Assessment of the Effects of Whole Body and Regional Soft Tissue Composition on Bone Strength and Development in Females

ASSESSMENT OF THE EFFECTS OF WHOLE BODY AND REGIONAL SOFT TISSUE COMPOSITION ON BONE STRENGTH AND DEVELOPMENT IN FEMALES.

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Deepika R. Laddu

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Deepika R. Laddu entitled, "Assessment of the effects of whole body and regional soft tissue composition on bone strength and development in females" and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Dr. Scott Going Date: June 4th, 2013

Dr. Craig Stump Date: June 4th, 2013

Dr. Linda Houtkooper Date: June 4th, 2013

Dr. Cynthia Thomson Date: June 4th, 2013

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College. I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: June 4th, 2013
Dissertation Director: Dr. Scott Going

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SIGNED: Deepika R. Laddu

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DEDICATION

To my family and friends for their endless love and support.

In memory of my late uncle who always told me to laugh, love and most of all, enjoy life.

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GLOSSARY OF ACRONYMS

ACRONYM	DEFINITION
Metabolic disease/dysfunction	
T2DM	type 2 diabetes mellitus
IR	insulin resistance
Statistical analysis	
MLR	multiple linear regression
ANCOVA	analysis of covariance
SEE	standard error of estimates
CVs	coefficients of variation
Dietary variables	
FA	fatty acids
PUFA	polyunsaturated fatty acids
LCPUFA	long-chain polyunsaturated fatty acids
n-3 LCPUFA	omega-3 long chain polyunsaturated fatty acids
n-6 LCPUFA	omega-6 long chain polyunsaturated fatty acids
ALA	alpha-linolenic acid, 18:3ω-3
LA	γ-linoleic acids,18:2 ω-6
AA	arachidonic acid, 20:4ω-6
EPA	eicosapentaenoic acid, 20:5ω-3
DHA	docosahexaenoic acid, DHA, 22:6ω-3
COX	cyclooxygenases
LOX	lipoxygenases
PGE_2	two-series prostaglandin
LTB_4	four-series leukotrienes
LTC_4	four-series leukotrienes
RevD1	E-1 series resolvins
Bone-regulating transcription factors	
Cbfa-1	core binding factor-1
IGF-1	insulin growth factor-1
IGFBP	IGF-binding protein
OPG	osteoprotegerin
RANK	receptor activator of nuclear factor-kB
PPAR	peroxisome proliferator activator receptors (α, β/δ, γ; PPAR-γ1, PPAR-γ2)
RANK	receptor activator of nuclear factor-kB ligand
Gla-OCN	inactive osteocalcin
Glu-OCN	activated/carboxylated osteocalcin
Inflammatory cytokines	
IFN-γ	interferon-γ
IL-1β	interleukin-1 beta
IL-4	interleukin 4
IL-6	interleukin 6
IL-11	interleukin 11
TNF-α	tumor necrosis factor-α
M-SCF	macrophage colony stimulating factor
Bone variables	
BSI	bone strength index
SSI	strength-strain index
Crt	cortical bone
Trab	trabecular bone
Crt Thick	cortical thickness
CSA	cross sectional area
MCSA	muscle cross sectional area
EC	endosteal circumference
PC	periosteal circumference
BMC	bone mineral content
aBMD	areal bone mineral density
vBMD	volumetric bone mineral density

GLOSSARY OF ACRONYMS-*continued*

Analysis methods	
YAQ	youth administered food frequency questionnaire
PYPAQ	past year physical activity questionnaire
MRI	magnetic resonance imaging
DXA	dual-energy x-ray absorptiometry
pQCT	peripheral quantitative computed tomography
ROI	region of interest
CT	computed tomography
PHV	peak height velocity
Anthropometry and Body Composition	
WC	waist circumference
HC	hip circumference
WHR	waist-to-hip ratio
BMI	body mass index
Ht	standing height
Wt	body weight
TBFM	total body fat mass
TBLM	total body lean mass
SAT	subcutaneous adipose tissue
VAT	visceral adipose tissue
IAAT	intra-abdominal adipose tissue
AFM	Android fat mass
SKM	skeletal muscle

GLOSSARY OF DEFINITIONS

Bone metabolic diseases	
Osteopenia	Considered to be a precursor of osteoporosis, osteopenia is characterized by bone mineral density is lower than normal for an individual. Specifically, is defined as a s a bone mineral density T-score between -1.0 and -2.5.
Osteoporosis	A widespread metabolic bone disease characterized by accelerated bone loss, low bone mass, micro-architectural deterioration, leading to reductions in bone strength and increased fracture risk. Clinical diagnosis of osteoporosis includes a T-score of -2.5 or above.
Fat-bone hormones	
Adiponectin	An adipocyte-derived hormone that is released exclusively by the adipose tissue, and is known for its anti-inflammatory properties . Synthesis and secretion of adiponectin is influenced by the amount of total body lean mass and is inversely related to fat mass. Additional roles of adiponectin include regulation of metabolic homeostasis and insulin sensitivity, glucose metabolism and fatty acid catabolism.
Leptin	An adipocyte-derived hormone that is conventionally known as the "satiety hormone regulator, and energy metabolism. Leptin is also a key regulator of bone metabolism by binding with leptin receptors on hypothalamic neurons to produce a sympathetic-dependent biphasic effect on bone remodeling. Depending on the signaling cascade activated, leptin will have either an osteogenic or anti-osteogenic influence on the skeleton.
GH/IGF-1 axis	Produced and secreted by the pituitary gland , growth hormone (GH) has anabolic effect of promoting protein synthesis, gonadal steroid hormone synthesis and secretion, increasing muscle growth and promoting lipolytic fat oxidation; increases in adiposity suppresses GH production. IGF-1 is also the most abundant growth factors secreted by osteoblasts and stored in bone, and therefore contributes to bone mineral accrual and attainment of peak bone mass. Both GH and IGF-1 are required for required for increases in both longitudinal bone growth and bone size during puberty
Estrogen	An androgenic steroid metabolized by the ovary, adrenal cortex and adipose tissue that is primarily responsible for the female sex characteristics. Estrogen is a fat storing hormone that gradually increases with weight (fat) gain as well as during the pubertal transition in females. An abundance of receptors located on osteoblasts OB, OC, and osteocytes, indicating the role of estrogen in modulating bone metabolism. Receptors located specifically in bone marrow cells regulate osteoclastogenesis. Estrogens overall appear to decrease the rate of bone turnover, there by influencing osteoclastic activity. Estrogen deprivation, notably occurring during peri- and post menopause results in an increase in bone remodeling sites, increasing the risk of osteoporosis
IFN-γ	A cytokine involved in systemic inflammation
IL-1	A cytokine involved in systemic inflammation
IL-1β	A cytokine involved in systemic inflammation
IL-4	A cytokine involved in systemic inflammation
IL-6	A cytokine involved in systemic inflammation
IL-11	A cytokine involved in systemic inflammation
TNF-α	A cytokine involved in systemic inflammation
Receptor activator of nuclear factor kappa-B ligand (RANKL)	Member of the tumor-necrosis factor family, RANKL is a primary regulator of bone-resorbing activity and survival of the mature osteoclast. RANKL can also serve as a ligand for osteoprotegerin, a mechanism involved in reducing osteoclastogenesis.
Osteoprotegerin (OPG)	OPG is a soluble decoy receptor for RANK-L, and it functions to reduce RANK-L/RANK-mediated osteoclastogenesis by competitively binding to RANK-L binding sites normally reserved for RANK receptors on precursor and later-stage osteoclasts.
Type-1a collagen	organic component of bone comprising of ~90% collagen fibrils that is synthesized by osteoblasts, and secreted as the osteoid, and then assembled extracellularly. Collagen fibrils are arranged in an overlapping manner, to allow for space between adjacent fibrils, and the formation of intermolecular cross links, thereby producing a stable porous structure, providing bone its resiliency and tensile like properties and ultimate yield strength.

GLOSSARY OF DEFINITIONS-*continued*

Bone Cells	
Basic Multicellular unit (BMU)	A collection of cells derived from different sources that are primarily involved in the basic bone remodeling process, otherwise known as the "ARF sequence": activation? resorption? formation. The 4 cellular elements that represent one BMU include osteoblasts, osteocytes, bone lining osteoids, and osteoclasts that are involved in removing old packets of bone (via osteoclasts) and replacing or forming new bone tissue (via osteoblasts).
Osteoblast	Primary bone cell derived from mononuclear mesenchymal stem cell lineage involved in regulating bone formation and bone remodeling. Osteoblasts are actively involved in secreting prostoglandins, as well as alkaline phosphatase, which aids in mineralization, osteocalcin, and other matrix proteins
Osteoclast	Multinucleated bone cells originating from the hematopoieticstem cell lineage that are primarily responsible for regulating bone resorption and bone remodeling. The phagocytic-like mechanism of the osteoclast to degrade bone is primarily carried out by their secretion of acidic enzymes (e.g., tartrate resistant acid phosphatase (TRAP), hydrochloric acid, and collagenase) and lysosomal proteases (i.e., cathepsin K).
Osteoid	secreted by osteoblast during bone formation phase as several different proteins (e.g., alkaline phosphatase, osteocalcin). The osteoid is the unmineralized organic component of the matrix composed predominantly of collagen type 1a fibrils. Collagen cross linking of these fibrils provides bone its flexibility and tensile like properties. The osteoid later gets transported to bone surface to serve as bone lining cells
Osteocyte	osteocytes are osteoblasts that become embedded into the bone matrix, and are commonly known as the "mature bone cells". Osteocytes are responsible for matrix synthesis and mineralization by catalyzing the crystallization of calcium and inorganic phosphorous to form hydroxyapetite crystals, therefore giving bone resiliency and structural strength. Osteocytes also serve as "bone sensing" cells or "mechano sensors" as they are the first cells to react to micro-tears and damage at the bone surface and thus regulate the bone's response to stress and mechanical load. Osteocytes make up the majority of bone cells.
Bone Strength Variables	
Mechano-transduction	The conversion of a physical force into a biochemical or cellular response
stress/strain	Is defined as the force per unit area and is equal in magnitude but opposite in direction to the applied load. Bone stress can be categorized as either (1) tensile, occurring when 2 forces act along a straight line in opposite directions; (2) compressive, occurring when 2 forces act along a straight line in the same direction; or (3) shear, occurring when 2 forces are acting parallel to each other but not in the same line. Mechanic forces that are applied to bone are generally a combination of the 3 stresses, resulting in a bending or torsion. Deformation that occurs as a result of the applied force is known as strain, which is equal to the change in length divided by th original length.
Young's modulus (modulus of elasticity)	Is defined as the ratio of stress divided by strain. At low levels of stress, a linear relationship exists between stress and strain. The linear portion of the stress-strain curve is known as the elastic region, in which the removal of the load does not cause permanent strain or deformation. The plastic region is defined as the point at which the curve becomes nonlinear, as permanent deformation occurs even after the load is removed. Stressing a bone beyond the plastic region will result in failure, such as a fracture. The maximum stress at the point of failure will determine the ultimate strength of a bone.
Bone Material and Structural Variables	
Bone mass	The amount of bone tissue (mineral content) in bone or the entire skeleton.
Bone strength	Bone strength is conventionally defined as the amount of loading force required to cause the material to fail under a certain loading condition. Bone strenght is characterized by both materialistic (BMC, BMD) which provides bone its compressive strength and ridigity and resistance to stress and mechanical loading, and structural properties (CoThick, CSA, PC, EC), which quantifies the amount of bone surface and its distribution about bending and torsion axes; in essence, geometry allows bones to carry loads effectively but remain relatively light.
Bone-Strength Index (BSI, mg^2/cm^4)	BSI is used to estimate the bone's as the ability of bone to withstand compression at metaphysis region. BSI is calculated as the product of the metaphyseal total area and total vBMD squared: Bone strength index = Total area x Total $vBMD^2$.

GLOSSARY OF DEFINITIONS-*continued*

Strength-Strain Index (SSI, mm^3)	SSI is used to estimate the bone's ability to resist torsional strain and bending forces at diaphyseal regions. Diaphysis SSI is calculated by the Stratec software (version 6.0), which is based on the integrated product of the geometric properties (i.e., sectionmodulus) with the material properties of bone: Strength-strain index (SSI, mm3) = $\Sigma_{i=1;n}[((ri^2 \times a)/r_{max}) \times (\text{cortical vBMD/ND})]$.
BMC (g/cm)	The "degree of mineralization" is the extent to which the organic bone matrix has been filled with mineral. Specifically, it is the mass of mineral contained in an entire bone, or the mass of mineral per unit bone length.
areal Bone mineral density (aBMD)	aBMD is defined as the mineral mass of bone divided by its projection area in a given direction, and serves as a proxy measurement for bone strength(g/cm2 of hydroxyapatite (HA)). aBMD is measured directly for each pixel in the region of interest (ROI) by comparing the X-ray attenuation of that pixel to a reference standard. aBMD, assessed by DXA is measured directly as the mineral mass along a straight path between the X-ray source and detector in a given region of interest (ROI).
Volumetric bone mineral density (vBMD)	vBMD is assessed by pQCT represents the true volumetric bone mineral density, and is defined as the mass of mineral divided by the volume enclosed by the periosteal bone surface. The primary advantage of vBMD s that it is not confounded by bone growth or bone size.
Epiphysis	The epiphysis is the rounded articular end of a long bone at its joint with adjacent bone(s),that is formed by the secondary ossification center. During the period of longitudinal growth, epiphyses are separated from the main portion of the bone by a layer of cartilage, but then later become united to the main bone through ossification.
Diaphysis	The midsection (shaft) of a long bone, between the epiphyses. The diaphysis is also the location of primary bone ossification and is composed predominately of cortical bone.
Metaphysis	The wider portion of a long bone adjacent to the epiphyseal plate. During growth and maturation, the metaphysis begins to ossify near the epiphysis and diaphysis and is responsible for linear bone growth. Full maturation of the skeleton is completed when epiphyseal growth plates, located in the metaphysis are closed, and the metaphysis completely ossified into solid bone. This is the area of continued longitudinal bone growth until physical maturity, when the cartilage in the metaphysis is replaced by bone (ossification), bringing together the diaphysis and the epiphysis. Specifically, the metaphysis stops growing around 18 to 25 years of age.
Cortical bone	Referred to as "compact bone," cortical bone is one of the two types of osseous tissue bone that contributes approximately 80% of the mature skeleton. Cortical bone is located in regions subject to predominantly tensile stresses has a higher percentage of collagen fibers aligned along the bone long axis (diaphysis). The thick, dense composition of cortical bone provides mechanical strength, and be resistant to torsional and bending forces.
Trabecular bone	Synonymously known as "cancellous" or "spongy" bone is one of two types of osseous tissue. Trabecular bone, although more porous, and thus less dense, has a significantly higher surface area and is known to be more metabolically active and respond faster to metabolic loading strains, compared to cortical bone. Trabecular bone is found primarily at the end of long bones, such as near the epiphysis regions, and proximal to joints and within the interior of vertebrae. Its primary function is to provide resilience and shock absorption.
Periosteal Circumference (PC, mm)	Is the outer bone surface of all long bones
Endosteal Circumference (EC, mm)	Is the inner bone surface that lines the outer surface of the medullary cavity of all long bones.
Cortical thickness	Is the distance between the outer (periosteal) and inner (endosteal) bone surfaces of a long bone.
Cross-sectional area	The total surface area of bone in a cross-sectional slice, after excluding all the spaces occupied by the marrow cavity and other soft tissues within the pores.
Cross-sectional moment of inertia (CSMI)	Also known as the second moment of area, is defined as the distribution of material with in a cross-section of bone and is used to calculate bending stress within a cross-section of bone. Specifically, CSMI is inversely related to the stress within a cross-section that is subjected to bending strains, making it proportional to bending rigidity. In most bones, CSMI varies with the bending direction, or the distance from the neutral axis, and are not axially symmetric. The maximum and minimum CSMI's are oriented 90? apart.

GLOSSARY OF DEFINITIONS-*continued*

Section Modulus (Z)	Is defined as the maximum bending stress in a cross-section, which occurs at the furthest point from the neutral axis. Section modulus (Z)= CSMI/d_{max}
Bone Analysis Techniques	
peripheral quantitative computed tomography (pQCT)	A type of CAT scan that uses x-rays to make cross-sectional images of bone and has the ability to independently assess the geometric characteristics of total bone, cortical and trabecular bone. pQCT provides 3-dimensional estimates of volumetric BMD and bone cross-section related to bone size (area), mass (mineral content), apparent tissue density and geometry (spatial distribution of mass), and can capture modeling adaptations leading to both structural and architectural changes during growth that in turn can be used to estimate stress magnitudes and changes in bone strength.
Contour mode	Is used by the Stratec pQCT software to detect the outer edge of the bone. The operator selects a threshold value which will be used to separate the soft tissue from the outer edge of bone. Based on the defined threshold, the software will detect a voxel located on the outer bone edge and will then eliminate any voxel with a threshold below the entered value. In this manner, the soft tissue is removed from around the outer edges of the bone.
Cort mode	Is an algorithm used by the Stratec pQCT software to calculate cortical density and area. The algorithm removes all voxels within the ROI that have an attenuation coefficient below the default threshold of 710 mg/cm³. The remaining bone is analyzed and is considered to be purely cortical bone.
Peel mode	Is an algorithm used by the Stratec pQCT software to concentrically peel away a defined percentage of the outside area of bone at metaphyseal regions. The remaining inner region is analyzed and is considered to be purely trabecular bone.
Maturation Analysis	
Peak Height Velocity (PHV)	Is defined as age at which maximum rate of growth in stature is achieved during the adolescent spurt. In longitudinal studies, PHV is commonly used as an indicator of maturation status, serving as a landmark for characterizing intra- and inter-individual or group differences in growth, body sizes, and velocities of other body dimensions can be expressed.
Maturity Offset	Maturity Offset an algorithm based on estimated years from peak height velocity (PHV) using Mirwald's equation, accounting for interactions among anthropometric measures (i.e., height, weight, sitting height, leg length) and chronologic age. Positive maturity offset values represent years after PHV while a negative maturity offset value represents years before PHV.
Tanner Stage	Is a scale of physical development in children and adolescents. The scale defines physical measurements of development based on external primary and secondary sex characteristics, such as the size of the breasts and development of pubic hair.

ABSTRACT

Osteoporosis is a major public health concern with origins in childhood and is potentially linked to childhood obesity. This study used novel approaches in bone imaging to characterize skeletal development in girls and to assess the influence of whole body and regional soft tissue composition on bone material, structural and geometric properties, the primary determinants of bone strength, controlling for important covariates such as maturation, diet and physical activity.

Prospective analyses were conducted to assess associations between measures of total body fat (TBFM) and android fat masses (AFM) and skeletal muscle fat (SMF) content on bone mineral content, density and strength. The results showed that higher TBFM and AFM were inversely associated with changes in cortical bone sites of the femur and tibia. These findings suggest that gains in abdominal adiposity during the pre- and early- pubertal years may contribute to suboptimal bone development and skeletal fragility later in life.

The analyses also showed inverse associations between baseline muscle density of the thigh and calf with 2-year changes in bone strength and bone density of the metaphyseal and diaphyseal sites of the femur and tibia. This paradoxical relationship between SMF and bone outcomes was explained by subsequent analyses showing that girls exhibiting larger gains in muscle density experienced larger increases in bone density and strength compared to girls who did not significantly increase muscle density. These findings suggest that fatty infiltration of skeletal muscle contributes to suboptimal bone development in peri-pubertal girls.

Further longitudinal analyses were conducted to examine the individual effects of the muscle-bone unit components on 2-year changes in bone strength. These results showed that muscle size contributed to gains in bone strength, independent of its mechanostat effect on BMC.

These results underscore the importance of muscle size for promoting bone development and bone strength during growth.

A final set of analyses were conducted to examine the effects of dietary fatty acids on bone development. The results of these analyses suggest that while decreasing intakes of AA n-6 FA may benefit bone health, higher intakes n-3 FAs may benefit tibia bone density development in young girls.

CHAPTER 1

INTRODUCTION

The overall objective of this research was to examine the relative effects of total body and regional soft tissue composition (adiposity and skeletal muscle) and diet on bone mineral accrual and changes in geometry and strength indices in young females.

The proposed study was based on the following assertions: 1) osteoporosis is a major public health concern; 2) osteoporosis is, in part, a pediatric disorder, manifested in old age; 3) obesity during childhood and adolescence is the most common cause of abnormal growth acceleration and impaired bone growth [1]; 4) Osteoporosis and fracture risk in adulthood is a function of peak bone mass achieved at the end of adolescence [2-6].

Both bone mass and structural geometry are important determinants of bone strength and fracture risk. Achieving a high peak bone mass early in life predicts a higher bone mass and a lower fracture risk later in life. The effects of fat on bone may be determined by regional, rather than whole body fat mass, and therefore, regional adiposity likely plays a pivotal role in determining bone structural development and strength. Body composition versus body weight may be a stronger predictor of bone development. Muscle is the primary voluntary load on the skeleton such that increasing loads imposed by larger muscle forces precedes the developmental adaptations in bone strength and bone mass [7-9]; thus, adaptive increases in bone strength are secondary to gains in muscle size and muscle strength that occur during growth and development [9]. While genetic endowment can predict 60-80% of the variability in skeletal growth and development [10], modifiable factors such as nutritional status clearly influence full skeletal growth and expression of genetic potential. Dietary fatty acids (i.e. polyunsaturated fatty acids) influence bone cell activity, displaying both pro- and anti-inflammatory properties that may

modulate bone development throughout maturation via the synthesis and secretion of prostaglandins, and growth factors. The pre-pubertal and peri-pubertal years may present a "window of opportunity" for bone structural adaptations to body composition to be maintained into adulthood. As described in *background* and *significance*, there are data to support each of these points, although the effect of total and regional adiposity on bone macro-architectural structure (BMC, BMD and especially structural geometry) remains inconclusive due to a scarcity of prospective studies.

SPECIFIC AIMS

The **primary aims** were to:

Aim 1a: To examine the relationship between whole body and regional fat distribution (i.e., android fat mass) with bone mineral content (BMC), volumetric bone mineral density (vBMD), bone geometry and indices of bone strength in young girls.

Hypothesis 1a: Higher levels of total body fat (TBFM) and android fat (AFM) would be inversely associated with bone mineral content (BMC) and bone strength and density parameters at weight bearing bone sites in young girls

Aim 1b: To determine the relationship between skeletal muscle fat content and changes in bone mineral content (BMC), volumetric bone mineral density (vBMD), and indices of bone strength in young girls.

Hypothesis 1b: greater baseline skeletal muscle fat content (calf and thigh) would be inversely associated with 2-year gains in bone density and bone strength in weight bearing bone sites in young girls.

Aim 2: Determine the effects of muscle quality and the muscle cross sectional area in predicting bone strength in young girls.

Hypothesis 2: In addition to bone mineral mass, muscle cross-sectional area and muscle density, surrogates of muscle force and quality will be strong predictors of bone strength at weight bearing bone sites.

Aim 3a: To evaluate the influence of n-3 and n-6 LCPUFA consumption on bone structure (mass, density, thickness, and shape) and bone strength in young girls.

Hypothesis 3a*:* Higher intakes of n-3 FA will promote gains in bone strength, while higher consumption of n-6 FA will be negatively associated with bone development parameters.

Aim 3b: To determine the effects of varying ratios of n-6-to-n-3 LCPUFAs on bone strength and development in young girls.

Hypothesis 3b: Higher intakes of n-6/n-3 FA ratios will be inversely related to 2-year gains in bone strength and bone development.

Ancillary aim: To quantify and determine the validity of android fat mass as a predictor for visceral fat area at the umbilicus level, and develop equations to predict visceral fat mass, measured by magnetic resonance imaging (MRI) at the umbilicus level using android fat mass measured by dual-energy X-ray absorptiometry (DXA) and body composition and anthropometry data in young girls.

Hypothesis: Body composition assessment tools that interpret anthropometric data with regard to body fat distribution using DXA measures of android fat mass will be equally as valid at predicting visceral fat from MRI.

DXA derived prediction equations of visceral fat will help clarify that regional fat deposition is more strongly related to bone geometry, strength and development indices young girls.

PUBLIC HEALTH RATIONALE

Osteoporosis is conventionally accepted as a debilitating bone disease that is most discernible during old age. Due to the rising concern for optimizing skeletal development, osteoporosis is increasingly accepted as being a pediatric disorder that is manifested later in life [4, 11]. Currently, nearly 10 million Americans over the age of 50 have osteoporosis, while 18 million more are at risk of developing the disease[12, 13]. Women aged 50 years or more have a 30-40% estimated risk for incurring an osteoporotic fracture during their lifetime [14, 15]. The incidence of osteoporosis is projected to triple by the year 2040, as a result of increased life expectancy, expected population growth, and unhealthy lifestyles. Disruption of normal bone development during growth, resulting in impaired mineral accrual and lower bone strength, would likely increase the risk for developing osteoporotic fractures later in life [4, 16, 17]. A clinically relevant decrease in fracture risk of 20-40% may result if even small increases equal to 3-5% in bone strength were maintained. Thus, it is imperative that preventive strategies that optimize bone development and strength are initiated during childhood in order to prevent the development of osteoporosis and bone related injuries later in life.

Puberty is a critical period for bone development, with as much as 90% of bone mineral accrued by the end of adolescence [18] making it an opportune time to promote maximal bone density and bone geometry [6]. While genetic endowment can predict 60-80% of the variability in skeletal growth and development [19], modifiable factors such as nutritional status clearly influence full skeletal growth and expression of genetic potential. Puberty is also a critical time for growth of lean mass and increased adiposity. Obesity during this period has been linked to obesity in adulthood, as children who are

obese or overweight are 70% more likely to becoming obese as an adult [20]. Similarly, bone mass tracks into adulthood, and peak bone mass and bone strength achieved in adolescence or early adulthood are primary determinants of fracture risk [4, 21, 22]. Both indices are dependent on maximizing mineral accrual during growth. Some data suggests a link between childhood obesity and impaired bone growth; however, the precise role of excess adiposity on bone remains unclear. Moreover, body composition rather than weight per se, may be the strongest determinant of bone throughout life. Lean mass is a well- established determinant of bone size, geometry, mineral content and skeletal architecture in young children [23-26], having a positive influence on bone development [27-29]. Whether excess fat augments bone during development is unclear as information regarding the fat-bone relationship in children and youth is conflicting, with evidence suggesting that total fat mass has a positive[30-33]; negative [34, 35], or null effect [36] on bone mass and density (i.e.; BMC; aBMD). The conflicting results may also be due to differences in fat distribution, and the failure in studies to distinguish between regional fat distribution, which are likely to render different consequences for bone [16, 29, 37-39]. The pro-inflammatory cytokines secreted by so called "pathogenic" fat depots [16, 29, 37-39] are likely to induce negative systemic effects on surrounding musculature and joint tissues by creating a toxic inflammatory environment, promoting further breakdown of the skeleton, whereas adipose tissue stored at other sites may not have these effects.

Nearly 17% of the pediatric population are obese. Concern for the role of excess adiposity and its potential consequence for bone derives from observations that both overweight and obese children are over-represented in the number of fracture cases for given age [16, 35, 40-43]. In support of the notion that excess body fat may impair bone

development, some studies have shown that fractures occurring in childhood are linked to alterations in metabolic parameters associated with increased deposition of visceral fat mass, which may serve as the earliest signs of potential skeletal insufficiency [44, 45] and the risk for developing osteoporosis later in life [37]. Indeed, recent studies suggest that increases in adiposity, especially in abdominal visceral fat and skeletal muscle fat content, in childhood, may impair skeletal growth and bone mineralization [37]. Further, some evidence shows that individuals with high muscle fat content may also have high marrow adipose content [46], contributing to weaker bones, suggesting that the same metabolic processes may be regulating fat infiltration of the bone and muscle [47]. Therefore, it is important to study soft tissue composition, including lean and fat masses in order to understand the skeletal adaptive response to changes in both static (fat) and dynamic (muscle) loading, or the foundation for developing interventions designed to achieve optimal bone strength, maximize bone mass, and slow down the rate of bone loss typically seen in late adulthood.

Undoubtedly, nutrient intake is a critical determinant of soft tissue and bone development and structure in growing children [48]. Historically, nutrients such as calcium, vitamin D, and protein have been the subject of considerable research for the effects on bone health whereas other nutritional factors have received far less attention. New evidence suggests that consuming sufficient levels of essential omega-3 polyunsaturated fatty acids (n-3 PUFAs), alpha-linolenic acid (ALA, 18:3 n-3) and its lipid derivatives, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) supports bone health by modulating bone metabolism and reducing bone loss [48-50]. Bone protective effects of consuming a diet high in omega-3 remain

controversial. While there is abundant evidence illustrating the effects of consuming various omega-3 and omega-6 fatty acids on bone density, structure and strength, and bone biomechanical properties in animals [50-55], evidence showing that the influence of fatty acids on bone translate to the human skeleton are limited; importantly, the impact of dietary fatty acid intake on bone health during critical periods of skeletal growth such as the peri-pubertal years is not well characterized [56]. Given the critical role of nutrition during growth and development, it important to assess whether the possible bone-promoting properties dietary n-3 PUFA aid in enhancing various bone indices to achieve optimal bone growth and development in young girls.

Studies on the pediatric fat-bone relationship have been hampered by the technological limitations of using dual-energy X-ray absorptiometry (DXA) to assess bone parameters. DXA is inherently limited for use in youth, as estimates of areal bone mineral density (aBMD, g/cm^2), and bone mineral content (BMC, g) are influenced by increases in size (tissue depth) and rapid changes in stature that occur during growth, and therefore confound interpretation [39, 57]. Additionally, DXA is unable to distinguish between cortical and trabecular bone compartments, and thus cannot provide measures of bone geometry, structure or strength. Unlike DXA, application of low radiation dose imaging via peripheral quantitative computed tomography (pQCT) provides 3-dimensional estimates of the volumetric BMD and bone architectural features (cortical thickness, cross-sectional area; CSA) that in turn can be used to estimate bone strength. Given that small changes in either bone size or shape can lead to significant changes in bone strength, independent of changes in bone mass, estimation of both material (e.g., mineral density) and structural (e.g., geometry, size and shape) properties of bone are

necessary to accurately estimate bone strength [17]. Indeed, the ability of pQCT to measure trabecular bone, which is 8-times more metabolically active than cortical bone, makes it highly sensitive to changes in skeletal density [16] and strength.

In summary, the underlining purpose of this study was to determine i) the optimal age (or stage of maturation) for intervention, ii) whether changes in dietary fat consumption contribute to enhanced bone structural development and bone strength, ii) whether changes in whole body fat mass influence bone strength and development, iv) whether regional fat depots are a stronger determinant of bone structure and strength than whole-body fatness, v) whether structural changes, in response to diet and physical activity, contribute to enhanced bone strength, and vi) whether osteogenic adaptations to mechanical load occur in response to changes in fat mass or soft tissue composition, and if these adaptations are maintained in the long term, or merely represent an acceleration of a predetermined bone mass. Thus, the objective of the dissertation was to assess longitudinal influences of whole body and regional fat distribution (i.e., total body fat mass, android fat mass and skeletal muscle fat content) and lean soft tissue composition (i.e., skeletal muscle) on bone density, bone geometry, and indices of bone strength in young girls. Given the potential bone-enhancing and bone protective effects of omega-3 polyunsaturated fatty acids, longitudinal evaluation of dietary intake of these essential fatty acids on various bone indices was also evaluated. An ancillary study was conducted to assess whether DXA could be used as an alternative to MRI or CT to estimate visceral fat distribution; in this analysis, MRI estimates of visceral fat and DXA estimates of android fat in a mixed sample of adolescents and young adults males and females were used to assess whether visceral fat could be accurately estimated for android fat.

BACKGROUND AND SIGNFICANCE

Osteoporosis in adults: a growing public health concern

Osteoporosis is a widespread metabolic bone disease, characterized by skeletal fragility, low bone mass, and poor micro-architectural infrastructure, particularly at cancellous bone sites, which reduces overall bone strength and increases susceptibility to fracture [58]. Recent reports (2004) from the U.S. Surgeon General [13] and the National Osteoporosis Foundation (NOF) [12] estimate that nearly 10 million Americans over the age of 50 have osteoporosis, while 18 million more are at risk of developing the disease [12, 13]. In addition, 34 million Americans are at risk of osteopenia, or low bone mass, which increases the risk for osteoporosis, fractures and other bone-related complications later in life [59]. While bone mass and strength decrease during adulthood in both men and women, the prevalence of osteoporosis is higher in women than in men [60]. Epidemiological reports by the 2004 U.S. Surgeon general indicates that, in the United States, one out of every two women over 50 will experience an osteoporotic fracture in their lifetime, while prevalence is one in five for men [13].

Approximately 1.5 million osteoporotic fractures are reported each year, predominately occurring at the hip, spine and distal forearm. Notably, current estimates for lifetime risk for fracture in 50-yr-old white women in the U.S. is 17.5% at the hip, 15.6% at the vertebrae, and 16.0% at the distal forearm [14, 61]. Unfortunately, individuals who sustain a hip fracture often require long-term nursing care and most fail to return to their pre-fracture status. Osteoporotic fractures are associated with additional health-burdens such as chronic pain, disability and depression, diminished function to perform daily living activities, loss of independence and premature death [59]. The acute

and long term medical expenses associated with the cost of fractures impose additional burdens. For example, in the United States, costs of osteoporotic-related injury are expected to be as high as $20 billion per year, with hip fractures accounting for more than a third of the total cost [12, 60]. Thus, if fractures are undiagnosed or un-reported, the national costs of osteoporotic fractures will likely rise, given the accelerated increase in population growth prolonged life expectancy and unhealthy lifestyles

Pharmacological therapy (i.e., bisphosphonates, raloxifene, calcitonin, and selective estrogen receptor modulators), is traditionally used as the first line of therapy for osteoporosis [62, 63]. The therapeutic efficacy of pharmaceutical agents to delay or halt bone loss and reduce overall fracture risk is confirmed in clinical studies [62, 64-70]; bisphosphonates, in particular, are effective in reducing fractures in at-risk populations by 40% to 50% [64, 71].

Despite these established therapeutic benefits, treatment for osteoporosis is suboptimal, as long term safety issues have not been well established; in addition, the efficacy of oral preparations is limited because of low bioavailability, complications, and adverse effects from the gastrointestinal tract. The safety profile of these drugs is also a cause of concern, as agents such as tamoxifen and raloxifene are associated with two to three-fold increases in venous thromboembolic events, while more casual, lesser adverse events related to drug intake include leg cramps and hot flashes [63]. Appropriate drug selection, adherence and compliance are also noteworthy limitations, given osteoporosis therapy is a long-term procedure, and for most, will impose life-long burdens [65]**.** Ultimately, while pharmacological therapies are conventionally prescribed to decrease the risk of fractures, prevention strategies conducted earlier in life, such as improvements

in diet, nutrition, and body composition-- factors that promote bone mineral accrual and bone density and reduce bone loss, can help to maintain bone strength and reduce overall fracture risk later in life.

Osteoporosis: A Pediatric Disorder Manifested in old age

Osteoporosis has conventionally been considered to be a debilitating bone disease of the elderly. Nearly 30 years ago, osteoporosis was recognized to have pediatric antecedents, waiting to manifest itself later in life [4, 11]. Importantly, the attainment of an optimal (peak) bone mass and strength in early adulthood is a primary determinant of fracture risk [4, 21, 22, 72]. Puberty is a critical period for growth and development of bone, and optimization of bone strength. During this time, bone formation exceeds the rate of bone resorption resulting in acquisition of approximately 85–90% of peak bone mass by the end of puberty [4, 33, 50].

The dynamic and anatomic properties of bone allow it continue to remodel itself throughout a lifetime, as it adapts to the functional needs and mechanical loading imposed on it. Changes in growth velocity and total body length are dependent on the bone constituents, calcium carbonate, calcium phosphate, collagen and water, which together help to provide the strength and stiffness and rigidity of the bone. As long bones grow, remodeling occurs along the bone mass cortex which becomes deposited inside both the periosteal envelope and marrow cavity. The thickness of this cortex is determined by the growth ratio of the endocortical surface relative to the periosteal surface [27, 29, 73, 74]. In contrast, because volumetric BMD (vBMD) is a ratio of total bone mineral accrued relative to the total enlarging bone, vBMD remains constant or increases only slightly during growth [73]. In addition, development of the trabecular

network that is positioned at the growth plate and at the diaphyseal end of the bone is attributed to the increase in trabecular thickness that develops during puberty. Together, increases in bone size and the adaptation of bone geometry contribute to greater bone strength.

The majority of bone mineral acquisition that occurs during childhood and adolescence is driven by the critical convergence of genetic, hormonal, nutritional, and environmental stimuli that interact with each other to stimulate bone formation for the enhancement of linear growth and skeletal expansion [44]. Sex differences in bone development are established in the pre- and peri-puberty years, and have been shown to independently be associated with each of these factors. For example, in males, androgens, growth hormone, and the growth hormone insulin-like growth factor I axis are commonly known to have independent effects in stimulating periosteal apposition and thus, cortical width, whereas, in females, estrogen plays a dual and opposing role by stimulating endosteal apposition while inhibiting periosteal apposition, thereby resulting in a thinner, narrower bone cortex in girls compared to boys (2, 6, 20, 53).

Differences in bone between same sex individuals may relate to differences in age, sexual maturity, tempo and timing of pubertal development, and timing of maximal changes in bone width and consolidation of bone (measured as vBMD) [21, 75]. Evidence from longitudinal bone tracking studies found that the girls with a higher rate of pubertal maturation had a greater increase in bone mass at all sites two years after the onset of puberty as compared to the girls experiencing a slower maturation rate [21, 75, 76]. Similarly, Foley et al observed that over eight years, the majority of children who were in the lowest tertile of bone mass at baseline remained in that tertile as adolescents,

suggesting that the susceptibility to osteoporosis can be identified early in life [21]. Together, these results emphasize the importance of adolescence in terms of efforts aimed at maximizing bone mineral accrual, as observations indicate that over 25% of bone mineral is accrued in the two years surrounding peak linear growth [4]. This is approximately equal to the amount of bone mass a female will lose from 50 to 80 years of age [77]. Modeling adaptations, dependent of mineral accrual, leading to structural changes during growth contribute to bone strength independent of bone mass [78].

Past studies have been limited by the reliance on dual-energy x-ray absorptiometry (DXA) [34, 35, 79-82]. Results from DXA may be misleading in youth, since BMC and aBMD measured by DXA are influenced by size and the rapid changes that occur during growth confound interpretation [83]. Moreover, its 2-dimensional estimation of bone morphology (dimensions, geometry) does not allow for accurate estimations of whole bone strength and mechanical competence [4].

The application of low radiation dose, 3-dimensional imaging via peripheral quantitative computed tomography (pQCT), is more valid than DXA for quantifying soft tissue composition and other indices of bone strength including material (volumetric BMD; vBMD, mg/cm^3) and structural properties (cortical thickness, cross-sectional area; CSA) in children, without the confounding of growth [4, 57]. With pQCT, the contributions of changes in bone mass, density, and geometry to changes in indices of bone strength can be safely and accurately estimated in young children, although this technique has been rarely applied in prospective intervention studies in youth.

Clearly, efforts to optimize bone accrual and promote beneficial structural adaptations during childhood and adolescence are warranted. In order to have a maximal

effect, intervention strategies aimed at reducing the incidence of osteoporosis must start during childhood and adolescence. Given the lack of effective treatments for established osteoporosis, and in order to optimize peak bone mass and reduce future risk of osteoporosis, lifestyle-modifying prevention strategies that optimize bone before the onset of puberty and are maintained beyond the attainment of sexual maturity is warranted [50, 75].

EXPLANATION OF DISSERTATION FORMAT

The “Present Study” (Chapters 3–5) summarizes methods, main findings, an integrated summary and recommendations, conclusions, and limitations relevant to specific aims 1–3, and ancillary aim 1. Specific aims 1–3 are longitudinal analyses addressed in three manuscripts, and the ancillary aim is a cross-sectional analysis addressed in one published manuscript. These four manuscripts are included in this document (APPENDICES A–E, pp. xx--xxx). In addition, longitudinal results presented in specific aims 1 and 2 are currently under review.

CHAPTER 2

REVIEW OF THE LITERATURE

Bone Morphology and Skeletal Development

Structural components of bone

Three-dimensional geometry of bone is structurally designed to provide optimal bone strength while its material properties confer bone's stiff-yet flexible, lightweight-yet strong structure that makes the skeleton capable of providing movement, but also facilitate resistance to various types of mechanical strains imposed upon it.

Bone is classified into four distinct categories according to their shape: long bones include arm and leg bones, short bone defines bones of the wrists and ankles, flat bones include ribs and bones of the skull and irregular bones comprise of spinal vertebrae. Structurally, bone is defined by its cortical and trabecular bone, which can further be classified as primary (woven) or secondary (lamellar) bone [6]. Although both are similar in matrix structure and composition, cortical and trabecular differ is in mass, macroscopic appearance, and functionality. Each type of bone will be discussed in detail below.

Cortical bone often referred to as "compact" bone. Encompassing nearly 80% of the entire skeleton, cortical bone is distinguished from trabecular bone by its cellular makeup, predominantly composed of osteons, and its highly dense nature, giving rise to its higher mass-to-volume ratio [6]. Moreover, the cortical volumetric bone mineral density (vBMD) is determined by the number and average size of osteonal canals (porosity) and the mineralization density of the cortical tissue [84]. Cortical bone is fashioned into three primary envelopes: the outer-periosteal surface (periosteum), the endocortical, and intracortical components of its inner (endosteal) surface. Aside from the

articular cartilage, which insulates each end of the bone, acting as the joint between adjacent bones, the entire bone is enclosed in the periosteum. The periosteum is a hard, solid shell that provides bone with resistance against bending strains. Encompassed within the periosteum are additional layers of bone that give rise to its intricate microarchitecture and structural integrity. The epiphyseal surface, for example, is located at the end of each bone, composed highly of a spongy- trabeculae meshwork. Because the epiphyses is subject to the greatest compression forces [85], the composition of this cancellous bone abundantly localized within the epiphyses provides the bone with the greatest amount of elastic strength. Distal to the epiphysis is the metaphysis, which is formed as a result of epiphyseal growth plate fusion, marking an end to longitudinal bone growth. The diaphysis, commonly referred to as the shaft of long bone, is located between the metaphyses on each end.

Located within the cortical shell of the diaphysis is a hollow chamber known as the medullary cavity. The compact bone situated in the diaphysis is fashioned to expand the cortical shell outward, effectively creates space for a rigid, hollow chamber called the medullary cavity [85]. This cavity contains several empty spaces, or voids, along with various types of connective tissue and is continuous with trabecular (spongy) bones and is filled with a type of connective tissue called marrow [85, 86]. Yellow marrow, which is typically located in the medullary cavity of mature long bone, is generally functions as a fat-storing center, while red marrow, which predominately resides within the spaces of spongy bone, synthesizes various types of blood cells, most notably, red blood cells (RBCs) [85]. Importantly, the primary intent of the medually cavity provides the bone

with light and flexible properties, while the outward displacement of the cortical shell provides increases in bending strength, or a resistance to bending strains [86].

Trabecular bone comprises of the remaining 20% of bone in the skeleton and is made up of bone tissue known as trabeculae [84]. Generally, trabecular bone is less dense than cortical bone, but has ten-times more surface area and is approximately 8 times more metabolically active. Trabecular bone mineral density (vBMD) is determined by the thickness of the trabeculae, the average spacing between them, and the total amount of mineralized trabecular tissue [84]. As such, trabecular bone thickens or thins in response to bone formation or resorption that occurs on the trabeculae surface. Further, the tissue properties of trabecular bone are defined by the orientation of the trabeculae, and the vertical and horizontal distribution of trabeculae, and the connectivity established between trabeculae [84]. The interconnection of trabecular plates in addition to the number, thickness, spacing, distribution, and connectivity of trabecular plates reflect the trabecular network ultimately determine bone strength by providing bone with lightness and structural flexibility [86]. By virtue of its structure, the trabeculae network can absorb more energy than cortical bone by deforming before cracking, but is compromised in their ability to tolerate heavier loads. Consequently, a loss in trabecular connectivity has more deleterious effects on bone strength compared to thinner, but well connected trabeculae [86].

Bone loss occurs as soon as the third decade of life [21, 73], in which rate of bone resorption exceeds rates of bone formation. For women, menopause marks a critical period for changes in hormonal status, body composition, and importantly, accelerated bone loss, contributing to significant decrease in total and regional bone mineral densities

(BMD) [87]. Typically, significant declines in BMD are observed at the spine and proximal femur before menopause begins, [73, 88], followed by accelerating rates of bone loss for approximately 7 years after menopause begins [89]. The age-related bone degradation that occurs in peri and post-menopausal women can be explained by a delay in bone formation initiation, the consequent imbalance between modeling and remodeling, and the eventual break down of both the material and structural properties of bone [5, 73]. Remodeling, which is normally responsible for repairing surface microdamage is followed by modeling or "construction" of new bone and mineral deposition at the same site. However, during menopause, the rate of bone removal exceeds the rate of bone deposition, contributing to negative bone balance, trabecular and cortical thinning, increased porosity and an accelerated the loss of bone tissue connectivity. Reductions in estrogen contribute to the disruption in bone homeostasis, primarily by increasing the longevity of bone-resorbing osteoclasts [73, 90, 91]. Together, the combination of these factors influence the rate of remodeling as it moves from a low rate before menopause to a high rate after menopause [73, 92, 93], and consequently increases the susceptibility to higher structural damage, bone fragility, and pathogenesis of osteoporosis in women [73].

Bone Modeling and Remodeling

The skeleton is dynamically designed to be both functional as well as flexible in order to adequately support the body. Contrary to the previous dogma [58, 94], the skeleton is not inert as the cellular activity that is mediated by bone forming osteoblasts and bone resorbing osteoclasts function to maintain a level of homeostasis between the formation and removal of bone at various skeletal sites. In turn, the adaptive processes of bone modeling and bone remodeling remain continuously active throughout life, allowing

for the skeleton to evolve and adapt to meet the continuous demands imposed by dynamic (muscle) and static (fat) strains, and ultimately preserve an optimal balance between strength and weight. Importantly, communications between osteoblasts and osteoclasts that delegate bone turnover events is essential to allow for appropriate modifications in skeletal size, architecture, mass, and strength to occur during growth. The intricate processes of bone modeling and remodeling will be discussed in further detail below.

During early embryonic and post-natal growth, bone modeling occurs at accelerating rates, contributing to new bone formation, longitudinal bone growth, maximum accrual of bone mass and the attainment of peak height velocity. Indeed, bone *modeling* (formation) during childhood occurs at various skeletal sites and in absence of previous bone resorption, though the rate at which new bone is formed and laid down decelerates as skeletal maturation is achieved. Thereafter, bone *remodeling* predominates, representing a localized process that couples both bone formation and bone resorption, mediated by osteoblasts and osteoclasts, respectively.

Initiation of the bone remodeling events requires a response facilitated by the bone-sensing osteocytes. Osteocytes belong to the osteoblast cell lineage, and are the mechanosensors of bone deformation, as they are the first cells to detect the precise location and magnitude of damaged bone imposed by the prevailing loads. More specifically, apoptosis (cell death) of osteocytes presented at regions with significant microdamage are the forerunners of bone remodeling, as these cells provide the topographic location that initiates biochemical and hemotactic signaling that target osteoclasts to the damaged site [6]. Importantly, this localized regulation on remodeling facilitated by osteocytes is bidirectional, in that they not only herald osteoclastogenesis,

but the products formed from bone resorption likely play a role in up-regulating osteoblast precursors, modulating matrix synthesis and subsequently, bone formation. Mechanistically, the coordinated actions of osteoblasts and osteoclasts form a bone multicellular unit (BMU), in which these cells operate collectively and in a concerted fashion, to reconstruct small "packets" on bone at a time. During remodeling, for example, the BMU act predominantly on the three distinct compartments of endosteal (inner) envelope of cortical bone: endocortical, intracortical, and trabecular, and to a lesser extent, the periosteal (outer) envelope [6]. Upon signaling from the osteocytes, the BMU regulates the sequence of bone remodeling events, starting with osteoclast-mediated removal or "resorption" of old bone. Recruitment of proliferating osteoclast precursors to the damaged bone site eventually differentiate into pre-osteoclasts (OCPs). OCPs act as intermediates of the bone remodeling as they are responsible for activating signaling cascades involved in up-regulating RANKL presented on the surface of osteoblasts which proceed to bind to its receptor (RANK) found at the cytoplasmic surface of both OCPs and already-mature osteoclasts [95, 96]. This direct contact between RANKL/RANK induces the maturation of osteoclasts and subsequent osteoclastogenesis mediated degradation of the bone matrix at damaged skeletal sites. Pre-osteoblasts that differentiate from the previously recruited osteoprogenitor (mesenchymal) cells start refilling the excavated bone sites by centripetally depositing new bone as the un-mineralized matrix (osteoid). Maturation of the osteoblasts into osteocytes also promotes mineralization of the matrix, thereby completing one bone remodeling cycle [97-99]. The completion of this bone turnover at each skeletal site leads to necessary adaptive changes in bone size, shape, and distribution to accommodate

growth, development and increased mechanical loading imposed on the skeleton (**figure 1**; [13]).

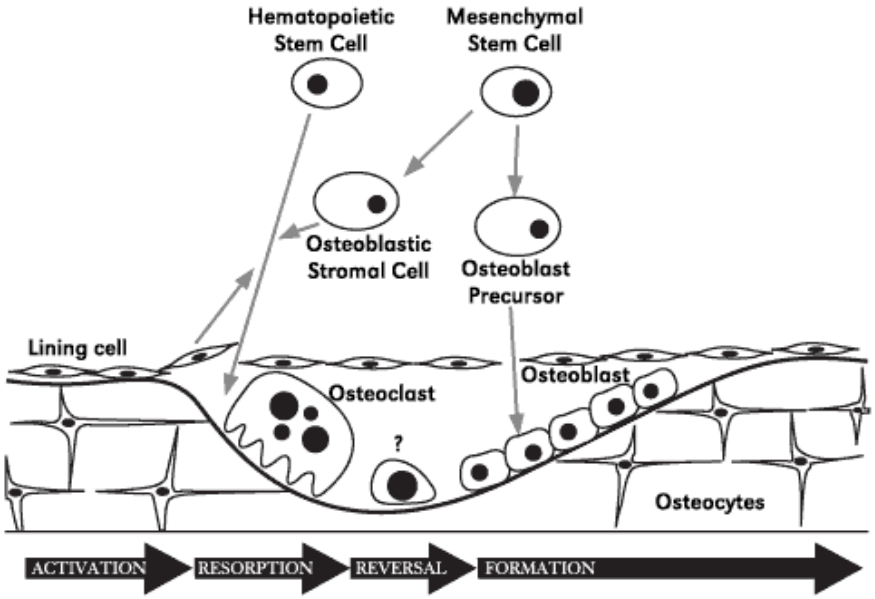

Figure 1. Bone Remodeling Pathway [13].

In the growing adult bone, bone remodeling by BMUs is constant, occurring at differ times and different skeletal sites according to the mechanical demands of the body. On average, one BMU can replace about 0.05 mm^3 of adult bone over a course of 4 months, and are coincidently responsible for 95% of bone turnover in adults [6, 8, 100]. However, the number of BMUs present in a specified volume of bone tissue and the rate at which bone remodeling is completed is dependent on several factors, such as the activation frequency and the longevity of each individual BMU [98, 101]. Activation frequency, or "birth rate" will determine how the number of BMUs active; e.g., a high activation frequency will yield a large number of BMUs [98]. The BMU longevity, synonymously termed the "sigma period," (σRC), specifically relates to the number of days it takes for a BMU to completely remodel a fixed two-dimensional slice through a

region of bone [98, 102]. For example, in cortical bone, the BMU would take approximately 120 days to pass through and remodel one plane, of which 20 days of this period will be dedicated to the activation and maturation of pre-osteoclasts and subsequent excavation of the bone resorption cavity (lacunae), 10 days will be spent on the formation of the un-mineralized matrix (osteoid) and quiescence period, followed by the final 90 days, in which layers of new mineralized bone will be centrally deposited by the osteoblast and osteocytes [98, 102].

Because BMUs work predominately on the cortical-endosteal and trabecular surfaces, over time, bone remodeling will induce the expansion of the marrow cavity and consequently, a removal of the bone trabeculae. During early adult life, BMUs are in a conservation mode of remodeling activity as a volume of new bone equally replaces the volume of damaged bone removed at one time. Significant decreases in bone mass and mineral density become more apparent with increasing age, as increased BMU activity (or a negative BMU balance) results more bone removal and less bone formation. Consistently, declines in overall muscle quality, which has been associated with whole-body muscle weakness [103, 104] and poor physical function [105] due to increased skeletal muscle fat infiltration has also been shown to contribute to also contribute to age related bone loss in older adults, declines in bone formation, as these factors are likely to alter the mechanical loading effect and the necessary adaptive changes in bone strength to accommodate the changes individual body weight. Generally speaking, bone modeling by BMUs do not form more bone than they can resorb and therefore are capable of only making cortices and trabeculae thinner but not thicker [98, 106].

In summary, the primary purpose of bone modeling and remodeling is to establish optimal bone mass and peak bone strength during growth and to maintain ultimate yield strength during aging. Moreover, the coordinated actions of bone modeling and remodeling are necessary for defining skeletal shape, repairing sites with compromised structural integrity and structure and lastly, for maintaining skeletal mass and preserving its morphology. Throughout life, increased mechanical loading from body weight and damage caused by fatigue are likely to develop; however the responsibility of BMU's to sense, respond to environmental changes are accomplished by initiating reconstruction of the material composition and macro-architecture and restoration of the entire skeleton. Alterations in body composition resulting from gains in adiposity and lean (muscle) tissue, which are hallmark characteristics of puberty and growth. Gains in lean mass, in particular, occur at rates faster than accumulating bone mass, however evidence in both adults [58, 107] and children [30-33]; indicate that both fat and lean masses collective act as mechanical loading imposed on the skeleton thereby augmenting bone mineral deposition and bone strength by mechanism of increased bone modeling process. Importantly the rate at which modeling and remodeling differ by stage of life, as modeling predominates during childhood in order to fashion the cortical and trabecular bone and excavate the bone marrow so that localized control of future bone remodeling and bone cell activities is present; modifications of these bone sites during growth will result in cortical and trabecular thickening. In contrast, during adulthood, continuous removal of damaged bone and successive replacement with new bone underscore the importance of remodeling to ensure a stable skeletal construct is being formed; consequently thinning of these bone structures as aging progresses. The rate at which

bone loss and micro-architectural bone degeneration occurs is dependent on local control from bone derived growth factors, cytokines and hormones that aid in maintaining homeostatic control on BMU-bone cell activities.

Biomechanical and Molecular Regulation of Bone Remodeling

Defining the components of the "Bone-Muscle Unit"

The structural and material properties that give rise to bone quality and ultimate bone strength underscore the prevailing notion of a tight, functional relationship between muscle and bone. Although the definition of the "muscle-bone" unit remains unclear, there are several lines of evidence showing that structural adaptations in bone size, and geometric shape and modifications in material composition influence the ultimate yield bone strength that is necessary to accommodate for imposing loads [6, 7, 9, 108-110]. Indeed, the functional relationship between bone and the muscle requires a continuous balance between bone strength and the mechanical forces that normally challenge bone stability [109]. Mechanistically, the muscle-bone unit is characterized by the ability of the bones to work independently but synchronously to adapt, fashion and refashion, and develop a stable structure so that each bone can tolerate static and dynamic loading without failure or compromised agility.

Teleologically, the muscle-bone unit has evolved to achieve the optimal bone structure to match usual mechanical loads, while adding new material only to sites undergoing the most stress. In order to achieve ultimate bone strength, bone must fulfill the contradictory properties of being stiff yet flexible, and relatively light yet strong. Coincidently, the foundation for an optimal bone structure is determined prior to skeletal maturation. Initial lengthening of new bone, for example, is facilitated by the synchronal

activities of periosteal apposition and excavation of the marrow cavity via endosteal resorption. Consequently, the loss of synchrony between periosteum and endosteum bone formation is necessary to fashion a wider, thicker cortex, and the development of intricate elliptical and triangular structures that locally adapt to the loads exposed on the skeleton. Additionally, enlarging the medullary cavity causes displacement of the cortex from its neutral axis, and this contributes to the perpetual increases in structural stiffness during growth. Further adaptations to various degrees of loading will continue by the selectively adding and removing bone from existing surfaces and reconfiguring new bone into the optimal shape, size and contours necessary to withstand the applied mechanical challenges.

Additionally, bone is also designed to take on the fundamental properties of building springs, as it must not only maintain its lightness but also achieve maximal bone strength. The cancellous (trabecular) bones of the axial skeleton, in particular, are characterized by their spongy, porous trabecular structure, which gives bone its light but strong attributes. Despite its lower vBMD, trabecular bone is an integral component of bone strength in that its trabeculae meshwork can act as shock absorbers, providing protection to bone by resisting deformation and preventing micro-crack formation. In turn, the greater peak strain that is consequently sacrificed for a higher tolerated stress load facilitates bone extension, flexion, and rotational movements.

Young's modulus/ultimate yield strength

The *modulus of elasticity* (*Young's modulus*) is commonly used to describe the relationship between stress and strain and how this modulus relates to the overall stiffness or rigidity of bone [111]. Because bone strength is made of material and structural

composites, this modulus can characterize the material strength, when ratios of the stress (x) over strain (y) are examined, and similarly, reflect the (whole) bone structural strength, when deformation (x) and load (y) relationships are evaluated; the primary difference being that material strength is not size dependent, where as structural strength is influenced by its geometric (three-dimensional) properties [84] (figure 2; [112]). In either model, the linear slope of this line represents the pre-yield material behavior upon which initial loading on the bone reflects the elastic or "material stiffness" component of bone. Under these conditions, release of the force will result in an elastic transition to its original shape. The point, at which the curve becomes "plastic" or nonlinear, reflects bone's material (or structure) to permanently deform, even after the load is removed. Further stress imposed that are beyond the "plastic region," will result in eventual fracture or break; significant deformation of the material is likely to occur before fracture results, however, the amount of material deformed at one site is variable [84].The ultimate yield strength of bone can herein be calculated by determining the maximum stress necessary to cause failure of resistance [84, 111].

