pregnant, I will not participate in the study.

If I have any questions, I can always call the researcher, Dr. Richard Lewis at the following number: 706-542-4901.

Sincerely,

Emma Laing, PhD, RD, LD
Department of Foods and Nutrition
University of Georgia
279 Dawson Hall

I was given the opportunity to complete a simple urine test for pregnancy:
(Check one): **YES**____ **NO**____

_____ _____
Signature Date

I refuse to take the pregnancy test:
(Check one): **YES**____ **NO**____

_____ _____
Signature Date

I understand the project described above. My questions have been answered and I agree to participate in this project. I have received a copy of this form.

_____
Signature of the Participant/Date

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

Additional questions or problems regarding your rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu**.**

APPENDIX B

PARENTAL PERMISSION

PARENTAL PERMISSION FORM

I, _____, give permission for my child, _____, to participate in the research titled "Supplemental Vitamin D and Functional Outcomes in Early Adolescence," which is being conducted by Drs. Richard Lewis and Emma Laing of the Department of Foods and Nutrition at The University of Georgia. Dr. Lewis may be reached in room 279 Dawson Hall at 706-542-4901. I understand that the participation of my child is completely voluntary. I can withdraw permission at any time without penalty or loss of benefits to which my child is otherwise entitled, and have the results of the participation, to the extent that which it can be identified as my child's, returned to me, removed from the research records, or destroyed. Refusal to participate will involve no penalty or loss of benefits to which my child is otherwise entitled.

1) The following points have been explained to me:
a) The reason for the research is to study the impact of vitamin D supplementation on biochemical markers of bone health in children. The benefits that my child and I can expect from participation are the assessment of diet, maturation, growth, and body composition (percentage of body fat and nonfat tissue). The type of information collected will provide important information about growing children and their potential to be healthy teens and adults. In addition, my child will gain individual health knowledge that may improve his/her quality of life and possibly detect a health problem. If vitamin D status and markers of bone health are improved in childhood through increased dietary vitamin D, the benefits may be realized long after the time my child is involved in the study. This information can be used to determine if a simple and inexpensive nutritional supplement can improve bone health during childhood, which would reduce the risk of osteoporosis later in life.

b) All measurements are being used for research purposes only, not medical purposes. However, if abnormalities are found in any measure, my child and I will be notified and referred to an appropriate health care professional.

c) Once enrolled in the study and following the completion of each testing session, my child will receive $50 for baseline, $50 for 3 weeks, $20 for 6 weeks, $20 for 9 weeks, and $60 for 12 weeks, for a potential total of $200 for the entire study. Payments will be distributed only if all testing sessions are completed for a given time point and supplements are taken as directed. My child will receive a certificate at study completion, birthday cards, reminder calls, and other non-monetary incentives such as UGA posters, magnets, key chains, etc., items of approximately $1 to $2 in value. Finally, all individual and group results will be presented to my child and me at the conclusion of the study.

2) The procedures are as follows:
a) Prior to enrolling in the study, my child will be mailed a sexual maturation self-assessment form to complete at home and mail back to the Bone and Body Composition Laboratory (BBCL). My child will compare his/her own appearance to pictures/drawings representative of each sexual maturation stage (i.e., drawings and photographs of genital areas) and circle the image he/she most closely resembles. If my child meets the criteria for inclusion for sexual maturation, he/she will be scheduled for the first testing session. Prior to any testing or participation, a permission form for me and an assent form for my child will be mailed/emailed

to me outlining the testing procedures that will be used during the study. My child and I will be instructed to sign these forms prior to our appointment. However, if I misplace or do not bring the signed forms upon our arrival to the laboratory, my child and I will be given the opportunity to reread these forms and ask any questions that we may have about the study before signing the forms. The researcher will then sign the respective forms. My child and I will be walked through all procedures and reminded that we are free to withdraw without penalty at any time.
b) Session 1 of testing will be conducted at five different time points [at the beginning of the study and after 3, 6, 9, and 12 weeks] and will require approximately 45 minutes. On the day of testing, my child and I will arrive in the BBCL in Dawson Hall at the scheduled time, following an overnight fast. My child will provide his/her second morning urine sample in a private restroom. A trained phlebotomist will insert a small tube (catheter) into a vein in my child's arm and will then draw approximately 30 mL of blood from my child's arm, after which he/she will be given a snack (15-20 minutes). My child's blood and urine will be analyzed for compounds that reflect how his/her bone health and vitamin D status responds to the supplements. Any unused portions of blood that is collected will be discarded after 10 years post completion of the study.