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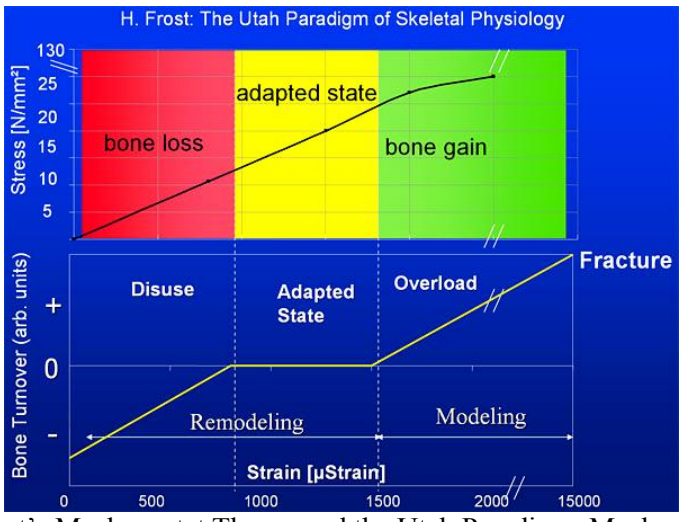

Figure 2. Harold Frost's Mechanostat Theory and the Utah Paradigm: Mechano-transduction mechanism [112]

The functional balance between bone strength and the mechanical forces that challenge bone stability have studied and compiled formally as the mechanostat hypothesis. Fundamentally, Frost's theory, described more extensively in the Utah Paradigm skeletal physiology, reveals a dependency of postnatal bone development on mechanical loading that contributes to subsequent structural strength adaptations during growth [8, 108, 112, 113]. In children, for example, gains in muscle mass occur at an accelerating rate, surpassing bone mass accumulation [97, 98]. Alterations or increases in the loading environment that contribute to deposition of new bone tissue include: (1) the growth and development of muscles, (2) increases in body mass, and (*3*) the lengthening of the diaphysis, intricately fashioned to meet these rapidly evolving mechanical demands [26, 98, 114]. Accordingly, mechanical stimulus that induces elastic deformation of bone activates a cellular response leading to increased bone modeling and remodeling processes. This response to stress, by bone, is initiated by the bone sensing osteocytes, as they proceed to recruit remodeling drifts (mediated by osteoclasts) that mediate the lengthening and shaping of the diaphysis of long bones [6] and subsequently, the migration of the modeling drifts (controlled by osteoblasts) which mediate changes in size, shape and location, ensuring that a proper amount of space is available to accommodate this newly refashioned bone [8, 113]. Importantly, before new bone is formed and laid down, the bone cells must recognize that the mechanical loading imposed is different than previous mechanical stimuli. In turn, bone cells have must have an internal memory of the previous mechanical environment in order to initiate a response to the new stresses imposed. Ensuing this onsite, localized recognition, bone cells can then process the information and proceed on to distinguishing the type of

mechanical challenge that is being applied. In most long bones, for example, bending loads creates a strain gradient, across the bone section and along the neutral axis [98, 115]. BMU recruitment to the site will lead to bone removal at the neutral axis; however, to prevent excessive bouts of bone resorption, each osteoclast cell along the bone section pre-sets a stimulus threshold, based on local loading history, above which a mechanical signal will causes a cellular response [98]. In response to the mechanical stimulus, osteoblast cells, as well as many other cell types, reorganize their cytoskeletons, thereby, reprogramming their mechano-sensitivity, such that accommodations to the new mechanical strain environment can be met [98].

A central component of bone remodeling homeostasis is the adaptive, feed-back loop that exists between the bone tissue strain, (deformation) and bone strength. Undoubtedly, changes in bone geometry and material composition likely have a genetic determination [6, 108, 111, 116]. However, once post-natal morphology is established, mechanical usage and stress imposed on the skeleton therein predominates as a mediator of muscle stress-bone operational unit, predicting up to 40% of post-natal bone strength [108]. A combination of factors including bone marrow mediators and signaling molecules, connect the muscle-bone unit together, while non-mechanical mediators, such as hormones, nutrition, behavior and environment specifically modulate the ultimate mechanostat effects on bone strength. Collectively, these factors participate in regulating the modeling and remodeling threshold, by determining when and where adaptive bone strength is needed, so that varying peak loads that can be tolerated. Importantly, because of this regulatory feed-back control loop, a linear relationship exists between muscle cross sectional area, a surrogate for maximum forces the muscle is able to produce under

physiological conditions, and the bone cross sectional area, a surrogate for bone strength, in healthy individuals [109, 117]. The stimulation on bone growth by the components of the muscle-bone feedback loop contributes to subtle changes in bone mass and geometry [118] as well as ultimate yield bone strength. Coincidently, this adaptive response to mechanical stress are the foundation for preventing or minimizing bone loss and treating bone diseases such as osteoporosis.

Although the bone adaptation process is considered to be lifelong, bone modeling is predominates during early childhood, and adolescence, and less so during mid-life and adulthood. In contrast, adulthood is characterized by increased remodeling rate and bone resorption that contributes to trabecular thinning and higher bone loss. Findings from epidemiological studies also note that more than 50% of the bone strength acquired during youth is lost at 80 years of age [108]. Therefore, growth is the most opportune time to modify bone material and structural composition, the primary determinants of bone strength, as adaptive bone strength achieved by bone modeling and remodeling becomes increasingly inefficient with age. Regional specificity of the mechanostat effects on bone also differ as bony regions closer the ground (i.e., weight bearing bones of the appendicular skeleton) also confer a greater osteogenic potential. Because weight-bearing bones are directly exposed to larger gravitational forces and loading, dynamic strains on bone aid in enhancing appositional bone growth. Specifically, bones distal to weight bearing sites are exposed directly to stresses from physical activity and therefore render the greatest anabolic response to mechanical loading. Other factors such as sex, and pubertal maturation timing and status also influence the relative and absolute changes in bone growth and adaptive bone strength.

Determinants of Bone Strength

Bone Material and Bone Structural Properties

Bone strength is characterized by its material properties and structural design. Bones' biomechanical properties of allow for it to respond to natural forces, such as propulsions against gravity, ground reaction and muscle contractions, by internalizing these stresses, and adapt its material composition to conform elastically, to match the type and rate at which the force is be applied [6, 111]. With regards to Newton's first law of thermodynamics, in which energy can be created nor destroyed, but can only be absorbed, when bone is exposed to mechanical or impact loading, bone absorbs any imparted energy by readily deforming its shape, similar to a spring, without cracking, breaking. In turn, this flexibility like quality of bone enables it to shorten and widen when compression forces are applied, or lengthen and narrow when tension or torsional strains are imposed. Further, the geometric dimensions of bone, including lengths of bending "arms" or levers, and the surface dimensions of bone cross-sections, collectively known as bone's cross-sectional geometry, are also determinants of how well bone responds to stress. The cross-sectional geometry in particular, quantifies the amount of bone surface and its distribution about bending and torsion axes [84]. Notably, the largest physiological loads imposed on bone result from muscle contraction. Because muscles are closely attached to bone joints, they must produce a large amount of force in order to move each lever arm (bone). Likewise, bone, must structurally modify its shape and size, and contours in order to adapt its strength and tolerate the high dynamic loading of muscle force [118]. Thus, any changes in one or both material and structural properties

can result in a significant change in bone strength, compromised skeletal integrity and increased bone fragility [86].

The mineralization of the bone tissue and the fashioning of the mineralized bone tissue is conducted strategically, to meet the bone's architectural requirements. Because of the intricate orientation of collagen fibers (organic component) and the mineralization of the bone matrix by hydroxyapatite (inorganic component) [84], the material composition of bone is a primary determinant of yield strength by allowing bone to be stiff and resist deformation. In turn, greater mineralization of the bone tissue results in larger material stiffness, while lower mineral content which compromises its peak loading ability, results in a lower resistance to deformation [6]. Though, amount of material needed to construct bone of various sizes does not differ. Rather, wider, tubular bones with narrow cross-sections are fashioned to have a thinner cortex, whereas larger bones with bigger cross sections are constructed to have a larger medullary cavity and thus, a larger marrow cavity [6]. Compared to smaller bones, larger bones confer a lower apparent volumetric bone mineral density; however, this displacement of its thinner cortex in long bones offers a greater resistance to bending (larger bending strength). Mathematically, this is because bending strength is proportional to radius^4 [6, 119]; thus, long bones are ideally designed to have greater stiffness than flexibility.

Lean Mass is a determinant of Bone Mass

Simple changes in weight are likely to alter bone properties. Accordingly, several studies in adults and children demonstrate that changes in bone properties, BMC and BMD, parallel the increases or decreases in body weight [30, 120-123]. Body composition rather than weight per se, may be the strongest determinant of bone

throughout life. Fat mass may be a causal, independent determinant of bone mass, but the evidence is conflicting, as the relationship between body weight on bone is possibly influenced by confounding factors such as lean mass. Lean mass is a well- established determinant of bone size, geometry, mineral content and skeletal architecture [23-26]. In adults, some studies have reported that fat mass is also a positive determinant of bone mass and size, independent of muscle mass [124-126]. In contrast, children with excess adiposity may experience dissociation between weight gain and accrual in bone mineral content [31], suggesting a mismatch between gains in body mass and appropriate skeletal adaptations during growth [23]. However, because the loading effect from dynamic strains from muscle are stronger than the static loading effect from fat mass, exposure to muscle weight and muscle forces on the skeleton is likely to predominate as a stimulator of bone strength and bone density in children and young adults [28, 78]. In support of this notion, one longitudinal analysis, Goulding et al (2000) demonstrated that in young girls, the detrimental effect of obesity on bone during the period of peak bone mass acquisition may persist up to 4 years from initial observations [41]. In children, skeletal adaptations may depend on appropriate gains in lean mass [78, 127] and not fat mass [28, 128]. Indeed, Petit and colleagues (2008) found that girls who gained weight over a six year period, experienced greater gains in aBMD compared to weight-stable girls. Nevertheless, bone strength, reflected in DXA measures of bone cross-sectional area (CSA) and section modulus (Z, cm^3), was low relative to total body weight [78]. Because the weight gain in these girls was largely explained by an increase in fat mass, these results suggest that the change in bone strength may be adequate for changes in lean mass, but not for increases in body weight. Similarly, longitudinal data reported by Wetzsteon et al (2008) showed

that although bone strength was higher in overweight compared to normal weight children and was appropriate for their higher lean mass, it was low relative to their larger fat mass and body weight [28]. These findings are important, as they indicate that stronger associations exist between bone strength and lean mass than fat mass, thereby supporting the critical role of the muscle-bone unit and the skeletal adaptive response to its mechanical loads. Importantly, the mismatch of bone strength and bone mineral accrual to body weight, may explain, in part, why the incidence of fractures during puberty are higher in overweight and obese children compared to their normal weight peers [16, 23, 28, 43]. Hence, when the mechanical loading effect of body weight on bone mass is adjusted for lean mass, fat mass may have no additive effect or a negative effect on bone mass in contrast with the positive effect of weight-bearing itself [122, 129-131]. Mechanistically, these results are supported by Frost's mechanostat theory owing to the conventional idea of a functional relationship between muscle and bone in which bone has the ability to adapt the dynamic strains imposed by muscle contractions, which serve as an osteogenic stimulus to bone [23-25, 132], but not static loads, which may be imposed by excess fat mass [23].

There are several lines of evidence showing that structural adaptations in bone size and geometry and modifications in its material composition, yield an ultimate bone strength necessary to accommodate imposing loads [6, 9, 108-110]. Given bone mass is a primary composite of bone strength, while dynamic strains imposed by muscle contractions are the primary stimulators of bone strength, it remains unclear whether muscle force, or muscle quality, or bone mass are stronger determinants of bone strength during growth. The objective of Aim 2 was to investigate the components of the muscle

bone unit and determine whether bone mass, muscle quality, muscle size is a stronger predictor of bone strength in young girls. It is hypothesized that the independent influences of muscle quality and bone mass, respectively, muscle force, will predict bone strength at weight bearing bone sites more strongly than muscle cross-sectional area, alone.

Adiposity and bone accrual-still an established paradigm?

Mechanisms of Disease: is osteoporosis the obesity of bone?

Although generally considered to be two distinct diseases with multi-factorial etiologies, recent evidence suggests an epidemiologic, clinical, and pathophysiologic link between obesity and osteoporosis [58, 133]. For example; 1) both disease are independently effected by environmental, behavioral and genetic factors and it is possible that interaction between these factors influence the development of both diseases; 2) Acquisition of bone mass, bone strength and structural development are dependent on the skeleton's ability to adapt to the stresses imposed upon it as the composition of muscle mass and fat mass are likely to have different loading effects that may promote or hinder bone strength at various skeletal sites [13]; 3.) Osteoporosis and obesity typically develop with normal aging; osteoporosis is also highly associated with incidence of increased bone marrow adiposity; 4) Adipocytes (cells responsible for storing energy) and osteoblasts (the bone formation cells) derive from a common progenitor—the mesenchymal stem cell (**figure 3)**.

The precise mechanisms underlying the relationship between fat and bone remain unclear [16]; however, literature suggests that the interaction between obesity and bone metabolism initiates in the bone marrow microenvironment. For example, adipocytes

and osteoblasts originate from a common progenitor, mesenchymal stromal cells, which have pluripotent capabilities and an equal propensity to differentiate into either energy storing or bone forming cells. The balance of this differentiation is predominately regulated by the peroxisome proliferator-activated receptor (PPAR)-γ pathway and the beta-catemin (β-catenin)/Wnt signaling pathway, as well as TGF-α, leptin, and estrogen, additional factors that are related to osteogenesis and adipogenesis [58, 134-137]. Activation of PPAR- γ (i.e., PPAR-y2) pathway promotes differentiation of mesenchymal stromal cells into adipocytes, thereby playing a key role in initiating adipogenesis, as well as fat redistribution [58, 138] where as the and Wnt signaling, respectively promotes osteoblast differentiation while also inhibiting adipogenic differentiation [58]. The complexity of the fat-bone relationship is underscored by these competing pathways, as the regulation of these interacting pathways determines to the ultimate effect of fat mass on bone [58, 133] (**figure 3)**.

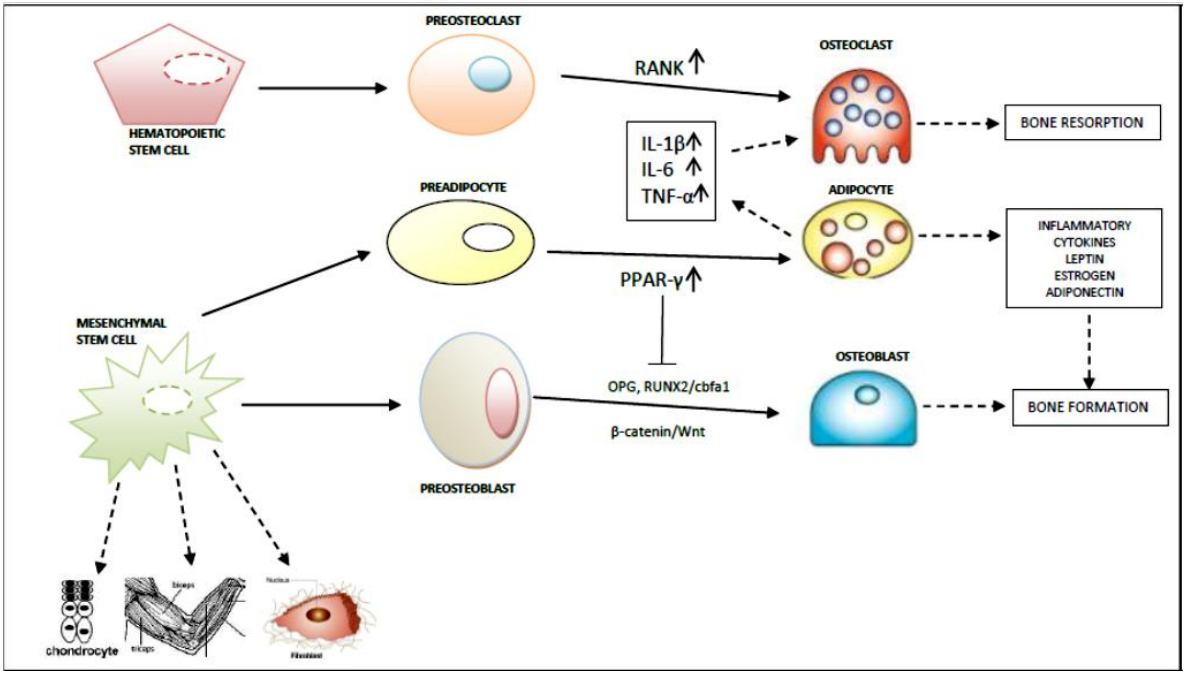

Figure 3. Mechanisms of the potential "fat-bone" interface.
Several potential mechanisms explaining the "fat-bone" interface have been proposed. Bone-resorbing osteoclast derive from a hematopoietic stem cell lineage, while mesenchymal stem cells are cable of differentiating into either "pre-osteoblasts" or "pre-adipocytes," or can be secreted in the peripheral tissues contributing to chrondocyte, myoblast, or fibroblast synthesis. Phenotypic expression of the osteoblast is dependent on key activation factors including bone morphogenetic protein 2 (BMP2), transforming growth factor-β (TBF- β), and transcription factor, osterix, while commitment of the progenitor cells to the adipocyte lineage requires activation of CCAAT/enhancer binding proteins (CEBP) α, β and δ, and peroxisome proliferative activated receptor (PPAR)γ2. Inflammatory cytokines produced and secreted by adipocytes including leptin, estrogen, and adiponectin can have a stimulatory effect on bone formation, whereas secretion of pro-inflammatory cytokines can stimulate osteoclast activity thereby enhancing bone resorption processes. BMP, bone morphogenetic protein; CEBP, CAAT/enhancer binding proteins; DLX, distal-less homeobox; IL, interleukin; MCSF, macrophage colony stimulating factor; MSX, MSH homeobox homolog; OPG, osteoprotegerin (tumor necrosis factor ligand super family, member 11); PPAR, peroxisome proliferative activated receptors; RANK, receptor activator of nuclear transcription factor κB (tumor necrosis factor receptor superfamily, member 11a); RANKL, receptor activator of nuclear transcription factor κB ligand (tumor necrosis factor receptor superfamily, member 11); RUNX, runt-related transcription factor; TGF, transforming growth factor. Figure modified from Rosen, Bouxsein et al 2006 [44].

A "fat-bone" paradigm?

Conventional wisdom in adults posits a positive fat-bone relationship, claiming that adiposity protects bone against fracture risk by mechanisms of increased mechanical stress on bone and positive metabolic effects on mineral accrual by hormones secreted by adipocytes [58, 78, 107]. Whether excess fat augments bone during development is unclear as information regarding the fat-bone relationship in children and youth is conflicting with some evidence suggesting that total fat mass has a positive [30-33]; negative [34, 35], or null effect [36] on bone mass and density (i.e., BMC, aBMD). To illustrate, results by Farr et al (2010) demonstrated that in prepubescent females, total body fat is positively related to bone strength variable, strain index (SSI), and this relationship was consistent across both tibia and femur weight bearing sites [122]. Similar findings support the idea that adiposity augments total-body as well as upper and lower limb bone mass by enhancing bone mineral accrual and periosteal apposition in pre-pubescent children [31, 121, 124]. In contrast, Pollock et al (2007) noted an 8% lower tibia SSI [131] in high fat adolescents verses their normal weight peers [131]. Significant decreases in tibia cortical thickness and cross sectional area were also observed among higher fat verses normal weight individuals [131]. Results from The Avon Longitudinal Study of Parents and Children (ALSPAC) project from Bristol in the UK, concluded that after appropriately adjusting for height and lean mass, fat mass showed strong positive relationships with total body-less-head bone mass and area in pre-pubescent girls, but this relationship was reversed as girls progressed through puberty [16, 124]. Bone tracking studies conducted by Foley et al (2006) examined that while higher levels of lean mass were positive stimulators of spinal and hip bone density in pre-pubescent boys and girls,

increases in percent body fat mass either attenuated or reversed the positive osteogenic influences of lean mass on bone in both sexes [16, 21]. Together, these studies suggest that the role of fat mass on bone is dependent on maturation status, some evidence suggests excess fat mass maybe detrimental to the growing skeleton peri- and post-puberty, an observation that has frequently been reported in obese children [139].

Implications for total body fat on pediatric bone:

Chronic weight gain that leads to obesity in adulthood is linked to obesity adolescence, just as osteoporosis risk in adulthood is a function of peak bone mass achieved at the end of adolescence [3, 27, 78]. Coincidently, obese adults also have an increased risk of lower limb fractures. Some data suggests a link between childhood obesity and impaired bone growth as evidence shows that a large proportion of children who incur a fracture [23, 40, 41, 140] or repeated fractures have a high body mass index (BMI) or increased body fat mass [16, 43]. Consistent with this premise, epidemiological data indicates that fracture incidence is nearly doubled in obese children, with increases from 16% in normal weight to 33% in overweight and obese children [42, 122]. Reports also indicate that overweight girls have been reported to have a higher incidence of forearm fractures compared to age-matched controls [140]. Results from a longitudinal analysis also showed that obese children, primarily with excess fat mass, and with no history of prior fracture were at a higher risk of experiencing a new fracture. These findings coincide with early reports by Goulding et al (2000), showing that obese children with prior fracture history had an increased risk of reoccurring fractures that may persist up to 4 years from initial observations [16, 41, 141]. Recent evidence suggests that childhood obesity may lead to reductions in cortical thickness (relative to periosteal

circumference), and cortical area thereby compromising bone strength and increasing fracture risk [27, 142]. Probable causes for this occurrence are likely attributed to gait abnormalities, which increase the likelihood to falling; additional evidence suggests that obese children have lower bone mass and bone strength relative to body size, increasing their risk for fracture. Moreover, several studies have observed that pre-pubescent obese children not only experience accelerated growth but typically, these children tend to be more advanced in bone age and are above average in size and height, which may contribute to higher fracture risk [1, 143]. At any age, overweight and obese children are over-represented in the number of fracture cases [16, 35, 40-43]. Given that nearly 20% of the pediatric population is obese, these findings are concerning insofar as the disruption of normal bone development during growth may result in impaired mineral accrual and lower bone.

During puberty children do not experience gains in weight and in bone mineral content at the same time. In fact, clinical observations note that children who are classified as obese or overweight experience dissociation between weight gain and accrual in bone mineral content [31], suggesting a mismatch between gains in body mass and appropriate skeletal adaptations during growth [23]. Moreover, obese or overweight children are also likely to experience an earlier progression into puberty, causing an earlier development of skeletal architecture thereby making their skeleton vulnerable to fractures [35]. Conventional belief is that body weight and body composition are the strongest determinants of bone throughout life, though, it is important to distinguish between the mechanical loading effects of fat mass and lean mass on bone. Indices of body weight, and particularly lean mass, has been shown to positively influence size,

geometry, mineral content and architecture of skeleton and largely reflect the skeletal adaptations, and remodeling to accommodate the loading. However, the relative influence of fat mass on skeletal development, independent of lean mass remains unclear [128]. While several reports conclude that overweight and obese children do exhibit higher aBMD, BMC, and higher bone strength [16, 29], gains in various bone parameters do not translate to reduced fracture risk as additional results show that obese children have lower volumetric bone mineral density (vBMD, mg/cm3) and geometric parameters relative to their body mass [23, 57, 122]. Rather adaptations of their skeletal frame may depend on appropriate gains in l*ean mass* [78, 127] and not *fat mass [28, 128]*. In fact, Petit and colleagues found that when compared to weight-stable adolescent girls, girls who gained weight over a six year period experienced a greater increase in aBMD, yet bone strength was low relative to total body weight, potentially increasing their risk for fracture later in life [78]. The mismatch between growth variables may largely be explained by excess adiposity rather than by reduced lean mass, and therefore make the skeleton of obese children highly vulnerable to experiencing and sustaining fractures [35]. Evidence from one longitudinal study that examined the effect of fat mass gained over a 7-yr period on bone indicated that children in the higher quartile of fat mass had reduced bone strength but similar cortical bone thickness as normal weight children [27]. Should the longitudinal effect of greater gains in fat mass persist over time, it is expected that the reductions in cortical geometry will occur, such that cortical thickness relative to periosteal circumference will be too small, thereby compromising bone strength. Adaptive responses to long-term gain in fat mass will result in increases in endosteal apposition but subsequent reductions in periosteal expansion, resulting in smaller cortical

thickness, decreased bone strength and increased risk of fracture [27]. Indeed, similar longitudinal results reported by Wetzsteon et al (2008) indicate that although bone strength was higher in overweight compared to normal weight children, strength was appropriate for the higher lean mass but was considered low relative to their larger fat mass and body weight [28]. Mechanistically, these results are supported by Frost's mechanostat theory in which bone has the ability to adapt the dynamic strains imposed by muscle contractions, which serve as an osteogenic stimulus to bone ([23-25, 132], but not static loads, which may be imposed by excess fat mass [23]. These findings are important, because it can be inferred that stronger associations between bone strength and increased muscle force, and not fat mass, may also partially explain why children with higher fat mass have increased risk of fractures [28]; when the mechanical loading effect of body weight on bone mass is adjusted for lean mass, fat mass may have a *no additive or a* negative effect on bone mass in contrast with the positive effect of weight-bearing itself [122, 129-131]. Nonetheless, ongoing research that investigates significant determinants of bone development and strength and identifies factors that can optimize peak bone mass during early life is a necessary contribution to the primary prevention of osteoporosis [144]. Given the increasing prevalence of childhood obesity and its potential adverse effect on skeletal development, it is important to address early prevention strategies in order to optimize peak bone mass during growth with the aim of reducing the risk of developing osteoporosis later in life.

Effects of regional fat distribution on bone strength

Individual differences in fat distribution and a failure to distinguish between fat depots, may explain the conflicting results regarding the fat-bone relationship, as the

location and pathological nature of each fat depot is likely to have different consequences for bone [16, 29, 37-39]. Recent literature suggests that phenotypic characteristics of regional fat depositions are stronger predictors of fracture risk and osteoporosis development than total body fat alone [17, 37, 145]. However, evidence supporting the relationship between visceral abdominal fat (VAT) and bone are mixed [146], with some studies suggesting abdominal fat has a positive correlation with total body bone mass [90, 146] and others reporting negative associations with bone density, content, structure, and strength [37, 38, 129, 146, 147]. While several studies have observed that obese children have higher fat deposition in the trunk area. Consequently, these children also experience an earlier onset of puberty, are further advanced in maturation and skeletal development compared to their normal weight counterparts [17, 80, 81]. Indeed, recent studies suggest that increased visceral fat deposition in childhood may impair skeletal growth and mineralization. In support of this notion, some studies have shown that fractures occurring in childhood are linked to alterations in metabolic parameters associated with increased deposition of visceral fat mass, which may serve as the earliest signs of potential skeletal insufficiency [44] and the risk for developing osteoporosis later in life [37]. In support of this premise, evidence from Garnett et al (2004) showed that greater levels of central rather than total adiposity might be deleterious for developing bone [80]. Similarly, Pollock et al (2010) reported that in a cohort of overweight children who were pre-diabetic or non-pre-diabetic, although total body fat was positively associated with bone mineral content (BMC), visceral fat was inversely related to BMC suggesting that differences in bone amongst these children might be explained by the amount of various pathogenic fat regions [17]. Farr et al (2011) also suggested that a negative relationship

between bone and visceral adipose tissue (VAT) and fat within skeletal muscle in children [83]. Given that childhood obesity incidence of childhood obesity has never been higher [148], these findings suggest the relationship between fat mass and bone may be determined by site-specific pathogenic fat depots and ectopic fat, rather than total body fat alone [16].

"Fat-Bone" Interface: Deciphering a hormonal regulation on bone development

The differential effects of various fat depots on metabolic risk factors is well described and it is likely that differences in patterns of regional fat deposition also explain the complex interaction between adipose tissue and bone [37]. While it is clear that adipose tissue exerts independent effects on bone remodeling through the release of bone-regulating hormones (i.e., leptin, aromatase, adiponectin, 11-β-HSD1, IGF-1, TNF-α, IL-6), the primary role of adipose tissue on bone metabolism is likely dependent on the type of adipose tissue and site at which adipose tissue has accumulated in the body [37, 38, 107, 146]. Indeed, results from both animal and adult human studies illustrate that fat depots know for their pathogenic nature (e.g., visceral adiposity) secrete different pro-inflammatory cytokines acting through a bone-fat-pancreas axis [149, 150], and ultimately the effects of adipose tissue on bone may be dependent on the bone site and fat depot; the positive effect of adiposity on bone development through its mechanical loading effect may be counteracted by the production of pro-inflammatory cytokines secreted by pathogenic fat depots, a response that is associated with insulin resistance which is increased in individuals with type-2 diabetes [37, 149, 151].

Several studies have noted the opposing effects of visceral (VAT) and subcutaneous (SAT) fats on the appendicular skeleton [37, 38, 152, 153]. Though the

precise mechanism explaining this contrasting behavior between subcutaneous and visceral fat depots is unclear, the pathogenic nature of these fat depots and their ability to secrete a variety of different adipokines and hormones that regulate downstream biological function and protein expression explains one possible link between fat and bone [37, 38, 87, 154] [37, 38]. Whereas visceral fat is negatively associated with bone structure and strength, subcutaneous fat may provide benefit to bone by protecting against the pathogenic nature of visceral fat by virtue of the expressing anti-inflammatory proteins (i.e., adiponectin, Il-10) that are potentially involved in protecting against the development of osteoporosis [37]. In support, results from in vitro studies showed that cells derived from intra-abdominal adipose tissue suppress osteoblast activity much more than cells from subcutaneous adipose tissue [37, 152]. Evidence in animal models and human cell culture studies also confirmed that subcutaneous fat is largely responsible for secreting anti-inflammatory cytokines, as well as leptin and aromatase enzymes, which have been shown to stimulate the proliferation and differentiation of osteoblasts [107, 136, 155-158], supporting positive relationship between subcutaneous adipose tissue and bone size and density [16, 34, 37, 38, 107] through its ability to enhance bone formation. In contrast, visceral adiposity is associated with provoking lipotoxicity by secreting higher levels of TNF , TNF- , and IL-6, all which are pro-inflammatory markers that directly suppress osteoblast differentiation while enhancing osteoclastogenesis and bone resorption activity [37, 44, 145]. Thus, increases in visceral adiposity associated with weight gain is directly involved in enhancing bone osteoclast production, but are also involved in disrupting metabolic homeostasis which is independently involved in weakening the skeleton by promoting pro-inflammatory cytokine release to bone.

Abnormal levels of circulating adipocyte-derived hormones such as adiponectin and leptin may explain the differences impaired bone development and increased fracture risk in obese children, as they have also been shown to be key initiators to early onset of puberty. Obesity, for example, is associated with chronic hypersecretion of "free" (unbound) leptin, and significant decreases in the production of anti-inflammatory cytokine, adiponectin. Aside from its central role in regulating appetite and energy expenditure, Leptin, however, appears to mediate biphasic, pleuritropic effect on bone. Though the role of leptin in bone metabolism is not fully elucidated, animal and cell-culture data have shown that leptin's stimulatory effects include [136, 149, 159] by up-regulating osteoblast proliferation and differentiation [136] and inhibiting osteoclastogenesis [159]; in contrast, additional data showing that leptin is negative regulator of bone mass and bone size [160] is explained in party by leptin's ability to enhance pro-inflammatory cytokine secretion at various skeletal sites [136, 161, 162]. Moreover, the precise actions of leptin are likely dependent on the current leptin status of the individual, as animals studies have shown that excess secretion of leptin, characteristic of obesity, is a negative regulator of bone mass [161] and bone metabolism [153]. Importantly, increased secretions of leptin and/or decreased adiponectin production also contributes to the up-regulation of pro-inflammatory cytokines, TNF-α, IL-6, CRP [81, 136, 150, 163], and increased macrophage accumulation and transport to peripheral bone tissue eventually resulting in altered bone turnover rates, enhanced bone resorption, and increased bone loss in obese individuals.

In summary, these results give conclusive evidence of the potential adverse effects of increased adiposity on the developing bone. While much of the current

available information addresses the effects of fat mass on adult bone health and fracture risks, there is considerable evidence suggesting that fractures occurring in childhood are linked to alterations in body composition resulting in disproportionate fat distribution, particularly increased visceral adiposity, and further evidence suggests that these may serve as early signs of skeletal insufficiency [70]. Importantly, the allocation of adiposity, in particular, visceral adipose tissue, may outweigh the beneficial effects of total fat mass on bone [37, 38, 152, 164], insofar as the response of the skeleton to local and systemic pro-adiogenic hormones may explain the complex relationship between fat and the developing bone in obese populations.

Previous cross-sectional observations have illustrated inverse associations between fat mass and weight-bearing bone strength in both young girls [57] and adults [47]. Given that metabolic derangements associated with obesity are closely related with a central (abdominal), rather than a peripheral (gluteo-femoral) fat pattern [165], the extent of the relationship between total body and regional fat masses on bone strength, and bone geometry in growing children remains unclear. Thus the objective of aim 1a and 1b was to examine longitudinal associations between total body fat (TBFM), android fat (AFM), and skeletal muscle fat (SMF) content, with weight bearing bone parameters in young girls. It is hypothesized that higher levels of total body fat and android fat, and greater skeletal muscle fat content (calf and thigh) would be inversely associated with gains in bone strength in weight bearing bone sites in young girls.

Influence of Long chain Fatty acids on bone maintenance and development

Biochemistry of Absorption, Metabolism and bioactivity of omega-3 and omega-6 LCPUFAs

Optimal nutrient intake is an important determinant of growth and development. Long chain poly-unsaturated fatty acids (LCPUFAs), in particular, have received attention with regards to their important role in preventing chronic diseases (i.e., cancer, cardiovascular disease (CVD), type-2 diabetes) [166]. There is some evidence that suggests that the benefits of these fatty acids may also extend to bone [53, 167, 168]. Indeed, LCPUFAs are noted for their role as potential inflammatory mediators, as several studies propose that the effects of LCPUFAs on metabolic bone diseases and skeletal outcomes, such as osteoporosis, may be due, in part, to their immune-modulating and anti-inflammatory actions [167, 169, 170].

In humans, LCPUFAs have two primary physiological roles. First, LCPUFAs function as phospholipids in membrane to provide support in creating the optimum lipid bilayer structure, and to facilitate intercellular communication. Second, LCPUFAs provide the precursors of bioactive lipid metabolites that are capable of mediating both, autocrine and paracrine activities and signaling cascades throughout the body [167]. The two families of essential fatty acids, alpha-linolenic acid (ALA, 18:3 n-3) and γ-linoleic acids (LA, 18:2 n-6), have specifically been shown to mediate the effects of LCPUFAs on bone metabolism by serving as precursors for the conversion into the biologically active LCPUFAs, including, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), and arachidonic acid (AA, 20:4 n-6). Further metabolism of EPA, DHA and AA, result in the production of lipid metabolites that are classified as either eicosanoids (e.g., prostaglandins, leukotrienes, thromboxanes, E-series resolvins), derived

from either 20-carbon n-3 (ALA) or n-6 (LA) LCPUFAs, or docosanoids, derived primarily from 22-carbon n-3 LCPUFAs [168]; with respect to bone, specific eicosanoid products of AA include two-series prostaglandins (i.e., PGE_2), and four-series leukotrienes (e.g.,LTC_4, LTB_4) [171, 172]. Moreover, the balance of n-3 and n-6 bioactive compounds derived from ALA or LA parent compounds is regulated by the competition for the conversion enzyme, Δ-6 desaturase, which is necessary for initiating elongation and desaturation reactions for the endogenous formation of downstream n-7 and n-9 metabolites that also participate in bone modeling pathways [173]. In turn, because ALA and LA quantitatively compete for binding to the Δ-6 desaturase enzyme, increasing the consumption of ALA not only increases the bioavailability of n-3 EPA or DHA, but can also inhibit the formation of AA derived eicosanoids [172, 174]. Consequently, higher intake of LA n-6 FAs may result in the quantitative advantage of n-6 to block the conversion of ALA into EPA, and alternately, increase concentrations of AA and its potent lipid metabolites into circulation.

Long-chain polyunsaturated fatty acids: Selected mechanisms of action on bone

The various signaling pathways that are controlled by long-chain fatty acids and their respective bioactive metabolites converge to regulate prostaglandin synthesis, and downstream bone modeling processes. Pro-inflammatory and anti-inflammatory cytokines that are secreted from n-3 and n-6 FAs are known regulators of osteoblast and osteoclast differentiation and activity. Indeed, the balance of these cytokines is crucial for mitigating pathogenesis of skeletal insufficiencies and osteoporosis later in life. For example, higher levels of n-6 or n-3 FA intake can result in the formation of oxidation products catalyzed by the activities of cyclooxygenases (COX), lipoxygenases (LOX),

and cytochrome P450–like epoxygenases [168, 175]. COX enzymes, specifically, are responsible for converting AA and EPA into prostaglandins belonging to the 1-2- and 3-series [176]. Although two isoforms of COX genes have been identified (COX-1, COX-2) COX-2 is the inducible form of this enzyme, as it preferentially has a higher specificity for AA than EPA, and therefore synthesizes prostaglandins belonging to the 2- series (PGE2) rather than 3- series. Further, because AA metabolizes into n-6 FA lipid mediators, AA is indirectly capable of upregulating COX-2, which initiates a feed-forward effect to further stimulate pro-inflammatory prostaglandin synthesis, including PGE_2.

Given its biosynthesis and secretion is from bone cells, PGE_2 appears to be the primary and most potent mediator of bone cell function and bone metabolism, with capabilities exerting localized and systemic effects on various bone tissues. Upon its synthesis from AA, PGE_2 displays both biphasic, and dose-dependent properties, under low concentrations, PGE_2 appears as a potent activator of several osteogenic transcription factors, including core binding factor-1 (Cbfa-1) and IGF-1 [53, 168, 177] expression, which serve as key stimulators of osteoblastogenesis (**figure 4A**). For example, in response to mechanical loading, low levels of PGE_2 are released by mechano-sensing" osteocytes and matured osteoblasts residing within bone, in turn, initiating bone remodeling processes for the repair, removal and replacement of old bone with new bone [53, 178]. In contrast, higher concentrations of PGE_2 promote osteoclastogenesis by stimulating expression of both receptor activator of nuclear factor-κβ ligand (RANK-L) and RANK, or by inhibiting expression of OPG, the primary protein involved in controlling osteoclast number and preventing RANK-L/RANK-mediated

osteoclastogenesis [50, 53, 179, 180] (**figure 4B**). Results from dose response studies in animal models showed that prolonged exposure [181] and high doses [182] of PGE_2 resulted in higher bone resorption, decreased trabecular bone volume, while in contrast prolonged exposure to lower doses of PGE2 contributed to significantly higher bone formation rates [182]. In-vitro studies using murine spleen cells have also shown that in bone osteoclast cells increasing doses of PGE_2 stimulated osteoclastogenesis, via increasing both osteoclast number, and osteoclast longevity by preventing its apoptosis. Additionally, higher PGE_2 levels increased excavation of the resorptive pit in bone lacunae thereby enabling frequent resorption events to take place [183, 184]. Together, these results infer that while higher consumption of n-6 FA promotes greater AA concentration and increased PGE_2 synthesis increase bone resorption, modulation of PGE_2 synthesis by higher n-3 FA exposure may augment bone formation.

PGE_2 also influences the synthesis and the actions of insulin-growth factors (IGF). IGF-1, in particular, is the predominant growth factor derived by bone tissue and facilitates in both autocrine and paracrine signaling events to regulate bone metabolism. The primary function of IGF-1 is to stimulate new bone cell formation, such as pre-osteoblasts (osteocytes) increasing both, the number of bone forming osteoblasts, as well as the amount of cells responsible for building the bone matrix [168]. IGFs are initially secreted and deposited into the bone matrix; upon its release from the matrix during osteoclast mediated bone resorption, IGF-1 is then capable of anabolically regulating bone tissue within the bone matrix by enhancing Type-1a collagen expression while reducing collagen degradation [168, 185], suggesting that it IGF-1 may have may reflect an influence on bone modeling and bone mass acquisition in adults [186]. Coincidently,

IGF1 and bone forming osteoblast cells modulate the expression of one another. Cell culture studies have shown that osteoblasts have a high affinity for extracellular IGF-binding proteins (IGFBPs), which modify the ligand-receptor interaction by stabilizing IGF-I with its receptors [168, 187]. The secretion and activity of IGF-1 in bone is highly dependent on the concentrations of PGE_2 [185]; because PGE_2 is also capable of stimulating the expression of necessary IGFBPs, this suggests that the anabolic capacity of PGE_2 to upregulate osteoblast proliferation, increase endogenous production of IGF-1 from osteoblasts [168], promote the stability of IGF-1 to its receptor and finally modulate osteoblast activity and responsiveness to IGF-1 is crucial for subsequent bone remodeling phases [168, 188]. Additionally, the functional significance of IGFs, along with other bone growth factors is crucial for establishing bone homeostasis and includes coupling bone formation and bone resorption processes to maintain bone remodeling homeostasis, preserve bone and prevent loss during ageing as well as optimize peak bone mass and mineral acquisition during growth and development stages later in life [189].

PGE_2 synthesis also regulates the secretion and recruitment of pro-inflammatory cytokines to the bone surface. Cytokines that are specifically involved in modulating bone modeling and remodeling phases include interferon-y (IFN-y), interleukins (IL-1, IL-4, IL-6 and IL-11), and tumor necrosis factor-a (TNF-α) and macrophage colony stimulating factor (M-SCF); the predominant role of these cytokines is to promote osteoclast-mediated bone resorption, through mechanisms involving increased osteoclast differentiation, proliferation, and increased longevity of osteoclasts by inhibiting its apoptosis [48, 136, 190]. Furthermore, IL-1, a known pro-inflammatory marker of bone pathologies, such as rheumatoid arthritis, has also been noted for its role in inhibiting

chrondocyte proliferation, while inducing cartilage degradation and this response appears to be mediated by PGE_2 [171, 191].

Increasing evidence of a positive feedback loop between pro-inflammatory cytokine production and PGE_2 synthesis may explain the relationship between osteolytic cytokine production, such as TNF-α, IL-6 and IL-1β in the presence of higher PGE_2 concentrations. For example, high levels of PGE_2 can result in oxidation IL-6 and while both PGE_2 and IL-6, are involved in inducing each other's production [168] results from in-vitro co-culture studies show evidence of cross talk between COX-2/PGE_2 and IL-6 pathways to promote osteoclastogenesis via up-regulation of osteoclast mediated RANK expression and inhibition of RANKL/RANK binding and osteoprotegerin (OPG) activity in osteoblast cells [168, 192]. In-vitro analysis using human osteoblast cells showed that when co-cultured with a selective COX-2 inhibitor, both PGE2 and IL6 productions were suppressed. Additionally, in the presence of anti-PGE_2 antibodies IL-1β which stimulates the synthesis of IL-6 synthesis was also significantly reduced [171, 193]. More recent evidence in in-vitro osteoblast cells exposed to n-3 and n-6 FAs showed that AA-induced mRNA expression of pro-inflammatory IL-1a, IL-1 β, and TNF-α cytokines was inhibited by presence of EPA in cell culture[168, 192, 194]. Bhattacharaya (2007) interestingly showed that ageing mice fed fish oil rich in n-3 PUFAS for 6-months resulted in decreased IL-6 and TNF-α expression as well as significantly increased bone density [194]. Together, these results suggest a tight regulatory control between PGE_2 synthesis and pro-inflammatory cytokine production is dependent on the active COX pathway; inhibition of COX not only abrogates downstream pathway, but results in diminished PGE_2 synthesis as well.

Early human intervention studies also found significant reductions in pro-inflammatory IL-1, IL-2 and TNF-α in cartilage tissue were observed following dietary supplementation with fish oil containing both EPA and DHA [171, 195]. Similarly, Caughey et al found that 4-week use of flaxseed in domestic food preparation, suppressed TNF-α and IL-1β by 30% in healthy volunteers [196]. Results from clinical trials showed that decreases of 38.5% to 40.6% of macrophage IL-1 production were observed in rheumatoid arthritis (RA) patients given dietary fish oil supplementation [189, 197]; further investigation by Kremer and colleagues (1996) indicated that because some patients discontinued use of their non-steroidal anti-inflammatory drug (NSAID), but responded better to given sources of n-3 FAs, these results suggested that the therapeutic actions of n-3 FA supplementation on RA and related bone diseases may be related to its ability to biosynthesize eicosanoids such as 3-series prostaglandins (PGE_3) [189]. To illustrate, activation of PGE_3, which has a lower pro-inflammatory capacity compared to PGE_2; thus by increasing intake of n-3 FAs, thereby suppressing AA formation and reducing the concentrations of PGE_2, this contributes to a stimulatory effect from low dose PGE_2, prevents further release of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF-α) that are directly involved in simulating bone resorption, and reduces COX and LOX metabolites that contain additional pro-inflammatory potential. Because low dose PGE_2 can stimulate the release of IGF-1and indirectly promotes new cartilage and collagen synthesis for new bone to be laid down, PGE_2 may also be involved in the treatment of bone diseases with dietary n-3 PUFA intake. As such, these results provide evidence that increasing overall n-3 FA intake and thus modifying the balance of n-6 relative to n-3 FAs, benefits bone health by inhibiting the adversary effects of n-6 PUFA

induced pro-inflammatory cytokine production.

Much like prostaglandins, leukotrienes (LTs) generated from either AA mediated 5-lipoxoygenase (5-LO) enzymatic reactions or EPA mediated lipoxygenase can also act as localized regulators of bone metabolism. Though differences in their action on bone are apparent, by which lipoxygenase (LOX) generated lipid mediators from EPA (resolvins, lipoxins, protectins and docosanoids) have both anti-inflammatory and pro-resolving activities while 4-series leukotrienes (i.e., $LTC_{4,}$ LTB_4) derived from AA are synthesized primarily by inflammatory cells, such as polymorphonuclear leukocytes, macrophages, and mast cells [198] and therefore exert pro-inflammatory effects on bone. For example, evidence from early animal studies suggests that the primary function of $LTC_{4,}$ LTB_4 is to stimulate bone resorption while inhibiting osteoblast mediated bone formation [199, 200]. In-vitro results show that LTC_4, LTD_4, and its lipid mediators stimulated isolated avarian osteoclasts that resorbed calcified bone matrices [200], and similarly, enhanced bone resorption, osteoclast number in the calvarieae of mice [201]. The role of 4-series LTs on bone forming osteoblasts is not as clear, as it is with $PGE_{2,}$ but there is some evidence indicating that LTs can affect bone formation in a similar does-dependent manner. Whereas low levels of LTB_4 specifically have been shown to promote bone formation, higher levels of LTB_4 resulted in stimulated bone resorption [168, 172, 175]. Moreover, because LT can interact with prostaglandins to regulate osteoblast activity, and because dietary n-6 and n-3 FAs regulate lipoxygenase activity, it is possible that elevated LTB_4 production is associated with higher n-6 intake may be attenuated with increased consumption of n-3 FAs [171]. Furthermore, studies in mouse bone marrow cultures indicate the pro-resolving activities of LTs, particularly, n-3

derived resolving E1 (RvE1) and their ability to act in a localized manner to decrease osteoclast growth and differentiation while also preventing formation of the resorption pit, and thus bone resorption [202]. Poulesson and colleagues [53] showed that AA and AA-derived 5-LO lipid mediators were higher in bone marrow of ovariectomized female rats (OVX) compared to sham rats; however, after supplementation with DHA and EPA not only resulted in increased concentration of EPA and DHA in the bone marrow, but also enhanced production of LOX mediators generated from these respective n-3 FAs, thereby suggesting that the consequences on bone metabolism induced by high intakes of AA and production of its respective lipid mediators can be reversed by the anti-inflammatory, pro-resolving profile of LOX enzymes [53]. Given the important role of plasma antioxidants in the pathogenesis of osteoporosis [48], these studies, together, provide some evidence of how n-3 FAs may prevent and even reverse bone loss through the antioxidant capacities mediated by resolvins and lipoxins, to turn off activation of various n -6 FAs, such as AA, mediated pro-inflammatory cytokine production (e.g., IL-1, IL6, TNF-α) [48, 50, 168, 190].

Several other mechanisms that explain the relationship between n-3 and n-6 PUFAs and bone turnover have been proposed. To illustrate, n-3 FAs may promote osteoblastogenesis by preventing of the formation of products that *inhibit* osteoblastogenesis. For example, it is well established that reactive oxidative species (ROS) and oxidative stress play an active role in promoting bone loss by stimulating bone resorption and inhibiting bone formation. Generally, osteoclastogenesis is controlled, in part by two proteins osteoblasts associated proteins, RANKL, and osteoprotegerin (OPG), also known as the osteoclastogenesis inhibitory factor (OCIF), which is a soluble,

membrane bound protein receptor secreted by osteoblasts. Because OPG can act as a decoy receptor and bind to RANKL, preventing the interaction of RANKL/RANK explains one method for which OPG blocks osteoclast formation and differentiation as well as osteoclast mediated bone resorption [96]. Coincidently, data from animal [171, 199] and human studies have indicated the ability of n-3 PUFAs to decrease production of the n-6 FA derived eicosanoid, PGE_2 [167, 171, 203] but increase bone formation markers, alkaline phosphatase and osteocalcin [171] may explain one mechanism for which n-3 FAs promote bone formation pathways. Evidence from studies in baby chicks who were fed oil high in n-3 FA, verses soy-bean oil rich in n-6 FA, had higher serum bone alkaline phosphatase (ALP) activity, contributing to higher rates of bone formation [199] while decreases in urinary biomarkers of bone resorption have also been noted in adult studies [170, 204]. Given PGE_2's inhibitory effect on OPG production, to thereby reduce OPG/RANKL ratios [205, 206], additional evidence from randomized control trials showed that subjects consuming dairy products fortified with n-3 PUFAs had significantly large increases in OPG and osteocalcin synthesis compared to controls [207]. Similarly, in rheumatoid arthritis patients, increased serum levels of OPG, but decreased levels of serum RANKL, TNF-α, and RANKL/OPG were noted following fish oil supplementation, suggesting that improvements in bone quality may occur by n-3 PUFAs ability to decrease the inflammatory response by targeting TNF-α as well and inhibiting free RANKL to bind to RANK and initiate subsequent bone resorption pathways [208]. Bone protective effects of n-3 FA may also include modulating calcium balance to increase overall calcium absorption, and altercations of the peroxisome proliferator activator receptor-gamma (PPAR-y) pathway to control stem cell differentiation of

osteoblasts over adipocytes and in turn enhance osteogenesis. To illustrate, long-chain n-3 FAs have been shown to increase intestinal calcium absorption by increasing Ca-ATPase, the primary enzyme responsible for active calcium absorption in the intestine [209, 210]. In support, studies in rats indicated that dietary supplementation of n-3 FAs lead to increased calcium transport across the basolateral membrane and reduced calcium fecal excretion [48, 168].

n-3 PUFAs and their bioactive metabolites also regulate transcription of a various genes and downstream signaling pathways via the action of PPARs [53], specifically, the PPAR-1y isoform. Enhancement of osteoblast differentiation is mediated through modulation of the PPAR-y pathway, which controls both lipid peroxidation and adipogenesis PPAR-y2 as well as osteoblastogenesis (PPAR-y1) [53]. Specifically, it is believed that lipid mediators of the n-3 PUFAs target molecular mediators acting as PPAR-y1 agonists that preferentially promote mesenchymal stem cell differentiation into adipocytes over osteoblasts [53]. In turn, increased intake n-3 FAs indirectly reduces PPAR-y2 expression in adipose tissue and promotes osteoblastogenesis via PPAR- y1 activation [48, 53] (**figure 5**).

In summary, results from animal and human studies support the bone protecting roles of n-3. Improvements in calcium absorption and transport to bone, enhanced synthesis of bone formation biomarkers and increased bone mineral density at various skeletal sites have been reported, suggesting that intake of n-3 FA may also benefit children during developing by optimizing peak bone mass and achieving peak height velocity. However, given the differentiating effects of n-3 and n-6 FAs and lipid mediators on bone metabolism, additional research investigating the optimal intake of n-3

PUFAs or ratio of n-3 and n-6 PUFAs required to maximize bone protective effects in aging populations, and enhance bone formation in developing populations are warranted. Given the increasing consumption of n-6 FAs, and the limited intakes of n-3 FAs in typical western diets, the importance of a high fat intake in the increasing prevalence of childhood and adult obesity remains controversial, while qualitative changes (i.e. the fatty acid composition) have been largely disregarded. The purpose of Aim 3 was to evaluate the effects from consuming various long-chain polyunsaturated fatty acids (LCPUFAs) and ratios of n-3 and n-6 FA intake on indices of bone strength and bone development.

Omega-3 and Omega-6 FA role in bone development: Review of animal studies

Evidence from animal studies indicates that both *n*-6 and *n*-3 FA are necessary for growth and development [189]. In fact, early studies by Borland and Jackson have shown that animals deficient in certain types of EFAs developed severe osteoporosis and bone related injuries compared to non-deficient animals [168, 211].

Currently, the effects of LCPUFAs on the developing bone in animals are conflicting, concluding either a positive [212-214], negative [215] or null effect [199, 216] on measured bone outcomes. More importantly, evidence from animal studies show that n-6 and n-3 LCPUFAs appear to have contrasting actions in modulating bone metabolism positing that the primary role of n-6 PUFA appears to regulate bone resorption and bone formation in dose-dependent manor [55, 171, 189, 217], while the stimulating effects of n-3 FAs on bone formation and mineral accretion at cortical and trabecular bone sites occur via competitively regulating concentrations of AA in circulation [55, 189, 218]. To illustrate, Lau and colleagues (2009) found that higher consumption of higher levels of n-3 from EPA and DHA and thus, lower n-6/n-3 FA

ratios showed favorable effects on bone strength, peak load, and mineral accumulation in the bone matrix of the femur in healthy young male and female mice [212]. Positive association between ω-3 PUFAs intake and increased bone calcium content and bone formation rates have also noted, suggesting a possible role of ω -3 PUFA in preventing the formation of lipid peroxidation, which normally inhibits osteoblastogenesis [48, 51]. Indeed, a large sum of the literature has noted the positive effects of n-3 LCPUFAs on bone modeling in growing animals, as these findings are conclusively supported by observations of reduced bone mineral loss [51, 171, 189, 212, 214]. In support to the above findings, evidence in Japanese quail showed that high dose and long-term exposure to n-6 FA impaired mature bone mechanical properties while equivocal doses and exposure time to n-3 FAs promoted higher bone mineral content and collagen cross-link concentrations in the underlying bone matrix [219]. Green et al (2004) also noted that long term intakes and higher n-3 relative to n-6 dosage resulted in higher femur bone mineral density, reduced plasma osteocalcin (bone turnover marker), and reduced PGE_2 release from femur in rats [220]. Reinwald et al (2004) interestingly reported that repletion of n-3 FAs via long-term supplementation (14 months) of EPA**,** reversed bone structural deficits in previously deficient growing rats [214], while modest improvements in the structural and mechanical properties of cortical bone in female mice [221]**.** Similarly, Watkins et al (2001) showed that in growing rats, higher dietary concentrations intakes of n-3 EPA and DHA resulted in higher concentration of the n-3 FA in bone compartments and this was associated with increased serum levels of the key bone formation marker, alkaline phosphatase (ALP) [213]. Contrasting arguments regarding the bone-promoting effects from LCPUFA consumption have also been reported. Cohen

et al, reported no effect bone mineral density (BMD) or bone strength in the femur and vertebrae from consuming flaxseed oil diet, rich in ALA, in young female mice [212, 216]. Similarly feeding piglets with *n*-3 FA rich diet containing high amounts of flaxseed oil had no effect on body growth and bone mass was observed in piglets fed a [216, 222]. In male rats that were fed a high fish oil diet (60 g/kg menhaden oil plus 10 g/kg soybean oil) from weaning through puberty, neither positive nor negative associations on bone mass, BMC, BMD or biomechanical bone strength of femurs and vertebrae was found [215]. Interestingly, feeding the same fish oil diet to young female rats resulted in decreased bone growth and vertebral peak load [215]. Together, these findings suggest that effects of n-3 FAs on bone may be more apparent at the cellular level, by modulating inflammatory and prostanoid pathways. The n-3 FAs induced bone-protective and bone-strengthening effects on the skeleton appear to be mediated in part by decreasing the synthesis of PGE_2 synthesis from the metabolism of n-6 FA, which may also result in down-regulation of cyclooxygenase-2 (COX-2) activity in local tissues [212]. Thus, the evidence from animal feeding studies further suggest modulating the dietary ratio of n-6/n-3 FA, and the synthesis of PGE_2 will result in optimized bone modeling, and the attainment of maximized bone strength, bone density and mineralization in the growing skeleton [212].

There is some data that suggests that maternal intakes of various n-6 and n-3 FA ratios that influence neonatal bone metabolism may potentially persist into adulthood [55, 172]. For example, LCPUFA-deficient male rats that were later weaned onto ordinary chow consisting of a various mixtures of LCPUFAs and followed into adult life showed significantly higher body weight and improved cortical bone mineral content (BMC),

area, and thickness compared with non-deficient controls [223]. Deficiencies in both n-6 and n-3 FA resulted in decreased BMD and altered mineralization in the adult offspring [18], suggesting that early and pre-natal PUFA intake may influence the expression of genes that regulate bone modeling/remodeling events and therefore may influence bone development as well as modify the risk of osteoporosis in their offspring later in life. Indeed, longitudinal evidence in female rats who were given different types of EFAs but otherwise placed on given iso-energetic diets during pregnancy and lactation, showed that bone development in adult offspring *differed* in relation to the amounts of EFA [223] or the varying ratios of n-6 and n-3 PUFAS [217, 223] given to mothers during peri-natal period. In a similar study by Korotokova et al (2004), rats were fed a pre-and peri-gestational diet of n-3 FA, n-6 FA, or mixture of n-6 and n-3 (n6+n3) which consisted of higher quantities of n-3 FA. Results showed that increases in femoral length and cortical bone content in addition to enhanced cortical cross-sectional bone area was observed in the adult offspring of mothers fed the n-6+n-3 diet, compared to offspring from the n-6 FA and the n-3 FA groups; interestingly, peri-natal dietary intake of n-3 FAs, had negative effects on cortical and femoral bone morphology, showing decreases in femoral length and cortical cross-sectional bone content, area and thickness in rats compared with those in the n6+n3 group [55]. Increased cortical bone radial growth, and increased bone strength, was also reported in n-6+n-3 offspring. These results suggest that a balanced maternal diet of both n-6 and n-3 fatty acids promote optimal bone growth and development in offspring, which may ultimately reduce fracture risk and skeletal fragilities later in life. Moreover, The beneficial effects of a mixed n-6 and n-3 FA may be explained, in part, by the higher n-3 consumption, and its direct effect on reducing

bone tissue via reducing eicosanoid synthesis (mainly PGE_2 and LTB_4) or indirectly, by regulating the synthesis of hormones, such as insulin growth factor-1 (IGF-1), which can directly stimulate new bone cell formation by enhancing osteoblast differentiation, increasing type-1a collagen expression and preventing collagen degradation in the underling bone matrix [55]. In summary, the results from these studies demonstrate that both the total quantity of LCPUFA intake and the composition of dietary LCPUFA are important for achieving the maximal bone mass mineralization. Furthermore, while both n-3 and n-6 are critical for proper bone development, higher intakes of n-3 and lower doses of n-6 FAs is optimal to elicit stimulatory effects on the bone matrix. Further evidence regarding the ideal quantities of n-6 and n-3 FAs for maximizing bone formation is warranted.

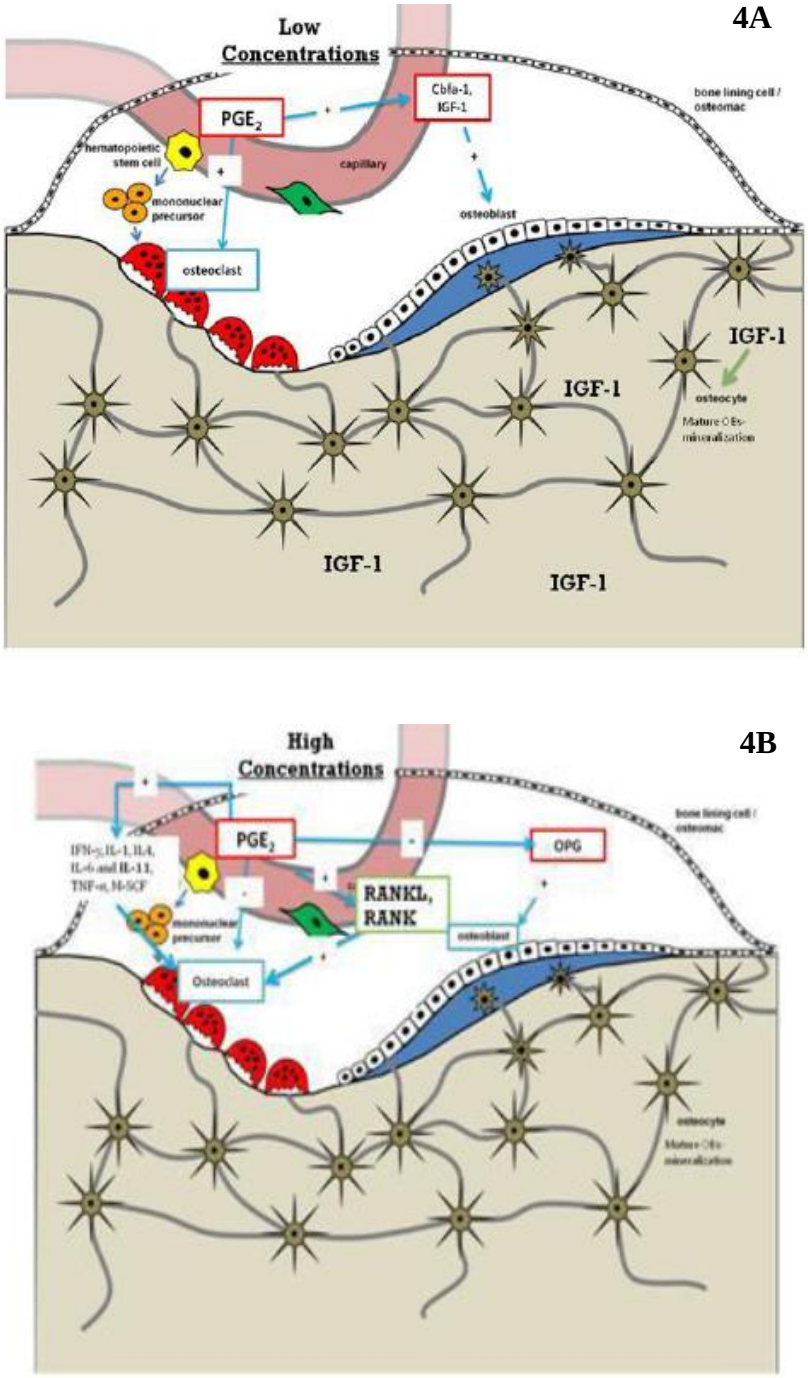

Figure 4. Potential mechanisms for the biphasic, dose-dependent effects of n-6 LCPUFA on bone formation and bone resorption pathways. Regulatory functions of n-6 on bone are carried out by the synthesis of n-6 metabolites, prostaglandin 2-series (PGE_2) and 4 series leukotrienes (LTB_4, LTC_4). **Panel A)** at lower concentrations, PGE_2 acts as potent activator of osteogenic transcription factors (Cba1, IGF-1) to increase osteoblast expression and increasing bone formation rates; **Panel B**) at higher concentrations, PGE_2 can enhance the synthesis of bone turnover proteins (i.e., RANKL, RANK) and the secretion of pro-inflammatory markers (IL6, TNF-α, MCSF,IL-1B, INF-y) to the bone surface, ultimately creating an inflammatory environment that contributes to the degradation of the bone matrix.

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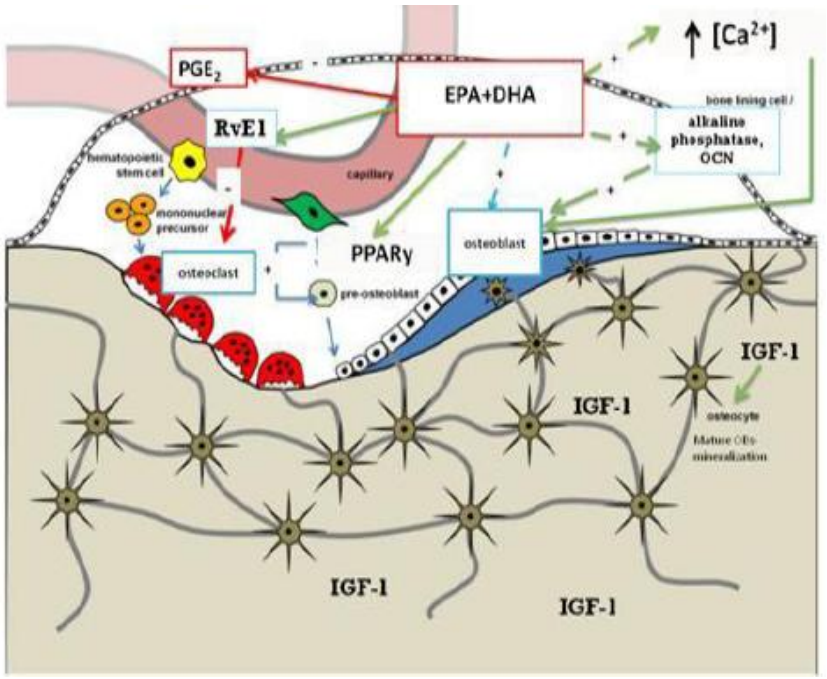


Figure 5. Potential mechanisms for the stimulatory effects of n-3 LCPUFAs on bone pathways. Mechanisms by which n-3 FA stimulate bone formation at both, cortical and trabecular bone occur by competitively regulating concentrations of n-6 AA in circulation and downstream PGE_2 synthesis. Increased expression of bone formation proteins, osteocalcin, bone-specific alkaline phosphatase, and matrix synthesis proteins (i.e., collagen 1a fibers) contribute to increased bone mineral accretion and gains in bone density. Positive association between ω-3 PUFAs intake and increased bone calcium content and higher bone formation rates have also noted, suggesting a possible role of ω -3 PUFA in increasing cell membrane unsaturation and preventing the formation of lipid peroxidation, which normally inhibits osteoblastogenesis. In-vitro studies show that n-3 LCPUFAs can regulate gene transcription and signaling pathways controlled by PPAR- γ (PPAR- γ 1, PPAR- γ 2), which are known to mediate osteoblastogenesis or adipogenesis activity. Although n-6 lipid metabolites can potentially act as PPAR- γ 2 agonist that preferentially direct cell differentiation into adipocytes, higher doses of n-3 LCPUFAs may competitively inhibit n-6 induced activation of PPARy2 in adipose tissue, and instead induce PPAR- γ 1-mediated transcription of bone forming-osteoblasts.

Omega-3 and Omega-6 FA role in bone maintenance in humans

Given the current dietary shift from higher saturated fat (SFA) consumption to higher intakes of n-6 PUFAs and increased ratio of *n*-6/*n*-3 FA in western diet, the biological importance of LCPUFA intake on bone development and bone maintenance in humans remain unclear. Moreover, evidence in humans is conflicting and is limited to observational studies conducted in postmenopausal women [48, 118, 224-227] and/or in men [204, 228, 229]. Results from these studies suggest that consumption of a variety of LCPUFAS was positively associated with bone mineral accrual, and improved bone microarchitecture. For example, in a recent study

examining the dietary influence of n-6 (AA) and n-3 (EPA; DHA) on bone maintenance in post-menopausal women [230, 231], it was observed that individuals consuming a larger n-6 verses n-3 PUFA intake had the lowest BMD [230], suggesting that dietary consumption of n-6 FAs may be detrimental to the skeleton. In contrast, results from the Rancho Bernardo study found that after controlling for standard osteoporotic covariates (i.e., age, body mass index, calcium intake (diet plus supplements), current exercise (≥3 times per week), smoking status (never, past, or current), alcohol intake, use of thiazides and thyroid hormones), a higher dietary ratio of n-6/n-3 FA was associated with lower BMD in the hip in all women; additionally, higher n-6/n-3 intakes were negatively associated with lumbar spine BMD in the spine of elderly women who were not using hormone therapy (HT), whereas reductions in the n-6/n-3 FA ratio were associated with increased bone strength in elderly men and women [228]. Given the inverse relationships between higher n6/n3 FA intake and bone density occurred at more bone sites in women who were not using HT, compared to HT users, it is possible that fatty acids may potentiate the effects of hormone therapy by increasing calcium absorption- thereby explaining the bone-protecting benefit in women receiving HT [228]. In summary, these results suggest that relative quantities of dietary LCPUFAS may play a pivotal role in preserving skeletal integrity and may aid in primary prevention or therapeutic regimens for treating or managing osteoporosis and related bone diseases.

Although limited, there is some evidence in adults that argues against the benefits of LCPUFAs on bone mass and bone formation, suggesting that PUFA intake neither hurts nor harms the skeletal frame [226]. For example, Bassey colleagues [226] showed that intake of essential fatty acids (EFAs) and their long-chain derivatives did not affect bone biomarkers in pre- and post-menopausal women, while a similar study showed that consumption of EFAs via

supplementation of 40 g flaxseed/d (9.1 g ALA) for 12 months did not effect on changes in bone mineral density in menopausal women following [227]. Since BMD changes slowly over time, it is likely that a longer follow-up would be necessary for assessing the slight effect of flaxseed on bone metabolism. Data from 137,486 postmenopausal women in the Women's Health Initiative reported that consumption of small amounts of EPA+DHA, but higher *n*-6 FAs was associated with a modest decrease in total fracture risk, and a higher EPA+DHA intake was associated with a small increase in risk of fractures [232]. Orchard and colleagues [232] reported that this unexpected positive correlation may be due to the low consumption of EPA+DHA n-3 FAs (mean intake=0.13 g/d), which were significantly lower than the minimal recommendations of 0.3-0.5g/d established by national [233, 234] and global health organizations [235], coupled with the lack of data on fish-oil or n-3 FA supplementation use by women in this study. Furthermore, limited variations in the range of EPA + DHA consumed in this cohort may also explain the associations between higher n-3 intakes and fracture risk [232]. Similar findings reported in the Framingham Osteoporosis Study showed that higher ALA consumption was associated with lower hip fracture risk in women, but not men, whereas protective associations between higher AA consumption and reduced hip fracture risk were observed only in men [236], Likewise Macdonald et al (2004) also concluded that higher PUFA intake was associated with bone loss at the lumbar spine and femoral neck in peri-menopausal women [237]. The higher saturated fat/higher sucrose intakes and the higher f n−6 relative to n−3 FAs consumed by these women may offer an explanation to these conflicting results. Moreover, given the efficiency in converting ALA to EPA and DHA is low; this conversion appears to be further compromised when higher intakes of n-6 FAs or saturated fatty acids are consumed in the diet, due to quantitative and competitive advantage of n-3 FAs to bind to essential desaturase enzymes that

are necessary for ALA elongation and desaturation into its respective bioactive metabolites [52, 53].

In summary, results from epidemiological studies regarding PUFAs and risk of total fracture or hip fracture remain equivocal. Though, the findings from animal and human studies suggest that n-3 and the n-6 FAs may have contrasting bioactive functions on bone and it is also possible that the shift in the balance between n-6 and n-3 may affect the synthesis and secretion of pro-inflammatory cytokines, which might further augment bone resorption and inhibit bone formation rates [168]. Furthermore, the impact of n-3 FA consumption or supplementation to promote positive changes in BMD, may be more detectable in more vulnerable populations such as the elderly or diseased or in youth.

Omega-3 and Omega-6 FA role in bone development in children

During childhood and adolescence, the bone formation occurs by bone modeling and remodeling phases. Bone growth during childhood is regulated by interacting factors including hereditary, which determine an individual's genetic potential, environmental stimuli and nutrition. Different nutrients, such as fatty acid consumption in particular have been shown to affect bone mass and bone development in children [218, 238-240]. For example, in a study conducted in growing females residing in Norway, a country whose population who frequently consumes fish and therefore has a lower dietary n−6/n−3 FA ratio compared to the U.S. population, dietary total PUFAs are positively associated with the change in bone mineral density of the ultradistal radius [238]. Cross-sectional analysis in healthy eight year old children consuming varying ratios of n-6 and n-3 FAs showed positive associations between %AA and whole body aBMD but negative correlations between percent total n-6 FAs and lumbar spine aBMD after controlling for sex and anthropometric variables [218]; interestingly, positive

association between DHA concentrations and lumbar spine BMD were found only in children of the uppermost tertile of weight and BMI, suggesting that body weight may be an interacting factor when evaluating the fatty acid-bone relationship [218]. Longitudinal analysis by Hogstrom and colleagues [49] found that n-3 FAs, especially DHA was positively associated with total and spinal BMD and bone mineral accrual in adolescent males. Prospective associations in four-year old children demonstrated that percent EPA and DPA (relative to total fatty acid concentration) were positively associated with bone mineralization and dual-x-ray absorptiometry (DXA)-derived whole body and regional bone density measures. Additional analysis in this study revealed that AA was inversely related to whole body BMC and aBMD, and relationships held constant after potential confounding factors, such as diet, were included in the models [172]. In toto, these results support findings in animals suggesting that both n-6 and n-3 FAs are crucial for maximizing bone mineral accrual and achieving of peak skeletal development; thus, the balance of n-6 to n-3 FAs may also serve as a stronger predictor of childhood bone metabolism and skeletal fractures later in life. Moreover, because many adult-onset bone and joint related diseases (i.e., osteoporosis) are due to skeletal fragility, weak bone strength and increased bone loss, increasing consumption of essential nutrients, such as LCPUFAs that aid in optimizing bone accrual during childhood and adolescence, may prevent the development of osteoporosis and one related pathologies later in life [189].

CHAPTER 3

METHODS

Study design

The parent study of this dissertation, the "Jump-In: Building Better Bones: study, was a school-based, group randomized, controlled trial with the long term goal being to assess the two year effects of high-impact jumping exercises on vBMD, bone mass, bone geometry and strength in pre- and early pubertal girls. Participants were recruited from fourteen elementary and four middle schools from the Catalina Foothills and Marana school districts in Tucson, Arizona. Schools were matched on school demographics (enrollment; mobility rates; socioeconomic status, reflected in percent enrollment qualifying for free and reduced lunch; race/ethnicity) and block randomized to the exercise intervention (9 schools) or control (9 schools) groups (**Table 1**). The study protocol was approved by the University of Arizona Human Subjects Protection Committee and was conducted in accordance with the Helsinki Declaration. All parents/guardians and participating girls provided written informed consent.

Table 1: Descriptive Characteristics of Girls at baseline, 12-month, and 24-month assessments

	Baseline	12-Month	24-Month
Age (years)	10.7±1.1	11.6±1.1	12.7±1.1
Maturity Offset (Years)	-1.1±1.0	-0.2±1.1	0.7±1.0
Height (cm)	144.5±9.7	151.1±9.9	157.0±9.0
Weight (kg)	39.2±10.4	44.2±11.3	50.1±11.9
BMI (kg/m^2)	18.5±3.3	19.1±3.4	20.2±3.6
Femur Length (cm)	34.0±3.0	35.2±2.8	36.8±2.5
Tibia Length (cm)	33.2±2.8	34.9±2.7	36.4±2.5
Total body fat mass (kg)	11.3±6.3	13.0±6.8	15.4±7.6
Total body Lean Mass (kg)	25.7±5.0	29.0±5.6	32.1±5.5
Total body percent fat (%)	27.8±8.5	28.4±8.3	29.8±8.2

Values are presented as mean ± SD. P = participated in lab measurements; C = completed all lab measurements
Baseline: P=509; C= 503; 12-Month: P=349; C=346; 24-Month: P=285; C=231

Study participants

Baseline data from 509 healthy girls, aged 8–13 years, who were participants in the "Jump-In: Building Better Bones" study was considered for this dissertation. Any female in school grades 4 or 6 in the participating schools were eligible to volunteer. Exclusion criteria included learning disabilities (identified by schools) that made it impossible to complete questionnaires or otherwise unable to comply with assessment protocols; medications, medical conditions, or a disability that limited participation in physical exercise [241]; and the inability to read and understand English. Girls were asked to participate in laboratory measurements at baseline, 6 months, 12 months, and 24 months. Descriptive characteristics of girls who completed baseline (n = 503), 12 month (n= 346), and 24 month (n = 231) measurements are shown in Table 1. A total of 3 cohorts were recruited and enrolled in the study between the Fall of 2007 and the Fall of 2009. A CONSORT diagram showing the progress of participants through the two year study is presented in **figure 6**.

Following informed consent, guardians completed a health history questionnaire, which included questions on participant ethnicity and race. Sample ethnicity was 23% Hispanic and 77% non-Hispanic. Sample race was 88% white, 6% Asian, 3% black or African American, 1.2% Latino, 0.6% Native American or Alaska Native, 0.6% Native Hawaiian or other Pacific Islander, and 0.4% other. According to U.S. National Center for Health Statistics/Centers for Disease Control and Prevention percentiles for body mass index (BMI, kg/m^2) [148], 3% of the baseline sample was underweight (BMI <5th percentile), 74% of the baseline sample was healthy weight (BMI 5th–85th percentile), 15% of the baseline sample was overweight (BMI 85th–95th percentile), and 8% of the baseline sample was obese (BMI >95th percentile). Baseline maturity offset values indicated that girls were on average 1.1 years prior to peak height velocity (PHV),

with a range from 3.2 years prior to PHV to 1.4 years post PHV. Baseline Tanner stage distributions were 32% pre-pubertal (stage I, n = 164), 61% early pubertal (stages II–III, n = 308), 5% peri-pubertal (stage IV, n=25) and 2% post pubertal (stage V, n=8). Because no significant differences in bone parameters were observed between control and intervention, the data from all participants were collapsed into a single group and examined for influences of soft tissue composition, physical activity, maturation and diet on 2-year changes in bone parameters in this large sample of early and peri-pubertal girls.

Anthropometry

Measures of body habitus (body mass, standing height, sitting height and bone lengths) were obtained following standardized protocols [242]. Body mass was measured to the nearest 0.1 kg using a calibrated scale (Seca, Model 881, Hamburg, Germany). Both standing height and sitting height were measured at full inhalation to the nearest millimeter (mm) using a calibrated stadiometer (Shorr Height Measuring Board, Olney, MD). Femur and tibia lengths were measured on the non-dominant leg. Femur length (nearest mm) was measured from the apex of the lateral epicondyle (base of the patella) to the inguinal crease. Non-dominant tibia length (nearest mm) was measured from the proximal end of the medial border of the tibial plateau to the distal edge of the medial malleolus. Coefficients of variation (CVs) for femur and tibia lengths in our laboratory (n = 509) were 0.44% and 0.23%, respectively. For each anthropometric variable, two measurements were taken and averaged. Both measurements were repeated if the first two trials differed by more than 4 mm for height, sitting height and bone lengths, and 0.3 kg for body mass, and the average of the second set of measures was used [57, 122].

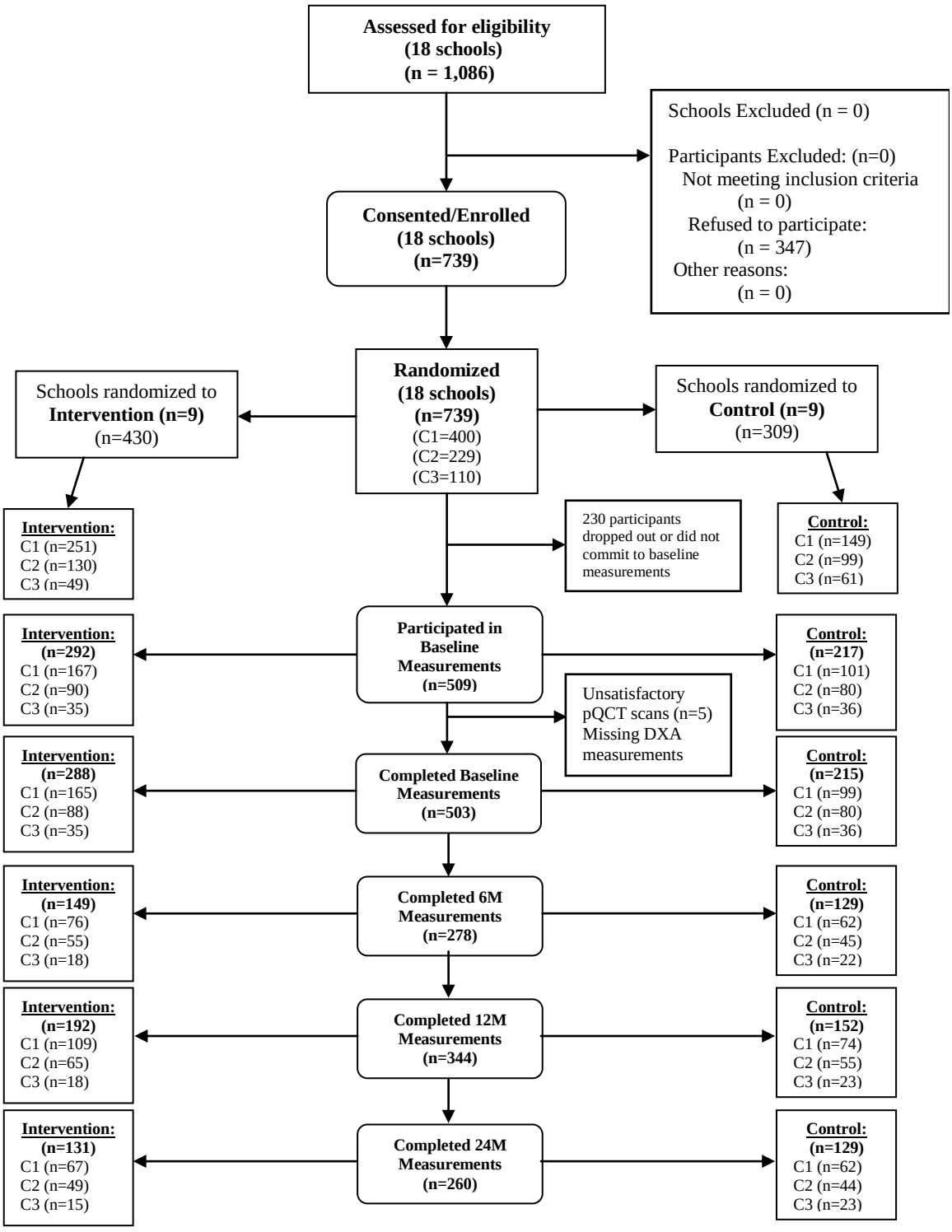


Figure 6. CONSORT flowchart describing the progress of participants through the "Jump-In: Building Better Bones" study. C1= cohort 1; C2= cohort 2; C3 = cohort 3.

Physical Maturation

Maturity was assessed using a self-report (with assistance available) questionnaire of breast development based on Tanner stages (Tanner), in which girls were asked to rate their Tanner stage based on breast development and also report their menarcheal status [243]. This validated questionnaire provides illustrations of stages of development and has been shown to agree with physician exam and grading. Despite the common use of Tanner staging in developmental studies, it is limited in its ability to accurately assess maturation [244]. Therefore, maturity offset was used an alternative index of maturation status, following methods of Mirwald et al [245]. Maturity Offset is calculated from gender specific algorithms that are used to estimate years from peak height velocity (PHV) derived from data from a 6-year longitudinal study in boys and girls [18]. These algorithms include interactions among anthropometric measures (i.e., height, weight, sitting height, leg length) and chronologic age to derive a maturity-offset value. The following equation was used to derive maturity offset in our sample of young females: Maturity offset (y) = -9.376 + 0.0001882*Leg Length (cm) and Sitting Height (cm) interaction + 0.0022*Age (y) and Leg Length (cm) interaction + 0.005841*Age (y) and Sitting Height (cm) interaction – 0.002658*Age (y) and Weight (kg) interaction + 0.07693*Weight (kg) by Height (cm) ratio [18, 245]. Positive maturity offset values represent years after PHV, while a negative maturity offset value represents years before PHV. In Mirwald's sample, the maturity offset equation for girls explained 89% of the variance in years from PHV [57, 122, 245].

Baseline Tanner stage and maturity offset values for our sample are shown in **Table 2**. Two fifth grade girls had been recruited into the study at the time analyses were completed for aims 1-3. They reported tanner stages similar to sixth grade girls and that were significantly different than fourth grade girls. Therefore, they were included in the sixth grade sample for aims 1-3. In the following analyses (Aims 1-3), maturation status was assessed using maturity offset

over the more conventional method of Tanner staging due to its reliance on objective anthropometric measurements of linear growth.

Table 2: Somatic and sexual maturation at baseline

	Total Sample (n=505)	4th Grade (n=227)	6th Grade (n=228)
Maturity Offset (y)	-1.1±1.1	-1.9±0.5	-0.1±0.6
PHV (% post)	19.2	0	42.5
Tanner Stage (1-5)	2.1±1.0	1.6±0.7	2.7±0.9
Pre-pubertal			
Tanner stage 1 (No.Girls/ %)	164 / 32.5	149 / 65.6	15 / 6.6
Early Pubertal			
Tanner Stage 2 (No. Girls/ %)	171 / 33.9	100 / 44.1	71 / 31.1
Tanner Stage 3 (No. Girls/ %)	137 / 27.1	27 / 11.9	110 / 48.2
Peri-pubertal			
Tanner Stage 4 (No. Girls/ %)	25 / 5.0	1 / 0.4	24 / 10.5
Post pubertal			
Tanner Stage 5 (No. Girls/ %)	8 / 1.6	0	8 / 3.5
Menarche (% post)	10.1	0.4	22

Values are mean ± SD unless otherwise noted. Somatic characteristics: Peak height velocity; sexual characteristics: Tanner staging; PHV= peak height velocity;
Values are for participants with plausible dietary data only

Physical activity assessment-*Past year physical activity questionnaire (PYPAQ)*

The Past Year Physical Activity Questionnaire (PYPAQ) has been validated in adolescents [246]. A modified version of the PYPAQ has been developed in our laboratory [247] to include a more comprehensive list of 41 activities common to youth. In brief, PYPAQ surveys all sport and leisure-time physical activity in which the respondent engaged at least 10 times in the past year outside of physical education class. The modified questionnaire was administered in an interview with the participant and guardian, during which participants were asked to record the average duration, weekly frequency, and the number of months of participation for each activity. Total PYPAQ score was computed using a modified equation from Shedd and colleagues [248] which incorporated weight bearing load, frequency and duration of each activity into the equation:

PYPAQ score = $\sum_{1-n}$ (duration (minutes/session) × frequency (days/week) × intensity (METs (27)) × load (peak strain score)), where n was the number of activities a subject reported during the past year. Individual scores for PA duration, frequency, and load were calculated using the following equations: Duration = $\sum_{1-n}$ (average minutes/session); Frequency = $\sum_{1-n}$ ([months/12] × days/week); Load = $\sum_{1-n}$ (peak strain score), where n was the number of activities a subject reported during the past year. PA intensity, commonly reported as the metabolic equivalent (MET, where 1 MET = 3.5 mL × kg^{-1} × min^{-1}) value of an activity, was not included in the PYPAQ equation to protect against collinearity because intensity and load were highly correlated (r = 0.94), and because peak strain is more relevant to bone than METs. Peak strain scores (PSS) reported by Groothausen and colleagues (1997) were used to increase the **c**ontribution of bone-relevant loading activities to the overall score [249]. Jumping activities (e.g. basketball, volleyball, gymnastics) were assigned a PSS of 3; activities that involve changing directions quickly and sprinting (e.g. tennis soccer,) were assigned a PSS of 2; all other weight-bearing activities were assigned a PSS of 1 (e.g. walking, hiking, golf). Low-impact activities that fell between categories were given a PSS of 1.5 (e.g. aerobics, dance). Non-weight-bearing physical activities (e.g. cycling, swimming) were assigned a PSS of 0.5. Sports and leisure-time activities that did not have a PSS previously reported in the literature were assigned the same value as the most similar activity.

Dietary Assessment

Total energy (kcals/day) and nutrient intakes [Aim 1, 3- total fat intake g/day; Aim 2- major fatty acid groups (g/day) including PUFA, n-3 and n-6 PUFAs, and n-6/n-3 ratio and individual fatty acids (g/day) (alpha-linolenic acid, ALA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA; Linoleic acid, LA; arachadonic acid, AA; Aim 2, 3- total calcium,

mg/day], were assessed using the semi-quantitative Harvard Youth/Adolescent Questionnaire (YAQ) [250] (**Table 3**). The YAQ is a self-administered (with assistance available) food-frequency questionnaire (FFQ) consisting of 152 questions with estimated serving sizes and frequencies of intake for 131 foods and dietary supplement use during the previous year. Acceptable validity and reproducibility of the YAQ have been established [250, 251] in children and adolescents and it has been used in studies reporting significant associations between nutrition and various outcomes such as childhood obesity, physical activity, and bone density[252].

At baseline, participants completed the YAQ at school or at home with assistance available from a parent/guardian or a trained technician, and with guidance of written instructions. All YAQs were reviewed by a trained study staff for completeness and coding for nutrient analysis was completed by Channing Laboratories (Boston, MA) following the standard coding procedures [251]. Baseline data for total energy (kcal/day) and total calcium (mg/day) and fatty acids (g/day) were used in the analysis for Aim 3. For Aim 1, average total energy (kcals/day) and average total fat intake (g/day) and were calculated from baseline, 6-month, 12-month and 24-month data. Analyses for Aim 3 used average intakes of total energy (kcal/day) and total calcium (mg/day), computed using baseline and 2-year values for each diet variable. Participants who had missing baseline data were excluded from all analyses.

Table 3: Dietary intake of Jump-In participants at baseline

Nutrient	Total Sample (n=464)	4th Grade (n=256)	6th Grade (n=208)	RDA
	Mean±SD	Mean±SD	Mean±SD	
Total Energy, kcal/d	1762±687 (364-5432)	1838±702 (364-5432)	1669±657 (435-4668)	1600-2000
total Fat, g/d	62±26 (10-215)	65±27 (10-194)	58±25 (15-215)	25-35%
Monounsaturated fat, g/d	22±10 (3-83)	23±10 (3-75)	21±9.3 (5-83)	NA
Saturated fat, g/d	22±10 (4-79)	23±10 (4-71)	21±10 (5-79)	≤10%
Polyunsaturated fat, g/d	12±5 (1-36)	12±5 (1-30)	11±5 (3-36)	0.5g/d
Baseline Arachidonic fatty acid - grams	0.1±0.1 (0.0-0.3)	0.1±0.1 (0.0-0.3)	0.1±0.05 (0.0-0.3)	NA
Docosahexaenoic fatty acid (DHA), g/d	0.1±0.1 (0.0-0.8)	0.1±0.1 (0.0-0.8)	0.04±0.04 (0.0-0.4)	NA
Eicosapentaenoic fatty acid (EPA), g/d	0.02±0.04 (0.0-0.6)	0.02±0.05 (0.0-0.6)	0.02±0.02 (0.0-0.1)	NA
Linolenic fatty acid, g/d	1.1±0.5 (0.1-3.1)	1.1±0.5 (0.1-2.6)	1.0±0.5 (0.3-3.1)	1.0-1.2 g/d
Linoleic, g/d	10±5 (1-31)	11±5 (1-27)	10±4 (2-31)	10g
Omega 3 (EPA+DHA), g/d	0.1±0.1 (0.0-1.2)	0.1±0.1 (0.0-1.2)	0.1±0.1 (0.0-0.4)	0.2-0.25 g/d
Calcium, mg/d*	1021±453 (120-2385)	1056±482 (120-2385)	978±411 (279-2024)	1300
Vitamin D, IU/d*	284±184 (21-1051)	297±192 (21-1051)	268±171 (23-953)	600
Total Long Chain n-3 Fatty Acid (EPA+DPA+DHA), g/d	0.1±0.1 (0.0-1.3)	0.1±0.1 (0.0-1.3)	0.1±0.1 (0.0-0.5)	NA

Values are for participants with plausible dietary data only; values in ( ) represent the range.
Dietary Recommended Intakes (DRI) established by the IOM and DGA
*values include intake from diet and supplement sources.

Bone and Body Composition Assessment

Bone Variables

Measures of bone strength (Aims 1-3), bone density (Aims 1-2) and bone geometry (Aim 1) were assessed using peripheral quantitative computed tomography (pQCT; XCT 3000, Stratec Medizintechnik GmbH, Pforzheim, Germany, Division of Orthometrix; White Plains, NY, USA) at the 4% and 20% femur and 4% and 66% tibia sites relative to the respective distal growth plates on the non-dominant limb. pQCT was also used to assess regional bone mineral content (BMC) measured at the same sites [253] (Aim 3). Scout scans were performed to locate the distal growth plates, with the scanner programmed to find the sites of interest based on skeletal lengths. Slice thickness was set to 2.3 mm and voxel size was set to 0.4 mm. Scanner speed was set at 25 mm/s. Additional details regarding pQCT bone measurements, image processing, calculations, and analysis are published elsewhere [122, 254] .

At the distal metaphyseal regions of the femur and tibia, contour mode 3 (169 mg/cm^3) was used to estimate total bone, and peel mode 4 (650 mg/cm^3 with a 10% peel) was used to ensure that only trabecular bone was captured. Because of the difficulties in interpreting metaphyseal bone density measurements from a single slice [151], three pQCT slices were averaged at the distal 4% femur and tibia regions. At the diaphyseal 20% femur and 66% tibia sites, contour mode 1 (710 mg/cm^3) and cort mode 2 (710 mg/cm^3) were used (**Table 4**).

Table 4: Summary of Filters used in Bone and Soft-tissue analysis using pQCT.

Skeletal Site	Location	Filter Technique
Bone		
Total Bone	**distal 4% metaphyseal region of femur and tibia**	contour mode 3 (169 mg/cm^3)
Trabecular Bone		peel mode 4 (650 mg/cm^3 with a 10% peel)
Cortical Bone	**diaphyseal 20% femur;**	contour mode 1 (710 mg/cm^3)
Cortical Bone	**diaphyseal 66% tibia**	cort mode 2 (710 mg/cm^3)
Soft-Tissue		
Adipose Tissue	**20% femur (thigh) and 66% tibia (calf)**	peel mode 2 (<40 mg/cm^3)
Muscle/Bone Tissue		peel mode (≥40 mg/cm^3)

All measurements were conducted relative to respective distal growth plates on the non-dominant leg.

The metaphyseal regions were chosen to represent skeletal sites predominantly comprised of trabecular bone whereas the diaphyseal regions were chosen to represent skeletal sites predominantly comprised of cortical bone. The 66% distal tibia and 20% distal femur were chosen because these sites are associated with the "bipennate muscles" of the calf and thigh, and therefore represent a site of the largest muscle girth in which the muscle contractile forces may stimulate bone mineral accrual and bone strength; furthermore, these sites have a higher amount of cortical density versus trabecular density. Additionally, the 20% distal femur was chosen because it represents a diaphyseal site that allowed sufficient distance between the body of the subject and the gantry of the pQCT to minimize radiation exposure to the ovaries and allowed girls to sit comfortably to minimize motion artifact. Cortical parameters were not assessed at metaphyseal regions because the spatial resolution of the pQCT device used in this study is not sufficient to analyze cortical shells of less than 2 mm [253].

Bone parameters measured at distal metaphyseal regions of the femur and tibia included trabecular vBMD (mg/cm^3) and bone strength index (BSI, mg^2/mm^4). Bone parameters measured at diaphyseal regions of the femur and tibia included cortical vBMD (mg/cm^3) and strength-strain index (SSI, mm^3) (**figure 7**). BSI estimates the bone's ability to withstand compression at

metaphyseal regions, and is calculated as the product of the metaphyseal total area and the square of total vBMD: Bone strength index (BSI, mg^2/mm^4) = total area x total $vBMD^2$ [255]. SSI is used to estimate the bone's ability to resist torsion and bending forces at diaphyseal regions. Diaphyseal SSI is calculated using Stratec software (Version 6.0) [254], which is based on the integrated product of the geometric properties (i.e., section modulus) with the material properties of bone: Strength-strain index (SSI, mm^3)= , n ($[(r^2_i \text{ x } a)/r_{max}]$ x (cortical vBMD/ND) [248]; section modulus is calculated as $(r^2_i \text{ x } a)/r_{max}$, where a is the area of a voxel (mm^2), r is the distance of a voxel from the center of gravity (mm), and r_{max} is the maximum distance of a voxel from the center of gravity (mm). The material properties of bone are calculated as the quotient of measured cortical density (cortical vBMD, mg/cm^3) and normal physiologic cortical density (ND,1200 mg/cm^3).

Axial compression tests on the 4% distal tibia and 4-point bending tests on the 66% distal tibia of human cadaveric tibiae conducted by Kontulainen and colleagues, showed that BSI and SSI explained 85% and 76% of the variance in failure properties, respectively [255]. To our knowledge, there is no mechanical testing data on the distal 4% and 20% femur sites. pQCT data acquisition and analyses followed guidelines provided by Bone Diagnostics, Inc. (Fort Atkinson, WI, USA). All pQCT scans were performed by a single operator, while a second operator analyzed all scans using the Stratec software (version 6.0, [254]). The pQCT instrument was calibrated and quality assurance procedures were completed daily in order to ensure precision of measurements.CVs previously reported from our laboratory [83, 122] were less than 1.1% for vBMD, bone geometry, and indices of bone strength (BSI and SSI).

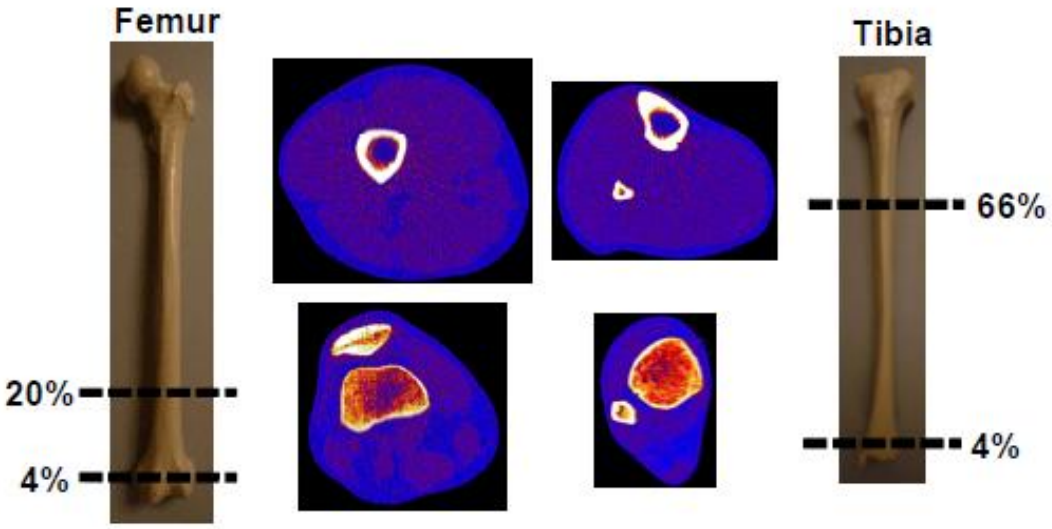


Figure 7**.** Representative pQCT images of distal metaphyseal and diaphyseal regions of the femur and tibia.

BMC (g) of the total body less head (TBLH), L2–L4 lumbar spine (LS) vertebrae in the anteroposterior plane, femoral neck (FN), and total femur and tibia of the nondominant leg were assessed with dual-energy X-ray absorptiometry (DXA) using a GE Lunar Prodigy (software Version 5.60.003) fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). Subjects were positioned following the standard manufacturer protocols. Because of the inherent limitations of aBMD in children and adolescents [256] and because the head is not responsive to environmental stimuli such as physical activity[257], TBLH-BMC has been proposed by the International Society for Clinical Densitometry (ISCD) as the most appropriate DXA-derived outcome measure of bone status in youth [258].

Soft Tissue composition

Regional soft tissue composition was assessed at the 20% femur (thigh) and 66% tibia (calf) sites relative to the respective distal growth plates of the non-dominant limb using pQCT (Table 4). Edge detection and threshold techniques were used to separate tissues (i.e., adipose, muscle, and bone) based on attenuation characteristics, which are directly related to tissue

composition and density (24,25). Images were filtered prior to being analyzed using contour mode 3 (-101 mg/cm^3) and peel mode 2 (40 mg/ cm^3) to separate adipose (<40 mg/cm3) and muscle/bone (40 mg/cm3), respectively. Images were filtered subsequently with a 7x7 image filter that clearly defined the edge of the muscle and eliminated all bone above 120 mg/cm^3, ensuring that muscle density was a direct result of the soft tissue within the edge of the muscle. Although this technique does not distinguish between intra- and extramyocellular fat compartments, previous studies in adults using Proton magnetic resonance spectroscopy (MRS) in adults [259], as well as in youth [260] have indicated that composite measures of IMCL and EMCL such as skeletal muscle density, are sufficient indices of skeletal muscle fat content. Thus muscle density was used as a composite index of fat content within the intra- and extramyocellular stores. Soft tissue parameters obtained at the calf and thigh regions included muscle cross-sectional area (MCSA, mm^2, Aim 3), muscle density (mg/ cm^3, Aims 1 and 3). CVs for MCSA, and muscle density at the calf region are 1.4% and 0.9%, respectively, whereas CVs for the same parameters at the thigh region are 1.2% and 0.4%, respectively.

Soft tissue mass and composition, including total-body fat mass (kg; Aims 1-3), and abdominal (android) fat mass (kg; Aim 1; Ancillary Aim) were obtained from dual energy x-ray absorptiometry (DXA) scans using the GE Lunar Prodigy (software Version 5.60.003) fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). Android fat mass (AFM), available from the manufacturer's automated ROIs, is defined as the area enclosed between a demarcation above the iliac crest to a second mark at 20% of the total distance between the iliac crest and the base of the skull (**figure 8**). Subjects were positioned following the standard manufacturer protocols. All participants were scanned on the same machine, and DXA scans were performed and reviewed by certified technicians. The Lunar Prodigy was calibrated daily according to the standard

procedures for maintenance and use as recommended by the manufacturer. DXA CVs for precision for measuring soft tissue composition in our laboratory have been previously reported [261, 262].

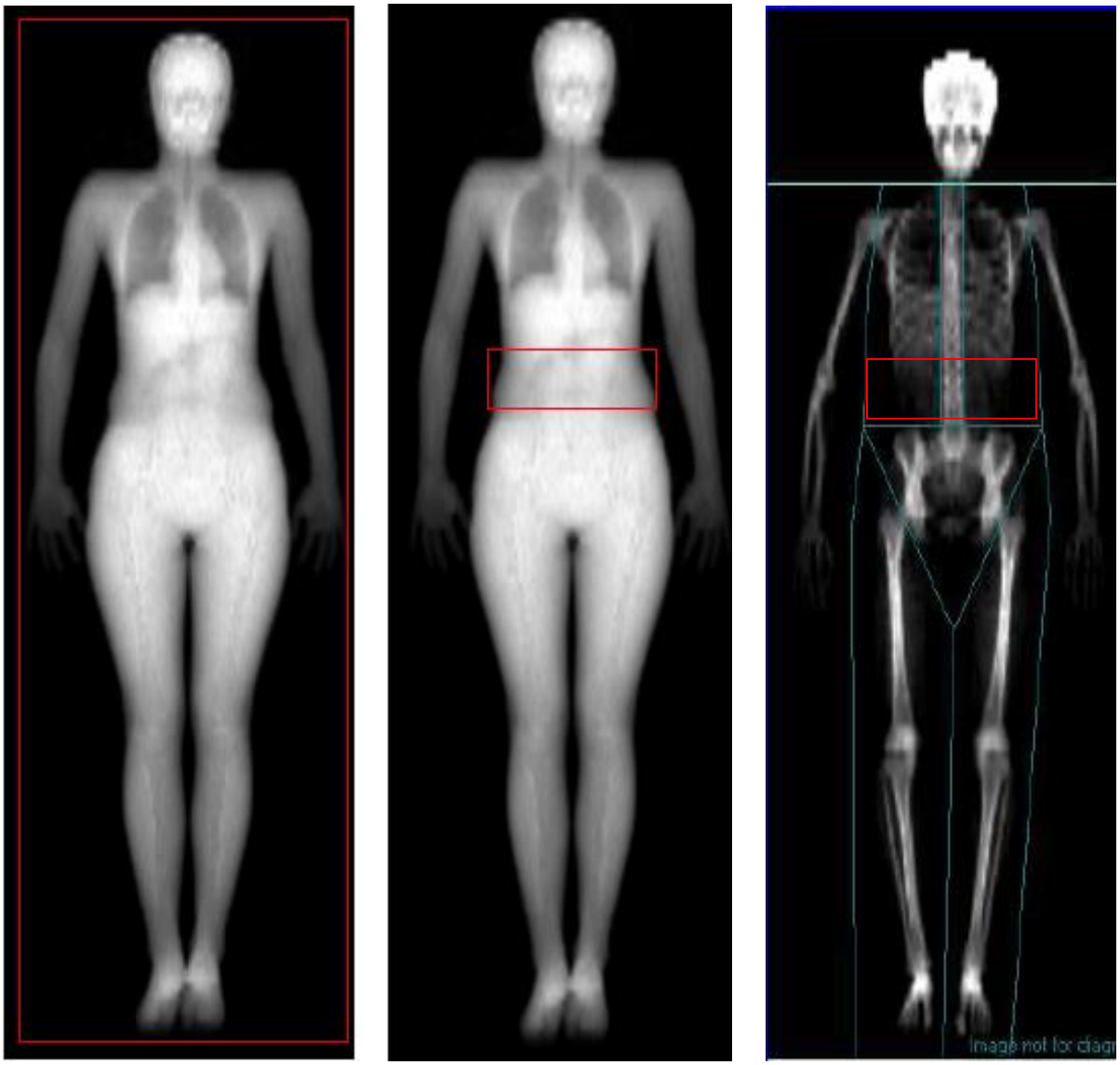


Figure 8. Representative DXA images of total body fat mass (TBFM) (left) and android fat mass (AFM) (middle) and the boundaries that define total body and android fat masses (right).

Statistical Analysis

The "Jump-In" study was the longest randomized controlled trial of the effects of a school-based high-impact jumping intervention on bone parameters using pQCT in young girls. The results from the 2-year exercise implementation showed that the exercise intervention did not affect bone parameters in this sample of early and peri-pubescent girls. Consequently, the data from all participants were collapsed into a single group and examined for influences of soft tissue composition, physical activity, maturation and diet on 2-year changes in bone parameters in this large sample of young girls. Data were checked for outliers and normality using histograms, and skewness and kurtosis were calculated for all variables. Baseline values of BSI and SSI were moderately skewed. Both log transformed and untransformed analyses were performed and all results were similar; thus, the untransformed data are reported for clarity. All bone variables represented as the change using baseline and 24 month data for each respective bone parameter were normally distributed; thus, no transformations were applied. Skewed nutrient intakes [AA, PUFA, omega-3, sum LCPUFA, total n-3 FA, total n-6 FA, ratio (LA/ALA), ratio (AA/omega-3), ratio (total n-6/totaln-3)] were normalized using log transformations or square root transformation (EPA, DHA); transformed data were used in all subsequent regression analyses. Descriptive statistics (means, SDs, and ranges) were calculated for the entire sample. Paired t-tests were used to assess significant differences between baseline and 2-year study visits for each variable (Aims 1a, 1b and Aim 2). Significant differences were observed for all variables except for diet (calorie, fat intake) and physical activity score. To quantify the unadjusted relationships between bone parameters and potential covariates, bivariate correlations were computed using Pearson's correlation coefficients (r) for continuous and Spearman's rho for categorical variables. Multiple linear regressions were used to regress changes in bone variables on each independent variable of interest after adjusting for influential model covariates chosen based on known biological and

biomechanical effects that significantly influence linear growth and body composition [6, 21, 247, 263, 264]. Prior to all multiple regression analyses, all regression models were assessed for linearity, normality and homoscedasticity using residual plots. To protect against collinearity, possible interactions between independent variables were explored in all models. Adjusted R^2 (Aim 3) represents the proportion of variance in the dependent variable explained by the statistical model. Partial correlation coefficients were used in order to determine the individual relationship between an independent variable and dependent bone outcome, after controlling for other model covariates. Analysis of covariance (ANCOVA) was used to compare changes in bone outcomes among respective tertiles of the independent variables of interest after adjusting for the same covariates included in the regression models. Because of the differences in units for pQCT bone outcome variables, results were normalized to the highest tertile by setting the highest tertile to 1.0 (Aims 1 and 3). In Aim 2, results were normalized to the lowest tertile by setting the lowest tertile to 1.0 and the middle and highest tertiles to greater than 1.0. Normalization of values was necessary due to the differences in units among pQCT variables. Bonferroni post hoc tests were used to adjust for multiple comparisons among tertiles of the independent variable of interest. Lastly, ANCOVA analyses were conducted to determine the estimated means (±SE) of the change in MD across the tertiles of baseline MD of the thigh and calf (Aim 1b), after adjusting for baseline model covariates. The level of statistical significance was set at $P<0.05$ (two-tailed). All analyses were performed using the Statistical Package for the Social Sciences for Windows, Version 20.0 (SPSS, Chicago, IL,USA).

CHAPTER 4

MAIN FINDINGS

The main results of longitudinal analyses addressing the three primary aims are summarized in this chapter and in three manuscripts (under review) provided in **APPENDICES A-D**. An ancillary aim was also addressed, based on a cross-sectional analysis of data from another source, which was focused on developing an algorithm for estimating abdominal VAT. The results of that analysis have been published [265] (**Appendix E**). Additional results and specific details pertaining to each aim are outlined in the three manuscripts in the appendices (**APPENDICES A-E**).

Specific Aim 1: Relationship between whole body and regional fat distribution on weight bearing bone geometry, density, and bone strength: a pQCT study in young girls.

The objective of specific aim 1 was to examine the effects of total body and regional adiposity (i.e., android fat mass and skeletal muscle fat content) and lean mass (i.e., skeletal muscle) with bone mineral content (BMC), volumetric bone mineral density (vBMD), bone geometry, and indices of bone strength in young girls. The individual effects of total body and android fat masses (specific aim 1a) and skeletal muscle fat content of the thigh and calf (specific aim 1b) were independently evaluated using a subsample of the Jump-In: Building Better Bones" study (n=260).

Descriptive statistics for baseline and 2 year data are given in **Table 5**. Based on body mass index (BMI, kg/cm^2), at baseline, 3.5% of the sample was underweight (BMI<5th percentile), 75% of the sample was healthy weight (BMI 5th to 85th percentile), 13.5% of the sample was overweight (BMI 85th to 95th percentile), and 7.7% of the sample was obese (BMI>95th percentile) [148]. On average, girls were 1.2 years away from achieving PHV at baseline, ranging from 3.2 years prior to PHV to 1.04 years post PHV. Dietary fat and caloric

intakes were consistent from baseline to 24-months (data not shown). Average calorie intake

Table 5: Sample Descriptive Characteristics ($\bar{x}$± SD)

	Baseline (n=260)	**24-months (n=260)**	**% change[a]**
Age (years)	10.6 ± 1.1	12.7 ±1.1	-
Maturity Offset (years)	-1.2 ± 1.0	0.70 ±1.0	-
Tanner (%; 1/2/3/4/5)	34/34/27/5/0	1/4/13/37/36/8	-
Menarche (%; Post)	7	47	-
Height (cm)	144.1 ± 9.8	156.7 ± 9.1	8.7[a]
Weight (kg)	38.6 ± 9.8	50.0 ± 12.0	29.6[a]
BMI (kg/cm^2)	18.34 ± 3.2	20.2 ± 3.7	9.9[a]
Femur Length (cm)	34.0 ± 3.0	36.7 ± 2.5	8.1[a]
Tibia Length (cm)	33.1 ± 2.9	36.4 ± 2.5	9.9[a]
Total energy intake (kcal)	1719± 647	1703.8 ± 490.4	-0.9
Total fat intake (g)	60.2 ± 25.6	59.7 ± 21.5	-0.9
Physical Activity Score	5229.2 ± 4589.7	5263.7 ± 5593.5	0.7
Total body fat mass (kg)	11.0 ± 6.0	15.2 ± 7.8	38.9[a]
Total body lean mass (kg)	25.4 ± 4.9	32.0 ± 5.5	26.0[a]
Android fat mass (kg)	0.8 ± 0.5	1.0 ± 0.7	31.7[a]
TBLH-BMC (g)	1032.6 ± 315.0	1495.5 ± 418.2	44.8[a]
Thigh muscle density (mg/cm^3)	76.3 ± 1.5	77.5 ± 1.5	1.6[a]
Calf muscle density (mg/cm^3)	79.0 ± 1.2	80.0 ± 1.2	1.2[a]
Femur BSI (mg^2/mm^4)	94.5 ± 26.8	123.8 ± 36.2	31.0[a]
Femur SSI (mm^3)	1315.4 ± 389.7	1874.8 ± 508.1	42.5[a]
Femur Total Density (ave) (mg/cm^3)	275.1 ± 33.4	290.0 ± 40.6	5.4[a]
20% Femur Cortical Density (mg/cm^3)	1045.8 ± 23.1	1067.2 ± 32.5	2.0[a]
4% Femur Trabecular Density (mg/cm^3)	236.7± 31.9	246.5 ± 36.8	4.2[a]
Tibia BSI (mg^2/mm^4)	50.7 ± 12.8	68.1 ± 19.6	34.4[a]
Tibia SSI (mm^3)	1151.8 ± 320.8	1590.9 ± 408.4	38.1[a]
Tibia Total Density (ave) (mg/cm^3)	294.7 ± 34.7	322.4 ± 46.6	9.4[a]
66% Tibia Cortical Density (mg/cm^3)	1028.2 ± 32.4	1056.9 ± 37.6	2.8[a]
4%Tibia Trabecular Density (mg/cm^3)	222.3 ± 25.5	229.8 ± 30.7	3.4[a]

Values are presented as mean ± SD. *P* values represent paired samples *t*-Test for difference between the baseline and 2-year study visits. TBLH= total body less head; BMC= bone mineral content (g) BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3)
[a] Significant at P<0.0001

(1711±541 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans (DGA) [234]. Average fat intake (31%±4.0%) met the dietary recommendations (25-35%) established by the American Heart Institute [266] and DGA [234].

As expected, significant increases in age, maturity, height, body weight, body mass index

(BMI), femur length, tibia length, total body lean mass, total body fat mass and lean mass, and calf and thigh muscle density and femur and tibia bone strength and bone density indices were observed (all p values < 0.0001) from baseline to the 2-year follow-up. Total calorie and fat intakes and physical activity did not change from baseline to the 2-year follow-up (all $p<0.05$).

Specific Aim 1a: Determine the relationship of total body and android fat mass with bone mineral content (BMC), volumetric bone mineral density (vBMD), and indices of bone strength in young girls.

The objective of specific Aim 1a was to examine longitudinal associations between total body fat (TBFM), and android fat (AFM), with weight bearing bone parameters in young girls. It was hypothesized that higher levels of total body fat (TBFM) and android fat (AFM) would be inversely associated with gains in bone mineral content (BMC) and bone strength and density at weight bearing bone sites in young girls.

Multiple linear regression analysis was used to regress changes in 2-year bone variables on baseline measures of total body and android fat masses after controlling for important covariates. In all models, bone outcomes are the changes in the respective parameter from baseline to 24 months. Covariates for the two models included baseline maturity offset, height, and whole-body lean mass, and average (baseline to 24-month) physical activity and dietary calorie and fat intakes. **Table 6** shows the (partial r) in comparison to the simple correlation (person's r) for the individual contribution of TBFM and AFM on bone strength and bone density (e.g., volumetric bone mineral density, vBMD) after controlling for the covariates.

Baseline TBFM and AFM were positively related to change in TBLH-BMC (all $p<0.0001$). Both baseline measures of TBFM and AFM were significantly and positively associated with changes in trabecular vBMD (all $p<0.002$) at metaphyseal regions of the femur, while negative associations were noted between TBFM and AFM and changes in cortical vBMD

($p<0.020$; $p<0.006$, respectively) at diaphyseal regions of the tibia. Regression analyses examining change in pQCT bone strength are also summarized in **table 6.** Change in BSI measured at the metaphyseal region of the femur was positively associated with both baseline TBFM and baseline AFM ($r= 0.17$-0.19; $p<0.002$-0.005). Similar trends were noted with change in SSI at diaphyseal regions of the tibia, having a positive relationship with baseline TBFM ($r=0.18$; $p<0.003$) and baseline AFM ($r=0.14$, $p<0.023$), while no associations were observed between baseline TBFM or AFM and changes in BSI at metaphyseal regions of the tibia or between changes in diaphyseal regions of the femur (SSI) (**Table 6)**.

Table 6: Bivariate relationships and partial correlations from multiple linear regression of baseline TBFM and AFM on changes in bone parameters

	Baseline TBFM		**Baseline AFM**	
Bone Variable	Pearson's r	partial r	Pearson's r	partial r
Δ TBLH-BMC (kg)	0.34[a]	0.25[a]	0.32[a]	0.24[a]
4% Femur				
Δ BSI	0.26[a]	0.19[b]	0.23[a]	0.17[b]
Δ total vBMD	0.26[a]	0.06	0.22[a]	0.03
Δ Trab vBMD	0.08	0.20[b]	0.09	0.20[a]
20% Femur				
Δ SSI	0.24[a]	0.11	0.21[b]	0.10
Δ Cort vBMD	0.27[a]	-0.05	0.21[b]	-0.09
4% Tibia				
Δ BSI	0.34[a]	0.10	0.29[a]	0.06
Δ total vBMD	0.31[a]	-0.02	0.27[a]	-0.04
Δ Trab vBMD	0.19[b]	0.09	0.17[b]	0.09
66% Tibia				
Δ SSI	0.22[a]	0.18[b]	0.18[b]	0.14[b]
Δ Cort vBMD	.130[b]	-0.15[b]	0.09	-0.17[b]

All bone outcome variables were calculated as the change occurring from baseline to 24-months. TBFM=total body fat mass (kg); AFM= android fat mass (kg); TBLH= total-body less head; BMC= bone mineral content (g); BSI=bone strength index (mg^2/mm^4); Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); SSI=strength–strain index (mm^3); Model covariates: baseline maturity offset, height and total-body lean mass and average diet (calorie, fat intake) and physical activity score.