For possible analysis in the future, a portion of the blood will be saved in order to assess vitamin D-related genes that may influence how my child's blood work responded to the supplements. Any information that is discovered from this genetic testing is related to research only (i.e., response of the vitamin D receptor gene to various levels of supplementation) and will not be used as therapy or diagnostic testing. This information will help the researchers advance their knowledge about the role of vitamin D in children. Therefore, the researchers do not intend to contact me or my child, now or in the future, regarding any future DNA testing. A new Federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against my child based on his/her genetic information. This law generally will protect my child in the following ways: Health insurance companies and group health plans may not request my child's genetic information obtained from this research. Health insurance companies and group health plans may not use my child's genetic information when making decisions regarding his/her eligibility or premiums. Employers with 15 or more employees may not use my child's genetic information obtained from this research when making a decision to hire, promote, or fire my child or when setting the terms of my child's employment. All health insurance companies and group health plans must follow this law by May 21, 2010. All employers with 15 or more employees must follow this law as of November 21, 2009. I am aware that this new Federal law does not protect my child against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

c) My child and I will be instructed on the proper use of the provided supplements. We agree to follow the instructions on the label of the supplements. I understand that the supplement is either 0 IU vitamin D_3 (i.e., the placebo), 400 IU vitamin D_3, 1,000 IU vitamin D_3, 2,000 IU vitamin D_3, or 4,000 IU vitamin D_3, none of which can cause harm to my child if taken properly. If supplementation causes noticeable, negative side effects, my child may opt to continue the study without taking supplements, or he/she may discontinue the study completely. When we return to the BBCL for follow-up testing sessions, my child and I will return the remaining tablets and receive a new bottle of tablets (except at the final visit). We will also be asked to return the

supplement compliance calendars. In order to minimize over-consumption of vitamin D from outside sources, my child will be asked to refrain from taking any vitamin, mineral or herbal supplements during the study. My child will be instructed to follow his/her normal dietary habits and will not be asked to refrain from fortified food products. My child will also be instructed to follow his/her normal physical activity patterns during the course of the study.

d) Session 2 of testing will be conducted at the beginning of the study and at 12 weeks only and will require approximately 3 to 4 hours. First, my child and I will complete a general information/health questionnaire, diet and physical activity questionnaires (approximately 15 minutes). We will also be given a three-day diet record to be mailed back to the BBCL in a stamped, self-addressed envelope provided by the researcher. My child's body composition will then be measured using two non-invasive bone- and muscle-scanning machines (30-40 minutes) and muscle strength will be assessed using a hand-grip dynamometer (1-5 minutes). I understand that a trained laboratory technician under the supervision of Dr. Richard D. Lewis will conduct all measurements. To assess if the supplements alter calcium absorption, an important measure of bone health, my child will have his/her blood drawn once following an overnight fast (an additional 5 mL of blood during Session 1). My child will receive a breakfast that includes a beverage containing 150 mg calcium and a stable calcium isotope tracer, ^{44}Ca. The ^{44}Ca isotope is safe and will cause no harm to my child. For the following 3 hours my child will not be allowed to consume any additional food or beverage apart from the water that is provided. Three hours after consuming the beverage, the phlebotomist will draw another 5 mL of blood from the catheter. The catheter will then be removed.

e) Session 3 of testing will be conducted at the beginning of the study and at 6 and 12 weeks only and will require approximately 20 minutes. My child and I will complete diet and sun exposure questionnaires (approximately 15 minutes). My child's height, sitting height, leg length, and body weight will then be measured (5 minutes).