[a] $P<0.001$; Pearson's r for continuous variables.

[b] $P<0.05$; Pearson's r for continuous variables.

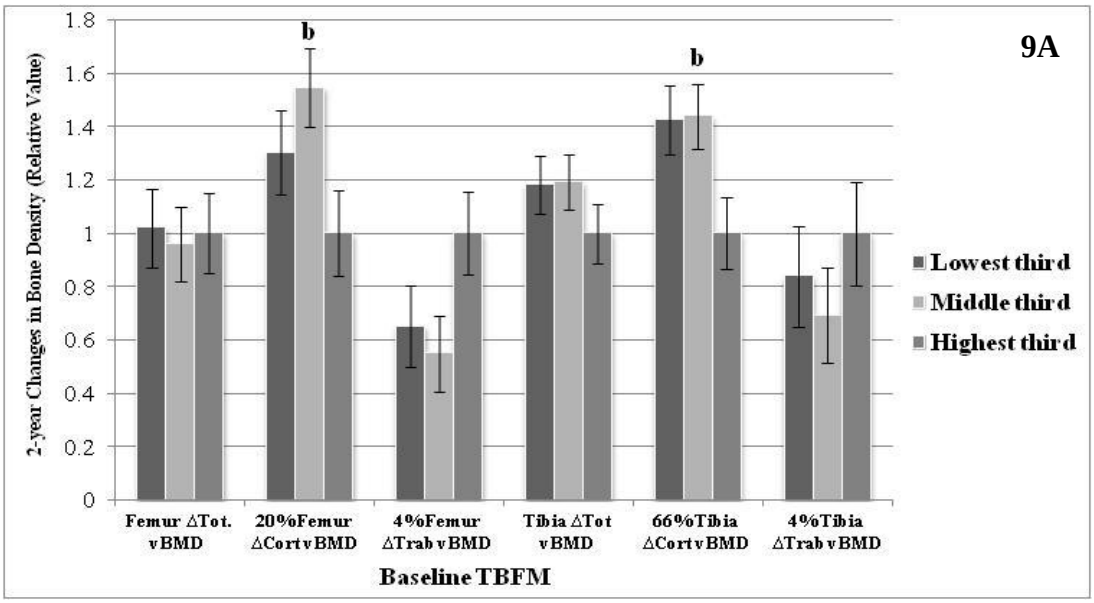
Comparison of bone strength and bone density parameters across tertiles of body composition

In order to understand the seemingly contradicting associations between fat masses and 2-year changes in bone outcomes, analysis of covariance (ANCOVA) was used to compare femur and tibia BMC, vBMD, and bone strength indices among respective tertiles of baseline TBFM, and AFM after adjusting for important model covariates. **Figures 9-10** show the normalized adjusted means (±SE) for changes in volumetric bone mineral density (vBMD) (**figure 9**), and bone strength (**figure 10**). The changes in DXA measures of total-body less head bone mineral content (TBLH BMC) across tertiles of TBFM and AFM are given in **figure 11**. The results were normalized to the highest tertile by setting the highest tertile to 1.0.

Analysis of Covariance (ANCOVA) showed that girls in the middle thirds of TBFM had significantly higher femur and tibia cortical vBMD compared to girls in the highest thirds of TBFM ($p<0.05$). These relationships were generalized, as similar trends were present with AFM; specifically girls in the middle compared to the highest thirds of AFM gained significantly more femur and tibia vBMD ($p<0.01$). No significant differences were observed among tertiles of TBFM and AFM and changes in bone strength indices (all $p>0.05$)**.**

Further analyses were conducted to determine if a curvilinear relationship existed between fat mass and changes in bone density parameters (**Table 7**). To prevent high collinearity (VIF>10), the linear and quadratic fat mass variable were not included in the same model. Results from multiple linear regression analyses showed that, after adjusting for baseline maturity offset, height, whole-body lean mass, and average diet (calorie; fat intake) and physical activity, TBFM ($r=0.21$) and AFM ($r=0.21$) were positively associated with trabecular vBMD measured at the distal metaphyseal femur (all $p<0.0001$). By contrast, significant negative associations between TBFM ($r= -0.15$, $p<0.02$) and AFM ($r= -0.15$, $p<0.01$) and tibia cortical vBMD. Together these results

suggest a possible curvilinear relationship exists between levels of fat mass and changes in bone density, particularly at the diaphyseal region of the tibia.



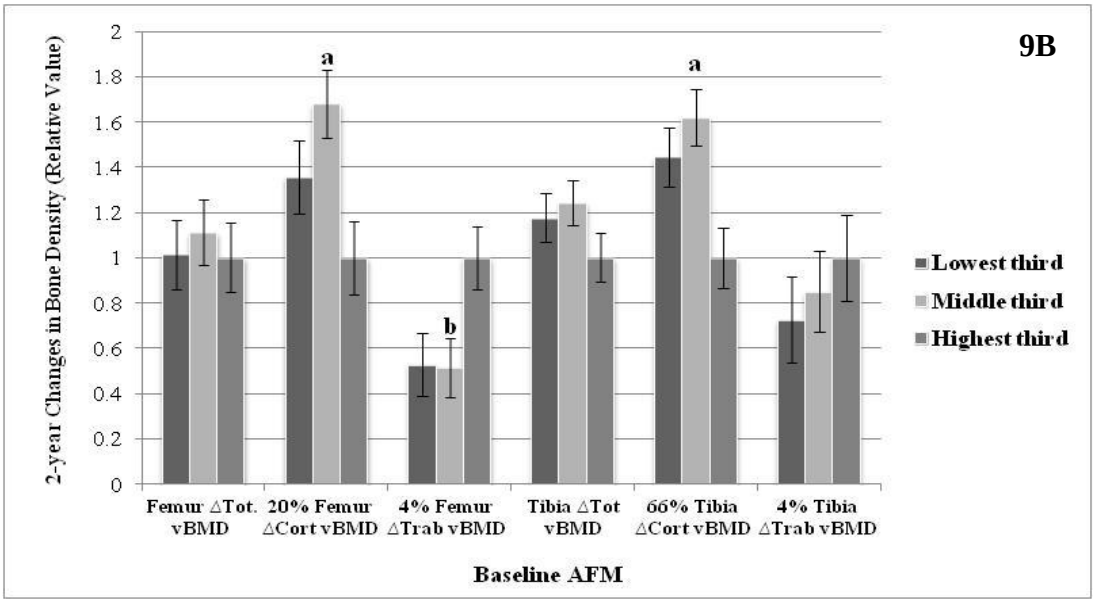
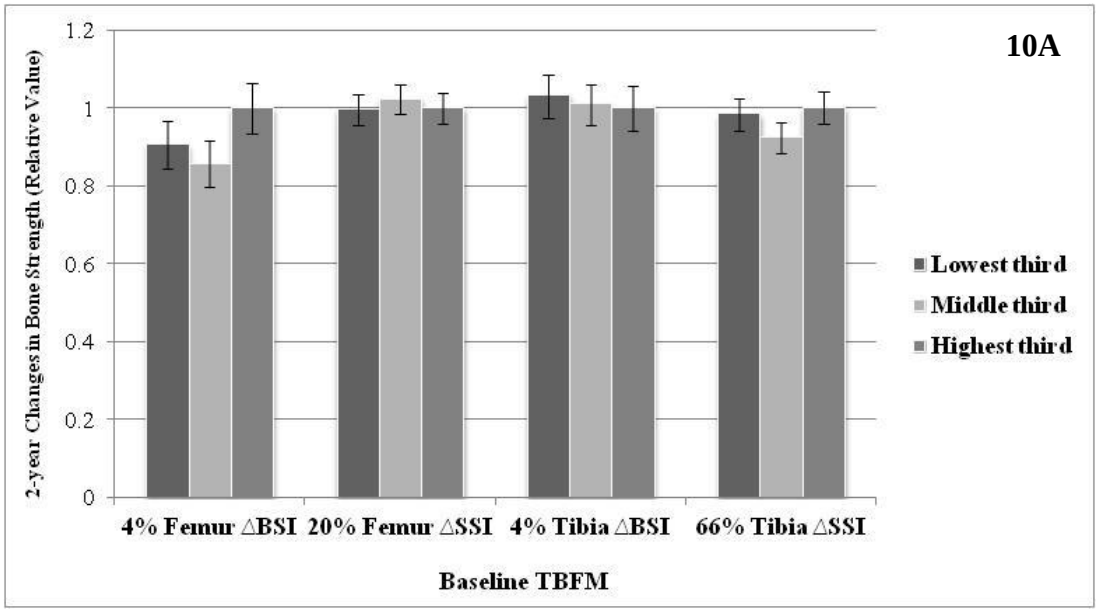


Figure 9. Estimated marginal means±SE for changes in femur and tibia volumetric bone density parameters across thirds of baseline total body (TBFM; A) and android (AFM; B) fat masses. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline fat masses were evaluated by ANCOVA using baseline covariates: maturity offset, height, total body lean mass, and average diet (calorie, fat intake) and physical activity score. Tot BMD= total (average) volumetric bone mineral density (mg/cm3); Cort BMD= cortical volumetric bone mineral density (mg/cm3); Trab BMD=trabecular volumetric bone mineral density (mg/cm3).

[a] Significantly different (P<0.01) from *highest* tertile; ANCOVA

[b] significantly different (P<0.05) from *highest* tertile; ANCOVA



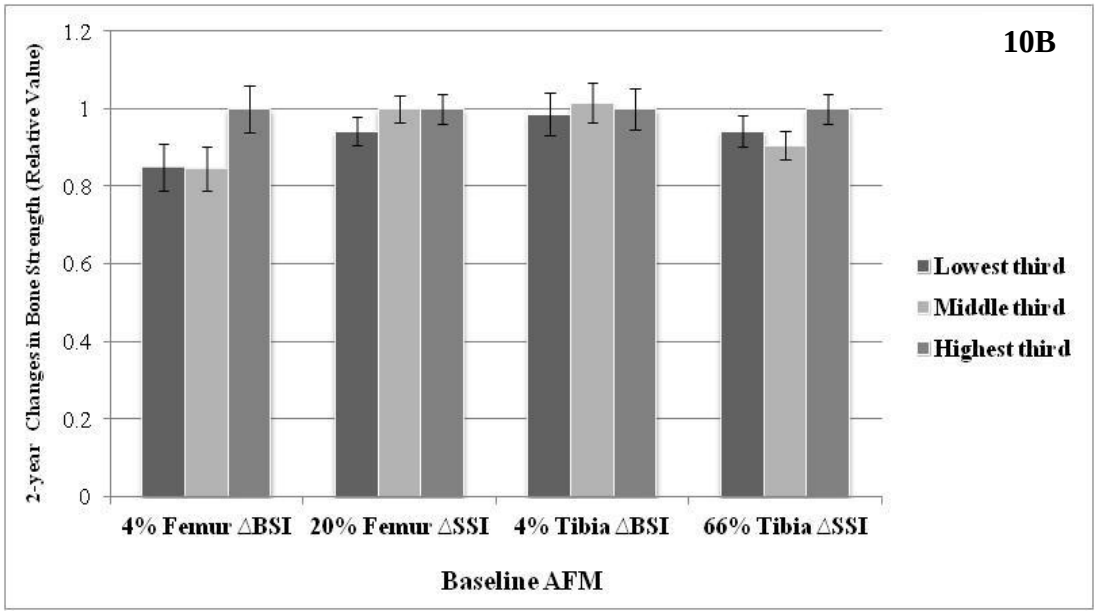


Figure 10**.** Estimated marginal means±SE for changes in femur and tibia bone strength indices across thirds of baseline total body (TBFM; panel A) and android (AFM; panel B) fat masses. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline fat masses were evaluated by ANCOVA using baseline covariates, maturity offset, height, total body lean mass, and average diet (calorie, fat intake) and physical activity score. BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3).

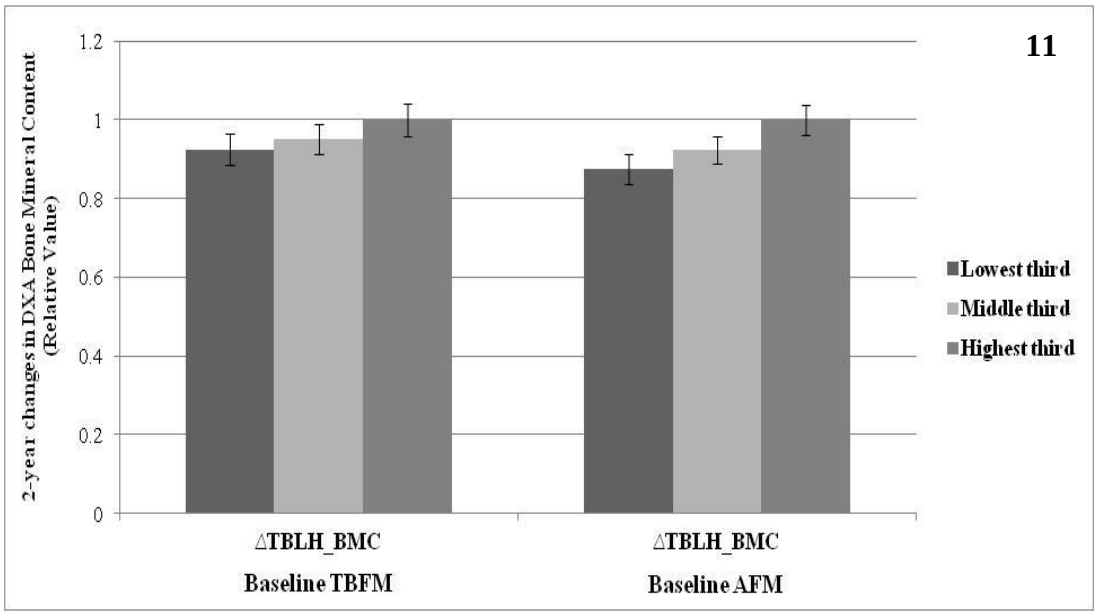


Figure 11. Estimated means±SE bone mineral content pQCT femur and tibia parameters. Outcome bone parameters presented as the change (baseline to 24-months) in femur and tibia bone densities. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of fat mass were evaluated by ANCOVA using baseline covariates: maturity offset height or bone length (femur bone length; tibia bone length, respectively), and averaged covariates (baseline to 24 months): dietary caloric intake; dietary fat intake; physical activity score. TBFM=total body fat mass (kg); AFM= android fat mass (kg); TBLH= total body less head; BMC = bone mineral content (g)

Table 7: Assessment of Curvilinear Relationship between baseline TBFM and AFM and changes in bone geometry

	$(TBFM)^2$		**$(AFM)^2$**	
Bone Variable	partial r	p-value	partial r	p-value
4% Femur Δ Trab vBMD	0.21	0.00	0.21	0.00
20% Femur Δ Cort vBMD	-0.02	0.80	-0.06	0.36
Tibia Δ Total vBMD (ave)	0.01	0.85	-0.01	0.85
66% Tibia Δ Cort vBMD	-0.15	0.02	-0.15	0.01

All bone outcome variables were calculated as the change occurring from baseline to 24-months. TBFM=total body fat mass (kg); AFM= android fat mass (kg); Trab BMD= Trabecular volumetric bone density (mg/cm^3); Cort BMD=cortical volumetric bone mineral density (mg/cm^3); Model covariates: baseline maturity offset, height and total-body lean mass and average diet (calorie, fat intake) and physical activity score.

Analysis of Bone Geometry

In order to determine if the relationships between fat masses and bone strength and bone density were explained by changes in bone geometry, a primary determinant of bone structure and

strength, additional multiple regression analyses were performed on a subsample (n=236) of girls who had completed baseline and 2-year bone geometry variables. Multiple linear regression analyses were performed to regress 2-year changes in bone geometry assessed at the femur and tibia diaphyseal (20% femur, 66% tibia) and metaphyseal (4% femur and tibia) sites on baseline measures TBFM and AFM (**Table 8)**. In all models, baseline maturity offset, height, and whole-body lean mass, and average (baseline to 24-month) physical activity and dietary calorie and fat intakes were included as independent covariates. Positive associations were found between baseline TBFM and changes in periosteal circumference (partial $r=0.20$; $p<0.003$) and endosteal circumference (partial $r=0.26$; $p<0.0001$) measured at the diaphyseal femur, as well as with periosteal circumference measured at the distal metaphyseal femur (partial $r=0.20$; $p<0.002$). By contrast, negative associations were noted between TBFM and cortical thickness at the femur diaphysis (partial $r= -0.14$; $p<0.05$). Similar to the changes observed at the femur, baseline TBFM was positively associated with change in periosteal circumference of the diaphyseal tibia (partial $r=0.13$; $p<0.05$). TBFM was not significantly associated with any changes in cortical thickness or endosteal circumference of the tibia (**Table 8**).

Overall, the directions of the associations between baseline TBFM and changes in femoral and tibial bone geometry were similar to those observed between baseline AFM and changes in bone geometry. In brief, significant, positive associations were found between baseline AFM and greater gains in bone geometry at the distal metaphyseal femur, including periosteal circumference (4% site) (partial $r=0.22$; $p<0.0001$). In addition, AFM was positively related with gains in periosteal (partial $r=0.18$; $p<0.005$) and endosteal (partial $r=0.23$; $p<0.0001$) circumferences measured at the diaphyseal (66% site) femur, but was not significantly associated with changes in cortical thickness. Similar to the associations between AFM and changes in

femoral bone parameters, positive relationships were found between AFM and changes in tibial bone geometry indices. AFM was positively associated with periosteal and endosteal circumference (partial r=0.15; partial r=0.16, all p<0.05) at the tibial diaphysis but was not significantly related with bone geometry at the metaphyseal tibia (**Table 8**).

Table 8: Bivariate relationships and partial correlations from multiple linear regression of baseline TBFM and AFM on changes in bone geometry (n=236)

	Baseline TBFM		**Baseline AFM**	
Bone Variable	Pearson's r	partial r	Pearson's r	partial r
4% Femur				
Δ PerCirc	-0.13	0.20[b]	-0.09	0.22[a]
20% Femur				
Δ EndCir	0.01	0.26[a]	0.00	0.23[a]
Δ PerCirc	-0.02	0.20[b]	-0.02	0.18[b]
Δ CortThick	-0.08	-0.14[c]	-0.06	-0.10
4% Tibia				
Δ Percirc	-0.24[b]	0.11	-0.21[b]	0.11
66% Tibia				
Δ EndCirc	-0.04	0.12	0.00	0.15[c]
Δ PerCirc	-0.09	0.13[c]	-0.05	0.16[c]
Δ CortThick	-0.07	-0.03	-0.08	-0.05

All bone outcome variables were calculated as the change occurring from baseline to 24-months. TBFM=total body fat mass (kg); AFM= android fat mass (kg); PerCirc= periosteal circumference (mm); EndCir= endosteal circumference (mm); CortThick= cortical thickness (mm); Model covariates: baseline maturity offset, height and total-body lean mass and average diet (calorie, fat intake) and physical activity score.

[a] P<0.001; Pearson's r for continuous variables.
[b] P<0.01; Pearson's r for continuous variables.
[c] P<0.05; Pearson's r for continuous variables.

Comparison of bone geometry parameters across tertiles of body composition

Estimated means (±SE) for the *change* in bone geometry parameters in the subsample of girls (n=236) were compared across tertiles of baseline TBFM and AFM, using ANCOVA after adjusting for baseline maturity offset, height, total- body lean mass, and average diet (calorie; fat

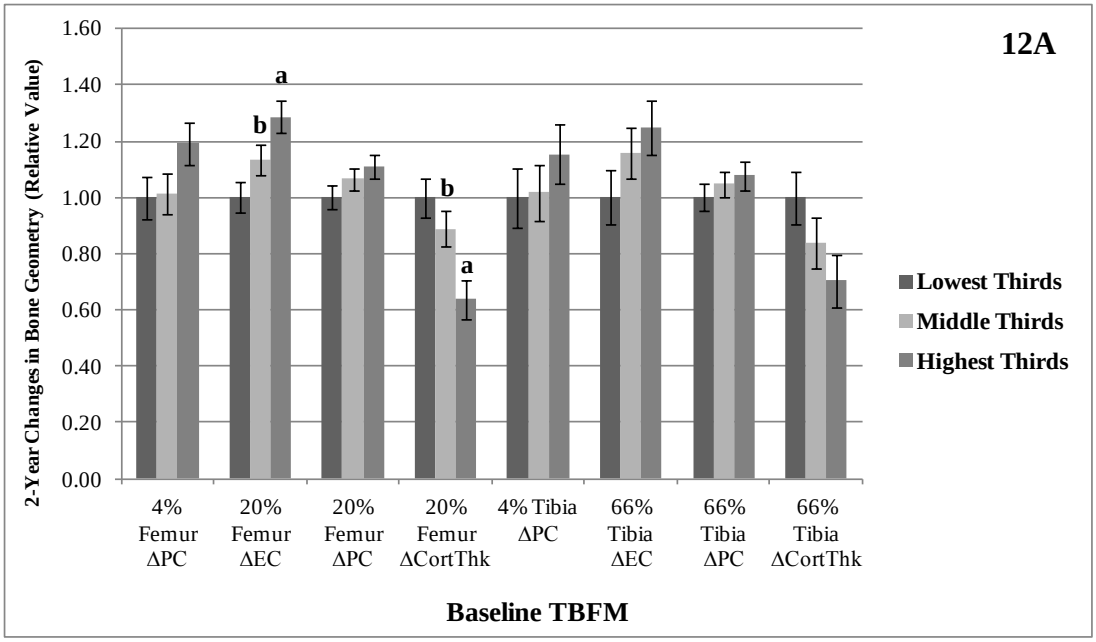
intake) and physical activity. **Figures 12** show the normalized adjusted means (± standard errors) for change in bone geometric parameters across tertiles of TBFM and AFM (**figure 12**). The results presented were normalized to the lowest tertile by setting the lowest tertile to 1.0.

Results from ANCOVA showed that girls in the highest thirds of TBFM increased their endosteal circumference of the diaphyseal femur more than girls in the middle and lowest thirds of TBFM (all $p<0.05$). In contrast, girls in the highest thirds of TBFM had significantly *less increase* femur cortical thickness than girls in the middle (by 39%; $p< 0.02$) and lowest thirds of TBFM (by 56%, $p<0.001$). There were no other significant associations found across tertiles of TBFM and changes in other bone geometry parameters at the metaphyseal or diaphyseal sites of the tibia (**figure 12A).**

Several significant differences in the 2-year changes in femur bone geometry parameters were observed across tertiles of AFM (**figure 12B**). Specifically, girls in the middle and highest thirds of AFM, respectively, experienced larger gains in periosteal circumference of the distal femur (4% site) (all $p<0.01$), whereas similar gains in periosteal circumference of the distal tibia (4% site) were observed in the highest versus lowest thirds of AFM. Importantly, these changes were not confined to the metaphyseal regions, but rather were more generalized across the weight-bearing regions of the diaphyseal femur (20% site). Girls in the middle and highest thirds of AFM showed significantly higher gains in endosteal circumference of the femur (all $p<0.01$). Further, girls in the middle thirds ($p<0.05$) and highest thirds of AFM ($p<0.01$) had significantly larger gains in periosteal circumference of the diaphyseal femur. By contrast, compared to girls in the highest thirds of AFM, girls in the lowest thirds and middle thirds had gained more femur cortical thickness (by 32% and 28%, respectively, all $p<0.05$). However, AFM was not

significantly associated with changes in any other bone geometry parameters at the diaphyseal sites of the tibia.

In conclusion, our results demonstrated that after controlling for growth, maturation, body composition (i.e., whole body lean mass), diet, and physical activity, baseline measures of total body and android fat masses were negatively associated with changes in bone density, particularly at cortical bone sites of diaphyseal femur and tibia. Furthermore, results illustrating the negative associations between total body and android fat masses and femur cortical thickness suggest a possible mismatch in growth in periosteal and endosteal circumferences that may lead to weaker bone strength and development. These longitudinal findings provide evidence that higher levels of fat mass may have detrimental effects on developing bone. Furthermore, excess gain in abdominal adiposity during the pre- and early- pubertal years may be not only serve as an important risk factor for metabolic dysfunction but may also contribute to suboptimal bone development and skeletal fragility later in life.



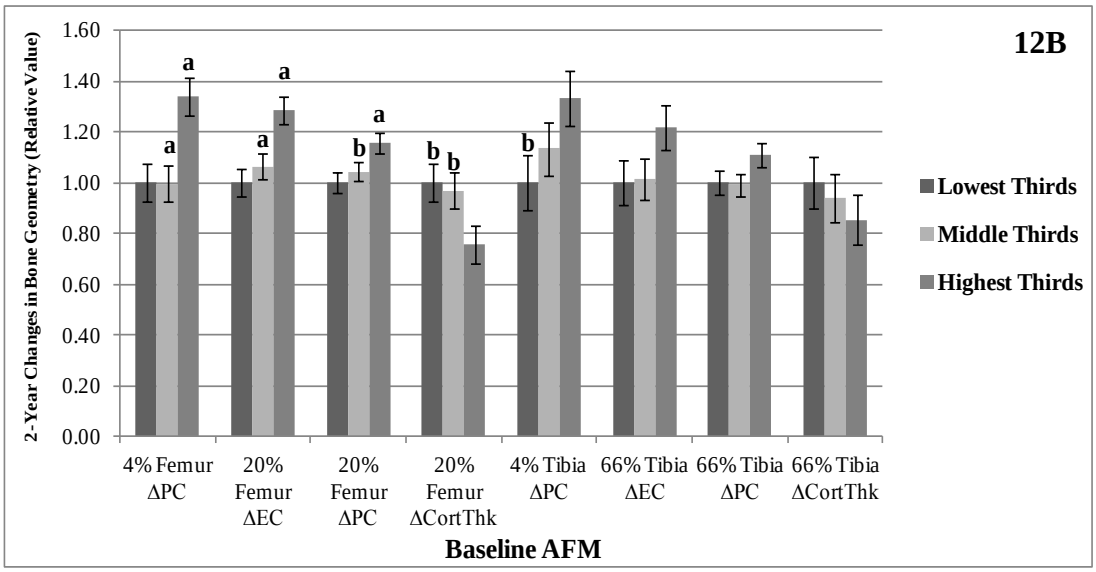


Figure 12. Estimated marginal means±SE for changes in femur and tibia bone geometry across thirds of baseline total body (TBFM; panel A) and android (AFM; panel B) fat masses. All bone outcome variables were calculated as the change occurring from baseline to 24-months. Bone outcome values were normalized to the lowest group by setting the lowest group values to 1.0, while higher values were set to greater than 1.0. TBFM=total body fat mass (kg); AFM= android fat mass (kg); PC= periosteal circumference (mm); EC= endosteal circumference (mm); CortThk= cortical thickness (mm); Model covariates: baseline maturity offset, height and total-body lean mass and average diet (calorie, fat intake) and physical activity score.

[a] Significantly different (P<0.01) from *lowest* tertile; ANCOVA

[b] Significantly different (P<0.05) P<0.05) from *highest* tertile; ANCOVA

Specific Aim 1b: Determine the relationship between skeletal muscle fat content and changes in bone mineral content (BMC), volumetric bone mineral density (vBMD), and indices of bone strength in young girls.

The objective of **Aim 1b** was to examine associations between baseline skeletal muscle fat content and changes in weight bearing bone parameters in young girls. It was hypothesized that greater baseline skeletal muscle fat content (calf and thigh) would be inversely associated with 2-year gains in bone density and bone strength in weight bearing bone sites in young girls.

Multiple linear regression analyses was performed with baseline measures of muscle density, maturity offset, bone length, average diet (calorie; fat intake) and average physical activity as independent variables. The results of the multiple linear regression analyses are shown in **Tables 9-10**, which illustrate the individual contribution (partial r) of thigh MD and calf MD on bone parameters after controlling for covariates. The simple correlations (Person's r) are also shown. In all models, changes in bone outcomes, calculated as the changes in the respective parameter from baseline to 24 month, were regressed on muscle density, adjusted for baseline maturity offset, bone length, whole body lean mass, diet (e.g., fat intake, calorie intake) and physical activity. Change in TBLH-BMC was inversely associated with baseline thigh ($r= -0.19$; $p<0.003$) and calf ($r = -0.24$; $p<0.0001$) muscle densities. Unexpectedly, inverse relationships were observed between thigh and calf muscle densities and changes in bone density, with significant associations found at the femur sites. Both thigh and calf muscle densities were negatively associated with change in total vBMD (all $p<0.05$) and with change in trabecular vBMD at metaphyseal sites of the femur ($p<0.002$) and tibia ($p<0.001$).

Results from regression analyses with change in bone strength as the dependent variable are summarized in **Tables 9-10.** Baseline thigh muscle density was inversely associated with change in BSI measured at the metaphyseal region of the femur ($r= -0.15$, $p<0.020$). Similar

associations were noted between calf MD and change in BSI at metaphyseal regions of the tibia ($r= -0.19$, $p<0.002$). Inverse associations were also observed between calf MD and the change in SSI at diaphyseal regions of the tibia ($r= -0.14$, $p<0.025$), whereas thigh MD was not significantly associated with changes in SSI at diaphyseal regions of the femur.

Comparison of bone parameters across tertiles of body composition

Analysis of covariance (ANCOVA) for estimated marginal means (95% CI) of the *change* in bone outcome parameters were compared across tertiles of baseline thigh and calf MD, respectively, after adjusting for baseline maturity offset, bone length (femur or tibia), whole body lean mass, and average diet (calorie; fat intake) and physical activity. **Figures 13 -15** illustrate the normalized adjusted means ± standard errors for change in vBMD (**figure 13**) and bone strength (**figure 14**) and TBLH-BMC (**figure 15**) across tertiles of thigh and calf MD. Change in bone outcome values were normalized to the highest group by setting the highest group values to 1.0 to account for the difference in units among pQCT. Girls in the lowest thirds of thigh MD gained 17% more TBLH-BMC ($p<0.013$) compared to girls in the middle thirds of thigh MD. Similarly, greater gains in TBLH-BMC were experienced in girls in the lowest versus middle (13%; $p<0.038$) and highest thirds of calf MD (20%; $p<0.001$). Girls in the lowest third had 23% greater change in femur BSI and 71% increase in total femur vBMD compared to the highest third of thigh MD. Greater gains in tibia BSI (19%) and total vBMD (24%) were also observed in the lowest versus the highest thirds of baseline calf MD. The largest increase in bone density was seen in trabecular vBMD at both femur and tibia metaphyseal sites (4% femur, 4% tibia). Girls in the lowest third of thigh MD had a 145% greater increase in femur trabecular vBMD compared to girls in the highest third of thigh MD. Similarly, girls in the lowest third of baseline calf MD had a 137% greater gain in trabecular vBMD at the tibia versus the highest third of baseline calf MD.

Table 9: Unadjusted bivariate Correlations and Partial Correlations from Multiple Linear Regression of 2-year Changes in Bone Parameters on Baseline Thigh Muscle Density (MD)

Baseline Thigh MD	Pearson's r	partial r
Δ TBLH-BMC (kg)	-0.13[b]	-0.19[b]
4% Femur		
Δ BSI	-0.11	-0.15[b]
Δ total vBMD	-0.14[b]	-0.17[b]
Δ Trab vBMD	-0.18[b]	-0.19[b]
20% Femur		
Δ SSI	0.02	-0.04
Δ Cort vBMD	-0.04	-0.06

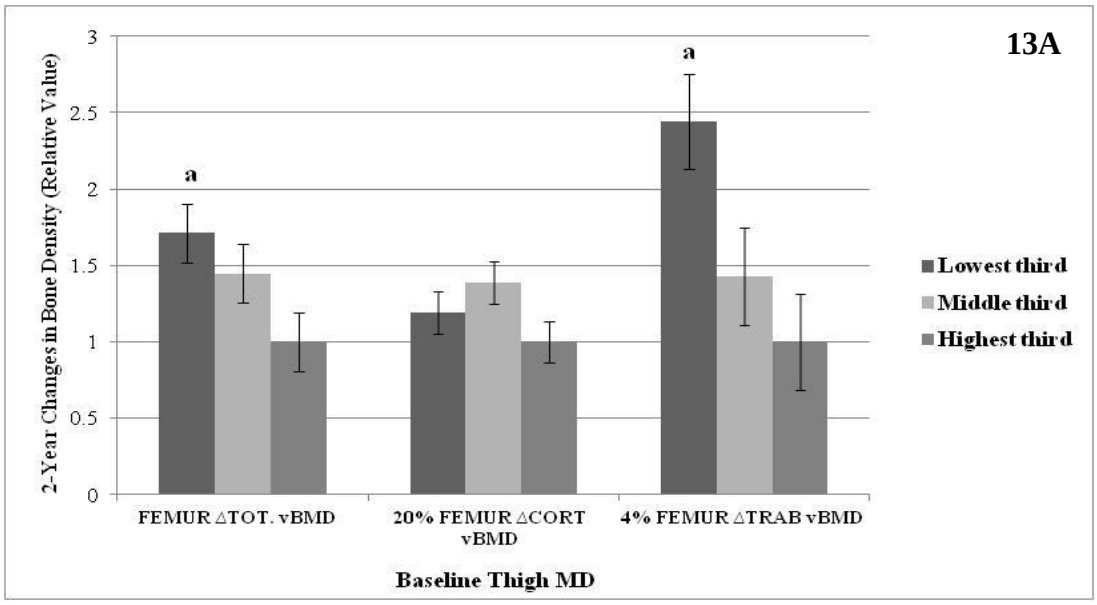
Table 10: Unadjusted bivariate Correlations and Partial Correlations from Multiple Linear Regression of 2-year Changes in Bone Parameters on Baseline Calf Muscle Density (MD)

Baseline Calf MD	Pearson's r	partial r
Δ TBLH-BMC	-0.22[a]	-0.24[a]
4% Tibia		
Δ BSI	-0.16[b]	-0.19[b]
Δ total vBMD	-0.09	-0.13[b]
Δ Trab vBMD	-0.21[b]	-0.22[a]
66% Tibia		
Δ SSI	-0.14[b]	-0.14[b]
Δ Cort vBMD	-0.02	-0.01

All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density (mg/cm^3); TBLH BMC= total body less head bone mineral content (g); BSI=bone strength index (mg^2/mm^4); Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); SSI=strength–strain index (mm^3). Model covariates= baseline covariates, maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score.

[a] P<0.001; Pearson's r for continuous variables.

[b] P<0.05; Pearson's r for continuous variables.



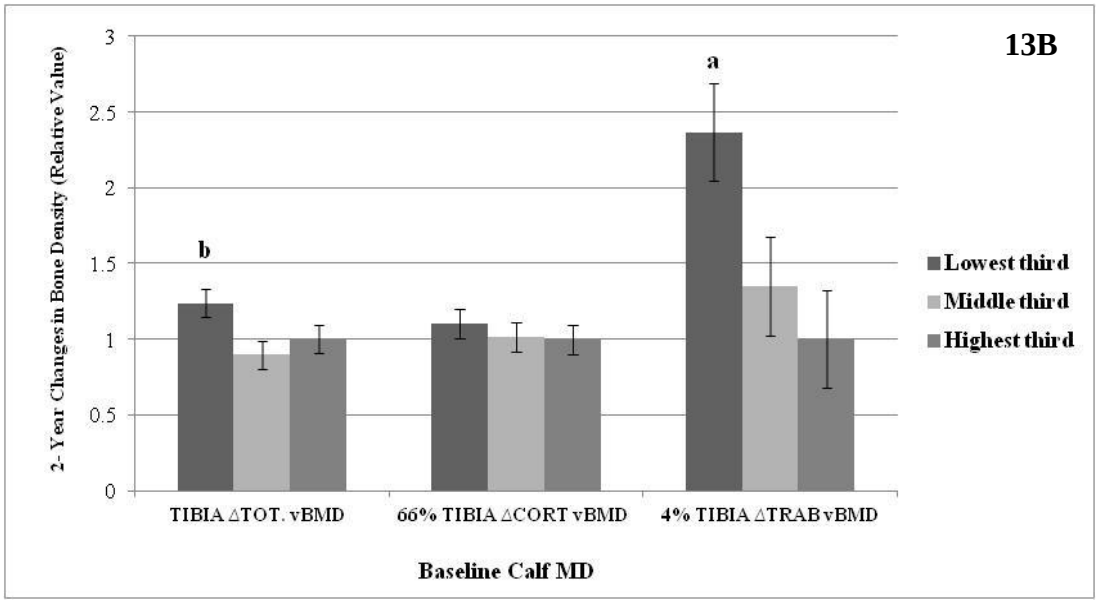


Figure 13. Estimated marginal means±SE for changes in femur and tibia volumetric bone density parameters across thirds of baseline thigh (panel A) and calf (panel B) muscle density. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline muscle density were evaluated by ANCOVA using baseline covariates: maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score. MD= muscle density (mg/cm3); Tot BMD= total (average) volumetric bone mineral density (mg/cm^3); Cort BMD= cortical volumetric bone mineral density (mg/cm3); Trab BMD=trabecular volumetric bone mineral density (mg/cm3).

[a] Significantly different (P<0.05) from highest tertile; ANCOVA

[b] Significantly (p<0.05) different from middle group; ANCOVA

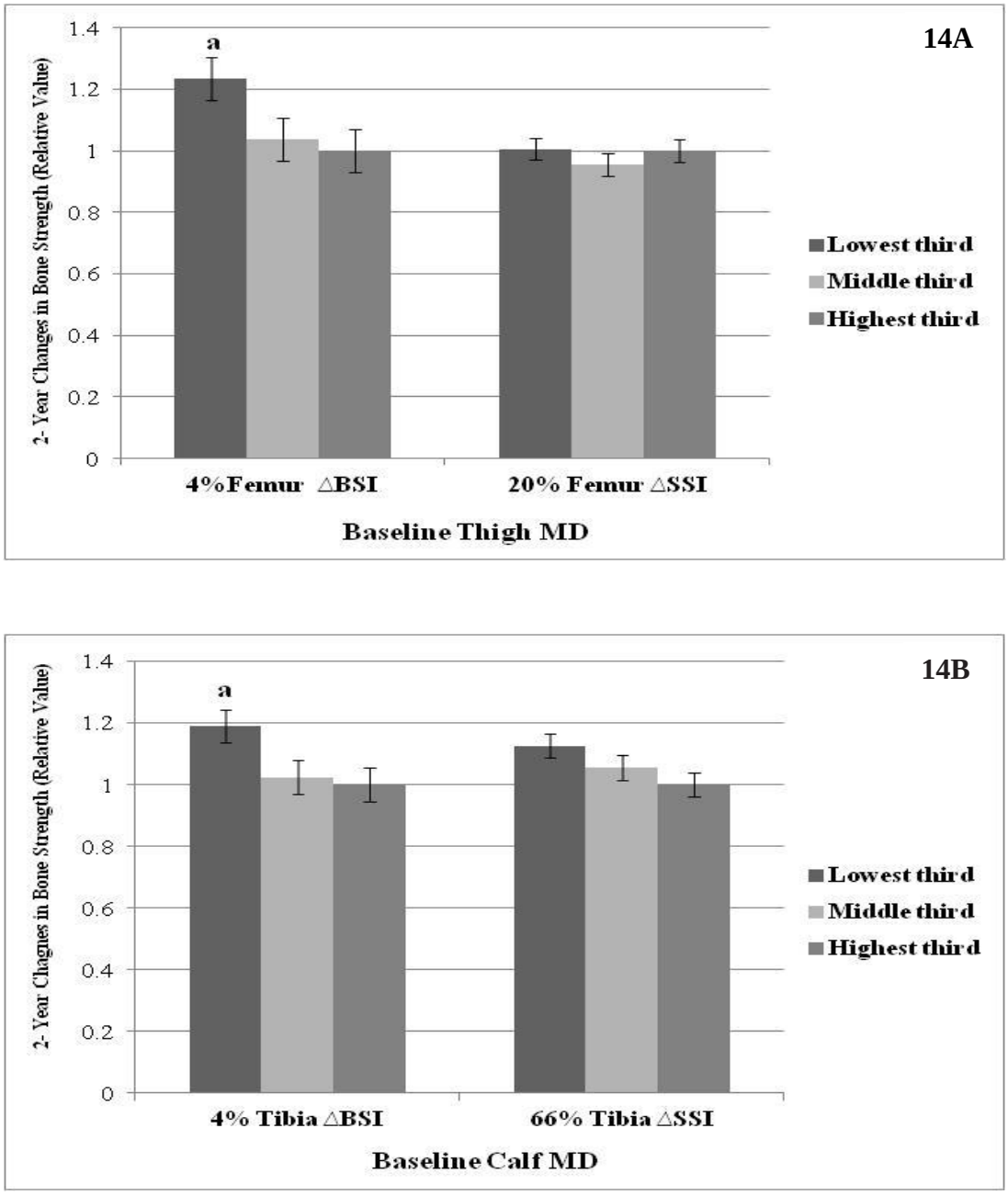


Figure 14. Estimated marginal means±SE for changes in femur and tibia bone strength indices across thirds of baseline thigh (panel A) and calf (panel B) muscle density. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline muscle density were evaluated by ANCOVA using baseline covariates, maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score. MD= muscle density (mg/cm3); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3)

[a] Significantly different ($P<0.05$) from highest tertile; ANCOVA

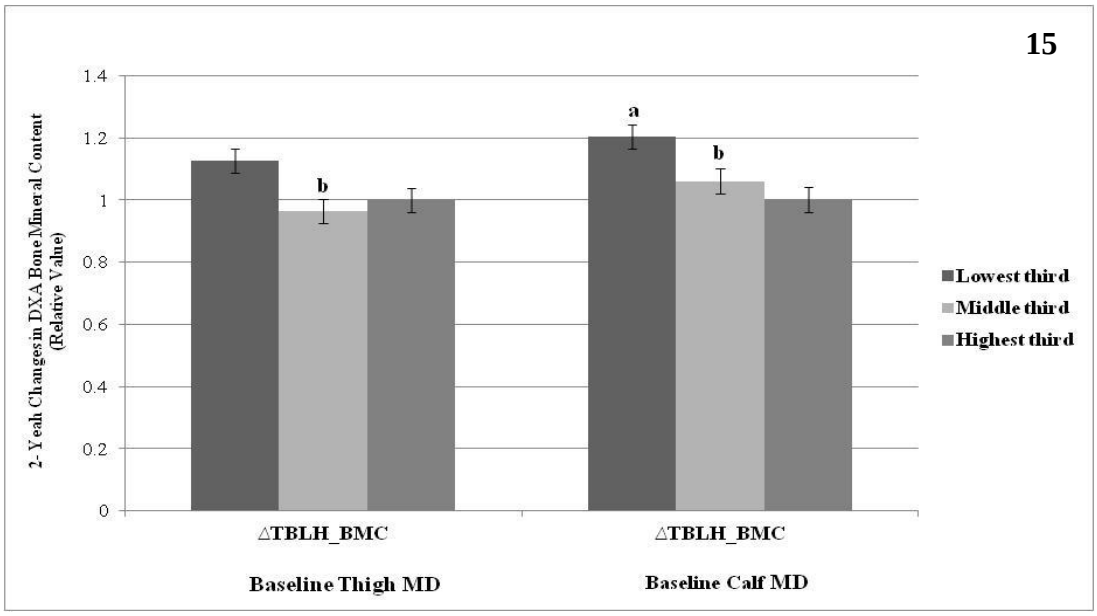


Figure15. Estimated marginal means±SE for change in DXA TBLH BMC by respective thirds of thigh and calf muscle density. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertile of muscle density were evaluated by ANCOVA using baseline covariates: maturity offset, bone length (thigh: femur bone length; calf: tibia bone length, respectively), and averaged covariates (baseline to 24 months): dietary caloric intake; dietary fat intake; physical activity score. MD= muscle density (mg/cm3); TBLH= total-body less head; BMC= bone mineral content (g);

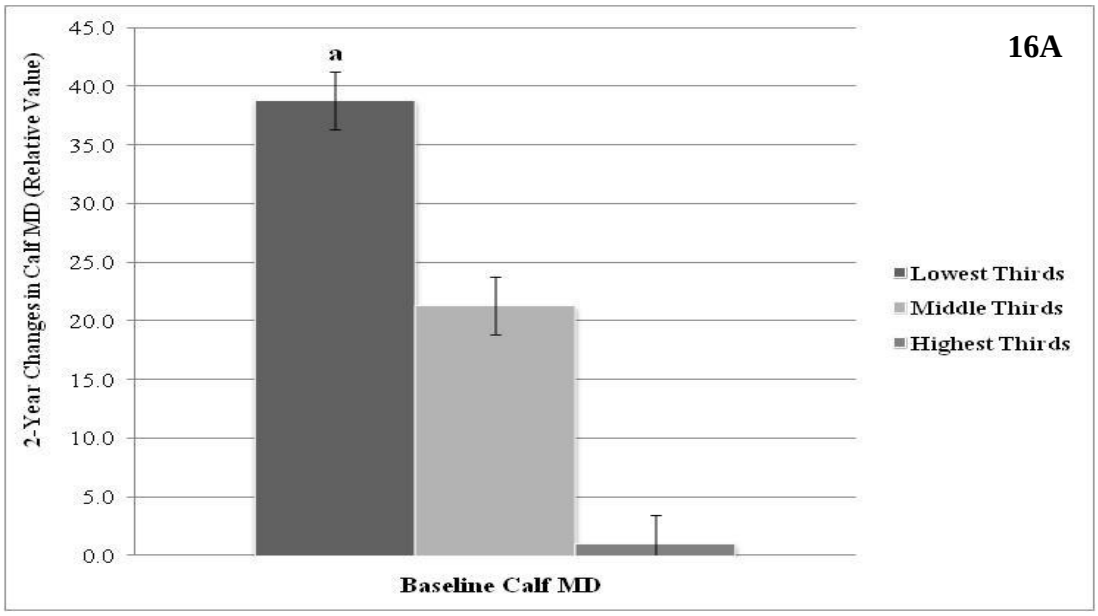
[a] Significantly (P<0.01) different from *highest* group; ANCOVA

[b] Significantly (P<0.05) different from *lowest* group; ANCOVA

In order to understand the inverse relationship between muscle density and changes in bone parameters, we also examined the change in MD across the tertiles of baseline MD of the thigh and calf (**figure 16**), after adjusting for baseline maturity offset, bone length (femur or tibia), whole-body lean mass, and average diet (calorie; fat intake) and physical activity. Girls in the lowest tertile of MD at baseline experienced the greatest gain in MD as well as the greatest gains in bone strength and density compared to girls in the middle and highest thirds (all $p<0.01$), as would be expected from our previous findings of a positive association between MD and bone strength [57], which

explains the seemingly paradoxical positive association between baseline muscle fat content and change in bone strength.

Estimated means (±SE) of the *change*s in the 2- year changes of adjusted covariates, maturity offset, diet (calorie, fat intake) whole body lean mass, bone length, physical activity, and additionally muscle-cross-sectional area (MCSA), a surrogate of muscle force/size. Interestingly, compared to the highest thirds, girls in the lowest thirds of thigh MD experienced the largest gains in femur bone length ($p<0.007$) as well as MCSA of the thigh (20% site) ($p<0.003$). Similarly, girls in the lowest versus highest thirds of calf MD experienced larger increases in tibia bone length ($p<0.005$), whole-body lean mass ($p<0.006$) and MCSA of the calf (66% site) ($p<0.002$) and were more advanced in skeletal maturation ($p<0.04$), which may partly explain the accelerated growth in bone parameters in these girls. Together, these results support our cross-sectional findings and confirm that larger gains in muscle density predicted greatest increases in bone strength and bone density at 2 years. Furthermore, because MCSA predominates as a stimulator of bone strength and bone density through the loading effect from dynamic strains from muscle contractile forces on the skeleton [28, 78], the larger growth in MCSA reported in the lowest versus highest MD may explain, in part, the largest increases in 2-year bone parameters.



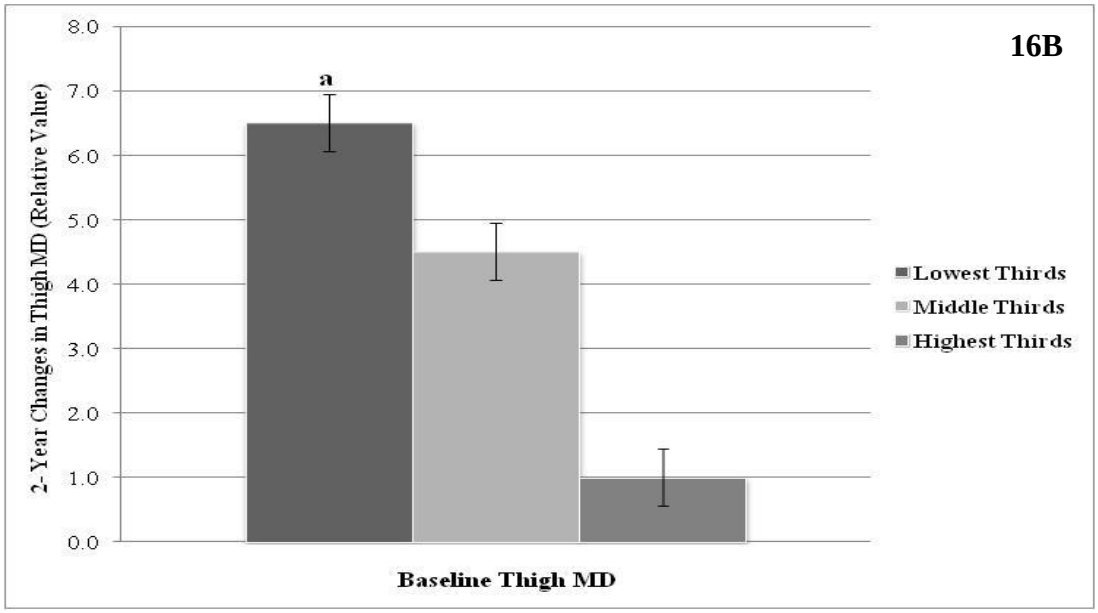


Figure 16. Baseline MD predicting 2-year changes in MD of the thigh (A) and calf (B). Estimated marginal means±SE for changes in thigh (panel A) and calf (panel B) muscle density across thirds of baseline thigh and calf MD. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline muscle density were evaluated by ANCOVA using baseline covariates, maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score. MD= muscle density (mg/cm^3).

[a] Significantly different ($P<0.0001$) from highest tertile; ANCOVA

Analysis of Bone Geometry:

Multiple linear regression analyses was performed on a subsample (n=236) of girls, with change in bone geometry as the dependent variable, with baseline measures of muscle density, maturity offset, bone length, average diet (calorie; fat intake) and average physical activity as independent covariates **Tables 11-12.** Results from regression show that baseline thigh MD was not significantly associated with periosteal circumference measured at the femur diaphyseal (20%) and distal metaphyseal (4% femur) sites, and endosteal circumference and cortical thickness of the femur diaphyseal region (all $p>0.05$). Calf MD showed negative associations with changes in periosteal circumference at diaphyseal (66% site) of the tibia ($r= -0.14$; $p<0.05$), but was not significantly associated with other bone geometry parameters at the distal metaphyseal (4% tibia) or diaphyseal regions.

In conclusion, our findings indicate that after controlling for linear growth, maturation, and important components of body composition (i.e., total body lean mass), girls who experienced the greatest gain in muscle density had the largest increases in bone density and strength compared to girls who did not significantly gain muscle density over the following 2 years. These findings support the premise that fatty infiltration of skeletal muscle contributes to suboptimal bone development in peri-pubertal girls.

Table 11: Bivariate relationships and partial correlations from multiple linear regression of baseline Thigh MD on changes in bone geometry (n=236)

Baseline Thigh MD	Pearson's r	partial r
4% Femur		
Δ PerCirc	-0.07	-0.07
20% Femur		
Δ EndCir	-0.05	-0.08
Δ PerCirc	-0.02	-0.04
Δ CortThick	0.08	0.08

Table 12: Bivariate relationships and partial correlations from multiple linear regression of baseline Calf MD on changes in bone geometry (n=236)

Baseline Calf MD	Pearson's r	partial r
4% Tibia		
Δ PerCirc	-0.13[b]	-0.11
66% Tibia		
Δ EndCirc	-0.09	-0.09
Δ PerCirc	-0.14[a]	-0.14[a]
Δ CortThk	-0.06	-0.05

All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density (mg/cm^3); EndCirc = endosteal circumference (mm); PerCirc = periosteal circumference (mm); CortThk = cortical thickness (mm). Model covariates= baseline covariates, maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score.

[a] P<0.05; Pearson's r for continuous variables.

Specific Aim 2: Longitudinal assessment of the effects of muscle quality and muscle size in predicting bone strength in young girls

Data were analyzed for 245 girls aged 9-13y to assess whether muscle size and muscle quality independently influence bone strength in addition to the influence of bone mineral mass in young girls. Baseline and 2-year descriptive statistics for the sample (n=245) are shown in table 12. Based on body mass index (BMI, kg/cm^2), 3.2% of the sample was underweight (BMI<5th percentile), 76% of the sample was healthy weight (BMI 5th to 85th percentile), 13% of the sample was overweight (BMI 85th to 95th

percentile), and 7.8% of the sample was obese (BMI>95th percentile) [148]. On average, girls were 1.1 years away from achieving PHV at baseline, ranging from 3.2 years prior to PHV to 1.04 years post PHV. Dietary fat and caloric intakes were consistent from baseline to 24-months. Average baseline caloric intake (1713±642 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans [234]. Average baseline calcium intake (including supplementation) (1015.6±437.3 mg) was 22% lower than the recommended levels (1300mg/d) established by the Institutes of Medicine [267] dietary recommendations.

Height, body mass, body mass index (BMI), femur and tibia lengths, total body lean mass, total body fat mass and lean mass all increased (all *P* values < 0.0001) from baseline to the 2-year follow-up as expected in young girls (**Table 13**). Calf and thigh MCSA and muscle densities, tibia and femur BMC ($p< 0.0001$) and bone strength indices also increased significantly ($p<0.0001$) over the 2-year period. Physical activity level (*1.8%*), total caloric intake (*5.1%*) and total calcium intakes (*5.6%*) did not change from baseline to 2 years ($p = 0.57$).

The associations between bone strength and muscle quality and the primary components of the "muscle-bone unit" (i.e., muscle force/size and bone mass) at weight bearing bone sites was assessed using pQCT in young girls. pQCT was used to measure changes in bone strength indices at distal metaphyseal and diaphyseal regions of the femur and tibia in addition to calf and thigh muscle density (MD; mg/cm^3), an index of skeletal muscle fat content reflecting muscle quality, and muscle cross-sectional area (MCSA; mm^2), surrogate of muscle force. Bone mineral content at diaphyseal regions of

the femur (20% site) and tibia (66%) was also measured by pQCT. It was hypothesized that muscle cross-sectional area and muscle density, would be predictors of bone strength, that in addition to bone mineral content, at weight bearing bone sites.

Table 13: Sample Descriptive Characteristics ($\bar{x}$± SD) at baseline and 2 years (n=245)

	Baseline (x±SD)	**24-Month (x±SD)**	**Percent Change**
Age	10.6±1.1	12.7±1.1	-
Maturity Offset	-1.1±1.0	0.7±1.0	-
Height (cm)	144.4±10.0	157.0±9.2	8.8
Body Weight (kg)	38.8±9.9	50.2±12.2	29.5
BMI	18.4±3.2	20.2±3.8	9.9
Femur Length (cm)	34.07±3.07	36.8±2.6	8.0
Tibia Length	33.2±2.9	36.4±2.5	9.8
Physical Activity Score	5291.7±4677.4	5196.7±4230.4	-1.8
Caloric Intake (kcal)	1713.0±642	1625.1±590.7	-5.1
Total Calcium (mg)	1015.6±437.3	958.4±423.2	-5.6
Lean Mass (kg)	25.5±5.1	32.2±5.6	26.2
Fat Mass (kg)	11.1±6.1	15.4±7.9	39.0
TBLH BMC (kg)	1.0±0.3	1.5±0.4	46.2
Thigh Muscle Density (mg/cm^3)	76.3±1.6	77.5±1.4	1.6
Calf Muscle Density (mg/cm^3)	79.0±1.2	80.0±1.2	1.2
20% Femur BMC (g)	230.1±48.1	297.6±61.6	29.4
20% Thigh MCSA (mm^2)	3555.5±716.3	4406.8±917.7	23.9
4% Femur BSI (mg^2/mm^4)	94.8±27.2	124.1±36.1	30.9
20% Femur SSI (mm^3)	1323.3±400.6	1888.6±523.5	42.7
66% Tibia Total BMC (g)	228.1±43.5	284.6±52.0	24.8
66% Tibia MCSA(mm^2)	3201.2±586.0	3878.8±667.1	21.2
4% Tibia BSI (mg^2/mm^4)	50.7±12.9	68.2±19.7	34.6
66% Tibia SSI (mm^3)	1159.7±327.8	1600.7±417.8	38.0

Values are presented as mean ± SD. Total calcium intake includes diet and supplement intake. *P* values represent paired samples *t*-Test for difference between the baseline and 2-year study visit; BMI = body mass index; MCSA = muscle cross-sectional area; TBLH= total body less head; BMC= bone mineral content (g); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3).

[a] All values significant at P<0.0001except physical activity score, calorie and calcium intake.

Multiple linear regression analysis was used to prospectively investigate the effects of bone mass, muscle quality, muscle size on 24-month bone strength in young girls. All regression models, which included calf or thigh muscle density, BMC and MCSA together, were used to test for potential independent associations between the muscle-bone unit components with 2-year changes in bone parameters. Each model was adjusted for influential covariates, which were calculated as the average values from baseline and 2-year data, and included maturity offset, bone length (femur or tibia), total-body fat mass, physical activity and dietary calorie and calcium intakes and the respective baseline bone parameter). Thigh muscle density, thigh MCSA and femur BMC were used together in all models that included femur bone parameters, and calf muscle density, calf MCSA and tibia BMC were included in all models that included tibia bone parameters. **Table 14** shows the partial r and *p* values for regression of averages of thigh and calf muscle densities and muscle-cross sectional area and BMC (femur and tibia) on 2-year changes in bone strength.

In models predicting bone strength at metaphyseal (4%) and diaphyseal (20%) regions of the femur, positive associations were observed between femur BMC and 2-year changes in femur BSI ($r=0.40$; $p<0.0001$) and femur SSI ($r=0.43$; $p<0.0001$)). Similar trends were noted between average tibia BMC and 2-year BSI ($r=0.44$; $p<0.0001$) and SSI ($r=0.60$; $p<0.0001$) at metaphyseal (4%) and diaphyseal (66%) regions of the tibia, respectively. Thigh MCSA was positively associated with change in femur SSI ($r=0.16$ $p<0.015$), and between average calf MCSA and tibia BSI ($r = 0.13$ $p<0.04$). Average thigh MD was not associated with any femur bone strength indices. Calf MD

was independently and negatively associated with tibia SSI (r= -0.11; p<0.08), but not with any other bone parameter.

Table 14: Independent associations from multiple linear regression of average muscle density, bone mass and muscle cross-sectional area on changes in bone strength

Dependent variables	**Average Thigh MD**		**Average Femur BMC**		**Average Thigh MCSA**	
	partial r	p-value	partial r	p-value	partial r	p-value
4% Femur ΔBSI	0.03	0.60	0.40	0.00	0.01	0.93
20%Femur ΔSSI	-0.04	0.56	0.43	0.00	0.16	0.01
Dependent variables	**Average Calf MD**		**Average Tibia BMC**		**Average Calf MCSA**	
	partial r	p-value	partial r	p-value	partial r	p-value
4% Tibia ΔBSI	-0.07	0.30	0.44	0.00	0.13	0.04
66% Tibia ΔSSI	-0.11	0.08	0.60	0.00	0.10	0.11

All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density; BMC= bone mineral content; MCSA = muscle cross-sectional area; BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3). All regression models were adjusted for covariates: maturity offset, bone length (femur or tibia), total-body fat mass, physical activity, calorie and calcium intakes and the respective baseline measure of bone strength.

*Significant at P<0.05; Pearson's r for continuous variables

ANCOVA, with averages of maturity offset, bone length, total-body fat mass, physical activity and dietary calorie and calcium intakes and the baseline bone parameter as covariates, was used to investigate possible differences in bone parameters assessed by pQCT among tertiles of bone mass (femur and tibia) along with muscle force and muscle densities of the calf and thigh. Analyses were also adjusted for the other muscle-bone component predictor variables at each respective bone site in order to understand how the different levels of the skeletal muscle parameters (i.e., muscle density and MCSA) or bone mass (i.e. BMC) relates to changes in bone strength. Girls in the highest third of

femur BMC gained 52% more femur BSI and 33% more femur SSI than girls in the lowest thirds of femur BMC (all $p<0.0001$); girls in the highest third of femur BMC also showed a significant 13% increase in femur SSI compared to girls in the middle thirds of femur BMC ($p<0.06$), whereas girls in the middle versus lowest thirds of femur BMC experienced a 43% increase in femur BSI and 23% increase in femur SSI over the follow 2-years (all $p<0.0001$). By contrast, girls in the lowest versus middle thirds of thigh MD gained 13% more SSI measured at the diaphyseal site (20% sites) of the femur ($p<0.05$). No significant differences in femur BSI or femur SSI were observed among tertiles of thigh MCSA over the 2-years ($p>0.05$).

Similar to trends observed across tertiles of thigh and femur muscle-bone unit components, 2-year gains in bone strength were also observed across tertiles of average calf MD and MCSA and tibia BMC (**figure 17)**. Specifically, Girls in the highest versus lowest thirds of average BMC of the tibia experienced the significantly larger increases in bone strength at the tibia BSI (37%) and tibia SSI (34%) (all $p<0.0001$), and similar 2-year gains in tibia BSI (by 17%; $p<0.06$) were found among the highest verses middle thirds of average tibia BMC. Furthermore, girls in the middle versus lowest thirds of tibia BMC gained 24% more tibia BSI over the following 2 years ($p<0.0001$). Interestingly, girls in the lowest thirds of average calf MD gained 13% more tibia SSI than girls in the middle thirds of calf MD ($p<0.03$). However, we did not observe any significant differences in the bone strength among tertiles of calf MCSA over the 2-year frame.

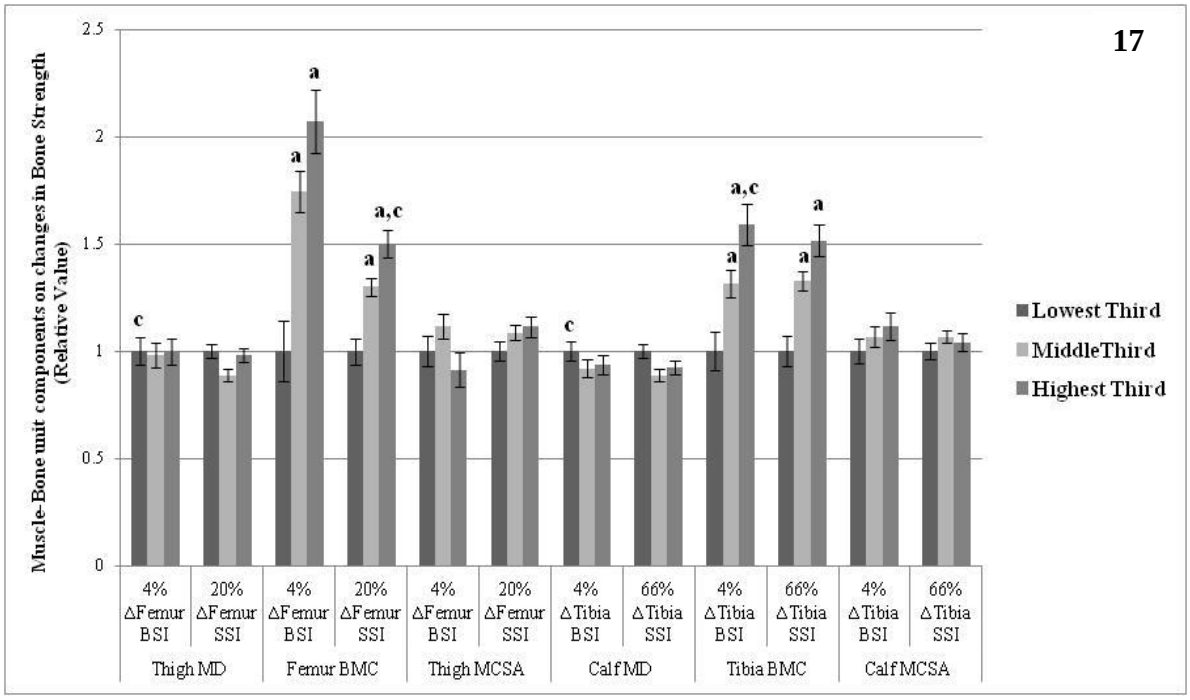


Figure 17. Estimated marginal means±SE for changes in femur and tibia bone strength indices across thirds of average muscle density, bone mass, and muscle size. All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density (mg/cm^3); BMC= bone mineral content (kg); MCSA = muscle cross-sectional area (mm^2); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3). Bone outcome values were normalized to the lowest group by setting the lowest group values to 1.0, while higher values set to greater than 1.0. Differences among groups across respective tertiles of the muscle density, bone mass and muscle cross-sectional area were evaluated by ANCOVA using average values for maturity offset, bone length (femur or tibia), total-body fat mass, physical activity and dietary calorie and calcium intakes and the respective baseline measure of bone strength.

[a] Significantly different than the lowest $p<0.01$
[b] Significantly different than the lowest $p<0.01$
[c] Significantly different than the lowest $p<0.05$

We also examined the changes in bone strength in girls who decreased in thigh (n=52) and calf (n=64) muscle densities versus girls who increased in thigh (n=193) and calf (n=181) muscle densities over the following 2-years. Changes in bone strength were larger in girls who increased versus decreased in muscle density at each weight bearing bone site, respectively. On average, larger changes in bone strength occurred at the distal metaphyseal (gain: 30.46±17.43 mg^2/mm^4; lost: 24.84±15.44 mg^2/mm^4) and diaphyseal

regions (gain: 569.19±216.97 mm^3; lost: 550.65±199.53 mm^3) of the femur regions compared to weight bearing sites of the tibia and in girls who gained versus decreased in thigh and calf muscle density **(Table 15)**.

Table 15: Mean (±SD) Changes in Bone Strength in Girls who Gained or Lost Muscle Density

	Δ Thigh MD	
Dependent Variable	Gained (n=193)	Lost (n=52)
4% Femur ΔBSI	30.46±17.43	24.84±15.44
20% Femur ΔSSI	569.19±216.97	550.65±199.53
	Δ Calf MD	
Dependent Variable	Gained (n=181)	Lost (n=64)
4% Tibia ΔBSI	18.33±9.57	15.24±9.93
66% Tibia ΔSSI	446.53±163.19	417.47±187.76

Values are presented as mean ± SD. All bone outcome variables were calculated as the change occurring from baseline to 24-months. BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3).

Analysis of Bone Density:

To examine the association between muscle bone unit components and bone density, multiple linear regression analyses was performed using change in bone density as the dependent variable, with average values for thigh and calf muscle density, femur and tibia muscle cross-sectional area and bone mineral content, and maturity offset, bone length (femur or tibia), total-body fat mass, physical activity and dietary calorie and calcium intakes and the respective baseline measure of bone geometry. Results showed that average femur BMC was positively associated with greater gains in bone geometry including femur total vBMD ($r=0.17$; $p<0.01$) and femur trabecular vBMD ($r=0.15$; $p<0.02$) but not with cortical vBMD at the diaphyseal femur site. Overall, the directions

of the associations between increased femur BMC and changes in femoral bone density parameters were similar to those observed between Tibia BMC and tibial bone density parameters. However, the magnitude of the associations at the tibia tended to be higher compared to associations at the femur (**Table 16**). In brief, positive relationships were found between tibia BMC and total vBMD ($r=0.15$; $p<0.03$) and trabecular vBMD ($r=0.20$; $p<0.002$). Neither femur BMC nor tibia BMC were associated with changes in cortical vBMD at the diaphyseal femur (20%) or tibia (66%) site. Notwithstanding these geometric adaptations, average MCSA and muscle density measured at the thigh (20% site) and calf (66% site), respectively was not significantly associated with any changes in total vBMD, trabecular, or cortical vBMD of the femur or tibia **(Table 16).**

Together, these findings demonstrate that on average, larger femur or tibia BMC, a measure of bone mass for a given age and maturity is associated with increases in bone strength and bone density at weight bearing bone sites during growth. Additionally, girls with larger muscle cross-sectional area (a surrogate for muscle force) was associated with greater increases in bone strength at the metaphyseal tibia and the diaphyseal femur bone sites. However, muscle density does not appear to be a significant, positive contributor to *gains* in bone strength when components of the muscle-bone unit are included in the model. Further analysis showed that girls who decreased in muscle density over the following two years did not significantly change bone strength, whereas girls who gained in thigh and calf muscle density experienced significant gains in bone strength and bone density at weight bearing sites of the femur and tibia, respectively. In addition, additionally, girls who increased calf MD experienced gains in tibia bone density parameters over the following 2-years. Together, these results suggest that subtle

adaptations in bone strength may occur in response to larger muscle force produced on the bone, while bone mineral accrual appears to be the strongest determinant of gains in bone strength growing children.

Table 16: Independent associations from multiple linear regression for average muscle density, bone mass and muscle cross-sectional area on changes in bone density

Dependent variables	**Average Thigh MD**		**Average Femur BMC**		**Average Femur MCSA**	
	partial r	p-value	partial r	p-value	partial r	p-value
Δ Femur Total Density (ave) (mg/cm^3)	-0.02	0.77	0.17	0.01	0.001	0.98
Δ 20% Femur Cort vBMD	-0.01	0.93	0.12	0.07	-0.02	0.82
Δ 4% Femur Trab vBMD	-0.02	0.76	0.15	0.02	-0.003	0.96

Dependent variables	**Average Calf MD**		**Average Tibia BMC**		**Average Tibia MCSA**	
	partial r	p-value	partial r	p-value	partial r	p-value
Δ Tibia Total Density (ave) (mg/cm^3)	-0.03	0.63	0.15	0.03	0.02	0.78
Δ 66% Tibia Cort vBMD)	0.08	0.20	0.06	0.34	-0.04	0.51
Δ 4%Tibia Trab vBMD	-0.07	0.26	0.20	0.002	0.12	0.08

All bone outcome variables were calculated as the change occurring from baseline to 24-months. BMC= bone mineral content; MCSA = muscle cross-sectional area; BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3); Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3). All regression models were adjusted for covariates: maturity offset, bone length (femur or tibia), total-body fat mass, physical activity, calorie and calcium intakes and the respective baseline measure of bone strength.

*Significant at $P<0.05$; Pearson's r for continuous variables

Specific Aim 3a: Assess the influence of n-3 and n-6 PUFA consumption on bone structure and bone strength in young girls.

Aim 3a was undertaken by to evaluate the independent influence of omega-3 (n-3) and omega-6 (n-6) long chain polyunsaturated fatty acids (LCPUFAs) consumption on bone structure (i.e., bone mass, bone density) and bone strength at diaphyseal and metaphyseal regions of the femur and tibia in young girls. It was hypothesized that higher intakes of n-3 FA will promote gains in bone strength, while higher consumption of n-6 FA will be negatively associated with bone development. Descriptive statistics for Aim 3a-b are given in **Table 17**. Based on body mass index (BMI, kg/cm^2), at baseline, 3.3% of the sample was underweight (BMI<5th percentile), 75% of the sample was healthy weight (BMI 5th to 85th percentile), 13.5% of the sample was overweight (BMI 85th to 95th percentile), and 8.2% of the sample was obese (BMI>95th percentile) [148]. On average, girls were 1.1 years away from achieving PHV, ranging from 3.2 years prior to PHV to 1.04 years post PHV. Average baseline caloric intake (1751.4±637.6 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans (DGA) [234]; average baseline calcium intakes (including supplementation) (1028.9mg±442.8mg) did not meet national recommended levels (1300mg/d) established by Institutes of Medicine [267] dietary recommendations.

Because epidemiological evidence is available for select FA, current national guidelines have established recommended intakes for FA that has been shown to modulate with disease outcomes. Recommendations for children and adolescents are relatively equal to those for adults and include ALA(1.0-1.2 g/d [234, 267], LA (10g/d) [234], omega-3 (EPA+DHA; 200-250mg/day) [234] and PUFA (500mg/d) [234, 267].

Table 17: Descriptive characteristics and pQCT bone parameters for sample (n=245)

	Mean
Caloric Intake (kcal)	1751±638
Ca^{2+} intake(mg)	1028.9±442.79
Maturity Offset	-1.14±1.03
Femur Length (cm)	34.05±3.05
Tibia Length (cm)	33.17±2.88
TBFM (kg)	11.17±6.07
TBLM (kg)	25.52±4.93
Fatty Acids	
ALA (g)	1.00±0.44
LA (g)	10.15±4.23
EPA (g)	0.02±0.02
DHA (g)	0.05±0.04
AA (g)	0.09±0.05
OMEGA-3 (g)	0.07±0.06
Total PUFA (g)	11.55±4.80
total n-3 (g)	1.07±0.46
total n-6 (g)	10.24±4.26
AA+omega3 (g)	0.16±0.09
AA/omega-3 (g)	2.05±1.56
LA/ALA (g)	10.45±2.06
total n-6/total n-3	9.84±1.97
Bone Measures	
Femur Metaphyseal site (4%)	
4% Femur BSI	95.27±26.94
Femur Total Density (ave)	275.43±33.69
4% Femur Trabecular Density (mg/cm^3)	237.78±31.97
Femur Diaphyseal site (20%)	
20% femur SSI	1323.07±392.71
20% Femur Cortical Density (mg/cm^3)	1045.16±23.45
Tibia Metaphyseal site (4%)	
4% tibia bsi	50.74±12.82
Tibia Total Density (mg/cm^3)	293.95±35.14
4% Tibia Trabecular Density (mg/cm^3)	222.44±25.91
Tibia Diaphyseal site (66%)	
66% Tibia SSI	1159.82±322.36
66% Tibia Cortical Density (mg/cm^3)	1027.53±31.55

[a:] Values include sources from diet and supplements
Dietary Recommended Intakes (DRI) established by the IOM **[267]** and DGA [234].
ALA, alpha-Linolenic acid (18:3n-3); PUFA, poly-unsaturated fatty acid; LA, Linoleic acid (18:2n-6); AA, Arachidonic acid (20:4n-6); omega-3 (EPA+DHA); total n-6/totaln-3, AA+LA/ALA+EPA+DHA

Clarity regarding recommendations for n-6/n-3 FA ratio is warranted as 5:1 n-6/n-3 is ideal; however, 10:1 n-6/n-3 FA ratio, which reflects the average ratio consumed in the Western diet, meets the upper level "safety" guidelines before it imposing an increased

risk for CVD and related disease outcomes. Suggested intakes (g/d) for individual n-3 (EPA, DHA) or n-6 FA (AA) have yet to be established (Table 2).

Understandably, the average intake of established recommended intakes of n-3 FA, including ALA (1.0 g/d±0.44), omega-3 FA (0.07g/d±0.05) by girls in this study was well below the reported recommendations; on average, PUFA intakes (11.55g/d ±4.8) met or exceeded recommended levels. In contrast, average intakes of LA (10.15g/d±4.23), and the n-6/n-3 ratio (9.8:1g/d ±1.7) met or were slightly higher than recommendations (**Table 22**; **APPENDIX F)**.

Multiple linear regression analysis was used to regress 2 year bone variables on baseline measures of select dietary fatty acids after controlling for important covariates. In each analysis, model covariates included baseline values of total calorie (kcal), total calcium (mg), TBFM, TBLM, bone length (femur or tibia) and the respective baseline bone measurement

Table 18 shows the partial r and *p* values for select dietary fatty acids on 24-month bone density parameters, along with adjusted R^2 values for regression models. Results from regression analysis indicated that a significant negative association was found between dietary intake of Arachidonic acid (AA) and cortical vBMD measured at distal regions of the femoral diaphysis ($p<0.05$; $r= -0.13$) but not with any other bone density or bone strength indices (**Table 18**). Other dietary fatty acids including EPA, DHA, ALA, LA, PUFA, omega3 (EPA+DHA), the sum of all LCPUFAs (AA+EPA+DHA), total n3 FA (ALA+ EPA+DHA),and total n-6 FA (LA+AA) were not significant with any 24-month indices of bone strength or bone density (all $p>0.05$).

Specific Aim 3b: determine the effects of ratios of n-6-to-n-3 LCPUFAs on bone strength and development in young girls.

Both n-3 and n-6 dietary fatty acids are necessary for growth and development. Given the increasing consumption of n-6 PUFA, and the limited intakes of n-3 PUFAS in typical western diets, specific Aim 3b was carried out in order to evaluate whether intake of higher LCPUFA ratios of n-6 to n-3 (n-6/n-3) FAs negatively influenced bone strength and bone development in girls. Specific ratios, chosen based on fatty acids that are commonly consumed by children and adults in North America, included LA/ALA, AA/ omega-3FA (EPA+DHA), and total n-6 (LA+AA)/ total n-3 (ALA+EPA+DHA). All dietary ratios were computed from the basic dietary fatty acid data collected from the YAQ. It was hypothesized that higher intakes of n-6/n-3 FA ratios would be inversely related to 2-year gains in bone strength and bone development parameters.

Table 18 presents the partial r, p-value, as well as the adjusted R^2 values for select multiple linear regression models that showed significant associations with 2-year changes in bone strength parameters. Adjustment for baseline covariates including total calorie (kcal), total calcium (mg), TBFM, TBLM, bone length (femur or tibia) and the respective baseline bone measurement, was conducted in all regression models. Dietary ratios, LA/ALA ($p<0.020$; $r=0.15$) and n-6/n-3 FA [AA+LA/ALA+EPA+DHA] ($p<0.01$; $r=0.17$) were positively related to average total vBMD of the tibia, but showed no favorable effect on other bone outcomes ($p>0.05$). The dietary ratio, AA/ omega-3FA, examined by multiple linear regression analysis were not significant with any bone strength indices.

Table 18: Regression coefficients for *select* baseline dietary fats regressed on 24-month bone density parameters

	AA		ratio LA/ALA		ratio total n6/total n3	
Bone Density	**Partial r**	**Model R^2_{adj}**	**Partial r**	**Model R^2_{adj}**	**Partial r**	**Model R^2_{adj}**
Femur Tot vBMD	0.02	0.78	0.01	0.78	0.00	0.78
20% Femur Cort vBMD (mg/cm^3)	-0.13[b]	0.54	0.04	0.53	0.04	0.53
4% Femur Trab vBMD (mg/cm^3)	0.07	0.76	0.00	0.76	0.00	0.76
Tibia Tot vBMD (mg/cm^3)	-0.08	0.76	0.15[b]	0.76	0.17[a]	0.76
66% Tibia Cort vBMD (mg/cm^3)	-0.03	0.62	-0.04	0.63	-0.04	0.62
4% Tibia Trab vBMD (mg/cm^3)	-0.01	0.77	0.04	0.77	0.03	0.77

All bone variables were normalized using log transformed, except for EPA and DHA, in which a square-root transformation was applied. Model covariates : baseline calorie intake (kcal), calcium intake (mg/day), maturation status, total body fat (kg), total body lean mass (kg), bone length (femur; tibia), and the respected estimated of the baseline bone outcome. Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); AA, Arachidonic acid (20:4n-6); ALA, alpha-Linolenic acid (18:3n-3); LA, Linoleic acid (18:2n-6); ratio total n-6/total n-3: LA+AA/EPA+DHA+ALA.

[a] P<0.01, Pearson's r for continuous variables

[b] P<0.05, Pearson's r for continuous variables

Comparison of bone parameters across groups of individual meeting dietary FA requirements

Analysis of Covariance (ANCOVA) was used to compare the 24M-bone parameters across groups of select FA intakes shows the adjusted marginal means (95% CI) for vBMD, geometry and bone strength indices across groups of select FA intakes that were categorized according to girls who met (FA=1) and did not meet (FA=0) recommended guidelines (**Table 23; APPENDIX F**). All models were adjusted for baseline maturity offset, bone length (femur or tibia), diet (calorie; calcium) and the respective baseline bone outcome variable.

Girls who consumed at least half the recommended values of omega-3 intakes had significantly higher total vBMD measured at the tibia but not with cortical or trabecular

vBMD sites or with any bone strength indices measured at the femur or tibia. Girls who met required levels of ALA compared to those who consumed less than the recommendations did not significantly differ in bone strength and bone density at 24-months. Similarly, no significant differences among girls meeting or not meeting LA or total n-6/n-3 ratio requirements were found.

Ancillary Aim: To assess the relationship between android fat mass and visceral fat area at the umbilicus level, and develop an equation to predict visceral fat mass, measured by magnetic resonance imaging (MRI) at the umbilicus level from android fat mass measured by dual-energy X-ray absorptiometry (DXA), body composition and anthropometric data in young girls.

The objective of this study was to develop prediction equations for estimating visceral adiposity (VAT) measured by magnetic resonance imaging (MRI) using anthropometric variables and measures of abdominal fat mass from DXA in adolescents and young adults. The DXA derived prediction equations will help clarify that regional fat deposition can accurately estimate VAT, and is equally and strongly related to bone geometry, strength and development indices in adolescents and young adults.

Prediction equations for estimating visceral adiposity (VAT) measured by magnetic resonance imaging (MRI) were developed using anthropometric variables and measures of abdominal fat mass from DXA in a mixed cohort of adolescents and young adults. Prediction of VAT from a manually drawn region of interest (ROI) spanning the abdomen (L1-L4) was compared to manufacturer's default regions (trunk and android regions) to assess whether prediction for the default ROI was as good as a manual ROI.

Descriptive characteristics for the entire sample and by sex are shown in **Table 19.** The sample was comprised of 35 males and 35 females (n=70) and included 2 Asians, 7 African Americans, 15 Hispanic and 46 non- Hispanic white subjects. The sample was a

mixture of underweight (n=10), normal weight (n=43), overweight (n=15) and obese (n=2) individuals based on BMI. The mean weight for the entire sample was 64.4kg.

Table 19: Descriptive characteristics by Gender and for the Total Sample

	Total Sample (n=70)			Males (n=35)			Females (n=35)		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Age (years)	19.19	3.67	11.0-25.0	19.54	3.93	12.0-25.0	18.84	3.41	11.0-24.80
Weight (kg)	64.43	14.96	28.9-119.7	70.10	17.26	28.9-119.70	58.77	9.49	39.2-78.20
Height (cm)	169.43	10.91	138.9-198.6	174.73	11.21	138.9-198.6	164.12	7.62	146.4-177.3
BMI (kg/m^2)	22.28	3.94	14.5-38.1	22.76	4.58	14.5-38.10	21.80	3.18	16.5-28.0
Waist (cm)	75.60	10.14	46.3-114.5	77.93	11.35	46.3-114.5	73.27	8.30	50.4-93.10
Android fat mass (kg)[1]	0.99	0.79	0.16-5.10	0.87	921.18	0.16-5.10	1.10	622.12	0.26-2.56
L1toL4 fat mass (kg)[1]	1.43	1.14	0.21-6.72	1.24	1215.52	0.21-6.72	1.62	1033.67	0.33-4.60
DXA percent fat[1]	22.10	11.05	4.8-47.4	15.97	8.48	4.8-41.0	28.23	9.93	11.6-47.40
VFA (cm^2)	27.18	16.24	6.0-111.6	28.53	19.99	6.0-111.6	25.83	11.49	8.4-63.50

[1]Measured by DXA
[2]Measured by MRI

A base model to predict MRI VAT from demographic covariates (e.g., age, race, and gender) was initially generated using multiple linear regression (R_{adj}^2=0.03). Age was the only significant predictor of VAT **(APPENDIX E)**. DXA measures of android fat mass (AFM) and anthropometric covariates (weight, height, BMI, waist) were then added (stepwise) to test their additional contributions **(APPENDIX E)**. Because AFM and L1-L4 are highly inter-correlated (r=0.982), they were tested in separate models for estimating VAT. Inclusion of anthropometry (weight, height, waist) to the base model significantly improved the R_{adj}^2 from 0.03 to 0.38, and reduced the SEE (cm^2) from 15.99

to 12.76 cm^2; in this model, only the anthropometric covariates, waist ($p<0.003$) and height ($p<0.004$) (and none of the demographic variables), were significant.

A base model to predict MRI VAT from demographic covariates (e.g., age, race, and gender) was initially generated using multiple linear regression ($R_{adj}^2=0.03$). Age was the only significant predictor of VAT. DXA measures of fat mass and anthropometric covariates (weight, height, BMI, waist) were then added (stepwise) to test their additional contributions **(Table 24-25; APPENDIX F,)**. Because AFM and L1-L4 are highly inter-correlated ($r=0.98$), they were tested in separate models for estimating VAT. Inclusion of anthropometry (weight, height, waist circumference) to the base model significantly improved the R_{adj}^2 from 0.03 to 0.38, and reduced the SEE (cm^2) from 15.99 to 12.76 cm^2; in this model, only the anthropometric covariates, waist circumference ($p<0.003$) and height ($p<0.004$) (and none of the demographic variables), were significant.

The model using only DXA-derived AFM ($p<0.0001$) explained 57% of the variance in VAT by MRI. Results from stepwise regression showed that only gender ($p<0.013$) was a significant predictor of VAT and inclusion of gender in the AFM model improved the R_{adj}^2 from 0.57 to 0.60 and reduced the SEE (cm^2) from 10.70 to 10.30 cm^2. Addition of body weight, ($p<0.046$) resulted in a further improvement in the prediction of VAT, increasing the $R_{adj}^2=0.62$ (SEE=10.06cm^2). Further analysis suggested an interaction between gender and AFM ($p<0.062$). The final model to predict VAT using AFM, gender and the interaction term explained 61% of the variance in VAT (SEE=10.10cm^2) **(Table 20)**.

Table 20: Multiple Regression Equations for Estimating VAT from AFM

Independent Variables	Regression Equation	R_{adj}^2	SEE (cm^2)
AFM	VAT=0.016AFM* + 11.815	0.57	10.70
AFM+Gen	VAT=0.016AFM + 6.360Gen* + 1.765	0.60	10.30
AFM+Gen+WT	VAT=0.019AFM - 4.880Gen* + - 0.245WT* + 24.08	0.63	10.06
AFM + Gen + (Gen x AFM)	VAT=0.015AFM* + 0.113Gen - 0.003(G x AFM)* + 12.78	0.61	10.10

VAT, visceral adipose tissue; AFM, android fat mass; Gen, gender; WT, weight
* Significant at $p \leq 0.05$

The model using only DXA-derived L1-L4 ROI ($p<0.0001$) explained 50% of the variance in VAT. Inclusion of gender ($p<0.014$) to the L1-L4 model increased the variance explained by the model to $R_{adj}^2 = 0.54$, and reduced the SEE from 11.51cm^2 to 11.08cm^2; however, in L1-L4 models, no other anthropometric or demographic variables were significant predictors of VAT. Further analysis revealed a significant interaction between gender and L1-L4 (gender*L1-L4). The final model to predict VAT using DXA L1-L4, gender and the interaction term explained 59% of the variance, and reduced SEE to 10.39cm^2 **(Table 21)**.

Table 21: Multiple Regression Equations for Estimating VAT from L1-L4 ROI

Independent variables	Regression Equation	R_{adj}^2	SEE (cm^2)
L1_L4 fat mass	VAT=0.010 L_{1-4}FM* + 12.653	0.50	11.51
L1_L4 fat mass+Gen	VAT=0.011 L1-4FM* - 3.40Gen* + 11.921	0.54	11.08
L1_L4 + Gen+ (Gen x L1_L4)	VAT= 0.010 L_{1-4} FM* + 1.883Gen - 0.004(Gen x L1-4 FM)* + 13.45	0.59	10.39

L_1-$_4$FM, L1-L4 fat mass; Gen, gender
* Significant at $p \leq 0.05$

Models including AFM were, overall, better at predicting VAT than L1-L4 ROI, and for any given model; AFM explained 2% to 7% more variance in VAT compared to L1-L4 ROI. In summary, DXA AFM and L1-L4 ROI provide acceptable estimates of VAT in adolescents and young adults. Estimation was improved with the inclusion of gender and weight in models with AFM. Because gender appeared to be a moderator in the prediction of VAT, particularly with DXA L1-L4, the utility of different DXA ROIs to predict VAT may be dependent on gender, an issue that needs investigation. AFM was a better predictor of VAT than the manually drawn L1-L4 ROI, although the difference was not large. Therefore, the combination of DXA-derived fat mass in the L1-L4 or android regions of interest with anthropometric measures (i.e., weight) are accurate methods of estimating visceral adipose tissue.

CHAPTER 5

DISCUSSION, SUMMARY AND CONCLUSIONS

This dissertation adds a number of novel findings to the knowledge base. In the study examining the effects of total body and regional fat masses on bone development (Aim 1a), results showed that larger levels of total body and abdominal adiposity are inversely associated with cortical vBMD measured at the diaphyseal regions of the femur and tibia. These results are consistent with cross-sectional findings from Taes et al [268] and Pollock et al [131], and longitudinal findings reported by Foley and colleagues [21] who specifically showed that weight gain from gain in fat mass was negatively associated with hip geometry, bone accrual and height-adjusted BMD in young children. Subsequent analysis suggested an inverse and curvilinear relationship between total body and android fat masses and cortical vBMD at the tibia, whereas a curvilinear, positive effect was observed between fat masses and femur trabecular vBMD. Thus, while moderate levels of adiposity may augment trabecular bone development, higher levels of adiposity may impair further cortical bone development in young girls. Importantly, the relationships between fat masses and bone strength and bone density were generalizable to bone geometry. TBFM and AFM were positively associated with periosteal circumference and endosteal circumference measured at the diaphyseal femur, as well as with periosteal circumference measured at the distal metaphyseal femur. Similar to the changes observed at the femur, baseline TBFM and AFM, were both positively associated with change in periosteal circumference of the diaphyseal tibia; positive associations between AFM and endosteal circumference of tibia were also found. By contrast, inverse associations were noted between TBFM, but not AFM, and cortical thickness at the femur diaphysis.

Together, these results show that higher levels of fat masses, particularly in abdominal regions, are detrimental to bone density, particularly at the diaphyseal region of the tibia. Furthermore, the negative associations between total body and android fat masses and femur cortical thickness suggest a possible mismatch in growth in periosteal and endosteal circumferences that may lead to impaired bone development and lesser bone strength.

In the study aimed to examine the associations of skeletal muscle density, surrogate for skeletal muscle fat content, with 2-year changes in bone development outcomes (Aim 1b), our results showed that larger increases in skeletal muscle density (and thus lower skeletal muscle fat content) predict greater 2-year increases in weight-bearing bone density and strength in girls. Regression results showed that baseline calf and thigh MD were inversely associated with BSI measured at the distal femur and tibia. Baseline thigh MD was inversely related to changes in femur total vBMD and trabecular vBMD, and similarly between baseline calf MD and changes in tibia SSI, total vBMD and trabecular vBMD. Follow-up analysis showed that girls who experienced the largest gains in muscle density over the following 2-years, had significantly greater increases in femur and tibia BSI, total femur vBMD and femur trabecular vBMD, compared to girls who did not significantly change in muscle density over the following 2-years. These results are consistent with a previous cross-sectional reported from our laboratory [57] as well as studies reported by Yerges-Armstrong and colleagues [47] who found that an index of skeletal muscle fat content, measured by pQCT, was inversely associated with vBMD of the tibia in older Afro-Caribbean men . Together, these findings suggest that

that fatty infiltration of the skeletal muscle may hinder optimal bone development during growth and may be an important risk factor for the development of fractures later in life

Evidence from the analysis that examined components of the muscle-bone unit on bone strength in growing children (Aim 2) showed that larger muscle cross-sectional area (a surrogate for muscle force) was associated with greater increases in bone strength at the metaphyseal tibia and the diaphyseal femur bone sites. Muscle density was not a significant contributor to *gains* in bone strength and bone density when other components of the muscle-bone unit were included in the model. Further evaluation showed that girls in the lowest thirds of thigh and calf muscle density had significantly higher femur and tibia (by 13%) bone strength at diaphyseal sites, compared to girls in the middle thirds of average thigh and calf muscle density (all $p<0.05$). In subsequent analysis, girls who decreased in muscle density over the following two years did not significantly improve bone strength or bone density, whereas girls who gained in thigh and calf muscle density gained bone strength at each weight bearing bone sites. As expected, BMC, a measure of bone mass, was associated with 2-year increases in bone strength and bone density parameters (except tibia cortical vBMD) at the distal metaphyseal and diaphyseal regions of the femur and tibia during growth. Strong linear relationships between BMC and muscle development in children and adults have been previously reported by Schenau and Frost (2008), suggesting that BMC is a function of muscle force or size [7, 269]. Furthermore, skeletal muscle force is well-established determinant of bone strength, and muscle contractile forces on bone are a function of muscle size, as previous reports by Petit et al (2005) indicate that higher muscle size generally parallels gains in muscle strength [84]. Our longitudinal findings support previous findings by Schoenau and

Rauch [269] as well as cross-sectional findings [57, 247] from our lab that suggest a close relationship between muscle contractile force and size and quality and bone strength. Together, these results suggest that subtle adaptations that lead to strengthening of bone (through increased bone mineral mass) likely occur in response to the loading stresses from skeletal muscle.

After evaluating the effects from consuming various long-chain polyunsaturated fatty acids (LCPUFAs) on indices of bone strength and bone development (aim 3), our evidence showed that higher consumption of AA was inversely associated with cortical vBMD measured at distal regions of the femoral diaphysis where as both LA/ALA and the ratio of n-6/n-3 FA were positively related to tibia total vBMD. Girls who met at least half the recommended values of omega-3 intakes had significantly higher total vBMD measured at the tibia. Girls who met required levels of ALA compared to those who consumed less than the recommendations did not significantly differ in bone strength and bone density at 24-months. Similarly, no significant differences among individual meeting or not meeting LA or total n-6/n-3 ratio requirements were found. The results of this study suggest that the role of n-3 FAs appear to neither benefit nor harm bone strength and development in young girls. Decreased intakes of AA n-6 FA may benefit bone health, where as consuming recommended ratios of select n-6 to n-3 FA ratios may benefit skeletal development in young girls.

DISCUSSION

The incidence of osteoporosis is projected to triple by the year 2040, reflecting increased longevity and unhealthy lifestyles. There is mounting evidence that suggests that bone mass tracks throughout life, as observations from recent studies indicate

longitudinal tracking of bone mass occurs across the pubertal growth spurt [4, 21, 270, 271]. In support of this notion, data from several longitudinal "bone tracking" studies suggest that osteoporosis-prone individuals can be identified even before puberty by virtue of having bone density values low for their age [270, 271]. Results by Foley et al (2009) showed that alterations in body composition is a significant contributor of altered tracking, in which weight gain from gain in fat mass was negatively associated with hip geometry, bone accrual and height-adjusted BMD in young children [21]. The degree to which body composition and environmental factors influence bone development remains unclear; nevertheless, prevention strategies including modification of lifestyle factors should begin before the onset of puberty and be maintained throughout this period of rapid growth and beyond the attainment of sexual maturity, in order to promote maximal bone mineral accrual [50] and to reduce future risk. An innovative aspect of this study was the use of pQCT, which allowed examination of structural changes as well as changes in vBMD. Moreover, characterization of changes in bone development allowed us to assess whether skeletal benefits continue to accrue over 2 years and whether differences in soft-tissue composition impact bone adaptations in pre-pubertal and early pubertal girls.

Regional fat distribution, particularly visceral abdominal fat and skeletal muscle fat is consistently and strongly associated with metabolic abnormalities including insulin resistance, impaired glucose tolerance (IGT), type 2 diabetes and increased inflammation [272, 273] in both children and adults [37, 38, 136, 274]; evidence suggests that these processes may be involved in maintenance of bone mass and structure [275], though the association between excess adiposity and bone in children remains complex. The

relationship is likely multi-factorial and influenced by variability in fat distribution, as different fat depots and ectopic fat have varying consequences for bone [16, 38, 156, 275]. While the relationship between fat and bone may be more influenced by site-specific fat depots and ectopic fat, rather than total body fat per se [16, 146, 275], the longitudinal relationship between these so-called "pathogenic" fat depots and bone is unclear. Recent studies have shown that increases in adiposity in childhood, especially in abdominal visceral fat and skeletal muscle fat content, may impair skeletal growth and mineralization [37, 38, 129]. In support of this notion, inverse relationships between visceral adiposity (VAT) and indices of bone structure and strength have been shown in adolescents [38] and in young women [37]. Moreover, some studies have shown that fractures occurring in childhood are linked to alterations in metabolic parameters associated with increased deposition of visceral fat mass, which may serve as an early sign of potential skeletal insufficiency [44]. Evidence from this study showed that while TBFM and AFM were positively associated with indices of bone strength and trabecular vBMD, high levels of TBFM or AFM may negatively impact cortical density at weight bearing bone sites and predict suboptimal bone development at weight bearing sites. Likewise, a similar, differential pattern in the relationship between fat masses and cortical and trabecular bone compartments has been reported in pre-menopausal women and young men [275]. Notably, the results of this study demonstrate the potential differences in findings from cross-sectional [122] and longitudinal analyses and suggest a potential change in the fat-bone relationship when examining the longitudinal transitions from early childhood to adolescence [16, 124]. Indeed, our results are compatible with findings reported by Wey and colleagues [27], who showed that in children and

adolescents (aged 8 to18 years), positive correlations between fat mass and bone strength and bone geometry observed in a cross-sectional analysis were attenuated and subsequently reversed in participants who gained excess fat mass when examined at follow-up. Together, the results from this longitudinal analysis provide evidence that higher levels of fat mass may have detrimental effects on developing bone, and thereby challenge the traditional paradigm that increased fat mass, by mechanism of its mechanical loading is *always* beneficial to the growing skeleton. Mechanistically, fat has two opposing effects on bone; while the extra mechanical loading from fat on bone contributes to periosteal expansion [276, 277] and greater bone mass [121, 124, 276]; increased inflammation associated with excess adiposity contributes to bone demineralization, and bone loss. Accumulation of abdominal visceral adipose tissue, in particular, is associated with the release of adipocytokines and subsequently, the hepatic release of acute phase response proteins such as C-reactive protein (CRP). CRP-induced recruitment of macrophages stimulates the secretion of inflammatory cytokines including monocyte chemotactic protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1) [58, 136, 275, 278], tumor necrosis factor alpha (TNF-α) and most potently, interleukin-6 (IL-6), which can independently act to increase osteoclast mediated bone resorption rates [136, 190]; likewise, the higher circulating levels of CRP is associated with higher serum N-terminal telopeptide of type 1 collagen (NTX), a marker of bone resorption [279], and indicator of lower bone mass [280]. Future studies are needed to examine the effects of different pathogenic fat depots on both weight bearing and non-weight-bearing skeletal sites in order to determine whether site-specific differences exist between various fat depots and bone. Coincidently, the effects of adipose tissue on bone may be dependent

on the individual's metabolic status. Thus, studies that prospectively examine the impact of varying levels of fatness on *bone development* in normal weight, overweight and obese children with pre-diabetes and children with "metabolically benign" obesity will provide insight into the ramifications of fat mass and associated metabolic impairments, on bone strength and development. In addition, an analysis of the child's adipocytokine profile will help to clarify the potential mechanisms associated with metabolic dysfunction and skeletal insufficiencies [272, 275].

Body composition, as opposed to weight per se, may be the strongest determinant of bone throughout life, as lean mass is a well-established determinant of bone size, mineral content, geometry and skeletal architecture [23]. Importantly, the mismatch of bone strength and mineral accrual to body weight may partly explain why the incidence of fractures during puberty is higher in overweight and obese children compared to their normal weight peers [16, 23, 28, 43]. In children, skeletal adaptations may depend on appropriate gains in lean mass [78, 127] and not fat mass [28, 128]. Indeed, Petit and colleagues found that girls who gained weight in the form of fat mass over a six year period experienced greater gains in aBMD compared to weight-stable girls. Nevertheless, bone strength, reflected in DXA measures of bone cross-sectional area (CSA) and section modulus (Z, cm^3), was low relative to total body weight [78]. Similarly, longitudinal data reported by Wetzsteon et al (2008) showed that although bone strength was higher in overweight compared to normal weight children and was appropriate for their higher lean mass, it was low relative to their larger fat mass and body weight [28]. These findings are important, as they indicate that stronger associations exist between bone strength and lean mass than fat mass, thereby supporting the critical role of the muscle-bone unit and the

skeletal adaptive response to its mechanical loads. To our knowledge, no other studies have used pQCT to assess the association of skeletal muscle fat content to changes in bone density and strength in young girls during growth. Evidence from our study showed that increases in skeletal muscle density, a surrogate for skeletal muscle fat content, predict 2-year increases in weight-bearing bone density and strength in girls. In support of this notion, evidence in adults suggests that individuals with high muscle fat content also have higher marrow fat content [46], which has been linked to a weaker skeleton [281], which may contribute to decreased bone strength and increased risk of fractures [45]. Although the reduction of muscle relative to gains in total fat deposition and regional fat distribution is more prevalent with aging [281], it is possible that fatty infiltration of skeletal muscle may serve as an important risk factor for bone health status in children and adolescents. Future studies that that utilize imaging techniques with higher spatial resolution, such as high-resolution pQCT (HR-pQCT) in children and adolescents will help in understanding the changes in bone microstructural parameters. In addition, characterization of bone turnover biomarkers to determine differences in bone formation and resorption rates in individuals with varying levels of skeletal muscle fat will provide some understanding of the relationship between fat and bone and whether this association is influenced by increased muscle mass.

Bone is constantly adapting its strength to maintain stability to support the imposing mechanical loads, as the coupling between muscle and bone relate to the acquisition of peak bone mass, bone strength and the growing musculature [7, 282, 283]. Muscle mass and presumably, quality, are associated with skeletal density and geometry through the dynamic forces imposed on the skeleton. Given that bone mass is a primary

material component of bone strength, and muscle strength and quality are determinants of bone mass, it remains unclear whether muscle force and muscle quality influences other aspects of bone strength in addition to bone mass, which would contribute to increases in bone strength during growth. Our findings showed that while BMC was the strongest determinant of skeletal adaptations in bone strength, when included in models with other muscle-bone unit components, larger muscle cross-sectional area (a surrogate for muscle force) was independently associated with increases in bone strength at the metaphyseal tibia and the diaphyseal femur bone sites. However, muscle density was not a significant contributor to *gains* in bone strength when included in regression models alone, or in combination with other predictors of bone strength. Because subsequent analyses showed that when compared to girls who lost muscle density over the following two years, and therefore did not significantly change their bone strength, girls who gained muscle density improved bone strength at both femur and tibia sites. Together, these findings suggest that slight gains in muscle density may promote small gains in bone strength, which may reduce the fracture risk and the development of osteoporosis later in life. Our results are congruent with findings by Schenau and Frost (2008) who reported a strong linear relationship between BMC and muscle development in children and adults, suggesting that BMC is a function of muscle force or size [7, 269]. Our data showing that larger MCSA, a surrogate for muscle force, predicts increases in bone strength [283] beyond the influence of bone mass is also in accordance to the Frost Mechanostat theory. Likewise, these results support previous cross-sectional findings that have examined the association between MCSA and measures of bone density, structure, and strength [122, 284], in which MCSA was significantly correlated with indices of bone strength in girls.

Further, longitudinal data from our laboratory suggest that particularly in children and adolescents, skeletal muscle cross-sectional area is strongly to bone strength, and therefore serves as an important determinant of bone development in girls (Farr et al, under review).

The health-promoting effects of n-3 fatty acids in relation to their prevention of various chronic diseases (e.g., coronary artery disease, type 2 diabetes, rheumatoid arthritis, and cancers) have been studied extensively [53, 168, 228]. There is some data that suggests that the benefits of LCPUFAs may extend to bone, as increasing epidemiological evidence has shown that low intakes of certain types of fatty acids contribute to bone loss [168, 171, 189, 213, 224, 228, 285]. Currently, in the United States, intakes of omega 3 fatty acids (EPA+DHA) are suboptimal, whereas the western diet is replete with omega-6 fatty acids (n-6 FA) [286]. Given the current dietary shift from higher saturated fat (SFA) consumption to higher intakes of n-6 PUFAs and increased ratio of *n*-6:*n*-3 FA in western diet [287], the biological importance of PUFA intake on bone development and bone maintenance is critical to assess in humans.

An additional unique feature of this study was the application of a validated FFQ to investigate the longitudinal influences of various dietary LCPUFAs on indices of bone strength and bone development in young girls. Our results showed a positive association between LA/ALA and total n-6/n-3 FA and bone development indices and no association with n-3 FAs. Although data in youth are scarce, these findings are consistent with previous studies reporting significant correlations between AA with total body bone density in healthy young children [218]. Data from Women's Health Initiative similarly reported that higher consumption of *n*-6 FAs were associated with a modest decrease in

total fracture risk in post-menopausal women, while higher n-3 intakes (EPA+DHA) were associated with a small but significant increase in risk of fractures [232]; however, similar to our study, low intakes of marine based sources of n-3 fatty acids (EPA+DHA) and the lack of data on fish-oil or n-3 FA supplementation use might have limited the findings in this study. In contrast, positive association between n-3 fatty acids and total body and spine BMD was found in a cohort of healthy young men [49]. Coincidently higher ALA consumption was associated with lower hip fracture risk in women, but not men, participating in the Framingham Osteoporosis Study, where as protective associations between higher AA consumption and reduced hip fracture risk were observed only in men [236]. Likewise Macdonald et al (2004) concluded excess PUFAs or an imbalance between the n−6 and n−3 fatty acids was associated with bone loss at the lumbar spine and femoral neck [237]. In toto, the results from these studies suggest that both n-6 and n-3 FA are necessary for optimal bone development, however, the ideal balance of n-6 to n-3 ratio that is needed to elicit a positive response on the skeleton and whether skeletal sites respond differently to the various n-3 and n-6 LCPUFAs remains unknown. Further, it is possible that while the type and quantity of LCPUFA consumed influence bone, the impact of n-3 FA consumption for promoting positive changes in BMD may be better detected in more vulnerable populations, including youth who are undergoing the pubertal transition and maturation.

Mechanistically, n-3 and n-6 LCPUFAs have both immune-modulating and anti-inflammatory actions [169, 170, 288]. The composition of the LCPUFA consumed has a direct impact on the type, quantity and the inflammatory nature of the eicosanoid released into circulation. For example, the bone-regulatory actions of n-6 LCPUFAs, carried out

by n-6 metabolites, prostaglandin 2-series (PGE_2) and 4 series leukotrienes (LTB_4, LTC_4), can control both bone resorption and bone formation pathways in a biphasic, dose-dependent manor. In contrast, n-3 illustrates stimulatory effects on bone formation at both, cortical and trabecular bone sites, in part, by competitively regulating concentrations of n-6 AA in circulation, and increasing the expression of bone formation proteins (e.g., osteocalcin, alkaline phosphatase) and matrix synthesis proteins (i.e., collagen 1 fibers). Because girls in this study were consuming n-6 FAs slightly above recommended levels, the bone promoting immune-modulating effects of lower dose- n-6 may explain the positive associations between LA/ ALA and n6/n3 and bone density. This dietary trend may also explain why longitudinal findings do not support the previous cross-sectional results from our lab that suggested a potential benefit on bone from consuming higher amounts of n-3 PUFA and lower intakes of n-6 LCPUFAs (unpublished). Future analyses will benefit from objective measures of blood serum concentrations of eicosanoids, prostaglandins, cytokines and bone turnover biomarkers in order to elucidate whether an altered inflammatory cytokine production has direct effects on bone. Furthermore because findings from the literature are inconclusive and dietary recommendations for LCPUFAs have not been clearly established, further evaluation regarding the optimal intakes and dietary ratios of n-6/n3 FA to optimize peak bone mass is warranted. The development of an overall diet quality index and nutrient assessment for bone health specific to children may serve as clinical, non-invasive method to evaluate dietary intake in relation to pediatric bone development during periods of rapid growth.

CONCLUSIONS

Specific Aim 1a: High levels of TBFM or AFM are negatively associated with cortical density at weight bearing bone sites and may impair development of cortical bone and predict suboptimal bone development in young girls.

Specific Aim 1b: Increases in skeletal muscle density predict greater 2-year increases in weight-bearing bone density and strength in girls.

Specific Aim 2: Muscle cross-sectional area (a surrogate for muscle size and force) is independently associated with increases in bone strength at the metaphyseal tibia and the diaphyseal femur bone sites. Also, increases in muscle density predict bone strength over 2 years.

Specific Aim 3: n-3 FAs appear to neither benefit nor harm bone development and bone strength in young girls. Decreased intakes of AA n-6 FA may benefit bone health, where as consuming recommended ratios of select n-6 to n-3 FA ratios may benefit skeletal development in young girls.

Ancillary Aim*:* A combination of DXA-derived fat mass (L1-L4 and android regions of interest) and anthropometric measures (i.e., weight) provides acceptable estimates of VAT in adolescents and young adults.

LIMITATIONS

The main limitation of the longitudinal analyses (Aim 1-3) conducted in this study was that the sample was limited to pre- and early pubertal predominantly normal-weight girls, and the results may not generalize to other populations. It is therefore possible that the fat-bone (Aim 1a and 1b) and muscle-bone relationship (Aim 2) were underestimated compared to what would be found in predominantly obese population or in a sample of

girls with a larger range of fat masses. Nevertheless, we clearly showed that higher levels of adiposity (total body and android fat) predicted lower cortical density (Aim 1a) and larger gains in muscle density were associated with significantly greater gains in bone strength and bone density at weight bearing sites (Aim 1b). An additional limitation of the study was that the utilization of pQCT to estimate bone measurements was limited to weight bearing bone sites (femur and tibia). pQCT cannot be used to measure the axial skeleton (e.g. spine) and more proximal femur (hip) sites, which may represent skeletal sites that are more susceptible to fractures later in life. Thus, we were unable to determine whether the influence of total body and regional adiposity on weight bearing sites differed from non-weight bearing sites (i.e., radius, ulna). Another concern is that maturational differences may have influenced our results. Maturation has been previously assessed in our laboratory using two methods, Tanner staging [289] and maturity offset [290]. Both methods have potential limitations; the Tanner stage data was obtained from a self-report questionnaire [289], whereas the Mirwald et al. [290] algorithm predicts years from PHV from cross-sectional data. In an attempt to reconcile whether choosing one method over the other influenced results, separate analyses were conducted with Tanner stage and maturity offset as measures of maturation. Results were similar (data not shown), thus, we report analyses with maturity offset based on its stronger association with bone parameters in this sample [122]. Furthermore, utilization of maturity offset over Tanner provided an objective and practical solution for the measure of biological maturity or bone age assessment [245], an important factor in skeletal studies of children and adolescents because there is a large range of physical maturation among individuals of the same chronological age. Lastly, it is important to

note that during the 2-year period of the present study, 52% of the girls included in this analysis participated in a school-based exercise intervention. In order to determine if baseline and 2-year bone outcomes were influenced by the intervention, all analyses were conducted with and without adjustment for the intervention. Paired sample T-tests were also run using baseline and 2-year data for the following covariates: physical activity scores, total body and regional adiposity, lean mass and muscle density at weight at the femur and tibia sites. There were no significant difference in body composition and physical activity covariates between control and intervention participants and analyses with and without adjustment for intervention were similar; therefore, we did not include the intervention effect in any of the analyses. Nevertheless, we acknowledge that this approach may not have completely removed all potential bias.

Our analyses aimed at evaluating the impact of adiposity on bone development (Aim 1a) were potentially limited by our use of DXA measures of android fat as a surrogate for visceral fat was a limitation since DXA android fat includes both subcutaneous and visceral adiposity. Given that visceral adiposity is more strongly related to skeletal fragility and insufficiency in children [37, 38], using a more direct estimate of VAT, such as obtained from magnetic resonance imaging (MRI), may have provided stronger associations with bone strength and bone development parameters. Previous work in our laboratory showed strong correlations between MRI-estimates of VAT and DXA-android fat ($r=0.78$) supporting its use as a reasonable surrogate of VAT [265].

For the regional adiposity and bone analyses (specific aim 1a and 1b) the most obvious limitation was that blood serum specimens were not available, and thus,

appropriate control for differences in hormone levels (e.g., estrogen, IGF-1, GH) and markers of metabolic dysfunction (i.e., insulin, glucose, HbA1c) and inflammatory adipocytokines (adiponectin, IL-6, TNF-α), which may confound the relationship between adiposity and bone, could not be assessed. Given, that visceral adiposity stimulates the secretion of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) which are known to negatively affect bone formation rates, while enhancing bone resorption rates; thus another potential limitation of this dissertation was that it was not possible to investigate the relationships between adiposity and markers of bone formation (i.e., PINP, OCN) and resorption (i.e., NTX, CTX, TRAPb), serum bone-regulatory factors (e.g., RANKL, OPG, adiponectin, and sclerostin).

In the investigation between muscle density and 2-year bone outcomes (specific aim 1b and 2), one limitation of this study was assessing the relationship between skeletal muscle fat content and 2-year bone parameters. Previous studies have used a single computed tomographic (CT) slice of the midthigh to estimate adipose compartments; however, in this study, single slices at the 20% femur and 66% tibia sites relative to the respective distal growth plates of the nondominant limb were obtained. Compared to the midthigh, these regions have smaller depots of adipose tissue. Nonetheless, strong correlations among adipose compartments of the calf and thigh have been reported using magnetic resonance imaging (MRI) [291]. Furthermore, a potential limitation of using pQCT to assess skeletal muscle fat content includes its inability to distinguish between intra- and extramyocellular (IMCL and EMCL, respectively) fat compartments. Undoubtedly, a stronger positive relationship between increases in skeletal muscle density (or lower levels of skeletal muscle fat content) and 2-year gains in bone strength

and bone density outcomes may have been observed if a more accurate measure of intramyocellular fat content was used. Ideally, both compartments should be measured, as they are likely intercorrelated and correlated with total-fat content. Results from Sinha et al showed that both intra- and extramyocellular fat stores, measured via 1H nuclear magnetic resonance spectroscopy, are significantly increased in obese compared with non-obese youth and that insulin resistance is significantly correlated with both intra- (r=0.72) and extramyocellular (r=0.68) fat content [260]. Previous studies in adults using Proton magnetic resonance spectroscopy (MRS) in adults [259], as well as in youth [260] have indicated that composite measures of IMCL and EMCL such as skeletal muscle density, are sufficient indices of skeletal muscle fat content. Importantly, abdominal visceral adipose tissue (VAT) was shown to be significantly associated with both intra- (r=0.73) and extramyocellular (r=0.86) fat content, together suggesting that both intra- and extramyocellular fat stores are related to central adiposity and insulin resistance. Thus, surrogates of both intra- and extramyocellular fat stores serve as appropriate composite indices of fat content within skeletal muscle. Despite the high contrast resolution, and quantification of fat depots with accuracy comparable to chemical analysis [292-296] than pQCT, its high cost, and accessibility prevents its use in larger scale studies. The application of low radiation dose, relatively low cost, low-invasive and fast speed of pQCT makes it more valid for quantifying soft tissue composition, and a feasible tool to safely estimate regional fat compartments of the calf and thigh as well as bone mineral content, and bone structure, vBMD, and bone geometry, the major determinants of bone strength [6].

One potential limitation of the analysis examining the muscle-bone components on bone strength (specific aim 2), is the low variability in growth of muscle density in this population may partly explain the low influence of muscle density on bone strength and development in this sample. Because bone variables are represented as the change occurring from baseline to 24-months, and since, girls experience, it is likely that girls achieved their maximum MD at baseline, resulting in small increases in bone strength and bone density outcomes. Future studies that include a larger increased variability in muscle density may contribute a larger impact on bone strength in young girls and may therefore predict fracture risk and the development of osteoporosis later in life.

The main limitation of the analysis relating dietary fatty acid intake with bone strength and bone density outcomes includes the utilization of the validated YAQ to assess long-chain polyunsaturated fatty acid (LCPUFA) intake. However, assessment of dietary and nutrient intake is challenging, especially in children. Although the semi-quantitative, YAQ has been validated as a reliable and adequate tools to assess children and adolescent habitual intakes over time [251], it has not been validated specifically for individual FAs (i.e. n-3, n-6, EPA and DHA). Given this inherent limitation, future studies that use FFQs that are validated for essential micronutrients, such as n-3 and n-6 metabolites are necessary for the evaluating the relationships between dietary patterns and disease outcomes. Furthermore, vitamin D status was not assessed in this sample, as difficulties in obtaining accurate measures of vitamin D status from FFQ are partly explained by the large variability in measuring intakes of vitamin D from foods and/or supplements, as well as from fortified foods, and the lack of data regarding individual sun exposure [297, 298]. Moreover, blood serum specimens for hormones known to directly

stimulate osteoblast-mediated formation (i.e., estrogen, insulin-growth factor I (IGF-1) were not available, presenting another potential limitation to the study. Notably, although supplementation and dietary intakes were combined to determine the total fatty acid intake, future studies that include populations that consume larger variations n-3 rich foods may have further strengthened this study.

FINAL THOUGHTS

Efforts to optimize bone accrual and promote beneficial structural adaptations during childhood and adolescence are warranted. To address this problem, we need to work harder at fostering childhood gains in bone mineral. Additional controlled trials are needed to define the optimal activity "prescriptions" for skeletal health. Interventions aimed at decreasing fracture risk through maximization of bone mineral accrual during the growing years are based on the assumption that beneficial adaptations persist into adulthood. Ongoing research that investigates the determinants of bone strength and development and identifying factors that can optimize peak bone mass and allow for appropriate skeletal adaptations during growth during early life are a necessary contribution to the primary prevention of osteoporosis. Furthermore, the alarming trend towards increasing childhood obesity warrant for a better understanding of the potential adverse effect of obesity on skeletal development. Thus, prevention strategies conducted earlier in life, such as improvements in diet, nutrition, and body composition, factors that promote bone mineral accrual and bone density and reduce bone loss, can also help to reduce risk of developing metabolic co-morbidities such as type 2 diabetes later in life. Moreover, given the current lack of effective treatments for established osteoporosis in adults, the emphasis of prevention strategies that includes modifying lifestyle factors

should be optimized before the onset of puberty and must be maintained throughout this period of rapid growth beyond the attainment of sexual maturity, in order to optimize peak bone mass and reduce future risk of osteoporosis [50].