3) Information from all testing sessions will be stored in locked filing cabinets. The discomforts or stresses that may be faced during this research are minor physical discomfort from blood draws and minor psychological discomfort from the questions about my child's diet or medical history. To minimize this stress, participants will be interviewed in private rooms. If undue discomfort occurs, my child has the right to discontinue the testing at any time.

4) The following foreseeable risks have been explained to me:
a) I understand that one of the foreseen risks to my child is discomfort during the blood draw. I understand that if a blood sample cannot be obtained after two attempts, no further attempts will be made.

b) I understand that another foreseen risk to my child is exposure to a small amount of radiation when assessing body composition with the bone- and muscle-scanning machines. The scans for the entire study will give a total radiation dose of 4.82 microseiverts (μSv). This dose is very small, as radiation doses from an adult chest X-ray ranges from 500 to 800 μSv and environmental background radiation per week totals 35 μSv. Thus, the total radiation exposure for the study is 0.5 to 1% of standard chest X-rays. In the event that information from any scan is lost or unusable, no additional scans will be performed.

Because our current knowledge of the risk of X-ray to the unborn child is limited, prior to conducting the bone and muscle scans, my child (if female) will sign a consent form developed for use with these machines that asks if she is currently pregnant or believes she may be pregnant. If my daughter is pregnant, she will be told that she cannot participate because the X-rays from the bone- and muscle-scanning machines pose a risk to the fetus. If my child expresses any doubts regarding pregnancy, a pregnancy test will be provided to complete in the privacy of her own home prior to DXA or pQCT testing. If the pregnancy test is refused or if determined to be pregnant, my daughter may maintain confidentiality by electing not to disclose the pregnancy test results to the research group, but must voluntarily withdraw from the study. Refusal will be documented. If my daughter and I elect to notify the research group of the pregnancy she/we will receive a referral to Dr. Andrew Muir, pediatric endocrinologist and study physician, or to our own primary care physician. Dr. Muir will also be available to medically evaluate my child if he/she reports any adverse reactions to the supplements.

My child's risk of vitamin D toxicity is minimal, but will be monitored by the research team who will perform blood and urine tests immediately following baseline, 3, 6, 9, and 12 week testing sessions. In addition, if my child reports any abnormal responses, or if blood and urine values suggest toxicity as described above, he/she will no longer receive supplements, but will be allowed to continue in the study if he/she desires.

5) The results of my participation and that of my child will be confidential and will not be released in any identifiable form without my child's prior permission and mine unless required by law. It is possible that the United States Food and Drug Administration may inspect my child's study records. My signature on this form authorizes that use of my data and my child's data in group analyses, which may be prepared for public dissemination and/or available to other researchers, without breaching my own or my child's confidentiality. To accomplish this, my child will be assigned a four digit subject participation code, which will be used on all data collected during my child's participation in this research. A master list with my child's name and corresponding code number will be kept separate from testing data and locked at all times. Records linking code numbers to names will be destroyed three years post-completion of this study. The final dataset will be stripped of any of my child's individual identifiers prior to release for sharing with other researchers. A link to the dataset (computerized spreadsheet) on our study website will be created and made available after the primary results from this study are accepted for publication in a research journal. A data-sharing agreement will be required from other researchers, which will stipulate that data will be used for research purposes only.

6) In order to process payments for my child's participation following each testing session (baseline, 3 weeks, 6 weeks, 9 weeks, and 12 weeks), the researcher(s) need to collect my child's name and mailing address on a separate payment form. This completed form will be sent to the Department of Foods and Nutrition business office and then to the UGA Business Office. The researchers have been informed that these offices will keep my child's information private, but may have to release my child's name and the amount of compensation paid to my child to the IRS, if ever asked. The researchers connected with this study have gone to great lengths to protect my and my child's private information and will keep this confidential in their locked files. However, they are not responsible once my child's name and mailing address leave their office/laboratory for payment processing.