APPENDIX A: LADDU DR, FARR JN, LAUDERMILK MJ, LEE VR, BLEW RM, STUMP C, HOUTKOOPER LB, FARR JN, LOHMAN TG, GOING SB. LONGITUDINAL RELATIONSHIP BETWEEN WHOLE BODY AND CENTRAL ADIPOSITY ON WEIGHT BEARING BONE GEOMETRY, DENSITY, AND BONE STRENGTH: A PQCT STUDY IN YOUNG GIRLS. *OSTEOPOROS INT*. (under review).

Correspondence to:
Deepika Laddu
Department of Nutritional Sciences
The University of Arizona
1713 E. University Blvd. #93
Tucson, AZ, USA 85721-0093
Phone: 602-615-7338
Fax: 520-621-8170
E-mail: dladdu@gmail.com

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Mini Abstract

Longitudinal relationships between total body and central adiposity with bone development parameters were assessed in young girls. Total body and android fat masses were negatively associated with cortical bone density of the femur and tibia. In conclusion, higher levels of fat mass may have detrimental effects on developing bone.

ABSTRACT

Childhood obesity may impair bone growth but the relationship between adiposity and bone remains unclear. Failure to account for fat pattern may explain the conflicting results. **Purpose:** The objective of this study was to examine longitudinal associations of total body fat (TBFM) and android fat (AFM), with weight bearing bone parameters in young girls. Subjects included 260 girls aged 8-13y at baseline from the "Jump-In: Building Better Bones" study. At baseline and 2 years, peripheral quantitative computed tomography was used to measure bone strength index (BSI, mg^2/mm^4), strength-strain index (SSI, mm^3) and volumetric bone mineral density (vBMD, mg/cm^3) at distal metaphyseal and diaphyseal regions of the femur and tibia. TBFM and AFM were assessed by dual-energy X-ray absorptiometry. **Results:** Baseline TBFM and AFM were positively associated with changes in femur BSI (r=0.193; r=0.174), tibia SSI (r=0.184; r=0.142), and femur trabecular vBMD (r=0.196; r=0.203), and inversely associated with tibia cortical vBMD (r= -0.146; r= -0.172). Analysis of Covariance (ANCOVA) showed that girls in the middle thirds of TBFM and AFM had significantly higher femur and tibia cortical vBMD than girls in the highest thirds of TBFM and AFM, respectively. All results were significant at $p<0.05$. **Conclusions**: High levels of TBFM or AFM may negatively impact cortical density at weight bearing bone sties and predict suboptimal bone development at weight bearing sites. NIH/NICHD #HD-050775.

Keywords: regional adiposity; bone development; girls, volumetric bone mineral density (vBMD); peripheral quantitative computed tomography (pQCT).

INTRODUCTION

Puberty is a critical period of development, marked by increases in bone mass, lean mass and fat mass. Obesity during this period is associated with increased risk for developing metabolic comorbidities, such as type-2 diabetes mellitus (T2DM) and cardiovascular disease. Some data also suggests a link between childhood obesity and impaired bone growth. Overweight and obese children at a given age, for example, are over-represented in the number of fracture cases [35, 41]. The precise role of excess adiposity on bone remains unclear. Peak bone mass and bone strength achieved in adolescence or early adulthood are primary determinants of fracture risk [4, 21] and both indices are dependent on maximizing bone mineral accrual during growth. Optimal bone development during puberty is critical as any disturbances that disrupt normal bone development during this time can lead to suboptimal bone strength, and likely increased risk for developing osteoporotic fractures later in life. Despite evidence of a link between obesity and impaired bone health in adults and children [16, 35, 43], a positive fat-bone relationship [121, 124] is still widely accepted. Studies in children have shown that children who incur a fracture [23, 41] or repeated fractures have a high body mass index (BMI) or increased body fat mass [16, 43]. Reduced cortical thickness (relative to periosteal circumference), cortical area, and compromised bone strength and increased fracture risk have also been reported [27]. In one longitudinal analysis, Goulding et al (2000) demonstrated that in young girls (aged 3-15 years) [41] the detrimental effect of obesity on bone during the period of peak bone mass acquisition may persist up to 4 years from initial observations.

The relationship between excess adiposity and bone in children is complex and data are conflicting, suggesting a positive [30, 31], negative [34, 35], or null effect [36] of total fat mass on bone mass and density. The relationship is likely multi-factorial and influenced by variability in fat distribution, as different fat depots and ectopic fat may have different consequences for bone [16]. Indeed, recent studies suggest that increased visceral fat deposition in childhood may impair skeletal growth and mineralization. In support of this notion, some studies have shown that fractures occurring in childhood are linked to alterations in metabolic parameters associated with increased deposition of visceral fat mass, which may serve as an early sign of potential skeletal insufficiency [44] and the risk for future osteoporosis [37]. Some studies have shown that children with excess adiposity may experience dissociation between weight gain and bone mineral accrual [31], suggesting a mismatch between gains in body mass and appropriate skeletal adaptations during growth [23]. Body composition, as opposed to weight per se, may be the strongest determinant of bone throughout life, as lean mass is a well-established determinant of bone size, mineral content, geometry and skeletal architecture [23]. Importantly, the mismatch of bone strength and mineral accrual to body weight may explain, in part, why the incidence of fractures during puberty is higher in overweight and obese children compared to their normal weight peers [16, 23, 28, 43]. In children, skeletal adaptations may depend on appropriate gains in lean mass [78, 127] while fat mass may have no additive effect or even a negative effect on bone mass [28, 126], in contrast with the positive effect of weight-bearing itself [129, 131].

Few studies have prospectively analyzed the effects of soft tissue composition on bone development and changes in bone strength in children [17, 27, 28, 37, 124]. The

purpose of this study was to determine the relationships of total body and central adiposity with bone development reflected in changes in volumetric bone mineral density (vBMD), and indices of bone strength in young girls. Past studies of bone development in children have been limited by the use of dual-energy x-ray absorptiometry (DXA) [34, 35, 79], which is confounded by changes in bone dimensions during growth and does not provide estimates of bone geometry, an important component of bone strength and fracture risk. A unique feature of this study was the use of peripheral quantitative computed tomography (pQCT), a relatively low radiation dose imaging technique that, unlike DXA, provides 3-dimensional estimates of volumetric BMD and bone architectural features (cortical thickness, cross-sectional area; CSA) and can therefore capture modeling adaptations leading to structural and architectural changes during growth [78]. To our knowledge, no studies have assessed the longitudinal relationship between total and regional (abdominal) body fat and indices of bone strength from pQCT in peri-pubertal girls, the critical phase of bone mineral accrual. Given that metabolic derangements associated with obesity are closely related with a central (abdominal) rather than a peripheral (gluteo-femoral) fat pattern [38], and because previous cross-sectional observations have illustrated inverse associations between fat mass and weight-bearing bone strength in both young girls [57] and adults [47], we hypothesized that higher levels of total body fat and android fat, would be inversely associated with gains in bone strength in weight bearing bone sites in young girls.

METHODS

Participants

The study protocol was approved by the University of Arizona Human Subjects Protection Committee and the study was conducted in accordance with the Helsinki Declaration. All guardians and participating girls provided written informed consent. Details regarding recruitment of the subjects and the baseline bone, soft tissue, diet and physical activity characteristics of the sample have been published previously [57]. At baseline, 509 healthy girls (aged 8 to 13 years) who were enrolled in 14 elementary and 4 middle schools in Tucson, Arizona were recruited as participants in the Jump-In: Building Better Bones study [57]. Exclusion criteria included the inability to read and understand English and learning disabilities (identified by schools) that made it impossible to complete questionnaires or made it difficult to comply with assessment protocols. Individuals taking medications known to affect bone metabolism or who had been diagnosed with medical conditions, or with a disability that limited participation in physical exercise as defined by the Committee on Sports Medicine and Fitness were also excluded [299]. Bone and soft tissue composition measures were available on 444 girls [57] at baseline, after elimination of 65 pQCT scans due to the presence of motion artifact. Of those girls, 260 girls had 2-year assessments of soft tissue composition and pQCT bone parameters and were included in the present analysis.

Anthropometry

Measures of anthropometry (body mass, standing height, sitting height and bone lengths) were obtained following standardized protocols [242]. Body mass was measured (nearest 0.1 kg) using a calibrated scale (Seca, Model 881, Hamburg, Germany).

Standing height and sitting height were measured at full inhalation (nearest millimeter, mm) using a calibrated stadiometer (Shorr Height Measuring Board, Olney, MD). Femur length and tibia lengths were measured on the non-dominant leg. Femur length (nearest mm) was measured from the proximal aspect of the patella to the inguinal crease. Tibia length (nearest mm) was measured from the proximal end of the medial border of the tibial plateau to the distal edge of the medial malleolus. Baseline coefficients of variation (CVs) for femur and tibia lengths in our laboratory (n=444 girls) are 0.34% and 0.51%, respectively [57]. For each anthropometric variable, two measurements were taken and averaged. Both measurements were repeated if the first two trials differed by more than 4 mm for height, sitting height and bone lengths, and 0.3 kg for body mass, and the average of the second set of measures was used.

Physical Maturation:

Maturation was assessed using maturity offset over the more conventional method of Tanner staging due to its reliance on objective anthropometric measurements of linear growth. Maturity Offset is based on estimated years from peak height velocity (PHV) using Mirwald's equation, [245]. These algorithms include interactions among anthropometric measures (i.e., height, weight, sitting height, leg length) and chronologic age to derive a maturity-offset value. Positive maturity offset values represent years after PHV while a negative maturity offset value represents years before PHV. In Mirwald's sample, the maturity offset equation for girls explained 89% of the variance in years from PHV [245].

Dietary Assessment

Dietary fat (grams/day) and total caloric intake (kcals/day) were assessed at baseline and 24-months using the Harvard Youth/Adolescent Questionnaire (YAQ) [250]. The YAQ is a self-administered food-frequency questionnaire (FFQ) designed to assess usual dietary intake and dietary supplement use during the previous year. Acceptable validity and reproducibility of the YAQ have been established [250, 251] and it has been used to examine associations between nutrition and health-related outcome measures including bone density [252, 300]. Participants completed the YAQ with assistance available. YAQs were reviewed and coded by trained study staff following standard coding procedures [251]. Nutrient analysis was completed by Channing Laboratories (Boston, MA).

Physical activity (PA)

Physical activity (PA) was assessed by the Past Year Physical Activity Questionnaire (PYPAQ) [246], a survey of all sport and leisure-time physical activity in which the respondent engaged at least 10 times in the past year outside of physical education class. The PYPAQ has been slightly modified to include a more comprehensive list of 41 activities common to youth [247]. The modified questionnaire was administered in an interview with the participant and guardian. Total PYPAQ score was computed using a modified equation from Shedd and colleagues [248], which incorporated weight bearing load, frequency and duration of each activity [247].

Bone and Body Composition Assessment

pQCT- Bone Measures

Changes in bone geometry, bone strength and volumetric bone mineral density

(vBMD) were assessed using pQCT (XCT 3000, Stratec Medizintechnik GmbH, Pforzheim, Germany, Division of Orthometrix; White Plains, NY, USA) at the 4% and 20% femur and 4% and 66% tibia sites relative to the respective distal growth plates on the non-dominant limb. Bone parameters measured at distal metaphyseal regions of the femur and tibia included trabecular vBMD (mg/cm^3) and bone strength index (BSI, mg^2/mm^4) . Bone parameters measured at diaphyseal regions of the femur and tibia included cortical vBMD (mg/cm^3) and strength-strain index (SSI, mm^3). BSI, calculated as the total area x total $vBMD^2$ [255], provides an estimate of the bone's ability to withstand compression at metaphyseal regions. SSI is used to estimate the bone's ability to resist torsion and bending forces at diaphyseal regions. SSI is the integrated product of the geometric properties (i.e., section modulus) with the material properties of bone: Strength-strain index (SSI, mm^3)= , n ($[(r^2_i \times a)/r_{max}] \times$ (cortical vBMD/ND); section modulus is calculated as $(r^2_i \times a)/r_{max}$, where a is the area of a voxel (mm^2), r is the distance of a voxel from the center of gravity (mm), and r_{max} is the maximum distance of a voxel from the center of gravity (mm). The material properties of bone are calculated as the quotient of measured cortical density (cortical vBMD, mg/cm^3) and normal physiologic cortical density (ND,1200 mg/cm^3).

Scout scans were performed to locate the distal growth plates, with the scanner programmed to find the sites of interest based on skeletal lengths. Additional details regarding pQCT bone measurements, image processing, calculations, and analysis are published elsewhere [122, 254]. CVs previously reported from our laboratory [122] were less than 1.1% for vBMD, bone geometry, and indices of bone strength (i.e., BSI and SSI). pQCT data acquisition and analyses followed guidelines provided by Bone

Diagnostics, Inc. (Fort Atkinson, WI, USA). All pQCT scans were performed by a single operator, while a second operator analyzed all scans using the Stratec software (version 6.0). The pQCT instrument was calibrated and quality assurance procedures were completed daily in order to ensure precision of measurements.

Dual Energy X-ray Absorptiometry (DXA)

Soft tissue mass and composition, including total-body mass, total-body fat mass, and abdominal (android) fat mass were obtained from whole-body dual energy x-ray absorptiometry (DXA) scans using the GE Lunar Prodigy (software Version 5.60.003) fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). Android fat mass (AFM), available from the manufacturer's automated ROIs, is defined as the area enclosed between a demarcation immediately above the iliac crest to a second mark at 20% of the total distance between the iliac crest and the base of the skull. Subjects were positioned following the standard manufacturer protocols. All participants were scanned by a certified technician, and all analysis was performed by a single technician. The unit was calibrated daily according to the standard procedures for maintenance and use as recommended by the manufacturer. DXA CVs for precision for measuring soft tissue composition in our laboratory have been previously reported [262].

Statistical Analysis

Data were checked for outliers and normality using histograms, and skewness and kurtosis were calculated for all variables. Changes in bone variables, (e.g., bone density, bone strength and bone geometry) were defined as the difference between baseline and 24-month measurements for each bone outcome. All changes in bone variables were normally distributed; thus no transformations were applied. Descriptive statistics (means,

SDs, and ranges) were calculated for the entire sample. Since no significant changes in dietary intakes of calories or fat or physical activity were observed over the 2-year study period, the average dietary intake values and physical activity (average PYPAQ score) were used as covariates. Associations were estimated from bivariate correlations using Pearson's r for continuous variables in order to determine simple relationships between bone outcome variables and covariates. Multiple linear regression analysis was used to regress changes in bone variables on baseline measures of TBFM and AFM after controlling for baseline measures of maturity offset, height, total-body lean mass, and average physical activity and calorie and fat intakes. Linearity, normality and homoscedasticity of residuals were assessed. Collinearity between covariates (correlation criteria= VIF $\geq$ 10) was also evaluated and covariates with the lowest VIF were included in the model. All regressions included maturity offset rather than age as previous reports from our laboratory have shown it has a stronger relationship with bone parameters [122]. The effect of baseline TBFM and AFM on bone outcomes were evaluated as separate models to determine whether the relationships between regional soft tissue composition and bone parameters differed from whole body fat mass.

Analysis of covariance (ANCOVA) was used to compare bone outcomes among respective tertiles of baseline TBFM and baseline AFM after adjusting for baseline maturity offset and height, baseline measures of total-body lean mass, and average physical activity, calorie and fat intakes. Because of the differences in units for pQCT bone outcome variables, data presented were normalized to the highest tertile by setting the highest tertile to 1.0. Bonferroni post hoc tests were used to adjust for multiple comparisons among tertiles of baseline TBFM and AFM. The level of significance was

set at P<0.05 (two-tailed). All analyses were performed using the Statistical Package for the Social Sciences for Windows, Version 20.0 (SPSS, Chicago, IL,USA).

RESULTS

Descriptive Characteristics

Descriptive statistics for baseline and 2 year data are given in Table 1. Based on body mass index (BMI, kg/cm^2) at baseline, 3.5% of the sample was underweight (BMI<5th percentile), 75% of the sample was healthy weight (BMI 5th to 85th percentile), 13.5% of the sample was overweight (BMI 85th to 95th percentile), and 7.7% of the sample was obese (BMI>95th percentile) [148]. On average, at baseline girls were 1.2 years away from achieving PHV, ranging from 3.2 years prior to PHV to 1.04 years post PHV. Dietary fat and caloric intakes did not change significantly from baseline to 24-months (data not shown). Average caloric intake (1711±541 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans [234]. Average fat intake (31%±4.0%) met the dietary recommendations (25-35%) established by the DGA [234].

As expected in healthy growing girls, significant increases in height, body weight, body mass index (BMI), femur length, tibia length, total body lean mass, total body and android fat and lean masses, calf and thigh muscle densities and femur and tibia bone strength and bone density indices were observed (all p values < 0.0001) from baseline to the 2-year follow-up.

Associations between body composition with bone change outcomes

Baseline TBFM and AFM were both positively correlated with changes in BSI at metaphyseal regions of the femur (r=0.23-0.26, p <0.0001) and tibia (r=0.29-0.34, all p

<0.0001) and changes in SSI at diaphyseal sites of the femur (TBFM: r=0.24, p <0.0001; AFM: r=0.21, p <0.05) and tibia (TBFM: r=0.22, p <0.0001; AFM: r=0.18, p <0.05). Baseline TBFM was significantly correlated with change in vBMD at diaphyseal sites of the femur (r=0.27; p <0.0001) and tibia (r=0.13, p <0.035) and trabecular vBMD at the metaphyseal region of the tibia (r=0.19, p <0.003). Baseline AFM was significantly correlated with the change in diaphyseal femur vBMD (r=0.21, p <0.001) (table 2).

Results from the multiple linear regression analyses are shown in table 2, which illustrates the individual contribution (partial r) of whole body and android fat masses on bone parameters after controlling for influential covariates in comparison to the simple correlation (Pearson's r). In all models, bone outcomes were regressed on TBFM and AFM, respectively, after adjusting for baseline maturity offset, height or bone length, total body lean mass, diet (fat intake; calorie intake) and physical activity. Baseline measures of TBFM and AFM were both significantly and positively associated with changes in trabecular vBMD (all p <0.002) at metaphyseal regions of the femur, while negative associations were noted between TBFM and AFM and changes in cortical vBMD (p <0.020; p <0.006, respectively) at diaphyseal regions of the tibia. Significant, positive associations were found between TBFM and AFM and change in BSI measured at the metaphyseal region of the femur (r= 0.17-0.19; p <0.002-0.005). Similar trends were noted for change in SSI at diaphyseal regions of the tibia, which had positive relationships with baseline TBFM (r=0.18; p <0.003) and AFM (r=0.14, p <0.023), while no significant associations were observed between baseline TBFM and AFM and changes in BSI at metaphyseal regions of the tibia or between changes in diaphyseal regions of

the femur (SSI). For all multiple linear regression analyses, substitution for percent body fat gave similar results as total body fat mass (data not shown).

Comparison of bone parameters across tertiles of body composition

Estimated means (±SE) for the *change* in bone outcome parameters were compared across tertiles of baseline TBFM and AFM, using ANCOVA after adjusting for baseline maturity offset, height, total- body lean mass, and average diet (calorie; fat intake) and physical activity. Figures 1-2 show the normalized adjusted means (± standard errors) for change in vBMD (figure 1) and bone strength (figure 2) across tertiles of TBFM and AFM.

Changes in cortical densities measured at diaphyseal sites (20% femur, 66% tibia) were significantly higher for girls in the middle versus highest thirds of baseline TBFM ($p < 0.05$). Indeed, these relationships were generalized, as similar trends were present with AFM; specifically, girls in the middle compared to the highest thirds of AFM gained significantly more femur and tibia vBMD ($p < 0.01$). No significant differences were observed among tertiles of TBFM and AFM and changes in bone strength indices (all $p > 0.05$).

DISCUSSION

In this longitudinal analysis, we investigated the effects of total body and abdominal fat mass (android fat) on bone development in young girls, aged 8-13 years. The results show that during a period of rapid mineral accrual, higher amounts of total body and regional adiposity may put girls at risk for suboptimal bone development, particularly at the diaphyseal region of weight bearing bones where cortical bone predominates. Previous cross-sectional findings from our lab showed evidence of a

positive, albeit weak association between TBFM and indices of bone strength and vBMD at the diaphyseal femur and tibia. The results from the present longitudinal study do not necessarily conflict with earlier findings, as simple Pearson's correlations indicated that both TBFM and AFM were positively correlated with nearly all bone strength and bone density parameters assessed at metaphyseal and diaphyseal sites of the femur and tibia. Several of these relationships were modified after adjusting for important covariates. Relationships with femur BSI, tibia SSI, and femur trabecular vBMD remained positive and significant, whereas negative associations were found between TBFM and AFM and cortical vBMD at the tibia (66% tibia). Comparisons of adjusted models using ANCOVA, across tertiles of baseline TBFM and AFM, indicated that girls in the middle thirds of TBFM (p <0.05) and AFM (p <0.01) had 44-55% and 62-68%, respectively better bone outcomes than girls in the highest thirds of whole body and regional fat masses. These results suggest a curvilinear effect of total and regional fat mass on bone development; while moderate levels of adiposity may augment bone development, higher levels of adiposity may impair further bone development in young girls. These results are consistent with cross-sectional findings from Taes et al [268] and Pollock et al [131], and longitudinal findings reported by Foley and colleagues who specifically showed that weight gain from gain in fat mass was negatively associated with hip geometry, bone accrual and height-adjusted BMD in young children [21]. Together, these results suggest that despite the increased mechanical loading imposed by higher body weight on bone, girls with higher body fat may be experiencing a mismatch between gains in body mass and skeletal adaptations in vBMD and bone structural parameters during growth [122].

Consequently, adiposity above a certain as yet undetermined level may predict sub-optimal bone development beginning in pre- and early pubertal girls.

The relationship between whole body and regional fat and skeletal development remains inconsistent. Importantly, the findings of this study demonstrate the potential differences in findings from cross-sectional and longitudinal analyses and suggest a potential change in the fat-bone relationship when examining the longitudinal transitions from early childhood to adolescence [16, 124]. Indeed, our results are compatible with findings reported by Wey and colleagues [27], who showed that in children and adolescents (aged 8 to18 years), positive correlations between fat mass and bone strength and bone geometry observed in a cross-sectional analysis were attenuated and subsequently reversed in participants who gained excess fat mass when examined at follow-up. The effects of fat mass on bone were influenced by the opposing effects of age and maturation; whereas fat mass may exert a positive effect on bone in pre-pubertal children, in peri- and post pubertal and older girls, fat mass may exert detrimental effects on the developing skeleton [16, 27]. In the present study, girls in the middle thirds of fat mass had significantly greater gains in vBMD, particularly at cortical sites of the diaphyseal femur and tibia, than girls in the highest tertile. Presumably, children with lower TBFM and AFM were "metabolically healthy," whereas children in the highest third of fat mass may have begun to experience metabolic complications (e.g., metabolic syndrome). In a recent study examining the relationship between regional fat deposition and bone in young, pre-pubertal children, Pollock and colleagues (2010) showed that overweight prepubescent children with pre-diabetes had lower total-body BMC, compared to overweight children without pre-diabetes [17]. Inverse relationships

between visceral adiposity (VAT) and indices of bone structure and strength have also been shown in adolescent girls and young women, aged 15 to 25 years [37]; similar negative relationships between bone strength and bone density parameters and VAT may exist in children [83]. Together, these findings provide some evidence that bone development may depend on fat distribution independent of total adiposity, and further an increased fat mass that persists into adulthood may lead to skeletal impairment along with the better-known metabolic abnormalities (i.e., Type-2 diabetes).

Past investigations of the fat-bone relationship in youth have been limited by their cross sectional study designs, as the bone-adiposity relationship at a given age may not accurately reflect what occurs over time [35]. Further, longitudinal studies of the effects of adiposity on bone have been limited by the failure to account for fat pattern, as regional adiposity, especially abdominal adipose tissue (VAT) and fat within skeletal muscle, is strongly related to metabolic derangements that may impair bone [27, 28, 78]. To our knowledge, this is the first study that investigated the longitudinal relationship between abdominal (android) fat and changes in bone structure and bone strength in children and adolescents.

This study was not without limitations. First, the sample of pre-and early pubescent girls were primarily of normal weight status; it is possible that the fat-bone relationship was underestimated compared to what would be found if a more obese population was included in the study. Nevertheless, we clearly showed that higher levels of adiposity (total body and android fat) predicted lower cortical density. Also, our use of DXA measures of android fat as a surrogate for visceral fat was a limitation since DXA android fat includes both subcutaneous and visceral adiposity. Given that visceral

adiposity is more strongly related to skeletal fragility and insufficiency in children [37, 38], using a more direct estimate of VAT, such as obtained from magnetic resonance imaging (MRI), may have provided stronger associations with bone strength and bone development parameters. Previous work in our laboratory showed strong correlations between MRI-estimates of VAT and DXA-android fat (r=0.78) supporting its use as a reasonable surrogate of VAT [265].

A significant strength of this study was the use of pQCT for measuring indices of bone strength, including material (volumetric BMD; vBMD, mg/cm^3) and structural properties (cortical thickness, cross-sectional area; CSA) thereby avoiding the confounding of growth [4, 57]. Unlike DXA, with pQCT, the contributions of changes in bone mass, density, and geometry to changes in indices of bone strength can be safely and accurately estimated in young children. Also our longitudinal design improves upon the limitations of past cross-sectional studies, by providing an opportunity to examine prospectively how adiposity and specific fat depots commonly associated with metabolic co-morbidities may lead to sub-optimal bone development. An additional strength of this study was appropriate control of diet and physical activity, maturation using gender-specific algorithms by Mirawald et al [245] to predict years from PHV, and whole body lean mass by direct analysis using DXA, rather than indirect assessment of body composition [35, 79, 124].

In conclusion, our results demonstrated that after controlling for growth, maturation, body composition (i.e., whole body lean mass), diet, and physical activity, baseline measures of total body and android fat masses were negatively associated with changes in bone density, particularly at cortical bone sites at diaphyseal regions of

weight-bearing bones (femur and tibia). Although the positive relationship between body fat and bone have been shown in cross-sectional analyses, these results highlight the potential differences between cross-sectional and longitudinal analyses and provide evidence that higher levels of fat mass may have detrimental effects on developing bone. The findings suggest that changes in body composition, such as too much gains in total body adiposity, particularly in the abdominal region, during the pre- and early- pubertal years may be not only serve as an important risk factor for metabolic dysfunction but may also contribute to suboptimal bone development and skeletal fragility later in life.

TABLES

Table 1: Sample Descriptive Characteristics ($\bar{x}$± SD)

	Baseline (n=260)	**24-months (n=260)**	**% change[a]**
Age (years)	10.6 ± 1.1	12.7 ±1.1	-
Maturity Offset (years)	-1.2 ± 1.0	0.70 ±1.0	-
Height (cm)	144.1 ± 9.8	156.7 ± 9.1	8.7[a]
Weight (kg)	38.6 ± 9.8	50.0 ± 12.0	29.6[a]
BMI (kg/cm^2)	18.34 ± 3.2	20.2 ± 3.7	9.9[a]
Femur Length (cm)	34.0 ± 3.0	36.7 ± 2.5	8.1[a]
Tibia Length (cm)	33.1 ± 2.9	36.4 ± 2.5	9.9[a]
Total energy intake (kcal)	1719± 647	1703.8 ± 490.4	-0.9
Total fat intake (g)	60.2 ± 25.6	59.7 ± 21.5	-0.9
Physical Activity Score	5229.2 ± 4589.7	5263.7 ± 5593.5	0.7
Total body fat mass (kg)	11.0 ± 6.0	15.2 ± 7.8	38.9[a]
Total body lean mass (kg)	25.4 ± 4.9	32.0 ± 5.5	26.0[a]
Android fat mass (kg)	0.8 ± 0.5	1.0 ± 0.7	31.7[a]
TBLH-BMC (g)	1032.6 ± 315.0	1495.5 ± 418.2	0.4[a]
Thigh muscle density (mg/cm^3)	76.3 ± 1.5	77.5 ± 1.5	1.6[a]
Calf muscle density (mg/cm^3)	79.0 ± 1.2	80.0 ± 1.2	1.2[a]
Femur BSI (mg^2/mm^4)	94.5 ± 26.8	123.8 ± 36.2	31.0[a]
Femur SSI (mm^3)	1315.4 ± 389.7	1874.8 ± 508.1	42.5[a]
Femur Total Density (ave) (mg/cm^3)	275.1 ± 33.4	290.0 ± 40.6	5.4[a]
20% Femur Cortical Density (mg/cm^3)	1045.8 ± 23.1	1067.2 ± 32.5	2.0[a]
4% Femur Trabecular Density (mg/cm^3)	236.7± 31.9	246.5 ± 36.8	4.2[a]
Tibia BSI (mg^2/mm^4)	50.7 ± 12.8	68.1 ± 19.6	34.4[a]
Tibia SSI (mm^3)	1151.8 ± 320.8	1590.9 ± 408.4	38.1[a]
Tibia Total Density (ave) (mg/cm^3)	294.7 ± 34.7	322.4 ± 46.6	9.4[a]
66% Tibia Cortical Density (mg/cm^3)	1028.2 ± 32.4	1056.9 ± 37.6	2.8[a]
4%Tibia Trabecular Density (mg/cm^3)	222.3 ± 25.5	229.8 ± 30.7	3.4[a]

Values are presented as mean ± SD. *P* values represent paired samples *t*-Test for difference between the baseline and 2-year study visits. TBLH= total body less head; BMC= bone mineral content (g) BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3)

[a] Significant at P<0.0001

Table 2: Bivariate relationships and partial correlations from multiple linear regression of baseline TBFM and AFM on changes in bone parameters

	TBFM		**AFM**	
	partial r	Pearson's r	partial r	Pearson's r
Δ 4%Femur BSI	0.19[b]	0.26[a]	0.17[b]	0.23[a]
Δ 20% Femur SSI	0.11	0.24[a]	0.10	0.21[b]
Δ Femur total vBMD	0.06	0.26[a]	0.03	0.22[a]
Δ 20% Femur Cort vBMD	-0.05	0.27[a]	-0.09	0.21[b]
Δ 4% Femur Trab vBMD	0.20[b]	0.08	0.20[a]	0.09
Δ 4%Tibia BSI	0.10	0.34[a]	0.06	0.29[a]
Δ66% Tibia SSI	0.18[b]	0.22[a]	0.14[b]	0.18[b]
ΔTibia total vBMD	-0.02	0.31[a]	-0.04	0.27[a]
Δ 66% Tibia Cort vBMD	-0.15[b]	0.13[b]	-0.17[b]	0.09
Δ 4% Tibia Trab vBMD	0.09	0.19[b]	0.09	0.17[b]

All bone outcome variables were calculated as the change occurring from baseline to 24-months. TBFM=total body fat mass (kg); AFM= android fat mass (kg); BSI=bone strength index (mg^2/mm^4); Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); SSI=strength–strain index (mm^3). Model covariates: baseline maturity offset, height and total-body lean mass and average diet (calorie, fat intake) and physical activity score.

[a] P<0.001; Pearson's r for continuous variables

[b] P<0.05; Pearson's r for continuous variables

FIGURE LEGENDS

Figure 1: Estimated marginal means±SE for changes in femur and tibia volumetric bone density parameters across thirds of baseline total body (TBFM; A) and android (AFM; B) fat masses. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline fat masses were evaluated by ANCOVA using baseline covariates, maturity offset, height, total body lean mass, and average diet (calorie, fat intake) and physical activity score. Tot BMD= total (average) volumetric bone mineral density (mg/cm^3); Cort BMD= cortical volumetric bone mineral density (mg/cm^3); Trab BMD=trabecular volumetric bone mineral density (mg/cm^3).

[a] Significantly different ($P<0.01$) from *highest* tertile; ANCOVA

[b] significantly different ($P<0.05$) from *highest* tertile; ANCOVA

Figure 2: Estimated marginal means±SE for changes in femur and tibia bone strength indices across thirds of baseline total body (TBFM; A) and android (AFM; B) fat masses. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline fat masses were evaluated by ANCOVA using baseline covariates, maturity offset, height, total body lean mass, and average diet (calorie, fat intake) and physical activity score. BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3).

FIGURES

Figure 1A) Total Body Fat Mass

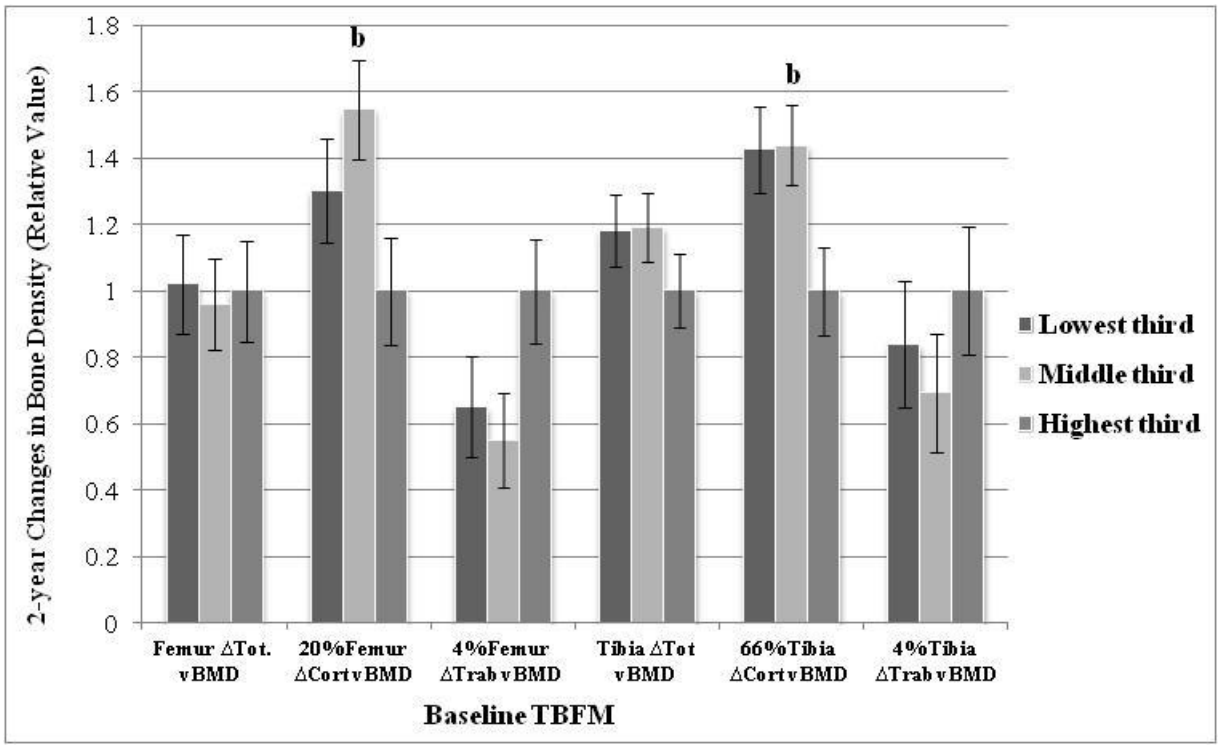


Figure 1B) Android Fat Mass

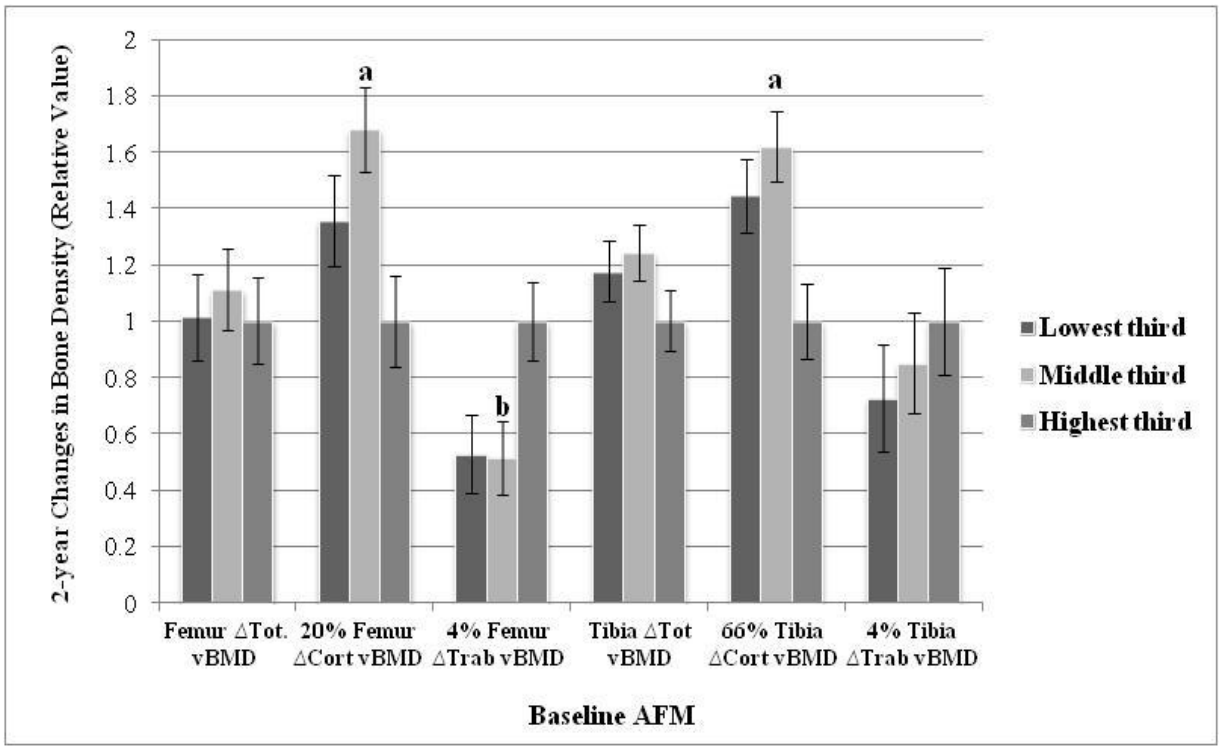


Figure 2A) Total Body Fat Mass

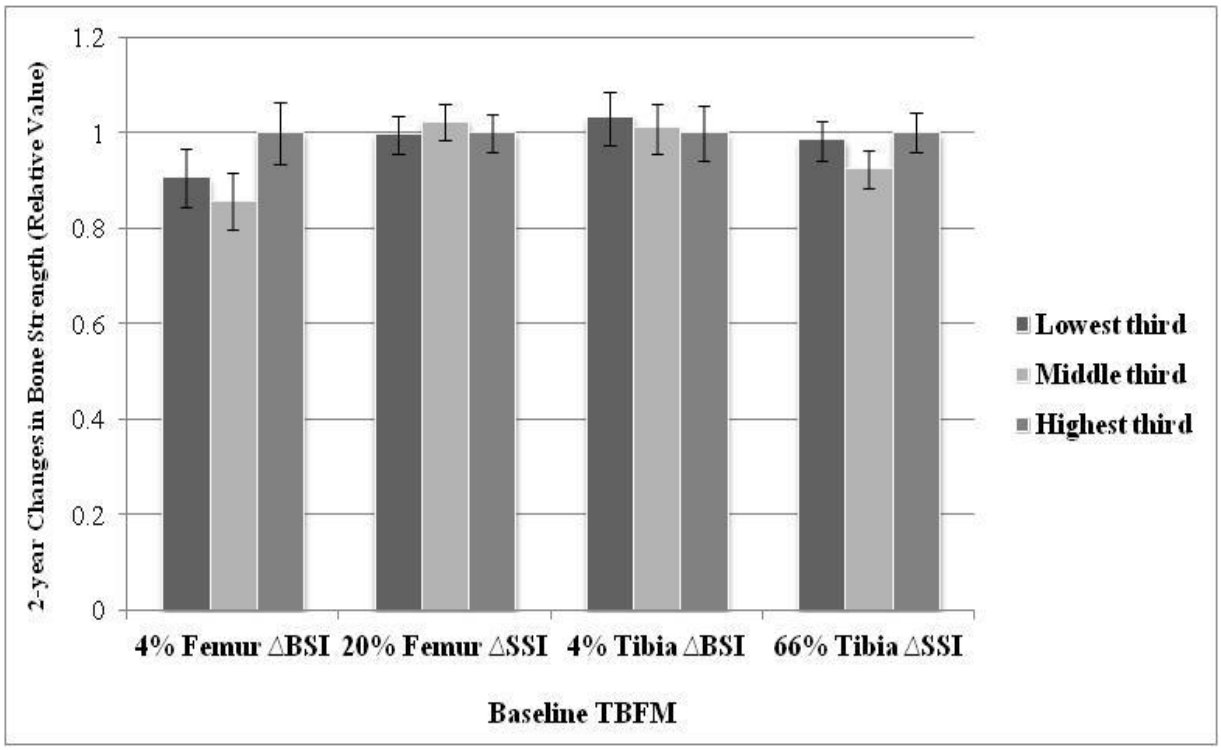
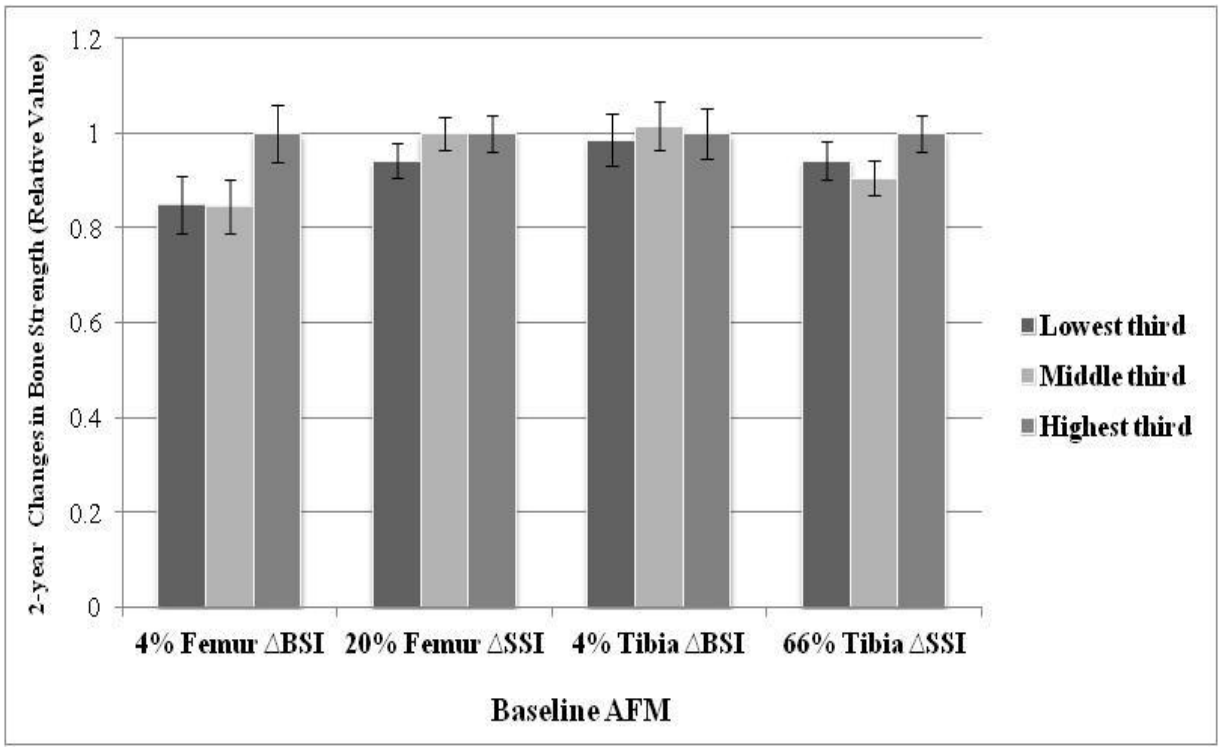


Figure 2B) Android Fat Mass



APPENDIX B: LADDU DR, FARR JN LEE VR, BLEW RM, STUMP C, HOUTKOOPER LB, FARR JN, LOHMAN TG, GOING SB. MUSCLE DENSITY PREDICTS CHANGES IN BONE DENSITY AND STRENGTH: A 2-YEAR PROSPECTIVE pQCT STUDY IN GIRLS

***BONE*. (under review).**

Author emails:

Deepika Laddu (dladdu@email.arizona.edu)
Joshua N. Farr (farr.joshua@mayo.edu)
Vinson Lee (vinsonl@email.arizona.edu)
Robert Blew (rblew@email.arizona.edu)
Craig Stump (cstump@deptofmed.arizona.edu)
Linda Houtkooper (houtkoop@cals.arizona.edu)
Timothy G. Lohman (lohman@email.arizona.edu)
Scott B. Going (going@email.arizona.edu)

Address of authors:
The University of Arizona
1713 E. University Blvd. #93
Tucson, Arizona 85721

Correspondence to:

Deepika Laddu
Department of Nutritional Sciences
The University of Arizona
1713 E. University Blvd. #93
Tucson, AZ, USA 85721-0093
Phone: 602-615-7338
Fax: 520-621-8170
E-mail: dladdu@gmail.com

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ABSTRACT

Fatty infiltration of skeletal muscle has been linked to metabolic disorders (i.e., type 2 diabetes mellitus) and poor physical function in children, adolescents and adults. Given the positive effects of skeletal muscle mass on bone growth, it is of interest to know whether fat infiltration of skeletal muscle predisposes children and adolescents to suboptimal peak bone strength that may track into later life. The objective of this study was to examine the ability of skeletal muscle fat content to predict changes in weight-bearing bone parameters in young girls. Two-year prospective data were collected on 260 girls, aged 8-13 years at baseline, who participated in the "Jump-In: Building Better Bones" study. Peripheral quantitative computed tomography was used to measure changes in bone strength indices [bone strength index (BSI), $mg^2/mm^4$and strength-strain index (SSI), mm^3] and bone density (volumetric bone density, vBMD, mg/cm^3) at distal metaphyseal and diaphyseal regions of the femur and tibia, as well as calf and thigh muscle density (MD, mg/cm^3), an index of skeletal muscle fat content. Multiple linear regression analyses with baseline measures of muscle density, maturity offset, bone length, whole-body lean mass, diet (calories, fat intake) and physical activity as independent variables showed that baseline calf and thigh MD were negatively associated with changes in distal femoral BSI (partial r= -0.15; p=0.020) and tibial BSI (partial r= -0.19; p=0.002). Inverse associations were also observed between baseline thigh MD and changes in femur total vBMD (partial r= -0.17; p=0.007), and trabecular vBMD (partial r= -0.19; p=0.002) of the distal femur. Similar associations were observed between baseline calf MD and changes in tibia SSI (partial r= -0.14; p=0.025), total vBMD (partial r= -0.13; p=0.038) and trabecular vBMD (partial r= -0.22; p<0.001). Analysis of

covariance showed that greater gains in femur BSI (23%), total femur vBMD (71%) and femur trabecular vBMD (145%) occurred in girls in the lowest versus the highest thirds of baseline thigh MD. Greater gains in tibial BSI (19%) total vBMD (24%) and trabecular vBMD (137%) were also observed in the lowest versus the highest thirds of baseline calf MD. In conclusion, larger increases in skeletal muscle density predict greater 2-year increases in weight-bearing bone density and strength in girls.
NIH/NICHD #HD-050775.

Keywords: skeletal muscle fat content; bone development; girls; volumetric bone mineral density (vBMD); peripheral quantitative computed tomography (pQCT).

INTRODUCTION

Peak bone mass and strength achieved in adolescence or early adulthood are primary predictors of fracture risk later in life [4, 21] and both indices are dependent on maximizing bone mineral accrual during growth. Notably, >90% of bone mineral is accrued by the end of adolescence [18] making this period the most opportune time to achieve maximal volumetric bone mineral density (vBMD) and promote adaptations in bone geometry, the primary determinants of bone strength [6]. Disruption of normal bone development during growth, resulting in impaired mineral accrual and lower bone strength, would likely increase risk for developing osteoporotic fractures later in life [4, 16].

The relationship between excess adiposity and bone in children is complex. The conflicting results that have been reported may be due to individual differences in fat distribution and a failure to distinguish between fat depots, which are likely to have different consequences for bone [16, 29, 37, 38] . Indeed, recent studies have shown that increases in adiposity in childhood, especially in abdominal visceral fat and skeletal muscle fat content, may impair skeletal growth and mineralization [35, 38, 129]. In support of this notion, evidence in adults suggests that individuals with high muscle fat content also have higher marrow fat content [46], which has been linked to a weaker skeleton [281]. Collectively, these findings suggest that the same metabolic processes may be regulating fat infiltration of skeletal muscle and bone [47]. T2DM and insulin resistance have been independently associated with fatty infiltration of skeletal muscle and fragility fracture in adults [301, 302] and children [17, 303] and adolescents [304] independent of overall adiposity. Given nearly 20% of the pediatric population is obese,

and for a given age, overweight and obese children are over-represented in the number of fracture cases [35], increased skeletal muscle fat may not only serve as an important risk factor for metabolic abnormalities [45, 260, 301], but also for suboptimal bone development in youth.

Previous studies have reported an inverse association between muscle density (mg/cm^3), which can be measured using peripheral quantitative computed tomography (pQCT), and skeletal muscle fat content [18, 21, 47, 105], Studies in older adults have shown that greater fatty infiltration of skeletal muscle is associated with reduced muscle strength and physical function, which may contribute to decreased bone strength and increased risk of fractures [45]. Studies in middle-aged and elderly men found that, after adjusting for hip BMD, a decrease in thigh muscle function was associated with 40% increase in hip fracture risk [47]. The relationship between fat and bone may be dependent on site-specific pathogenic fat depots and ectopic fat rather than total body fat per se [16]. Thus, through the reduction of muscle relative to gains in total fat deposition and regional fat distribution, which is more prevalent with aging [281], it is possible that fatty infiltration of skeletal muscle may serve as an important risk factor for bone health status in children and adolescents.

The purpose of this study was to determine the association of calf and thigh skeletal muscle fat content and lean mass (reflecting predominantly skeletal muscle) with changes in volumetric BMD (vBMD), and indices of bone strength for weight-bearing bones (femur and tibia) in young girls. A limitation of many past bone development studies in children has been the use of DXA, which is confounded by changes in bone dimensions during growth and does not provide direct measures of bone geometry. To

our knowledge, no studies have used pQCT to assess the association of skeletal muscle fat content and changes in bone density and strength in young girls during growth. pQCT provides 3-dimensional estimates of vBMD of trabecular and cortical bone compartments, which can be used to calculate indices of bone strength [255]. In a previous cross-sectional analysis, we found an inverse association between skeletal muscle fat content and bone strength of the femur and tibia in young girls [57]. Thus, we hypothesized that greater skeletal muscle fat content (calf and thigh) would be inversely associated with 2-year gains in bone density, and strength at weight-bearing skeletal sites in young girls.

METHODS

Participants

The study protocol was approved by the University of Arizona Human Subjects Protection Committee and the study was conducted in accordance with the Helsinki Declaration. All guardians and participating girls provided written informed consent. Details regarding recruitment of the subjects as well as the baseline bone, soft tissue, diet and physical activity characteristics of the sample have been published previously [57]. A total of 3 cohorts were recruited and enrolled in the study between the Fall of 2007 and the Fall of 2009. A CONSORT diagram showing the progress of participants through the two year study is presented in Figure 1. Depending on the enrollment time of the cohort, 24- month data was collected between December 2010 and April 2012. At baseline, 509 healthy fourth and sixth grade girls (aged 8 to 13 years), who were enrolled in 14 elementary and 4 middle schools in Tucson, Arizona, were recruited as participants in the Jump-In: Building Better Bones study [57]. Exclusion criteria included the inability to read and understand English and learning disabilities (identified by schools) that made it

impossible to complete questionnaires or made it difficult to comply with assessment protocols. Individuals taking medications known to affect bone metabolism or who had been diagnosed with medical conditions, or with a disability that limited participation in physical exercise as defined by the Committee on Sports Medicine and Fitness were also excluded [299]. The ethnic composition of the sample included 23% Hispanic and 77% was non-Hispanic participants, was represented of the population of Tucson, AZ and the surrounding area. Sample race was 89% white, 7% Asian, 2% black or African American, 0.3% Latino, 0.7% Native Hawaiian or other Pacific Islander, and 0.3% other. Baseline bone and soft tissue composition data were available on 444 girls [57], after elimination of 65 pQCT scans due to the presence of motion artifact. Of those girls, 260 girls had 2-year assessments of soft tissue composition and pQCT bone parameters and were included in the present analysis. The criterion for assessing the viability of a pQCT image, or if a repeat acquisition was required, was determined by rating the level of movement. Each scan was visually inspected and rated using a linear, ordinal scale of 1 to 5 to assess the level of motion artifact present. A score of 1 represents a scan with no movement and 5 represents extreme movement such that significant image streaking and disruption of the cortical shell was present. Images graded 4 or 5 are deemed to have unacceptable motion artifact for bone and soft tissue analysis. All motion artifacts were assessed by one of three trained staff or certified technicians.

Anthropometry

Anthropometric measures (body mass, standing height, sitting height and bone lengths) were obtained following standardized protocols [242]. Body mass was measured to the nearest 0.1 kg using a calibrated scale (Seca, Model 881, Hamburg, Germany). Both

standing height and sitting height were measured at full inhalation to the nearest millimeter (mm) using a calibrated stadiometer (Shorr Height Measuring Board, Olney, MD). Femur length and tibia lengths were measured on the non-dominant leg. To determine leg dominance, subjects were asked with which foot they would kick a ball when playing soccer/kickball. If the subject was uncertain, she was asked with what hand she writes, and that was determined to be her side of dominance. Femur length (nearest mm) was measured from the proximal aspect of the patella to the inguinal crease. Tibia length (nearest mm) was measured from the proximal end of the medial border of the tibial plateau to the distal edge of the medial malleolus. Baseline coefficients of variation (CVs) for femur and tibia lengths in our laboratory (n=444 girls) were 0.34% and 0.51%, respectively [57]. For each anthropometric variable, two measurements were taken and averaged. Both measurements were repeated if the first two trials differed by more than 4 mm for height, sitting height and bone lengths, and 0.3 kg for body mass, and the average of the second set of measures was used.

Physical Maturation

Maturation was assessed using maturity offset over the more conventional method of Tanner staging due to its reliance on objective anthropometric measurements of linear growth. Maturity offset is based on estimated years from peak height velocity (PHV) using Mirwald's equation [245]. These algorithms include interactions among anthropometric measures (i.e., height, weight, sitting height, leg length) and chronologic age to derive a maturity-offset value. Positive maturity offset values represent years after PHV while a negative maturity offset value represents years before PHV. In Mirwald's sample, the maturity offset equation for girls explained 89% of the variance in years from

PHV [245].

Dietary Assessment

Dietary fat (grams/day) and total calorie intake (kcals/day) were assessed using the Harvard Youth/Adolescent Questionnaire (YAQ) [250], The YAQ is a self-administered food-frequency questionnaire (FFQ) to assess usual dietary intake and dietary supplement use during the previous year; acceptable validity and reproducibility of the YAQ have been reported [250]. Previous studies have used this tool to examine associations between nutrition and health-related outcome measures, including bone density [252, 300]. Participants completed the YAQ with assistance available. All YAQs were reviewed and coded by trained study staff following standard coding procedures [251]. Nutrient analysis was completed by Channing Laboratories (Boston, MA) [251].

Physical activity

Physical activity (PA) was assessed by the Past Year Physical Activity Questionnaire (PYPAQ) [246], a survey of all sport and leisure-time physical activity in which the respondent engaged at least 10 times in the past year outside of physical education class. The PYPAQ has been slightly modified to include a more comprehensive list of 41 activities common to youth [247]. The modified questionnaire was administered in an interview with the participant and guardian. Total PYPAQ score was computed using a modified equation from Shedd and colleagues [248], which incorporated weight-bearing load, frequency and duration of each activity reported [247].

Bone and Body Composition Assessment

pQCT- Bone Measures

Changes in bone geometry, bone strength and volumetric bone mineral density

(vBMD) were assessed using pQCT (XCT 3000, Stratec Medizintechnik GmbH, Pforzheim, Germany, Division of Orthometrix; White Plains, NY, USA) at the distal 4% and 20% femur and distal 4% and 66% tibia sites relative to the respective distal growth plates on the non-dominant limb.

Trabecular vBMD (mg/cm^3) and bone strength index (BSI, mg^2/mm^4) were assessed at the distal metaphyseal regions of the femur and tibia, and cortical vBMD (mg/cm^3) and polar strength-strain index (SSI, mm^3) at the diaphyseal regions. BSI estimates the bone's ability to withstand compression at metaphyseal regions, and is calculated as the total area x total $vBMD^2$ [255]. SSI is used to estimate the bone's ability to resist torsion and bending forces at diaphyseal regions. Diaphyseal SSI was calculated using Stratec software as the integrated product of the geometric properties (i.e., section modulus) with the material properties of bone: Strength-strain index (SSI, mm^3)= , n ($[(r^2_i \times a)/r_{max}] \times$ (cortical vBMD/ND) [248]; section modulus is calculated as $(r^2_i \times a)/r_{max}$, where a is the area of a voxel (mm^2), r is the distance of a voxel from the center of gravity (mm), and r_{max} is the maximum distance of a voxel from the center of gravity (mm). The material properties of bone are calculated as the quotient of measured cortical density (cortical vBMD, mg/cm^3) and normal physiologic cortical density (ND,1200 mg/cm^3).

pQCT – Soft Tissue Measures

Soft tissue composition at the 20% femur (thigh) and 66% tibia (calf) sites of the non-dominant limb were assessed using pQCT. Tissue characteristics (i.e., adipose, muscle, and bone) were separated using edge detection and threshold techniques based on attenuation characteristics, which are directly related to tissue composition and density.

Details describing edge detection and image filtration for tissue analysis in our laboratory have been previously reported [57]. All images were filtered subsequently with a 7x7 image filter that clearly defined the edge of the muscle and eliminated all bone above 120 mg/cm^3; this ensured that muscle density was the only soft-tissue component being measured within the edge of the muscle. A limitation of pQCT is the inability to distinguish between intra- and extramyocellular fat compartments; however several controlled studies have established a clear relationship between lower muscle density and higher skeletal muscle fat content [105, 305]. Thus, muscle density was used as a composite index of skeletal muscle fat content in the intra- and extramyocellular stores [57]. CVs for muscle density (MD, mg/ cm^3) obtained at the calf and thigh regions were 0.9% and 0.4%, respectively.

Scout scans were performed to locate the distal growth plates, with the scanner programmed to find the sites of interest based on skeletal lengths. Slice thickness was set to 2.3 mm and voxel size was set to 0.4 mm. Scanner speed was set at 25 mm/s. Additional details regarding pQCT bone measurements, image processing, calculations, and analysis, are published elsewhere [83, 254]. Because repeat scanning of girls to establish the precision of pQCT was not considered ethical by the University of Arizona Human Subjects Protection Committee, separate study with adults, was conducted previously in this laboratory, to determine within-subject (n=29 per skeletal site) pQCT precision error (CV). After subject repositioning, CVs calculated as described by Glüer and colleagues [306] were less than 1.1% for vBMD, bone geometry, and indices of bone strength (i.e., BSI and SSI) [83]. pQCT data acquisition and analyses followed guidelines provided by Bone Diagnostics, Inc. (Fort Atkinson, WI, USA). All pQCT

scans were performed by a single operator, while a second investigator (J.N.F) analyzed all scans using the Stratec software (version 6.0). The pQCT instrument was calibrated and quality assurance procedures were completed daily in order to ensure precision of measurements.

Dual Energy X-ray Absorptiometry (DXA)

Soft tissue mass and composition, including total-body lean mass were obtained from DXA whole body scans. All participants were scanned by a certified technician, and all analyses were performed by a single technician. The unit was calibrated daily according to the standard procedures for maintenance and use as recommended by the manufacturer. DXA CVs for precision for measuring soft tissue composition in our laboratory have been previously reported [261, 262].

Statistical Analysis

Data were checked for outliers and normality using histograms, and skewness and kurtosis were calculated for all variables. All bone variables were normally distributed; thus, no transformations were applied. Descriptive statistics (means, SDs, and ranges) were calculated for the entire sample. Control for diet and physical activity has been suggested in studies of growing children, since nutrition [264] and physical activity [6, 247, 263], along with maturation [21], significantly influence linear growth and body composition . Since no changes in dietary intakes of calories or fat or physical activity were observed over the 2-year study period, the average values for calories, dietary fat, and physical activity (average PYPAQ score) were used as covariates. Pearson's correlation (r) coefficients for continuous variables were used to assess unadjusted relationships between the covariates and bone outcome variables. Multiple linear

regression was used to regress changes in bone variables on baseline measures of thigh and calf muscle densities after controlling for baseline measures of maturity offset, bone lengths (femur or tibia), whole-body lean mass, average physical activity and dietary total calorie and fat intakes. All regression models were assessed for linearity, normality and homoscedasticity using residual plots. To protect against collinearity, femur length or tibia length (without height) was included in regression models for thigh and calf muscle densities, respectively.

Analysis of covariance (ANCOVA) was used to compare changes in bone outcomes among respective tertiles of baseline thigh and calf MD, after adjusting for the same covariates included in the regression models. Because of the differences in units for pQCT bone outcome variables, results were normalized to the highest tertile by setting the highest tertile to 1.0. Bonferroni post hoc tests were used to adjust for multiple comparisons among tertiles of baseline thigh and calf MD. The level of statistical significance was set at $P<0.05$ (two-tailed). All analyses were performed using the Statistical Package for the Social Sciences for Windows, Version 20.0 (SPSS, Chicago, IL,USA).

RESULTS

Descriptive Characteristics

Descriptive statistics are given in Table 1. Based on body mass index (BMI, kg/cm^2), at baseline, 3.5% of the sample was underweight (BMI<5th percentile), 75% of the sample was healthy weight (BMI 5th to 85th percentile), 13.5% of the sample was overweight (BMI 85th to 95th percentile), and 7.7% of the sample was obese (BMI>95th percentile) [148]. At the 24-month follow up, 2.3% of the sample was underweight,

72.7% of the sample was healthy weight, 17.7% of the sample was overweight, and 7.3% of the sample was obese. On average, participants were 1.2 years away from achieving PHV at baseline, ranging from 3.2 years prior to PHV to 1.0 years post PHV. Average caloric intake (1711±541 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans (DGA) [234]. Average fat intake (31.4%±4.0%) met the dietary recommendations (25-35%) established by the American Heart Institute [266] and DGA [234].

As expected, significant increases in age, maturity, height, body weight, body mass index (BMI), femur length, tibia length, total body lean mass, total body fat mass and lean mass, and calf and thigh muscle density and femur and tibia bone strength and bone density indices were observed (all p values < 0.0001) from baseline to the 2-year follow-up.

Correlations between model covariates and bone change outcomes

Results from unadjusted bivariate analysis showed that baseline measures of maturity offset, bone lengths, and whole-body lean mass were positively correlated ($r = 0.17$ to $r = 0.61$ $p<0.05$) with all changes in bone parameters measured at the femur and tibia diaphyseal (20% femur, 66% tibia) and metaphyseal (4% tibia) sites, except for the distal femur (4%, trabecular density). Average physical activity score was correlated with change in SSI of the femur ($r=0.20$; $p<0.01$), and the change in cortical vBMD at diaphyseal sites of the femur and tibia ($r=0.13$-0.15; $p<0.05$). Baseline thigh MD (20% femur) was inversely correlated with changes in femur total vBMD ($r = -0.14$, $p<0.028$)

and trabecular vBMD (r= -0.18, p<0.004), whereas baseline calf MD (66% tibia) was inversely correlated with changes in most bone outcomes (Table 2).

Associations between body composition and bone change outcomes

The results of the multiple linear regression analyses show the individual contribution (partial r) of baseline thigh MD (Table 2) and baseline calf MD (Table 3) on bone parameters after controlling for covariates. Inverse relationships were observed between baseline thigh and calf muscle densities and changes in bone density, with significant associations found at the femur sites. Both baseline thigh and calf muscle densities were negatively associated with change in total vBMD (all p<0.05) and with change in trabecular vBMD at metaphyseal sites of the femur (p<0.002) and tibia (p<0.001).

Results from regression analyses with change in bone strength as the dependent variable are also summarized in Table 2 and Table 3. Baseline thigh muscle density was negatively associated with change in BSI measured at the metaphyseal region of the femur (r= -0.15, p<0.020). Similar associations were noted between baseline calf MD and change in BSI at metaphyseal regions of the tibia (r= -0.19, p<0.002). Inverse associations were also observed between baseline calf MD and the change in SSI at diaphyseal regions of the tibia (r= -0.14, p<0.025), whereas baseline thigh MD was not significantly associated with changes in SSI at diaphyseal regions of the femur.

Comparison of bone parameters across tertiles of muscle density

Estimated means (±SE) of the *change* in the pQCT bone variables were compared across tertiles of baseline thigh and calf MD, respectively, using ANCOVA after adjusting for baseline maturity offset, bone length (femur or tibia), whole-body lean mass,

and average diet (calorie; fat intake) and physical activity. Figures 2-3 show the normalized adjusted means ± standard errors for change in vBMD (figure 2) and bone strength (figure 3) across tertiles of thigh (Panel A) and calf MD (panel B). Participants in the lowest compared to the highest thirds of baseline thigh and, had significantly greater gains in total vBMD at the femur, whereas participants in the lowest versus middle thirds of baseline calf MD experienced a larger increase in total vBMD at the diaphyseal tibia. The largest increase in bone density was seen in trabecular vBMD at both femur and tibia metaphyseal sites (4% femur, 4% tibia). Participants in the lowest third of baseline thigh MD had a 1.45 fold greater increase at the distal femur in trabecular vBMD compared to participants in the highest third of thigh MD. Participants in the lowest third of baseline calf MD had a 1.37 fold greater gain in trabecular vBMD at the distal tibia versus the highest third of calf MD. Participants in the lowest third had 23% greater increase in femur BSI compared to the highest third of thigh MD. Similarly, participants in the lowest third of baseline calf MD gained 19% more BSI at the tibia versus girls in the highest third of calf MD. By contrast, baseline thigh and calf muscle density, respectively, was not significantly associated with changes SSI at the diaphyseal sites of the femur and tibia.

We also examined the estimated means (±SE) of the change in MD across the tertiles of baseline MD of the thigh (Figure 4A) and calf (Figure 4B), after adjusting for baseline maturity offset, bone length (femur or tibia), whole-body lean mass, and average diet (calorie; fat intake) and physical activity. Participants in the lowest tertile of MD at baseline experienced the greatest gain in MD as well as the greatest gains in bone strength and density compared to participants in the middle and highest thirds (all

$p<0.01$), as would be expected from our previous findings of a positive association between MD and bone strength [57], and which explains the seemingly paradoxical positive association between baseline muscle fat content and change in bone strength.

DISCUSSION

In this longitudinal analysis, we investigated the effects of skeletal muscle fat on bone development in young girls, aged 8-13 years. The results of this study show that baseline muscle density was inversely associated with change in bone outcomes, however participants exhibiting greater increases in muscle density, a surrogate for skeletal muscle fat content, also experienced larger increases in bone density and strength over the course of 2 years. These longitudinal data support cross-sectional findings [57, 247] and extend our knowledge of the relationships between adiposity and bone development in girls.

Lipid infiltration in the skeletal muscle, in tandem with loss of muscle mass and reduced skeletal loading, contributes to age related decline in skeletal muscle strength and function, decreased mobility, and an increased risk of falls and fractures [45]. Epidemiologic studies in older adults have reported relationships between muscle mass measurements and fractures that were related to lower BMD, functional decline, and metabolic dysfunction [307]. Visser et al (2000) also found positive associations between thigh muscle Hounsfield units (HU) and higher physical function score, assessed by the lower extremity performance test, after adjusting for important covariates including age, height and BMI [104] in black and white males and females. Lang and colleagues [45] similarly reported that in elderly men and women, a 1 SD decrease in thigh muscle HU resulted in a 50% increase in hip fracture risk. After appropriate adjustments for chronic disorders (e.g., diabetes, hypertension), the association of thigh muscle HU value with

hip fracture risk remained significant (40% risk), although the decline in risk supports the additional effect of metabolic dysregulation on bone maintenance and skeletal integrity [45].

Our previous cross-sectional work showed that higher skeletal muscle fat was associated with lower bone strength in pre- and early-pubertal girls, suggesting that skeletal muscle fat impaired bone development in our cohort. In the present study, gains in bone strength, and bone density measured at metaphyseal sites of the femur and tibia were associated with increases in muscle density. Importantly, the inverse associations between baseline muscle density and bone outcomes found in regression analysis can be explained in part by the fact that participants in the lowest muscle density group increased more in muscle density and had larger gains in bone strength and bone density at the thigh and calf, compared to the higher muscle density group. Given that lower muscle density is an index of greater skeletal muscle fat content [47], our findings suggest that fatty infiltration of the skeletal muscle may hinder optimal bone development during growth and may be an important risk factor for the development of osteoporosis later in life. Our finding in the present analysis that girls who increased muscle density also increased bone density and strength provides further support for the significant influence of muscle density on bone development. These results are consistent with previous studies reported by Yerges-Armstrong and colleagues [47] as well as a previous cross-sectional report from our laboratory [57].

Potential mechanisms that explain the loss of bone strength and physical function related to decreased muscle mass have been documented in the elderly. Skeletal muscle fat manifests itself as extramyocellular lipids that become embedded between muscle

fibers, and intramyocellular lipids contained in droplets of triglyceride that form on muscle cell membranes [45]. Increased infiltration of non-contractile components, such as lipids, into muscle tissue contributes to the loss of muscle bundle cross-sectional area and ultimately the atrophy of muscle mass and decrease in muscle function. In adults, age-related impaired skeletal muscle function can be explained in part by the increased storage of triglycerides and smaller lipid droplets that form along the muscle membrane, contributing to a loss of muscle strength and a decline in lower extremity performance [45]. The relationship between skeletal muscle fat content and muscle function in girls is unclear. Nevertheless, from our past and current analysis, it is clear that skeletal muscle density, an established surrogate of skeletal muscle fat infiltration, is an important factor to consider in understanding the development of trabecular bone.

Taken together with our findings, the results from several other investigations support the notion that mechanical loading effects of fat mass and lean mass on bone differ. Muscle mass and muscle quality are both associated with skeletal density and geometry and therefore play a dominant role in augmenting bone strength by the mechanism of increased mechanical loading on the skeleton [113]. Compared to fat mass, lean mass is likely to have a stronger positive influence on bone size, geometry, mineral content and architecture of skeleton and therefore adaptations to accommodate loading [28, 78]. By contrast, when the mechanical loading effect of body weight on bone is adjusted for lean mass, fat mass may have a no additive or a negative effect on bone mass in contrast with the positive effect of weight-bearing itself [129, 131]. Furthermore, it is possible that the relationships between fat mass and bone may differ at weight-bearing (i.e., femur and tibia) versus non-weight-bearing (i.e., radius) skeletal sites.

Most previous studies that have investigated the fat-bone relationship in youth have been cross-sectional by design [35, 81]. The longitudinal design of our study is a strength that provided an opportunity to determine how skeletal muscle density, a surrogate of skeletal muscle fat content, predicts changes in bone density and strength in girls. To our knowledge, this is the first study that investigated the longitudinal relationships between skeletal muscle density with changes in bone density, structure and strength in children and adolescents. Further studies will be necessary to determine how changes in muscle density predict fracture risk. This study is not without limitations. The most obvious limitation was that while muscle density is directly related to skeletal muscle fat content, it does not distinguish between intramyocellular (IMCL) and extramyocellular (EMCL) fat compartments. Previous studies in adults using proton magnetic resonance spectroscopy (MRS) in adults [259], as well as in youth [260] have indicated that composite measures of IMCL and EMCL such as skeletal muscle density, are sufficient indices of skeletal muscle fat content. Thus, muscle density from pQCT is a cost-effective, relatively low-radiation surrogate for muscle fat content that is feasible for relatively large-scale studies.

Strengths of this study include the longitudinal design which improves upon the limitations of past cross-sectional studies by providing an opportunity to assess whether "pathogenic" fat depots, such as fat within skeletal muscle which has previously been related to functional decline, muscle loss, and metabolic derangements, may impair bone development in young girls [28, 78]. Other strengths included a direct measure of whole-body lean mass by DXA, rather than indirect assessment of body composition [35, 79, 81, 124], and control for calorie and fat intakes as well as bone loading physical

activity using the modified PYPAQ [248], which has been proven to be a stronger predictor of indices of bone strength at weight bearing sites of the femur and tibia, compared to other PA assessment tools in youth (i.e., 3DPAR, pedometer) [247, 248].

In conclusion, our findings indicate that after controlling for linear growth, maturation, and important components of body composition (i.e., total body lean mass), girls who experienced the increases in muscle density had the largest increases in bone density and strength compared to girls who did not significantly gain muscle density over the course of 2 years. Although an inverse relationship between baseline muscle density and bone were observed in regression analysis, the results of this study highlight the influence of baseline muscle density on bone, and provide evidence that increases in muscle density have positive effects on the developing bone. These findings support the premise that fatty infiltration of skeletal muscle contributes to suboptimal bone development in peri-pubertal girls.

TABLES

Table 1: Sample Descriptive Characteristics (n=260)

	Baseline (n=260)	24-months (n=260)	% change[a]
Age (years)	10.6 ± 1.1	12.7 ±1.1	-
Maturity Offset (years)	-1.2 ± 1.0	0.70 ±1.0	-
Tanner (%; 1/2/3/4/5)	34/34/27/5/0	1/4/13/37/36/8	-
Menarche (%; Post)	7	47	-
Height (cm)	144.1 ± 9.8	156.7 ± 9.1	8.7[a]
Weight (kg)	38.6 ± 9.8	50.0 ± 12.0	29.6[a]
BMI (kg/cm^2)	18.3 ± 3.2	20.2 ± 3.7	9.9[a]
Femur Length (cm)	34.0 ± 3.0	36.7 ± 2.5	8.1[a]
Tibia Length (cm)	33.1 ± 2.9	36.4 ± 2.5	9.9[a]
Total energy intake (kcal)	1719.1± 646.5	1703.8 ± 490.4	-0.9
Total fat intake (g)	60.2 ± 25.6	59.7 ± 21.5	-0.9
Physical Activity Score	5229.2 ± 4589.7	5263.7 ± 5593.5	0.7
Total body fat mass (kg)	11.0 ± 6.0	15.2 ± 7.8	38.9[a]
Whole body lean mass (kg)	25.4 ± 4.9	32.0 ± 5.5	26.0[a]
Thigh muscle density (mg/cm^3)	76.3 ± 1.5	77.5 ± 1.5	1.6[a]
Calf muscle density (mg/cm^3)	79.0 ± 1.2	80.0 ± 1.2	1.2[a]
Femur BSI (mg^2/mm^4)	94.5 ± 26.8	123.8 ± 36.2	31.0[a]
Femur SSI (mm^3)	1315.4 ± 389.7	1874.8 ± 508.1	42.5[a]
4% Femur Total Density (mg/cm^3)	275.1 ± 33.4	290.0 ± 40.6	5.4[a]
20% Femur Cortical Density (mg/cm^3)	1045.8 ± 23.1	1067.2 ± 32.5	2.0[a]
4% Femur Trabecular Density (mg/cm^3)	236.7± 31.9	246.5 ± 36.8	4.2[a]
4% Tibia BSI (mg^2/mm^4)	50.7 ± 12.8	68.1 ± 19.6	34.4[a]
66%Tibia SSI (mm^3)	1151.8 ± 320.8	1590.9 ± 408.4	38.1[a]
4% Tibia Total Density (mg/cm^3)	294.7 ± 34.7	322.4 ± 46.6	9.4[a]
66% Tibia Cortical Density (mg/cm^3)	1028.2 ± 32.4	1056.9 ± 37.6	2.8[a]
4% Tibia Trabecular Density (mg/cm^3)	222.3 ± 25.5	229.8 ± 30.7	3.4[a]

Values are presented as mean ± SD. *P* values represent paired samples *t*-Test for difference between the baseline and 2-year study visit. BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3);
[a] Significant at P<0.0001

Table 2: Unadjusted bivariate Correlations and Partial Correlations from Multiple Linear Regression of 2-year Changes in Bone Parameters on Baseline Thigh Muscle Density (MD)

Baseline Thigh MD	Pearson's r	partial r
Femur Δ BSI	-0.11	-0.15[b]
Femur Δ SSI	0.02	-0.04
4% Femur Δ total vBMD	-0.14[b]	-0.17[b]
20% Femur Δ Cort vBMD	-0.04	-0.06
4% Femur Δ Trab vBMD	-0.18[b]	-0.19[b]

All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density (mg/cm^3); BSI=bone strength index (mg^2/mm^4); Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); SSI=strength–strain index (mm^3). Model covariates= baseline covariates, maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score.

[a] P<0.001; Pearson's r for continuous variables.

[b] P<0.05; Pearson's r for continuous variables.

Table 3: Unadjusted bivariate Correlations and Partial Correlations from Multiple Linear Regression of 2-year Changes in Bone Parameters on Baseline Calf Muscle Density (MD)

Baseline Calf MD	Pearson's r	partial r
Tibia Δ BSI	-0.16[b]	-0.19[b]
Tibia Δ SSI	-0.14[b]	-0.14[b]
4% Tibia Δ total vBMD	-0.09	-0.13[b]
66% Tibia Δ Cort vBMD	-0.02	-0.01
4% Tibia Δ Trab vBMD	-0.21[b]	-0.22[a]

All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density (mg/cm^3); BSI=bone strength index (mg^2/mm^4); Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); SSI=strength–strain index (mm^3). Model covariates= baseline covariates, maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score.

[a] P<0.001; Pearson's r for continuous variables.

[b] P<0.05; Pearson's r for continuous variables.

FIGURE LEGENDS

Figure 1. CONSORT flowchart describing the progress of participants through the "Jump-In: Building Better Bones" study. C1= cohort 1; C2= cohort 2; C3 = cohort 3.

Figure 2: Estimated marginal means±SE for changes in femur and tibia volumetric bone density parameters across thirds of baseline thigh (A) and calf (B) muscle density. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline muscle density were evaluated by ANCOVA using baseline covariates: maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score. MD= muscle density (mg/cm^3); Tot BMD= total (average) volumetric bone mineral density (mg/cm3); Cort BMD= cortical volumetric bone mineral density (mg/cm^3); Trab BMD=trabecular volumetric bone mineral density (mg/cm^3).

[a] Significantly different ($P<0.05$) from highest tertile; ANCOVA

[b] Significantly ($p<0.05$) different from middle group; ANCOVA

Figure 3: Estimated marginal means±SE for changes in femur and tibia bone strength indices across thirds of baseline thigh (A) and calf (B) muscle density. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline muscle density were evaluated by ANCOVA using baseline covariates: maturity offset, bone length, and

average diet (calorie, fat intake) and physical activity score. MD= muscle density (mg/cm^3); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3)

[a] Significantly different ($P<0.05$) from highest tertile; ANCOVA

Figure 4: Baseline MD predicting 2-year changes in MD of the thigh and calf. Estimated marginal means±SE for changes in thigh (A) and calf (MD) across thirds of baseline thigh and calf MD. Bone outcome values were normalized to the highest group by setting the highest group values to 1.0, while lower values were set to less than 1.0 and higher values set to greater than 1.0. Differences among groups for respective tertiles of baseline muscle density were evaluated by ANCOVA using baseline covariates: maturity offset, bone length, and average diet (calorie, fat intake) and physical activity score. MD= muscle density (mg/cm^3).

[a] Significantly different ($P<0.0001$) from highest tertile; ANCOVA

Figure 1: Consort Diagram

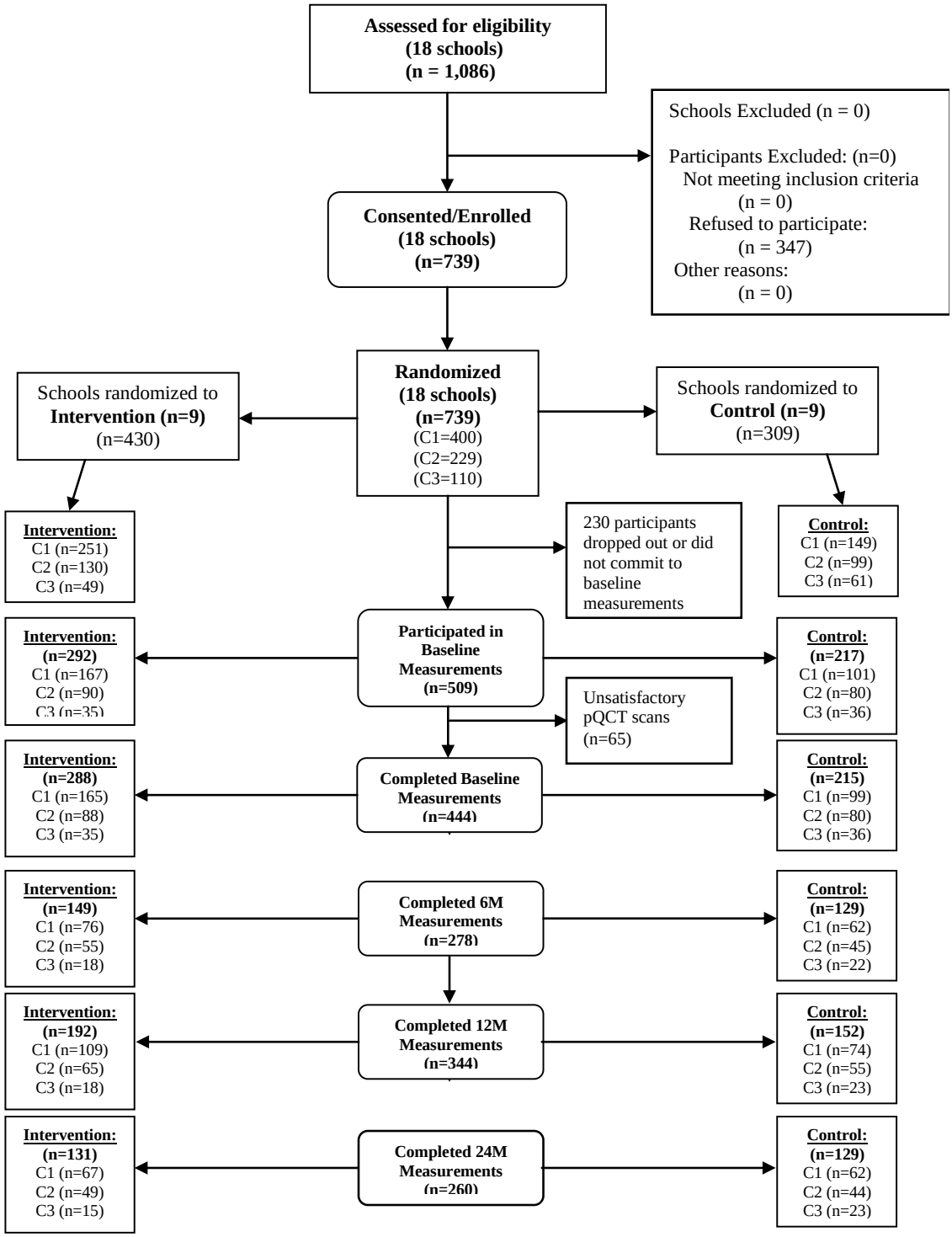


Figure 2A) Thigh MD

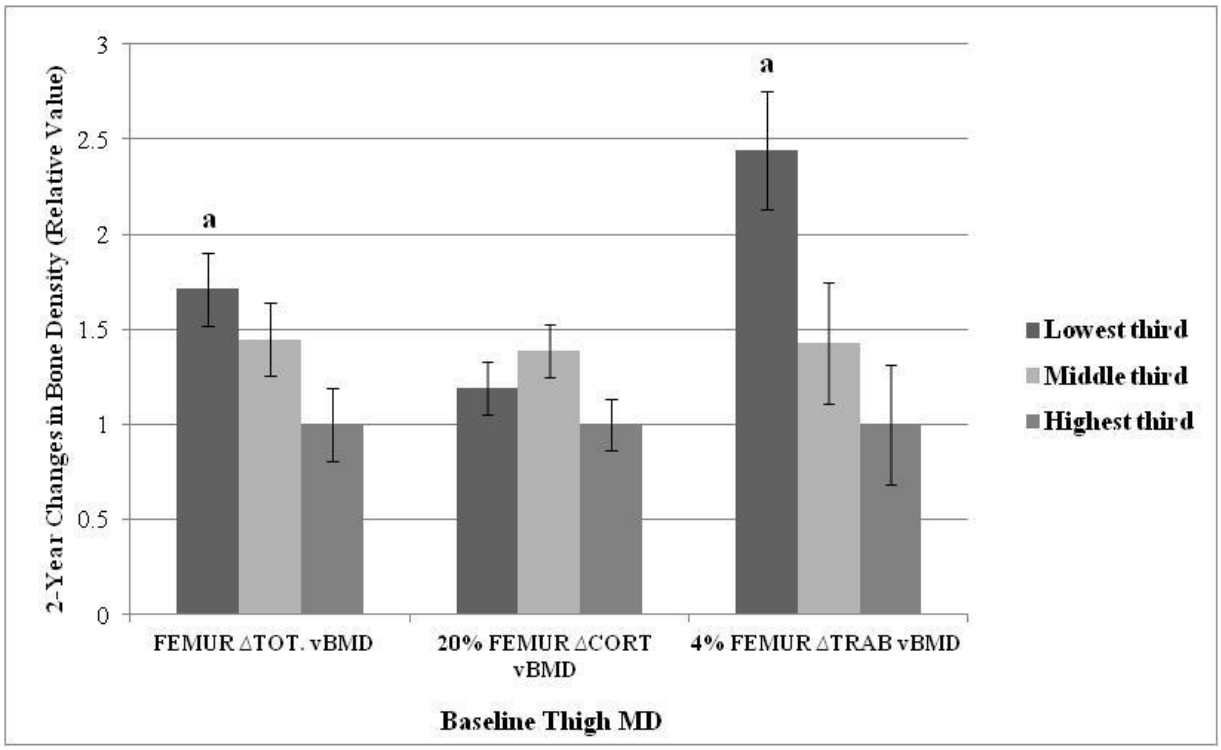


Figure 2B) Calf MD

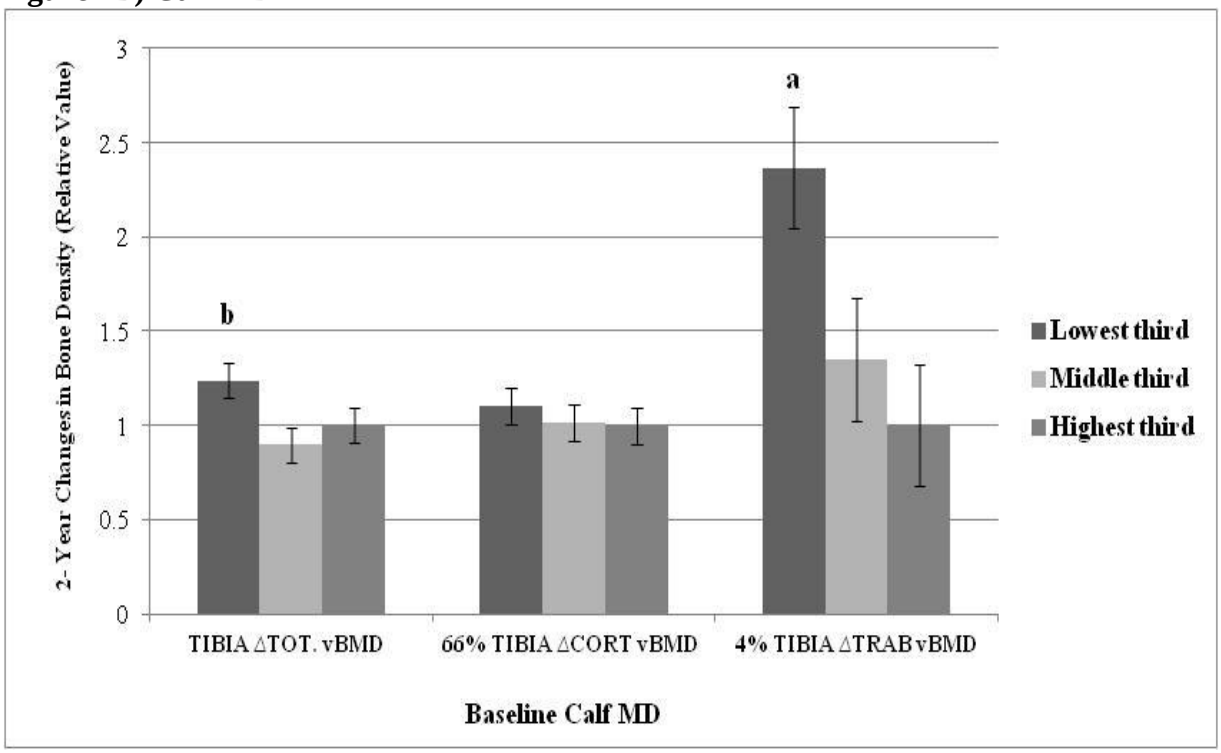


Figure 3A) Thigh MD

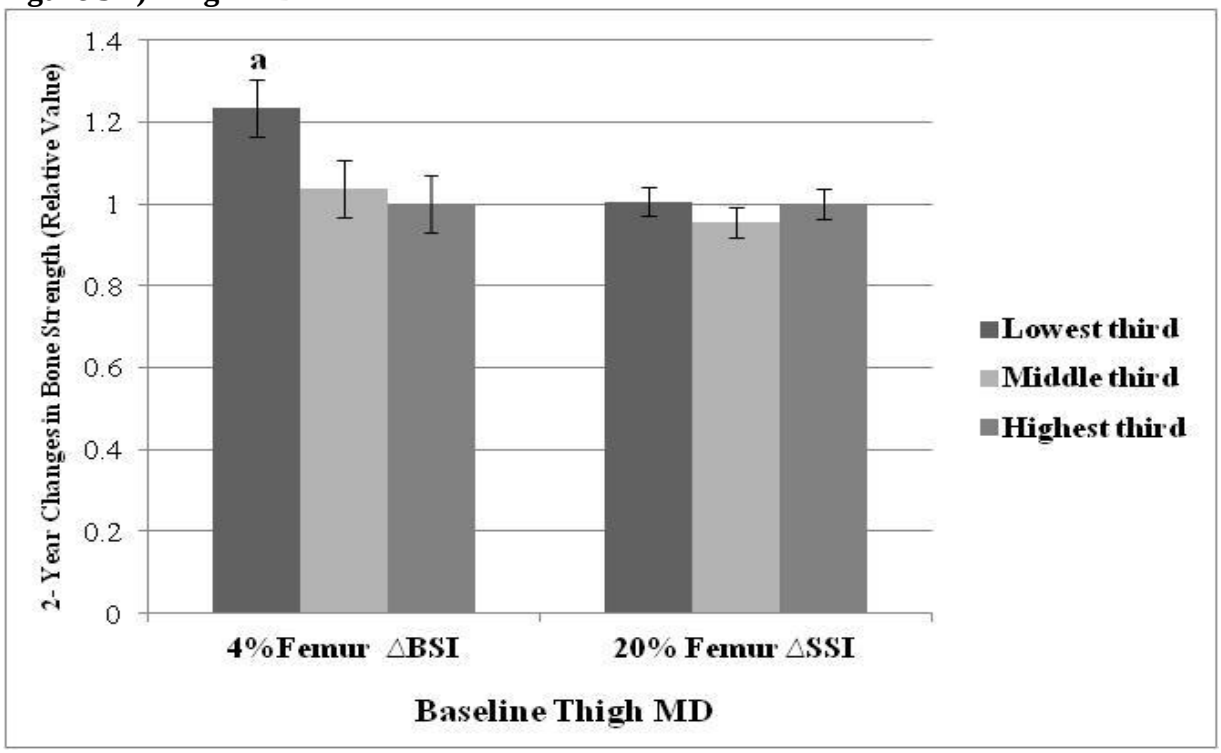


Figure 3B) Calf MD

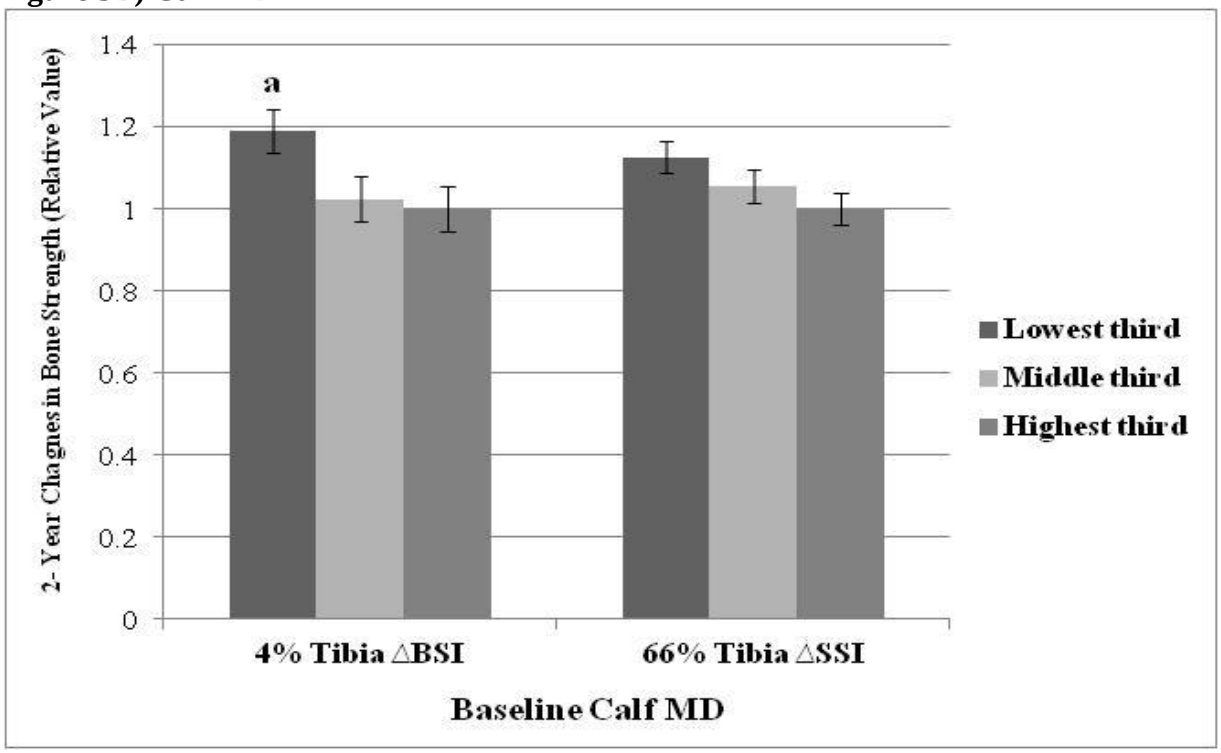


Figure 4A) Thigh MD

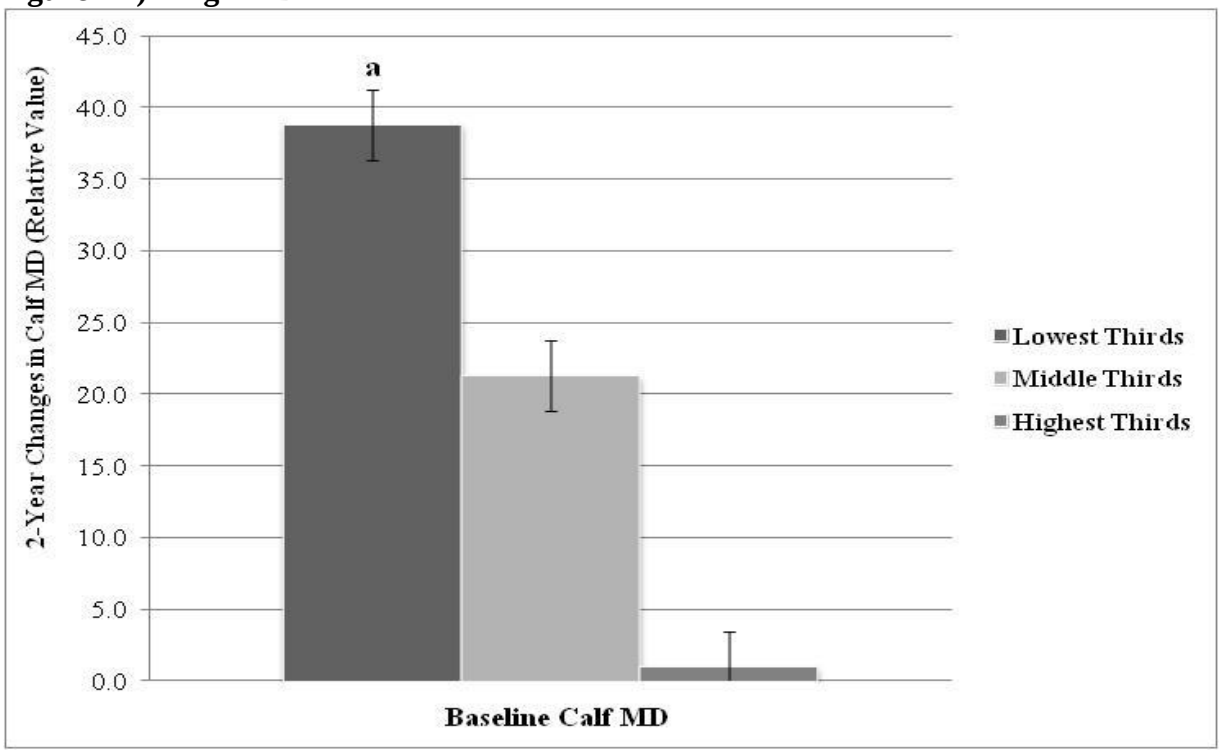
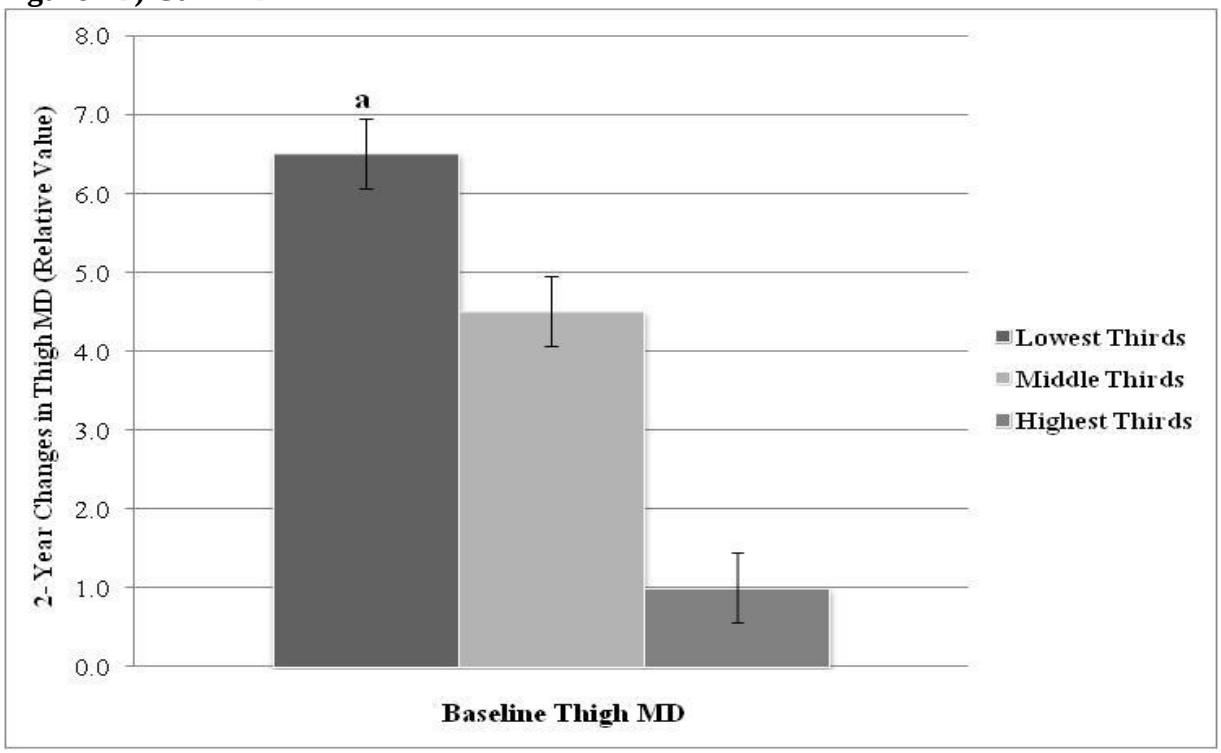


Figure 4B) Calf MD



APPENDIX C: LADDU DR, FARR JN, LEE VR, BLEW RM, LOHMAN TG, GOING SB. LONGITUDINAL ASSESSMENT OF CHANGES IN "MUSCLE-BONE UNIT"COMPONENTS ON BONE STRENGTH IN YOUNG GIRLS.

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Author emails:
Deepika Laddu (dladdu@email.arizona.edu)
Joshua N. Farr (farr.joshua@mayo.edu)
Vinson R. Lee (vinsonl@email.arizona.edu)
Robert M. Blew (rblew@email.arizona.edu)
Timothy G. Lohman (lohman@email.arizona.edu)
Scott B. Going (going@email.arizona.edu)

Corresponding Author:
Deepika Laddu
Department of Nutritional Sciences
The University of Arizona
1713 E. University Blvd. #93
Tucson, AZ, USA 85721-0093
Phone: 602-615-7338
Fax: 520-621-8170
E-mail: dladdu@gmail.com

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ABSTRACT

Bone mass is a material component of bone strength, and muscle strength and quality are determinants of bone mass; however it remains unclear whether muscle force or muscle quality is a direct determinant of bone strength during growth. Therefore, we investigated whether muscle size and muscle quality influences the bone strength beyond the influence of bone mineral mass in young girls. Longitudinal data from 245 girls aged 9-13y from the "Jump-In: Building Better Bones" study were analyzed. Peripheral quantitative computed tomography (pQCT) was used to measure changes in bone mineral content (BMC; g) and bone strength at metaphyseal and diaphyseal regions of the femur and tibia, along with calf and thigh muscle cross-sectional area (MCSA; mm^2), a surrogate for muscle force, and muscle density (MD; mg/cm^3), an index of skeletal muscle fat content. After adjustment for covariates, multiple linear regression analyses showed positive associations between femur BMC and changes in femur BSI ($r=0.40$; $p<0.0001$) and femur SSI ($r=0.43$; $p<0.0001$). Similar trends were noted between tibia BMC and 2-year changes in tibia BSI ($r=0.44$; $p<0.0001$) and SSI ($r=0.60$; $p<0.0001$). Positive associations were found between thigh MCSA and femur SSI ($r=0.16$ $p<0.015$), and between calf MCSA and tibia BSI ($r = 0.13$ $p<0.04$). Calf MD was inversely associated with tibia SSI ($r= -0.11$; $p<0.08$), but not with any other bone parameter. In conclusion, when included in models with BMC, muscle contractile force promoted bone strength, at diaphyseal sites of the femur and metaphyseal sites of the tibia, whereas muscle quality may impact skeletal adaptations in bone strength at weight bearing sites during growth. NIH/NICHD #HD-050775.

INTRODUCTION

Peak bone mass and bone strength achieved in adolescence or early adulthood are primary determinants of fracture risk [4, 21, 22] and both indices are dependent on maximizing mineral accrual during growth. Puberty is a critical period for bone development. Greater than 90% of bone mineral is accrued by the end of adolescence [18], making it an opportune time to promote maximal bone density and bone geometry [6]. Likewise, puberty is a critical time for growth of lean soft tissue mass and risk of increased adiposity. Body weight and exercise are critical factors for maintaining the normal mechanical stimulus on bone during growth [264]. Body composition, rather than weight per se, may be the strongest determinant of bone mineral accrual and maintenance. Muscle mass and presumably, quality, are associated with skeletal density and geometry through the dynamic forces imposed on the skeleton. Strong correlations between lean body mass, a surrogate of muscle mass, and bone mineral content (BMC), are well established, as reflected in observations that accrual of lean mass and bone mass during growth and development occur in a synchronized manner [9, 308]. Consistent with these observations, several studies have reported strong correlations between muscle area and cortical area of the radius in children, adolescents, and adults [109].

The notion that bone is constantly adapting its strength to maintain stability to support the imposing mechanical loads, and the coupling between muscle and bone, relating to the acquisition of peak bone mass, bone strength, and the growing musculature [7, 282, 283] are conventionally viewed in the context of the Frost Mechanostat theory [8, 26]. The Mechanostat theory postulates that muscle is the primary voluntary load on the skeleton such that increasing loads imposed by larger muscle forces

precedes the developmental adaptations in bone mass and bone strength [7-9]. Indeed, several lines of evidence show that structural adaptations in bone size and geometry along with modifications in material composition yield an ultimate bone strength necessary to accommodate imposed loads [6, 9, 108-110]. The functional relationship between bone and muscle require a continuous balance between bone strength and the mechanical forces that normally challenge bone stability [109]. Because muscle contractile forces act as the largest weight-bearing voluntary load on bone [7, 108], increases in muscle strength and thus the muscle force on bone directly influence bone strength acquisition during growth. Mechanistically, it can be postulated that the enhanced use and growth of muscle fibers during development act as an osteogenic stimulus on bone. As a result, the adaptive increases in bone strength are secondary to gains in muscle mass and muscle strength that occur during growth and development [9].

The bone-muscle unit, as it is conceived, should be strongly related to bone strength. Indices of the bone-muscle unit calculated as the ratio of bone mineral content to muscle cross-sectional area (MCSA) [118] or whole body lean mass [8, 9, 308], are limited by the assumptions inherent in ratios and the fact that muscle cross-sectional area provides no information concerning muscle quality. In youth, as in adults, fat infiltration in muscle may impair muscle contraction and limit force on bone. Given that nearly 20% of the pediatric population is obese, increases in skeletal muscle fat content in childhood, which occur along with increased adiposity, may impair skeletal growth and mineralization. Some evidence shows that individuals with high fat skeletal content tend to have lower bone strength with higher marrow adipose content [46]. Age related reductions in skeletal muscle mass and impaired muscle strength resulting in less force on

bone have been suggested to result in decreased bone strength in the elderly [45, 103, 309]. Lipid deposition in skeletal muscle is associated with increased muscle weakness, loss of mobility and increased skeletal fractures [45]. Indeed, cross-sectional studies in middle aged [47] and older adults [309] showed inverse associations between muscle adiposity and cortical volumetric bone mineral density (vBMD) of the tibia, while longitudinal studies [45] have shown that increased skeletal muscle fat infiltration predisposes to increased hip fracture in older adults. Consistent with these findings in adults, we have shown inverse associations between skeletal muscle density, a marker of skeletal muscle fat content, vBMD, and strength of weight-bearing bones in girls [310]. Disruption of normal bone development during growth, resulting in impaired mineral accrual and lower bone strength, would likely increase risk for developing osteoporotic fractures later in life. It is therefore possible that skeletal muscle fat infiltration may serve as an important risk factor for bone health status in children and adolescents and counter the positive effects of muscle cross-sectional area on bone. To date, the effect of increased skeletal muscle fat content on bone development in children has not been investigated.

At present, it remains unclear whether poor muscle quality and low muscle force serve as predictors of impaired bone strength in young children. Thus, the purpose of this study was to examine the components of the muscle-bone unit in growing children, with the aim of determining whether muscle size and muscle quality independently influence bone strength in addition to the influence of bone mineral mass in young girls. It was hypothesized that muscle cross-sectional area and muscle density, surrogates of muscle

force production and quality would be predictors of bone strength in addition to bone mineral content at weight bearing bone sites.

METHODS

Participants

Longitudinal data were analyzed from 245 healthy girls, aged 9 to 13, who were participants in the Jump-In: Building Better Bones study [57, 83, 122]. Fourth and sixth grade girls were recruited from 14 elementary and 4 middle schools in Tucson, Arizona. Exclusion criteria included the inability to read and understand English and learning disabilities (identified by schools) that made it impossible to complete questionnaires or made it difficult to comply with assessment protocols. Individuals taking medications known to affect bone metabolism or who had been diagnosed with medical conditions, or with a disability that limited participation in physical exercise as defined by the Committee on Sports Medicine and Fitness were also excluded [299]. The study protocol was approved by the University of Arizona Human Subjects Protection Committee and the study was conducted in accordance with the Helsinki Declaration. All guardians and participating girls provided written informed consent.

Anthropometry

Measures of body habitus (body mass, standing height, sitting height and bone lengths) were obtained following standardized protocols [242]. Body mass was measured (nearest 0.1 kg) using a calibrated scale (Seca, Model 881, Hamburg, Germany). Standing height and sitting height were measured at full inhalation (nearest millimeter, mm) using a calibrated stadiometer (Shorr Height Measuring Board, Olney, MD). Femur length and tibia lengths were measured on the non-dominant leg. Femur length (nearest

mm) was measured from the proximal aspect of the patella to the inguinal crease. Tibia length (nearest mm) was measured from the proximal end of the medial border of the tibial plateau to the distal edge of the medial malleolus. Coefficients of variation (CVs) for femur and tibia lengths in our laboratory (n=444 girls) are 0.34% and 0.51%, respectively [57]. For each anthropometric variable, two measurements were taken and averaged. Both measurements were repeated if the first two trials differed by more than 4 mm for height, sitting height and bone lengths, and 0.3 kg for body mass, and the average of the second set of measures was used.

Physical Maturation

Maturation was assessed using maturity offset over the more conventional method of Tanner staging due to its reliance on objective anthropometric measurements of linear growth. Maturity Offset is based on estimated years from peak height velocity (PHV) using Mirwald's equation, [245]. These algorithms include interactions among anthropometric measures (i.e., height, weight, sitting height, leg length) and chronologic age to derive a maturity-offset value. Positive maturity offset values represent years after PHV while a negative maturity offset value represents years before PHV. In Mirwald's sample, the maturity offset equation for girls explained 89% of the variance in years from PHV [57, 122, 245].

Dietary Assessment

Total calorie (kcals/day) and total calcium (mg/day) intakes were assessed at baseline and 24-months using the Harvard Youth/Adolescent Questionnaire (YAQ) [250]. The YAQ is a self-administered food-frequency questionnaire (FFQ) to assess usual dietary intake and dietary supplement use during the previous year; acceptable validity

and reproducibility of the YAQ have been established [250, 251]. It has been used to examine associations between nutrition and health-related outcome measures including bone density [252, 300]. Participants completed the YAQ with assistance available. YAQs were reviewed and coded by trained study staff following standard coding procedures [251]. Nutrient analysis was completed by Channing Laboratories (Boston, MA). Mean values of total calorie and total calcium intakes were calculated from baseline and 24-month assessments.

Physical activity

Physical activity (PA) was assessed by the Past Year Physical Activity Questionnaire (PYPAQ) [246], a survey of all sport and leisure-time physical activity in which the respondent engaged at least 10 times in the past year outside of physical education class. The PYPAQ has been modified to include a more comprehensive list of 41 activities common to youth [247]. The modified questionnaire was administered in an interview with the participant and guardian. Total PYPAQ score was computed using a modified equation from Shedd and colleagues [248], which incorporated weight bearing load, frequency and duration of each activity [247].

Bone and Body Composition Assessment

Baseline and 24-month measures of bone mineral content (BMC) and bone strength were assessed using pQCT (XCT 3000, Stratec Medizintechnik GmbH, Pforzheim, Germany, Division of Orthometrix; White Plains, NY, USA). Scout scans were performed to locate the distal growth plates, with the scanner programmed to find the sites of interest based on skeletal lengths. Contour mode 3 (169 mg/cm^3) was used to define the total bone, while Contour mode 1 (710 mg/cm^3), Peel mode 2 (710 mg/cm^3),

and Cort mode 2 (710 mg/cm^3) were used to define at the diaphyseal 20% femur and 66% tibia sites. Additional details regarding pQCT bone measurements, image processing, calculations, and analysis, are published elsewhere [122, 254]. Slice thicknesses were 2.3 mm, and voxel size was set at 0.4mm for all sites. Scanner speed was set at 25 mm/second. BMC was measured at the 20% femur 66% tibia sites relative to the respective distal growth plates on the non-dominant limb [253]. Bone strength index (BSI, mg^2/mm^4) was measured at the distal metaphyseal regions of the femur and tibia, and strength-strain index (SSI, mm^3) at the diaphyseal regions. BSI, calculated as described by Kontulainen [255] provides an estimate of the bone's ability to withstand compression at metaphyseal regions, whereas SSI provides an estimate of the bone's ability to resist torsion and bending forces and is calculated according to manufacturer's protocol (stratec software) as described previously [57]. CVs, previously reported from our laboratory [83, 122], were less than 1.1% for indices of bone strength. Scout scans were performed to locate the distal growth plates, with the scanner programmed to find the sites of interest based on skeletal lengths. Contour mode 3 (169 mg/cm^3) was used to define the total bone, while Contour mode 1 (710 mg/cm3), Peel mode 2 (710 mg/cm3), and Cort mode 2 (710 mg/cm3) were used to define at the diaphyseal 20% femur and 66% tibia sites. Additional details regarding pQCT bone measurements, image processing, calculations, and analysis, are published elsewhere [122, 254]. Slice thicknesses were 2.3 mm, and voxel size was set at 0.4mm for all sites. Scanner speed was set at 25 mm/second.

Soft Tissue Measures

Soft tissue composition at the 20% femur (thigh) and 66% tibia (calf) sites of the non-dominant limb were assessed using pQCT. Tissue characteristics (i.e., adipose,

muscle, and bone) were separated using edge detection and threshold techniques based on attenuation characteristics, which are directly related to tissue composition and density. Details describing edge detection and image filtration for tissue analysis in our laboratory have been previously reported [57]. All images were filtered subsequently with a 7x7 image filter that clearly defined the edge of the muscle and eliminated all bone above 120 mg/cm^3; this ensured that muscle density was the only soft-tissue component being measured within the edge of the muscle. A limitation of pQCT is the inability to distinguish between intra- and extramyocellular fat compartments; however several controlled studies using chemical phantoms, surrogates of limbs with known lipid concentrations, and biochemical studies using muscle biopsies samples have established a clear association between lower muscle density and higher skeletal muscle fat content [105, 305]. Muscle density was used as a composite index of fat content in the intra- and extramyocellular stores [57, 122]. CVs for muscle density (mg/ cm^3) obtained at the calf and thigh regions were 0.9% and 0.4%, respectively.

pQCT data acquisition and analyses followed guidelines provided by Bone Diagnostics, Inc. (Fort Atkinson, WI, USA). All pQCT scans were performed by a single operator, while a second operator analyzed all scans using the Stratec software (version 6.0). The pQCT instrument was calibrated and quality assurance procedures were completed daily in order to ensure precision of measurements.

Soft tissue mass and composition, including total-body mass, and total-body fat mass, were obtained from dual energy x-ray absorptiometry (DXA) scans using the GE Lunar Prodigy (software Version 5.60.003) fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). Subjects were positioned following the standard manufacturer

protocols. All participants were scanned by a certified technician, and all analyses were performed by a single technician. The unit was calibrated daily according to the standard procedures for maintenance and use as recommended by the manufacturer. DXA CVs for precision for measuring soft tissue composition in our laboratory have been previously reported [261, 262].

Statistical Analysis

Data were checked for outliers and normality using histograms, and skewness and kurtosis were calculated for all variables. All variables were normally distributed; thus, no transformations were applied. Dependent variables representing the changes in bone strength were calculated as the difference between baseline and 24-month measurements for each bone variable. Descriptive statistics (means, SDs, and ranges) were calculated, and a paired t-test was used to test for significant differences between baseline and 2-year values. Independent variables were expressed as the average of baseline and 2-year measures. Pearson's correlation coefficient for continuous variables was used to assess bivariate correlations between average muscle density, BMC and muscle cross-sectional area (MCSA) and 2-year changes in bone strength variables. Multiple linear regression analyses, which included calf or thigh muscle density, BMC and MCSA together, were used to test for potential independent associations between the muscle-bone unit components with 2-year changes in bone parameters. Each model included the following covariates: average values for maturity offset, bone length (femur or tibia), physical activity, calorie intake, dietary calcium intake, plus the baseline bone strength parameter. Thigh muscle density, thigh MCSA and femur BMC were used together in all models that included femur bone parameters, and calf muscle density, calf MCSA and tibia BMC

were included in all models that included tibia bone parameters. Linearity, normality and homoscedasticity of residuals were assessed. Collinearity between covariates (correlation criteria= VIF ≥ 10) was also evaluated and covariates with the lowest VIF were included in the model. All regressions included maturity offset rather than age as previous reports from our laboratory have shown it has a stronger relationship with bone parameters [122]. The level of significance was set at $P<0.05$ (two-tailed). All analyses were performed using the Statistical Package for the Social Sciences for Windows, Version 20.0 (SPSS, Chicago, IL,USA).