7) As a participant, my child assumes certain risk of injury. The researchers will exercise all reasonable care to protect my child from harm as a result of his/her participation. In the event of an injury as an immediate and direct result of my child's participation, the researchers' sole responsibility is to arrange transportation for my child to an appropriate facility if additional care is needed. The researchers will not provide any compensation or payment for medical care. As a participant, my child does not give up or waive any of his/her legal rights.

8) The investigator will answer any further questions that my child or I may have about this research, either now or during the course of the project. I understand the procedures described above.

My child was given the opportunity to complete a simple urine test for pregnancy:
(Check one): **YES**____ **NO**____

_____ _____
Signature Date

I refuse for my child to take the pregnancy test:
(Check one): **YES**____ **NO**____

_____ _____
Signature Date

I understand the procedures describe above. My questions have been answered to my satisfaction, and I agree to give permission for my child to participate in this study. I have been given a copy of this form.

Richard Lewis/Emma Laing _____ _____

Name of Researcher Signature Date
Telephone: 542-4901
Email: rlewis@fcs.uga.edu

_____ _____

Name of Parent or Guardian Signature Date

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

Additional questions or problems regarding your child's rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu.

APPENDIX C

HEALTH HISTORY QUESTIONNAIRE

Supplemental Vitamin D in Early Adolescence

Health History Questionnaire

Subject ID#_____
Interviewer _____
Date _____

Surgery/Medication/Fracture History

1. Please list major medical procedures, surgeries and/or injuries in your lifetime and related medications. Give the time of the procedure or injury and/or the frequency and duration of medication.

2. Have you ever gone through an extended period of time where you were bedridden or immobilized? YES or NO; *circle one*
   - If yes, how old were you and how long did this immobilization last?
   - Briefly explain the circumstances.

3. Are you currently taking any medications either prescribed by a doctor or over-the-counter (self-prescribed)? YES or NO; *circle one*
   - If yes, what medications?

4. Has any member of your family been diagnosed with any medical condition related to obesity or osteoporosis? YES or NO; *circle one*

5. Have you ever experienced a skeletal fracture in your lifetime? YES or NO; *circle one*

   - If yes, at what age did you experience a fracture?

   - In what type of circumstance did the fracture take place?

   - How was the fracture treated (casting, medication, rest, etc.)?

Other History

1. How would you rate your present health? ____Poor____Good____Fair____Excellent
2. Do you currently smoke cigarettes? ____ YES or NO; *circle one*
   a. If yes, on the average, about how many cigarettes a day do you smoke?
      ____1-5, ____6-14, ____15-24, ____25-35, ____35 or more
3. If you used to smoke but do not smoke now, how long did you smoke? _____years.
4. (*If Female*) At what age did you start your menstrual cycles? _____
5. (*If Female*) Are your menstrual cycles regular? YES or NO; *circle one*
   a. If not, how long have they been irregular? _____
6. (*If Female*) Have you ever used birth control pills? YES or NO; *circle one*
   a. How old were you when you began using birth control pills? _____

b. How long have you been using them? _____

7. (*If Female*) What periods of time did you stop using birth control pills? _____
(Please give dates, if applicable)

8. Are you on any nutritional supplements? _____

9. Are you currently dieting, or on a special type of weight loss program? YES or NO; *circle one*
   a. If yes, what program are you following? _____

10. Do you have any health problems that limit your physical activity? _____

11. How many hours, on average, do you spend watching TV, or on the computer? _____

APPENDIX D

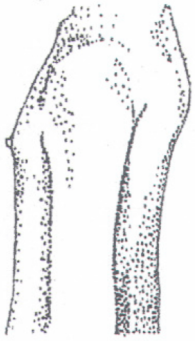
SEXUAL MATURATION QUESTIONNAIRES

Lewis, Richard D.

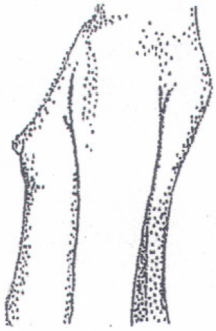
SEXUAL MATURATION QUESTIONNAIRE (GIRLS)

Subject ID#: _____
Date: _____

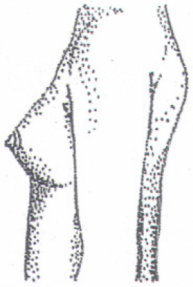
We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



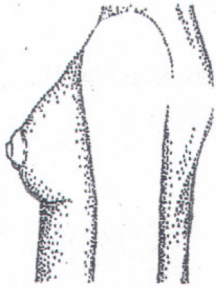
Stage 1: Elevation of papilla only.



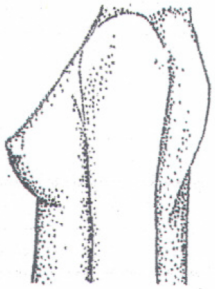
Stage 2: Elevation of breast and papilla as small mound, areola diameter enlarged.



Stage 3: Further enlargement without separation of breast and areola.



Stage 4: Secondary mound of areola and papilla above the breast.



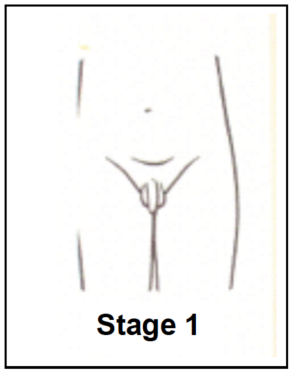
Stage 5: Recession of areola to contour of breast.

Thank you for answering this question. Please send this questionnaire back to the researcher in the stamped envelope provided.

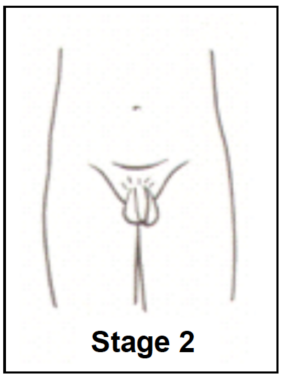
J. Tanner (1962) Growth and Adolescence, 2nd ed. Blackwell Scientific Publications, Oxford.

Sexual Maturation Questionnaire

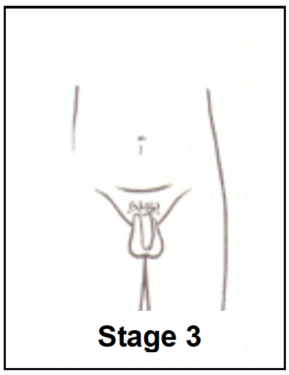
We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



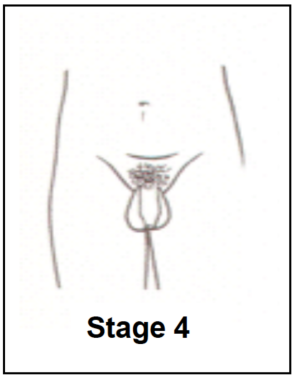
STAGE 1: The penis, scrotum, and testes are of the same size and proportion as in early childhood.



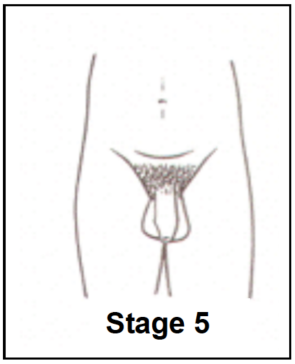
STAGE 2: The scrotum and testes have enlarged. The size of each testis can be judged by looking at the scrotum and also by feeling each testis through the skin of the scrotum. The skin of the scrotum becomes thinner, wrinkled and slightly red but this is difficult to see in a photograph. There is little or no change in the penis.



STAGE 3: The penis is longer than in early childhood but there is little change in thickness. The scrotum and testes are larger than in Stage 2. The scrotum now hangs down further below the base of the penis.



STAGE 4: The penis is further enlarged in length and breadth. The end of the penis becomes conical and there is an enlargement where this part (the glans) joins the rest of the penis. The scrotum and testes are further enlarged and the skin of the scrotum is darker.



STAGE 5: The penis, scrotum, and testes are adult in size and shape.