RESULTS

Descriptive Characteristics

Baseline and 2-year descriptive statistics for the sample (n=245) are shown in Table 1. Based on body mass index (BMI, kg/cm^2), 3.2% of the sample was underweight (BMI<5th percentile), 76% of the sample was healthy weight (BMI 5th to 85th percentile), 13% of the sample was overweight (BMI 85th to 95th percentile), and 7.8% of the sample was obese (BMI>95th percentile) [148]. On average, girls were 1.1 years away from achieving PHV at baseline, ranging from 3.2 years prior to PHV to 1.04 years post PHV. Dietary fat and caloric intakes were consistent from baseline to 24-months. Average baseline caloric intake (1713±642 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans [234]. Average baseline calcium intake (including supplementation) (1015.6±437.3 mg) was 22% lower than the recommended levels (1300mg/d) established by the Institutes of Medicine [267] dietary recommendations.

Height, body mass, body mass index (BMI), femur and tibia lengths, total body lean mass, total body fat mass and lean mass all increased (all *P* values < 0.0001) from baseline to the 2-year follow-up as expected in young girls (table 1). Calf and thigh MCSA and muscle densities, tibia and femur BMC (*P*<0.0001) and bone strength indices also increased significantly (*P*<0.0001) over the 2-year period. Physical activity level (*1.8%*), total caloric intake (*5.1%*) and total calcium intakes (*5.6%*) did not change from baseline to 2 years (*P* = 0.57).

Results from unadjusted bivariate correlation analysis showing the associations between bone-muscle unit components and changes in bone strength are summarized in table 2. Average femur BMC (20% site) (r= 0.45-0.55) was significantly correlated (all p<0.01) with changes in indices of bone strength measured at the femur metaphyseal and diaphyseal sites. Positive correlations between thigh MCSA (r=0.39-0.51) were significant (all p<0.01) with changes in bone strength parameters, whereas thigh muscle density was not significantly correlated with any changes in bone strength. Similarly, positive correlations were found between tibia BMC (66% site) (r=0.55-0.69) with changes in indices of bone strength measured at the tibia metaphyseal and diaphyseal sites (all p<0.01). Average calf MCSA (r=0.42-0.61) was significantly correlated with changes in bone strength parameters (all p<0.01). Calf muscle density was not significantly correlated with any changes in bone strength.

Associations between muscle-bone components and changes in bone strength

The results of the multiple linear regression analyses illustrates the independent contribution (partial r) of average thigh and calf muscle density, MCSA and BMC (femur and tibia) to 2 year changes in bone strength and bone geometry after controlling for

average maturity offset, bone length, total-body fat mass, physical activity and dietary calorie and calcium intakes and the baseline bone parameter (table 3). Positive associations were observed between femur BMC and changes in femur BSI (partial $r=0.40$; $p<0.0001$) and femur SSI (partial $r=0.43$; $p<0.0001$) measured at metaphyseal (4%) and diaphyseal (20%) regions of the femur, respectively. Thigh MCSA was positively associated with change in femur SSI (partial $r=0.16$ $p<0.015$), but not femur BSI, whereas no associations were found between thigh MD and changes in femur BSI and femur SSI.

Similar trends were observed between muscle-bone unit components and 2-year changes in tibia bone strength parameters. Positive associations were found between tibia BMC and 2 year changes in BSI (partial $r=0.44$; $p<0.0001$) and SSI (partial $r=0.60$; $p<0.0001$) at metaphyseal (4%) and diaphyseal (66%) regions of the tibia, respectively. Average calf MCSA was positively related to change in tibia BSI (partial $r = 0.13$ $p<0.04$) but not tibia SSI, whereas an inverse association was found between average calf muscle density and change in SSI of the tibia (partial $r= -0.11$; $p<0.08$).

DISCUSSION

In this longitudinal analysis, we investigated the influence of individual components of the muscle-bone unit, including bone mass, muscle size, and muscle quality, on changes in bone strength in young girls, aged 9-13 years. The results showed that greater femur or tibia BMC, a measure of bone mass is associated with increases in bone strength at weight bearing bone sites during growth. Additionally, larger muscle cross-sectional area (a surrogate for muscle force) was independently associated with greater increases in bone strength at metaphyseal tibia and diaphyseal femur bone sites.

However, muscle density was not a significant contributor to *gains* in bone strength when included in regression models alone, or in combination with other predictors of bone strength. Further analysis showed that girls in whom muscle density decreased over the following two years did not significantly change bone strength, whereas girls who gained in thigh and calf muscle density significantly improved bone strength at weight bearing sites of the femur and tibia (data not shown). While bone mineral accrual was the strongest determinant of gains in bone strength, other adaptations in response to muscle force occur that contribute to bone strength. Because the largest physiological loads are caused by muscle contractile force, our data are in accordance with the mechanostat theory, demonstrating that larger muscle size and force, along with bone mass, predict gains in bone strength [283].

Strong linear relationships between BMC and muscle development in children and adults have been previously reported by Schenau and Frost (2008), suggesting that BMC is a function of muscle force or size [7, 269]. Furthermore, skeletal muscle is well-established determinant of bone strength; contractile forces on bone are a function of muscle size and reports by Petit et al (2005) indicate that higher muscle size generally parallels gains in muscle strength [84]. Our longitudinal data support previous findings by Schenau and Rauch [269] as well as our previous cross-sectional findings [57, 247]. To our knowledge, this is the first study to assess the independent effects of muscle quality on changes in bone strength beyond the influences of muscle size and bone mass in young girls. These results underscore the importance of achieving peak bone mass in childhood for the prevention of fracture risk later in life. Given that diminished bone strength is a primary risk factor for fracture, subtle adaptations that lead to strengthening

of bone (through increased bone mineral mass) during growth likely occur in response to the loading stresses from skeletal muscle.

The overall function of the muscle-bone unit is to develop an optimal bone structure adding new material only to sites undergoing the most stress. The bone's response to the mechanostat effects differ by the skeletal site, as bony regions closer the ground (i.e., weight bearing bones of the appendicular skeleton), compared to proximal bone regions, have the greatest anabolic response to mechanical loading, resulting in greater osteogenic potential to increase bone mass and bone strength [98]. As expected, in this study, we showed that femur and tibia BMC, a measure for bone mass, was associated with gains in bone strength at metaphyseal and diaphyseal sites of the femur (partial r= 0.40-0.43) and tibia (partial r=0.44-0.60) (all $p<0.0001$). Furthermore, femur and tibia BMC were associated with gains in total and trabecular vBMD at each bone site. In addition, thigh MCSA, a surrogate of muscle force, contributed to the increase in bone strength at diaphyseal sites of the femur (partial r=0.16; $p<0.017$) while calf MCSA was significantly associated with changes in BSI at metaphyseal sites of the tibia (partial r=0.13, $p<0.04$). The influence of larger muscle force on femur bone strength is likely explained by the larger circumference of muscle located at the shaft of long bones. In contrast, because metaphyseal sites of the tibia are located closer to the ground and are therefore more exposed to the higher strain and frequency loads, the tibia metaphysis is likely to undergo a greater adaptive bone strength response compared diaphyseal sites of the tibia. Likewise, these results support previous cross-sectional findings that have examined the association between MCSA and measures of bone density, structure, and strength [122, 284], in which MCSA was significantly correlated (partial r = 0.55–0.82)

with indices of bone strength in girls. Further, longitudinal data from our laboratory suggest that particularly, in children and adolescents, skeletal muscle cross-sectional area is strongly related and bone strength [311], and therefore serves as an important determinant of bone development in girls (Farr et al, under review).

Clinical observations in young children have noted that inadequate muscle development interferes with adequate bone development [7, 9, 109]. To our surprise, thigh and calf muscle density were not associated with any changes in bone strength. In subsequent analysis, girls who lost muscle density over the following two years did not significantly change their bone strength, whereas girls who gained muscle density improved bone strength at both femur and tibia sites (all $p<0.01$).Moreover, girls experienced slight gains in thigh (1.22 ± 1.49 mg/cm^3) and calf (0.98 ± 1.44 mg/cm^3) muscle density, resulting in small increases in bone strength. Furthermore, it is possible that with growth related changes in body composition, greater variability in muscle density and greater gains in muscle density in this sample may contribute a larger impact on bone strength in young girls. These findings are important as they suggest that slight gains in muscle density may promote small gains in bone strength, which may reduce the fracture risk and the development of osteoporosis later in life.

This study was not without limitations. First, this sample included both pre-and early pubescent girls who were primarily of normal weight status. Although total body fat mass was accounted for, measurements in assessing localized fat depots such as skeletal muscle fat infiltration were limited. While pQCT can accurately measure skeletal muscle density, it is cannot distinguish between intramyocellular (IMCL) and extramyocellular (EMCL) fat compartments. Previous studies in adults using Proton magnetic resonance

spectroscopy (MRS) in adults [259], as well as in youth [260] have indicated that composite measures of IMCL and EMCL such as skeletal muscle density, are sufficient indices of skeletal muscle fat content and support the use of pQCT estimates of muscle density as a cost-effective, low-radiation dose imaging tool feasible for large-scale studies. Further, the limited range in growth of muscle density in this population may partly explain the low influence of muscle density on bone strength and development in this sample.

Previous studies assessing the muscle-bone relationship in children have been limited by the use of whole body or regional lean mass as a surrogate for muscle mass or muscle strength [9, 312, 313]; however, this provides an approximation rather than a precise measurement of the dynamic forces produced by muscle contractions on bone [118]. Moreover, whole body lean mass is more closely related to body size, rather than muscle strength, as this assessment provided by DXA includes organ mass and connective tissue into its calculation. Further, use of dual-energy x-ray absorptiometry (DXA) is confounded by changes in bone dimensions during growth, and does not provide estimates of muscle-cross-sectional area or site-specific measurements of bone mass, both of which are important component of bone strength and fracture risk. Thus, an important strength of this study was the use of pQCT, which can safely and accurately assess the contributions of site-specific changes in bone mass (BMC), and individual components of the muscle-bone unit (i.e., muscle density, muscle cross-sectional area) to changes in indices of bone strength during bone development. Additionally, pQCT estimates of muscle-cross sectional area at weight bearing bone sites at of the calf and thigh [9, 118, 312, 313] provide a more direct estimate of muscle force production. Lastly,

unlike past studies, we accounted for important covariates, including maturation and modifiable factors, such as diet (calories, calcium) and physical activity, which undoubtedly modify bone development during pubertal transitions.

In conclusion our results demonstrated that muscle cross-sectional area, a surrogate of muscle contractile force promoted bone strength, independent of bone mineral mass, at diaphyseal sites of the femur and metaphyseal sites of the tibia. While muscle density was not a significant contributor to changes in bone strength, further analysis showed that when compared to girls who lost muscle density over the following two years, and therefore did not significantly change their bone strength, girls who gained muscle density improved bone strength at both femur and tibia sites. As expected, BMC, an indicator of bone mass and an integral component of the muscle-bone unit, promotes the largest gains in bone strength and bone density at the femur and tibia in young girls, and the response to gains in BMC was generalizable across the weight-bearing skeleton, as gains in bone strength were seen at metaphyseal and diaphyseal sites of the femur and tibia. Together, these findings underscore the importance of muscle size and force in promoting peak bone mineral content, and directly stimulating bone strength, during growth and development.. Implementation of interventions that target muscle mass and increase muscle contractile force to optimize peak bone mass and bone strength during childhood may prevent the development of osteoporosis and bone related injuries later in life.

TABLES

Table 1: Sample Descriptive Characteristics ($\bar{x}$± SD) at baseline and 2 years (n=245)

	Baseline (x±SD)	24-Month (x±SE)	Percent Change
Age	10.6±1.1	12.7±1.1	-
Maturity Offset	-1.1±1.0	0.7±1.0	-
Height (cm)	144.4±10.0	157.0±9.2	8.8
Body Weight (kg)	38.8±9.9	50.2±12.2	29.5
BMI	18.4±3.2	20.2±3.8	9.9
Femur Length (cm)	34.07±3.07	36.8±2.6	8.0
Tibia Length	33.2±2.9	36.4±2.5	9.8
Physical Activity Score	5291.7±4677.4	5196.7±4230.4	-1.8
Caloric Intake (kcal)	1713.0±642	1625.1±590.7	-5.1
Total Calcium (mg)	1015.6±437.3	958.4±423.2	-5.6
Lean Mass (kg)	25.5±5.1	32.2±5.6	26.2
Fat Mass (kg)	11.1±6.1	15.4±7.9	39.0
TBLH BMC (kg)	1.0±0.3	1.5±0.4	46.2
Thigh Muscle Density (mg/cm^3)	76.3±1.6	77.5±1.4	1.6
Calf Muscle Density (mg/cm^3)	79.0±1.2	80.0±1.2	1.2
20% Femur BMC (g)	230.1±48.1	297.6±61.6	29.4
20% Thigh MCSA (mm^2)	3555.5±716.3	4406.8±917.7	23.9
4% Femur BSI (mg^2/mm^4)	94.8±27.2	124.1±36.1	30.9
20% Femur SSI (mm^3)	1323.3±400.6	1888.6±523.5	42.7
66% Tibia Total BMC (g)	228.1±43.5	284.6±52.0	24.8
66% Tibia MCSA(mm^2)	3201.2±586.0	3878.8±667.1	21.2
4% Tibia BSI (mg^2/mm^4)	50.7±12.9	68.2±19.7	34.6
66% Tibia SSI (mm^3)	1159.7±327.8	1600.7±417.8	38.0

Values are presented as mean ± SD. Total calcium intake includes diet and supplement intake. *P* values represent paired samples *t*-Test for difference between the baseline and 2-year study visit; BMI = body mass index; MCSA = muscle cross-sectional area; TBLH= total body less head; BMC= bone mineral content (g); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3).

[a] All values significant at P<0.0001except physical activity score, calorie and calcium intake.

Table 2: Bivariate relationships between bone-muscle unit components and changes in bone strength

Dependent variables	**Average Thigh MD**	**Average Femur BMC**	**Average Thigh MCSA**
4% Femur ΔBSI	-0.01	0.45[a]	0.39[a]
20% Femur ΔSSI	0.08	0.55[a]	0.51[a]

Dependent variables	**Average Calf MD**	**Average Tibia BMC**	**Average Calf MCSA**
4% Tibia ΔBSI	-0.05	0.69[a]	0.61[a]
66% Tibia ΔSSI	-0.04	0.55[a]	0.42[a]

All covariates represented as average data computed over 2 years. All bone variables are represented as the change from baseline to 24 months. MD= muscle density (mg/cm^3); BMC= bone mineral content (g); MCSA = muscle cross-sectional area (mm^2); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3)

[a] P<0.01; Pearson's r for continuous variables

Table 3: Independent associations from multiple linear regression of average muscle density, bone mass and muscle cross-sectional area on changes in bone strength

Dependent variables	**Average Thigh MD**		**Average Femur BMC**		**Average Thigh MCSA**	
	partial r	p-value	partial r	p-value	partial r	p-value
4% Femur ΔBSI	0.03	0.60	0.40	<0.001	0.01	0.93
20% Femur ΔSSI	-0.04	0.56	0.43	<0.001	0.16	0.01
Dependent variables	**Average Calf MD**		**Average Tibia BMC**		**Average Calf MCSA**	
	partial r	p-value	partial r	p-value	partial r	p-value
4% Tibia ΔBSI	-0.07	0.30	0.44	<0.001	0.13	0.04
66% Tibia ΔSSI	-0.11	0.08	0.60	<0.001	0.10	0.11

All bone outcome variables were calculated as the change occurring from baseline to 24-months. MD= muscle density; BMC= bone mineral content (g); MCSA = muscle cross-sectional area; BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3). All regression models were adjusted for covariates: maturity offset, bone length (femur or tibia), total-body fat mass, physical activity, calorie and calcium intakes and the respective baseline measure of bone strength.

APPENDIX D: LADDU DR, LEE VR, BLEW RM, LAUDERMILK MJ, ROE D, THOMSON CA, GOING SB. DIETARY FATTY ACID CONSUMPTION ON BONE DEVELOPMENT IN YOUNG GIRLS

Calcif Tissue Int.

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Author emails:

Deepika Laddu (dladdu@email.arizona.edu)
Vinson R. Lee (vinsonl@email.arizona.edu)
Robert M. Blew (rblew@email.arizona.edu)
Monica J. Laudermilk (mlaudermilk@coreperformance.com)
Cynthia A. Thomson cthomson@email.arizona.edu
Denise Roe droe@email.arizona.edu
Timothy G. Lohman (lohman@email.arizona.edu)
Scott B. Going (going@email.arizona.edu)

Corresponding Author:

Deepika Laddu
Department of Nutritional Sciences
The University of Arizona
1713 E. University Blvd. #93
Tucson, AZ, USA 85721-0093
Phone: 602-615-7338
Fax: 520-621-8170
E-mail: dladdu@gmail.com

ABSTRACT

Both n-3 and n-6 dietary fatty acids (FAs) are necessary for growth and development. Given the increasing consumption of n-6 FAs, and the limited intakes of n-3 FAs in typical western diets, the importance of a high fat intake in the increasing prevalence of childhood and adult obesity remains controversial, while qualitative changes (i.e. the fatty acid composition of fats) have been largely disregarded. The purpose of this study was to evaluate the effects from consuming various long-chain polyunsaturated fatty acids (LCPUFAs) and ratios of n-3 and n-6 FA intake on indices of bone strength and bone development. Two-year data collected on 245 girls, aged 8-13 years, who participated in the "Jump-In: Building Better Bones" study were analyzed. FA intake was assessed from the Harvard Youth/Adolescent Food Frequency Peripheral quantitative computed tomography was used to measure bone strength index (BSI, mg^2/mm^4) and strength-strain index (SSI, mm^3) and bone geometry (e.g., volumetric bone density, vBMD, mg/cm^3) at distal metaphyseal and diaphyseal regions of the femur and tibia,. Multiple linear regression analyses with baseline data of the dietary LCPUFA, total calorie (kcal/day), total calcium (mg/day), total body fat mass, total body lean mass, bone length (femur or tibia) and the respective baseline bone measurement as independent variables, dietary intakes of AA was negatively associated with cortical vBMD measured at distal regions of the femoral diaphysis (partial r= -0.13, $p<0.05$), where as both LA/ALA (partial r=0.15, $p<0.02$;) and the ratio of n-6/n-3 FA [AA+LA/ALA+EPA+DHA] (partial r=0.17, $p<0.01$) were positively related to average total vBMD of the tibia. AA, LA/ALA nor n-6/n-3 FA showed significant associations with indices of bone strength. Dietary fatty acids including EPA, DHA, ALA, LA, PUFA, omega3 (EPA+DHA), AA:omega-3 ratio, the sum of all LCPUFAs (AA+EPA+DHA), total n3 FA (ALA+ EPA+DHA), total n-6 FA (LA+AA) were also not significant with any 24-

month indices of bone strength or bone density. Analysis of covariance (ANCOVA) was used to compare bone parameter among tertiles of LCPUFAs. Girls who met at least half the recommended values of omega-3 intakes had significantly higher total vBMD measured at the tibia but not with any other bone density or bone strength indices. **Conclusion**: The results of this study suggest that the role of n-3 FAs appear to neither benefit nor harm bone strength and development in young girls. Decreased intakes of AA n-6 FA may benefit bone health, where as consuming recommended ratios of select n-6 to n-3 FA ratios may benefit skeletal development in young girls.

INTRODUCTION

Osteoporosis is a dehibilitating bone disease associated with increased bone fragility, micro-architectural deterioration, decreased bone strength and increased risk of bone fracture. Once considered a disease of the elderly, osteoporosis is now thought to have pediatric antecedents. Puberty is a critical period for bone growth and development, as maximizing bone mass and strength during skeletal growth is a primary determinant of fracture risk and the development of osteoporosis later in life [4, 21, 22, 72]. Indeed, the bone mineral acquired during childhood and adolescence is governed by the critical convergence of genetic, hormonal, nutritional, and environmental factors that interact to stimulate bone formation for the enhancement of linear growth and skeletal expansion [44]. While genetic endowment can predict 60-80% of the variability in skeletal growth and development [10], modifiable factors such as nutritional status clearly influence full skeletal growth and expression of genetic potential.

Historically, nutrients such as calcium, vitamin D, and protein have been the subject of considerable research for the effects on bone health whereas other nutritional factors have received far less attention. Long-chain polyunsaturated fatty acids (LCPUFAs) have recently been recognized for their role in the prevention of various chronic diseases (e.g., coronary artery disease, type 2 diabetes, rheumatoid arthritis, and cancers [53, 168, 228]. There is some data that suggests that the benefits of LCPUFAs may also extend to bone, as increasing epidemiological evidence has shown that low intakes of certain types of fatty acids contribute to bone loss [168, 171, 189, 213, 224, 228, 285]. Although the precise mechanism remain unclear, there is ample evidence that proposes that the two families of essential fatty acids, alpha-linolenic acid (ALA, 18:3ω-

3) and gamma-linoleic acids (LA, 18:2 ω-6), which are precursors for their biologically active LCPUFAs, eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3), and arachidonic acid (AA, 20:4ω-6) may modulate bone metabolism in part, due to their immune-modulating and anti-inflammatory actions [167, 169, 170].

In the United States, intakes of omega 3 fatty acids (EPA+DHA) are suboptimal. In contrast, the western diet is replete with omega-6 fatty acids (n-6 FA) as most individuals consume nearly 11-30 times more omega-6 fatty acids than omega-3 fatty acids (n-3 FA), far above the daily recommended n-6 intakes [286]. Given the current dietary shift from higher saturated fat (SFA) consumption to higher intakes of n-6 PUFAs and increased ratio of *n*-6:*n*-3 FA in western diet [287], the biological importance of PUFA intake on bone development and bone maintenance is critical to assess in humans.

To date, much of the investigation of LCPUFAs on bone have been in animals, with results indicating the need for both n-6 and n-3 LCPUFAs for achieving optimal bone growth and development [168, 171, 218, 314, 315]. However, evidence from animal studies suggests n-6 and n-3 LCPUFAs appear to modulate bone metabolism through competing and contrasting mechanisms. To illustrate, n-3 -3 has consistently shown to have a stimulatory effect on bone formation and mineral accretion at both cortical and trabecular bone sties [55, 171, 189, 212, 214, 218]. Positive association between ω-3 PUFAs intake and increased bone calcium content and bone formation rates have also been noted in rats [51], suggesting a possible role of ω -3 PUFA in preventing the formation of lipid peroxidation, which normally inhibits osteoblastogenesis [48, 51]. In contrast, n-6 LCPUFA primary role is to promote bone resorption, though numerous

studies show that n-6 eicosanoid metabolites, including two-series prostaglandins (i.e., PGE_2), and four-series leukotrienes (e.g.,LTC_4, LTB_4), can control both bone resorption and bone formation pathways in a biphasic, dose-dependent manor [55, 168, 171, 189, 217]. At lower concentrations, PGE_2 can act as a potent activator of osteogenic transcription factors (Cba1, IGF-1) that enhance bone formation rates while at higher concentrations, PGE_2 mediates increased synthesis of bone turnover proteins (i.e., RANKL, RANK) and pro-inflammatory cytokines (IL6, TNFα, MCSF,IL-1β, INF-γ) to the bone surface, together, contributing to the degradation of the bone matrix.

Results illustrating the effects on various patterns of LCPUFA intakes or n-3 FA supplementation on bone density and bone strength in humans are limited. The results from the few studies have recently evaluated the effects of various patterns of LCPUFA intakes or n-3 FA supplementation on bone density and bone strength have been equivocal, concluding either positive [49, 53, 204, 231, 314, 316], or no beneficial nor harmful effects [226, 227, 317] on bone outcome measures; however these results are limited to observational studies conducted in postmenopausal women [48, 225-227, 237, 318] and in men [204, 228, 229]. Likewise, the relationships between n-3 and n-6 FAs on bone in children and youth are equally scarce and complex, Indeed, one Norwegian study found that dietary total PUFAs were positively associated with the change in bone mineral density of the ultradistal radius in a cohort of growing females [239]. Interestingly, cross sectional analyses in healthy eight year old children consuming varying ratios of n-6 and n-3 LCPUFAs showed positive associations between %AA and whole body aBMD but negative correlations between %total n-6 fatty acids and lumbar spine aBMD; positive association between DHA concentrations and lumbar spine BMD

were found only in children of the uppermost tertile of weight and BMI, after controlling for sex and anthropometric variables [218]. Hogstrom and colleagues also found that n-3 fatty acids, particularly, DHA, was positively associated with total and spinal BMD and bone mineral accrual in adolescent males [49]. Together these results suggest that consumption of dietary FAs may modulate bone mass and bone development in children [218, 239, 240]. However, due to limited evaluation of the dietary FA and its effect on weight bearing bone sites (i.e. forearm, wrist, leg), use of DXA, which, unlike pQCT, is confounded by growth and is unable to estimates of bone geometry, an important component of bone strength [83], and the limited studies and inconclusive results in children, it is imperative to better understand the relationship between FA intakes and bone development, as nutrition is an important risk factor for bone health status as well as other metabolic diseases, that can be modified as early as childhood and adolescence.

To our knowledge, no studies have assessed the longitudinal relationships between dietary intakes of various LCPUFA and indices of bone development from pQCT in young girls during the critical phase of bone mineral accrual. Early cross-sectional evidence from our laboratory has indicated positive associations between bone mineral content, size and bone strength outcomes and EPA, and negative relationships with total PUFA, total n-6 and LA. However, it is unclear whether these relationships remain significant when assessing bone development. Thus the purpose of this study is to investigate whether consuming individual and varying ratios of n6 and n3 FAs during pre-or early puberty influence bone strength and bone development in young girls. We hypothesize that higher intakes of n-3 FA will promote gains in bone strength, while higher consumption of n-6 FA will be negatively associated with weight bearing bone

sites in young girls.

METHODS

Participants

Longitudinal data were analyzed from 245 healthy girls, aged 9 to 13, who were participants in the Jump-In: Building Better Bones study [57, 83, 122]. Fourth and Sixth grade girls were recruited from 14 elementary and 4 middle schools in Tucson, Arizona. Exclusion criteria included the inability to read and understand English and learning disabilities (identified by schools) that made it impossible to complete questionnaires or made it difficult to comply with assessment protocols. Girls taking medications which could alter bone metabolism or diagnosed with medical conditions, or with a disability that limited participation in physical exercise as defined by the Committee on Sports Medicine and Fitness were also excluded [299]. The study protocol was approved by the University of Arizona Human Subjects Protection Committee and the study was conducted in accordance with the Helsinki Declaration. All guardians and participating girls provided written informed consent. After informed consent, guardians completed a health history questionnaire with questions that also inquired about participant ethnicity and race [57, 122].

Anthropometry

Anthropometric measures (body mass, standing height, sitting height and bone lengths) were obtained following standardized protocols [242]. Body mass was measured to the nearest 0.1 kg using a calibrated scale (Seca, Model 881, Hamburg, Germany) and both standing height and sitting height were measured at full inhalation to the nearest millimeter (mm) using a calibrated stadiometer (Shorr Height Measuring Board, Olney,

MD). Femur length and tibia length were measured on the non-dominant leg. Nondominant femur length (nearest mm) was measured from the apex of the lateral epicondyle (base of the patella) to the inguinal crease. Non-dominant tibia length (nearest mm) was measured from the proximal end of the medial border of the tibial plateau to the distal edge of the medial malleolus. Coefficients of variation (CVs) for femur and tibia lengths in our laboratory are 0.34% and 0.51%, respectively (n=444 girls) [57]. For each anthropometric variable, two measurements were taken and averaged. Both measurements were repeated if the first two trials differed by more than 4 mm for height, sitting height and bone lengths, and 0.3 kg for body mass. If repeat measures were taken, then the average of the second set of measures was used in the analysis [57, 122].

Physical Maturation

Maturation status was assessed using maturity offset over the more conventional method of Tanner staging due to its reliance on objective anthropometric measurements of linear growth. Maturity Offset is based on estimated years from peak height velocity (PHV) using Mirwald's equation, [245] which was derived from data from a 6-year longitudinal study in boys and girls [18]. These algorithms include interactions among anthropometric measures (i.e., height, weight, sitting height, leg length) and chronologic age to derive a maturity-offset value. In females, Maturity offset (y) = -9.376 + 0.0001882*Leg Length (cm) and Sitting Height (cm) interaction + 0.0022*Age (y) and Leg Length (cm) interaction + 0.005841*Age (y) and Sitting Height (cm) interaction – 0.002658*Age (y) and Weight (kg) interaction + 0.07693*Weight (kg) by Height (cm) ratio [18, 245]. Positive maturity offset values represent years after PHV, while a negative maturity offset value represents years before PHV. In Mirwald's sample, the

maturity offset equation for girls explained 89% of the variance in years from PHV [57, 122, 245].

Dietary Assessment

Dietary fat and caloric intakes were assessed using the semi-quantitative Harvard Youth/Adolescent Questionnaire (YAQ) [250]. The YAQ is a self-administered food-frequency questionnaire (FFQ) consisting of 152 questions with estimated serving sizes and frequencies of intake for 131 foods and dietary supplement use during the previous year; acceptable validity and reproducibility of the YAQ have been established [250, 251] and it has been used in studies reporting significant associations between nutrition and various outcomes such as childhood obesity, physical activity, and bone density [252]. Participants completed the YAQ with assistance available from a trained adult. All YAQs were reviewed by a trained study staff for completeness and coding for nutrient analysis was completed by Channing Laboratories (Boston, MA) following the standard coding procedures [251].

The YAQ quantified dietary variables of interest including total calcium intake (mg), total energy intake (kcal), essential fatty acid parent compounds, ALA (grams), LA (grams), and the corresponding long-chain fatty acids, EPA (grams), DHA (grams), AA (grams), total PUFA (polyunsaturated fat; grams), the sum of omega-3 FA (EPA+ DHA; grams) and the sum of LCPUFA (AA+EPA+DHA; grams). Total n-6 FA (LA+AA; grams), total n-3 FA (ALA+EPA+ DHA, grams), and PUFA ratios including LA/ALA, AA/omega-3FA (EPA+DHA), and total n-6 (LA+AA)/ total n-3 (ALA+EPA+DHA) were computed for the basic data.

Bone and Body Composition Assessment

Changes in bone geometry, bone strength and volumetric bone mineral density (vBMD) were assessed using pQCT (XCT 3000, Stratec Medizintechnik GmbH, Pforzheim, Germany, Division of Orthometrix; White Plains, NY, USA) at the 4% and 20% femur and 4% and 66% tibia sites relative to the respective distal growth plates on the non-dominant limb. The metaphyseal regions were chosen to represent skeletal sites predominantly comprised of trabecular bone whereas the diaphyseal regions were chosen to represent skeletal sites predominantly comprised of cortical bone. Cortical parameters were not assessed at metaphyseal regions because the spatial resolution of the pQCT device used in this study is not sufficient to analyze cortical shells of less than 2 mm [253].

Bone parameters measured at distal metaphyseal regions of the femur and tibia included trabecular vBMD (mg/cm^3) and bone strength index (BSI, mg^2/mm^4) whereas bone parameters measured at diaphyseal regions of the femur and tibia included cortical vBMD (mg/cm^3) and strength-strain index (SSI, mm^3). BSI estimates the bone's ability to withstand compression at metaphyseal regions, and is calculated as the product of the metaphyseal total area and total vBMD squared: Bone strength index (BSI, mg^2/mm^4)=total area x total $vBMD^2$ [255]. SSI is used to estimate the bone's ability to resist torsion and bending forces at diaphyseal regions. Diaphyseal SSI was calculated using Stratec software (Version 6.0; [254], which is based on the integrated product of the geometric properties (i.e., section modulus) with the material properties of bone: Strength-strain index (SSI, mm^3)= , n ($[(r^2_i \times a)/r_{max}] \times$ (cortical vBMD/ND) [248]; section modulus is calculated as $(r^2_i \times a)/r_{max}$, where a is the area of a voxel (mm^2), r is

the distance of a voxel from the center of gravity (mm), and r_{max} is the maximum distance of a voxel from the center of gravity (mm). The material properties of bone are calculated as the quotient of measured cortical density (cortical vBMD, mg/cm^3) and normal physiologic cortical density (ND,1200 mg/cm^3).

Scout scans were performed to locate the distal growth plates, with the scanner programmed to find the sites of interest based on skeletal lengths. Slice thicknesses were set to 2.3 mm and voxel sizes were set to 0.4 mm. Scanner speed were set at 25 mm/s. Additional details regarding pQCT bone measurements, image processing, calculations, and analysis, are published elsewhere [122, 254]. CVs previously reported in our laboratory [83, 122] were less than 1.1% for vBMD, bone geometry, and indices of bone strength (ie, BSI and SSI). pQCT data acquisition and analyses followed guidelines provided by Bone Diagnostics, Inc. (Fort Atkinson, WI, USA). All pQCT scans were performed by a single operator, while a second operator analyzed all scans using the Stratec software (version 6.0, [254]). The pQCT instrument was calibrated and quality assurance procedures were completed daily in order to ensure precision of measurements.

Soft tissue mass and composition, including total-body mass, total-body fat mass, and whole body lean mass were obtained from dual energy x-ray absorptiometry (DXA) scans using the GE Lunar Prodigy (software Version 5.60.003) fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). Subjects were positioned following the standard manufacturer protocols. All participants were scanned on the same machine, and DXA scans were performed and reviewed by one of three certified technicians (MB, RB or HF). The Lunar Prodigy was calibrated daily according to the standard procedures for maintenance and use as recommended by the manufacturer. DXA CVs and precision in

measuring body soft tissue composition laboratory have been reported [261, 262].

Statistical Analysis

Data were initially checked for outliers and normality using histograms, and all variables were tested for skewness and kurtosis. Skewed nutrient intakes (AA, PUFA, omega-3, sum LCPUFA, total n-3 FA, total n-6 FA, ratio [LA/ALA], ratio [AA/omega-3], ratio [total n-6/totaln-3]) were normalized using log transformations or square root transformation (EPA, DHA); transformed data were used in all subsequent regression analyses. Total energy (kcal) and total calcium (mg) intakes were normally distributed and were therefore left untransformed, as were bone variables. All bone outcomes including bone density, bone strength and bone structure, respectively, are represented as 24-month measurements. Descriptive statistics (means, SDs, and ranges) were calculated for the entire sample. Appropriate control for maturation, in addition to total body fat, and lean masses, are necessary in studies of growing children, since the relationship between nutritional factors and bone accrual and linear growth during adolescence may be complicated by inter-individual variability in the onset and duration of maturation process [4].

Associations were estimated from bivariate correlations using Pearson's r for continuous and Spearman's rho for categorical variables in order to determine simple relationships between bone outcome variables and covariates. Multiple linear regression analysis was used to 24-month bone variables and baseline measures of dietary FAs, after controlling for baseline, maturity offset, total energy (kcal) and calcium (mg) intakes, total body fat mass (kg), and lean mass (kg), femur or tibia bone length (cm), and the baseline measure of the respective bone outcome variable. Linearity, normality and

homoscedasticity of residuals were assessed. Collinearity between covariates (correlation criteria= VIF $\geq$ 10) was also evaluated and the relevant covariates with the lowest VIF were included in the model. To protect against collinearity, femur length or tibia length without height was included in regression models for femur and tibia bone outcomes, respectively. All regressions included maturity offset rather than age as previous reports from our laboratory have shown a stronger relationship with bone parameters [122]. The effect of individual FAs, as well as the sums and ratios of FA intake on bone outcomes were evaluated as separate models to determine whether the relationships between higher n3 FA intake and bone parameters differed from higher n6 FA intakes.

Consumption of select n-3 and n-6 FAs including ALA, LA, total n-6/n-3 ratio, were then categorized into two groups based on if established dietary recommended intakes for these FAs were met (FA=1) or not met (FA=0). Because recommendations of omega 3 FA were not met by this sample, groups were made according to those who met half (FA=1) the recommended level and those who did meet this level (FA=0)meeting half the recommended intake Analysis of covariance (ANCOVA) on adjusted means was used to compare all bone outcomes among respective two-groups of these FAs, after adjusting for baseline covariates, maturity offset, total energy (kcal) and total calcium (mg) intakes, total body fat mass (kg), total body lean mass (kg), femur or tibia bone length(cm), and the respective baseline bone measure.. Bonferroni post hoc tests were also used to adjust for multiple comparisons among the two of ALA, LA, omega-3, 10:1 ratio [total n-6/totaln-3, respectively. The level of significance was set at $P<0.05$ (two-tailed). All analyses were performed using the Statistical Package for the Social Sciences for Windows, Version 20.0 (SPSS, Chicago, IL,USA).

RESULTS

Descriptive Characteristics

Descriptive statistics are given in Table 1. Based on body mass index (BMI, kg/cm^2), at baseline, 3.3% of the sample was underweight (BMI<5th percentile), 75% of the sample was healthy weight (BMI 5th to 85th percentile), 13.5% of the sample was overweight (BMI 85th to 95th percentile), and 8.2% of the sample was obese (BMI>95th percentile) [148]. On average, girls were 1.1 years away from achieving PHV, ranging from 3.2 years prior to PHV to 1.04 years post PHV. Average baseline caloric intake (1751.4±637.6 kcal) met the dietary recommendations for moderately active girls of this age (1600-2000 kcal) established by the 2010 Dietary Guidelines for Americans (DGA) [234]; average baseline calcium intakes (including supplementation) (1028.9mg±442.8mg) did not meet national recommended levels (1300mg/d) established by Institutes of Medicine [267] dietary recommendations.

Dietary intake

Because epidemiological evidence is available for select FA, current national guidelines have established recommended intakes for FA that has been shown to modulate with disease outcomes. Recommendations for children and adolescents are relatively equal to those for adults and include ALA(1.0-1.2 g/d [234, 267], LA (10g/d) [234], omega-3 (EPA+DHA; 200-250mg/day) [234] and PUFA (500mg/d) [234, 267]; Clarity regarding recommendations for n-6/n-3 FA ratio is warranted as 5:1 n-6/n-3 is ideal; however, 10:1 n-6/n-3 FA ratio, which reflects the average ratio consumed in the Western diet, meets the upper level "safety" guidelines before it imposing an increased

risk for CVD and related disease outcomes. Suggested intakes (g/d) for individual n-3 (EPA, DHA) or n-6 FA (AA) have yet to be established (Table 2).

Understandably, the average intake of established recommended intakes of n-3 FA, including ALA (1.0 g/d±0.44), omega-3 FA (0.07g/d±0.05) by girls in this study was well below the reported recommendations; on average, PUFA intakes (11.55g/d ±4.8) met or exceeded recommended levels. In contrast, average intakes of LA (10.15g/d±4.23), and the n-6/n-3 ratio (9.8:1g/d ±1.7) met or were slightly higher than recommendations (Table 2).

Analysis of Dietary Fatty Acid Intake and Bone Parameters

Correlations between model covariates and 2-year bone outcomes are illustrated in table 3. Calcium and calorie intake were not significantly correlated with any bone outcomes, whereas positive correlations were observed between TBFM (r=0.426 -0.484) and TBLM (r=0.621-0.862) and all bone strength indices measured at the metaphyseal regions of femur and tibia and diaphyseal sites of femur and tibia. Similar positive correlations were found between TBFM (r=0.19-0.22; $p<0.01$) and TBLM (r=0.15-0.50; $p<0.01$) and all vBMD outcomes measured at the metaphyseal and diaphyseal regions of the femur and tibia.

Correlations between dietary predictors and 2-year bone outcomes are summarized in table 4. In brief, correlations were found between FAs and 24-month indices of bone strength but not bone density. Negative correlations were found between BSI at metaphyseal regions of the tibia and LA/ALA (r= -0.172, $p<0.007$) and n-6/n-3 FA (r= -0.166; $p<0.009$). Both LA/ALA and n-6/n-3 FA ratios were also negatively correlated with SSI at diaphyseal regions of the tibia (all r= -0.144 $p<0.024$). Additional

negative correlations between n-6/n-3 FA and BSI at the metaphyseal femur (r= -0.147; p<0.022) and diaphyseal femur (r= -0.179; p<0.005) were noted. Correlations between other dietary variables and bone strength or bone density parameters were not significant.

The results of the multiple linear regression analyses are shown in table 5. Results from regression analysis concluded that after controlling for baseline covariates, total calorie (kcal), total calcium (mg), TBFM, TBLM, bone length (femur or tibia) and the respective baseline bone measurement, dietary intakes of AA was negatively associated with cortical vBMD measured at distal regions of the femoral diaphysis (p<0.045; r=-0.130), where as both LA/ALA (p<0.020; r=0.151) and the ratio of n-6/n-3 FA [AA+LA/ALA+EPA+DHA] (p<0.010; r=0.167) were positively related to average total vBMD of the tibia. AA, LA/ALA nor n-6/n-3 FA showed significant associations with indices of bone strength. Dietary fatty acids including EPA, DHA, ALA, LA, PUFA, omega3 (EPA+DHA), AA:omega-3 ratio, the sum of all LCPUFAs (AA+EPA+DHA), total n3 FA (ALA+ EPA+DHA), total n-6 FA (LA+AA) were also not significant with any 24-month indices of bone strength or bone density. Additionally, in all regression models, baseline TBFM was positively significant with 24-month BSI measured at metaphyseal sites of the femur (r=0.17-0.176; p<0.008), while TBLM showed positive associations with 24-month BSI of the metaphyseal site of the tibia (r=0.20-0.21; p<0.001) and SSI of diaphyseal femur (all r=0.19; p<0.004). TBFM was also significantly related with trabecular vBMD of the femur (r=0.16; p<0.014) but was negatively associated with tibia cortical vBMD (r= -0.18 to -0.19; p<0.004). In contrast, TBLM was positively related with cortical vBMD of the femur (r= 0.14; p<0.03) and trabecular vBMD of the tibia (r=0.19, p<0.004) in most, but not all regression models.

Comparison of bone parameters across groups of individual meeting dietary FA requirements

Comparisons of the 24M-bone parameters across groups of FA intake categorized according to those who met or did not meet recommended guidelines were performed using ANCOVA adjusting for baseline maturity offset, bone length (femur or tibia), diet (calorie; calcium) and the respective baseline bone outcome variable. Adjusted marginal means (95% CI) for vBMD, geometry and bone strength indices across groups of FA intake categorized according to those who met or did not meet recommended guidelines are reported in table 6. Girls who met at least half the recommended values of omega-3 intakes had significantly higher total vBMD at the tibia but not with other bone vBMD sites of the femur or tibia, nor was there any relationship with bone strength indices. Girls who met required levels of ALA compared to those who consumed less than the recommendations did not significantly differ in bone strength and bone density at 24-months. Similarly, no significant differences among individual meeting or not meeting LA or total n-6/n-3 ratio requirements were found.

DISCUSSION

The importance of a high fat intake in the increasing prevalence of childhood and adult obesity remains controversial, while qualitative changes (i.e. the fatty acid composition of fats) have been largely disregarded. The purpose of this study was to evaluate the longitudinal effects from consuming various long-chain polyunsaturated fatty acids (LCPUFAs) and ratios of n-3 and n-6 FA intake, indices of bone strength and bone development. To our knowledge, this is the first longitudinal study to investigate the

effects different LCPUFA patterns on bone strength and structural properties in pre-pubescent girls. The results from this study indicate that dietary intake of various types of n-3 LCPUFAs (e.g., ALA, LA, omega3,EPA, DHA, PUFA) are not associated with 24-month measures of bone strength, bone density and bone structural properties in girls who consumed moderate intakes in their diet. Furthermore, higher versus lower intakes of select n-3 LCPUFAs or n-6 LCPUFAs showed no favorable effect on bone outcomes. Interestingly, negative associations between AA and cortical vBMD measured at the 20% site of the femur diaphysis, whereas positive relationships between ratios, LA/ALA and total n-6/total n-3, and tibia total vBMD were noted. Together, these results suggest that although the role of n-3 FAs appears to neither benefit nor harm bone strength and development in young girls, further research is needed to investigate these differentiating effects of higher n-6 FA consumption on skeletal development and whether the bone promoting effects from various LCPUFA ratios continue throughout growth and maturation.

To date, most research concerning the role of n-3 or n-6 FA intake on bone [50, 142, 167, 212, 228] has focused on marine based sources of n-3 EPA and DHA [231, 319-322]. However, because ALA and LA serve as the predominant sources of n-3 and n-6 FAs in the US diet, investigation of the effects ALA and LA intakes, the sums of LCPUFAs (i.e., total n-3 FA, total n-6 FA) and ratios including ALA and LA (LA:ALA) on bone structural and strength properties were explored. No associations between individual n-3 LCPUFAs, E PA, DHA, ALA, LA, PUFA, omega3 (EPA+DHA), AA:omega-3 ratio, the sum of all LCPUFAs (AA+EPA+DHA), total n3 FA (ALA+ EPA+DHA), total n-6 FA (LA+AA), despite prior research from animal [48, 50, 54, 142,

323] and human studies [49, 172, 218, 228] demonstrating the importance of these higher n-3 and lower n-6 FAs for enhancing bone formation, bone mineral accrual and optimal bone development . Although analyses were run using n-3 LCPUFA variables that included both dietary and supplement use (if reported), the lack of association between any n-3 FA intakes and bone parameters may be explained by the low consumption of n-3 FA from marine sources, which is the easiest and most abundant source of n-3 FAs, in tandem with the low supplement use (i.e. multi-vitamin) and higher proportion of n-6FA consumed in the diet. While the dietary YAQ used in this study did include a large variation of EPA+DHA foods, on average, the consumption of omega-3 (EPA+DHA) was below the global recommendations of 500mg/day, established by the DGA [234]. Therefore, it is likely that the lower than recommended intakes of these n-3 LCPUFAs made it difficult to assess an association with bone density variables.

Interestingly, our findings indicate that body weight variables (i.e. TBFM, TBLM) may possibly outweigh any influence of dietary fatty acids on bone strength and development young girls. Results from bivariate analysis showed that while both TBFM and TBLM were positively correlated with all 24-month bone indices, only select fatty acids, including LA/ALA and n-6/n-3 FA were negatively correlated with BSI at the metaphyseal (20%) tibia, and SSI at the diaphyseal (66%) region of the tibia (table 3). Negative correlations between n-6/n-3 FA and BSI at the metaphyseal femur ($r= -0.147$; $p<0.022$) and diaphyseal femur ($r= -0.179$; $p<0.005$) were also noted. When evaluating the relationship between body composition and these particular FAs, LA/ALA ($r= -0.211$; $p<0.001$) and n-6/n-3 ($r= -0.177$; $p<0.005$) were negatively correlated with TBLM but neither were significantly correlated with measures of TBFM. Despite the negative

correlations between respective FA ratios and bone indices, results from linear regression analysis suggested positive associations between tibia total vBMD and LA/ALA ($r=0.15$; $p<0.02$) and n-6/n-3 FA ($r=0.17$; $p<0.01$), while negative associations were found between AA ($r=-0.13$; $p< 0.05$) and femur cortical vBMD. Accordingly, in all regression models, where the respective FA was the main predictor, either TBFM or TBLM were significantly associated with all bone strength and all bone density parameters indices, with the exception of tibia SSI and femur total vBMD, respectively. Further, in models where TBFM or TBLM were significant, TBFM explained 79% of variance in bone strength indices and 63-76% of the variance in bone structure parameters at weight bearing sites of the femur and tibia, while TBLM explained 83%- 87% of variance in bone strength and additionally 54-77% of variance in bone structural outcomes measured at femur and tibia skeletal sites. Together, these findings suggest that the change in direction of association between LA/ALA and n-6/n-3 in regression models can partly explained by the influence of TBFM and TBLM in the model, such that fat and lean masses may be stronger predictors of bone development than dietary variables, alone. These results are in agreement with earlier studies in our lab showing that positive cross-sectional [122] and longitudinal (unpublished) suggesting whole body fat and lean masses may augment bone density and bone strength by mechanisms of increased mechanical stress on bone, absorbing force associated with falls, and positive metabolic effects on mineral accrual by hormones secreted by adipocytes [58, 107].

Optimal intakes of total n-3 and n-6 FAs remain unclear. Conventionally, trends towards a positive relationship between ALA and omega-3 FA consumption and lower intakes of n-6:n3 FA and bone density have been reported in both children and

adolescents [218, 239, 240, 324], as well as in the elderly [53, 228, 320, 325]. However, results from our ANCOVA analysis showed no significant difference in bone strength and structural indices among individuals meeting or exceeding the dietary recommendations of ALA, LA, omega-3 and n-6/n-3 FA ratio and those who did not meet these guidelines. The current dietary shift from higher saturated fat (SFA) consumption to higher intakes of n-6 PUFAs and increased ratio of *n*-6/n-3 FA in the western diet [218], may also contribute to the higher intakes of n-6/n-3 FAs (10:1) observed in our cohort. Given that the ideal ratio of n-6/n-3 for bone health remains debatable, ranging from 4:1 to an upper limit of 10:1, the significantly higher consumptions of total n-6 versus total n-3 ($p<0.0001$), and average intake of 10:1 n-6/n-3 reported by this sample may underscore why substantial gains in bone strength bone density associated with higher n-3 intakes were not found in these young girls.

At present, the relationship between LCPUFA intake and bone in remain inconclusive. Our results showing positive associations between n-6 and bone development indices and no association with n-3 FAs were unexpected. However, our findings are consistent with previous studies reporting significant correlations between AA with total body bone density in healthy young children [218]. Data from Women's Health Initiative similarly reported that higher consumption of *n*-6 FAs were associated with a modest decrease in total fracture risk in post-menopausal women, while higher n-3 intakes (EPA+DHA) were associated with a small but significant increase in risk of fractures [232]; however, similar to our study, low intakes of marine based sources of n3 fatty acids (EPA+DHA) and the lack of data on fish-oil or n-3 FA supplementation use might have limited the findings in this study. Coincidently higher ALA consumption was

associated with lower hip fracture risk in women, but not men participating in the Framingham Osteoporosis Study where as protective associations between higher AA consumption and reduced hip fracture risk were observed only in men [236]. Likewise Macdonald et al (2004) also concluded excess PUFAs or an imbalance between the n−6 and n−3 fatty acids was associated with bone loss at the lumbar spine and femoral neck [237]. In toto, the results from these studies suggest that both n-6 and n-3 FA are necessary for optimal bone development, however, the ideal balance of n-6 to n-3 ratio that is needed to elicit a positive response on the skeleton and whether skeletal sites respond differently to the various n-3 and n-6 LCPUFAs remains unknown. Further, it is possible that while the type and quantity of LCPUFA consumed influence bone, the impact of n-3 FA consumption for promoting positive changes in BMD may be more detectable in more vulnerable populations, including youth who are undergoing the pubertal transition and maturation.

The overall balance of n-3 and n-6 bioactive compounds is regulated by the competition and binding to the Δ-6 desaturase enzyme, which is necessary for conversion of ALA or LA PUFA parent compounds into the endogenous formation of eicosanoid metabolites [173]. Notably, increasing the consumption of ALA and thus, the bioavailability of n-3 EPA or DHA, can competitively inhibit Δ-6 desaturase and the formation of AA derived eicosanoids [172, 174]; consequently the efficiency in converting ALA to EPA and DHA is limited and can be further compromised when higher intakes of n-6 PUFAs or saturated fatty acids are consumed in the diet. Thus, higher intake of LA n-6 PUFAs have a quantitative and competitive advantage of n-3 PUFAs to bind to the desaturase enzymes and increase AA and n6 eicosanoids

concentrations into circulation. The positive association between the ratio of LA/ALA and the 10:1 n-6/n-3 FA ratio and total density measured at the tibia (table 5) might support such interpretation. Further, the immune-modulating and anti-inflammatory actions n-3 and n-6 LCPUFAs are equally important to consider [169, 170, 288], as the composition of the LCPUFA consumed has a direct impact on the type, quantity and the inflammatory nature of the eicosanoid released into circulation. To illustrate, evidence from animal studies suggest that the bone-regulatory actions of n-6 LCPUFAs, carried out by n-6 metabolites, prostaglandin 2-series (PGE_2) and 4 series leukotrienes (LTB_4, LTC_4), can control both, bone resorption and bone formation pathways in a biphasic, dose-dependent manor (figures 1-2) [55, 168, 189, 217]. In contrast, n-3 illustrates stimulatory effects on bone formation at both, cortical and trabecular bone sites, in part, by competitively regulating concentrations of n-6 AA in circulation, and increasing the expression of bone formation proteins (e.g., osteocalcin, alkaline phosphatase) and matrix synthesis proteins (i.e., collagen 1 fibers) (figure 2). Because girls in this study were consuming n-6 FAs slightly above recommended levels, the bone promoting immune-modulating effects of lower dose n-6 may explain the positive associations between LA/ ALA and n6/n3 and bone density. However, because serum concentrations of eicosanoids, prostaglandins, hormones or cytokines was not assessed in this present study, we are unable to confer whether the higher intakes LA/ALA and n-6/n-3 or moderate intakes of omega-3 or PUFAs were related to an increased inflammatory cytokine production in these girls.

This study included both strengths and limitations. The primary strengths of this study included the use of pQCT for measuring various indices of bone strength including

material (volumetric BMD; vBMD, mg/cm^3) and structural properties (cortical thickness, cross-sectional area; CSA) in children, without the confounding of growth [4, 57]. Thus, unlike DXA, which is confounded by changes in bone dimensions during growth, with pQCT, the contributions of changes in bone mass, density, and geometry to changes in indices of bone strength, often reflected during bone development, can also be can also safely and accurately be estimated in young children. Adequate control for maturation using gender-specific algorithms by Mirwald et al [245], to predict years from PHV, which, despite yielding similar bone strength and density results when running analyses with Tanner stage, maturity offset was chosen because its association with bone parameters was consistently higher in this sample [122]. Appropriate control for whole body fat mass and lean mass by direct analysis using DXA, rather than indirect assessment of body composition [35, 79-81, 124, 326] allowed for proper assessment of total and regional body fat, and not body weight, on indices of bone strength and development. Lastly, utilization of the validated YAQ to assess dietary intake adds an additional strength to this study. However, assessment of dietary and nutrient intake is challenging, especially in children. Although the semi-quantitative, YAQ has been validated as a reliable and adequate tools to assess children and adolescent habitual intakes over time [251], it has not been validated specifically for individual FAs (i.e. n-3, n-6, EPA and DHA). Given this inherent limitation, future studies that use FFQs that are validated for essential micronutrients, such as n-3 and n-6 metabolites are necessary for the evaluating the relationships between dietary patterns and disease outcomes. Vitamin D status was not assessed in this sample, as difficulties in obtaining accurate measures of vitamin D status from FFQ are partly explained by the large variability in measuring

intakes of vitamin D from foods and/or supplements, as well as from fortified foods, and the lack of data regarding individual sun exposure [297, 298]. In addition, blood serum specimens for hormones known to directly stimulate osteoblast-mediated formation (i.e., estrogen, insulin-growth factor I (IGF-1) were assessed in this study. Notably, although supplementation was analyzed in addition to dietary consumption, including populations who consume larger variations n-3 rich foods may have further strengthened this study.

In conclusion, our results suggest that that n-6 but not n-3 FA intake appears to effect bone development in pre-and early-pubescent girls; however, the consumption of n-3 FAs were below the recommended levels whereas higher ratios of n-6/n-3 FAs consumed by this sample may also explain why longitudinal findings do not support the previous cross-sectional results from our lab that suggested a potential benefit on bone from consuming higher amounts of n-3 PUFA and lower intakes of n-6 LCPUFAs (unpublished). Because findings from the literature are inconclusive and dietary recommendations for LCPUFAs have not been clearly established, further evaluation regarding the optimal intakes and dietary ratios of n-6/n3 FA to optimize peak bone mass is warranted.

TABLES

Table 1: Descriptive characteristics and pQCT bone parameters for sample (n=245)

	Mean
Caloric Intake (kcal)	1751±638
Ca^{2+} intake(mg)	1028.9±442.79
Maturity Offset	-1.14±1.03
Femur Length (cm)	34.05±3.05
Tibia Length (cm)	33.17±2.88
TBFM (kg)	11.17±6.07
TBLM (kg)	25.52±4.93
Fatty Acids	
ALA (g)	1.00±0.44
LA (g)	10.15±4.23
EPA (g)	0.02±0.02
DHA (g)	0.05±0.04
AA (g)	0.09±0.05
OMEGA-3 (g)	0.07±0.06
Total PUFA (g)	11.55±4.80
total n-3 (g)	1.07±0.46
total n-6 (g)	10.24±4.26
AA+omega3 (g)	0.16±0.09
AA/omega-3 (g)	2.05±1.56
LA/ALA (g)	10.45±2.06
total n-6/total n-3	9.84±1.97
Bone Measures	
Femur Metaphyseal site (4%)	
4% Femur BSI	95.27±26.94
Femur Total Density (ave)	275.43±33.69
4% Femur Trabecular Density (mg/cm^3)	237.78±31.97
Femur Diaphyseal site (20%)	
20% femur SSI	1323.07±392.71
20% Femur Cortical Density (mg/cm^3)	1045.16±23.45
Tibia Metaphyseal site (4%)	
4% tibia bsi	50.74±12.82
Tibia Total Density (mg/cm^3)	293.95±35.14
4% Tibia Trabecular Density (mg/cm^3)	222.44±25.91
Tibia Diaphyseal site (66%)	
66% Tibia SSI	1159.82±322.36
66% Tibia Cortical Density (mg/cm^3)	1027.53±31.55

[a:] Values include sources from diet and supplements

Dietary Recommended Intakes (DRI) established by the IOM (60) and DGA (59).

ALA, alpha-Linolenic acid (18:3n-3); PUFA, poly-unsaturated fatty acid; LA, Linoleic acid (18:2n-6); AA, Arachidonic acid (20:4n-6); omega-3 (EPA+DHA); total n-6/totaln-3, AA+LA/ALA+EPA+DHA

Table 2: Comparison between FA recommended intakes and Average FA consumed by Sample

Fatty acid	DRI (g/d)	Sample Intake (Mean±S.D)
ALA	1.0-1.2 g/d	1.0 g/d±0.44
LA	10g/d	10.15g/d±4.23
Omega-3	0.2-0.25 g/d	0.07g/d±0.05
PUFA	0.5g/d	11.55g/d ±4.8
n-6/n-3 FA ratio	5:1 -10:1g/d	9.8:1g/d ±1.7

a: Values include sources from diet and supplements
Dietary Recommended Intakes (DRI) established by the IOM (60) and DGA (59).
ALA, alpha-Linolenic acid (18:3n-3); PUFA, poly-unsaturated fatty acid; LA, Linoleic acid (18:2n-6); AA, Arachidonic acid (20:4n-6); omega-3 (EPA+DHA); total n-6/totaln-3, AA+LA/ALA+EPA+DHA

Table 3: Bivariate Correlations between important covariates and 24-month pQCT bone measurements

Bone Variable	Caloric Intake (kcal)	Ca^{2+} intake(mg)	Maturity Offset	Femur Length (cm)	Tibia Length (cm)	TBFM (kg)	TBLM (kg)
Baseline Bone variables							
24M_4% Femur BSI	-0.09	-0.02	0.55[a]	0.46[a]	0.48[a]	0.43[a]	0.62[a]
24M_20% Femur SSI	-0.05	0.01	0.81[a]	0.79[a]	0.81[a]	0.47[a]	0.86[a]
24M_4% Tibia BSI	-0.07	0.01	0.65[a]	0.58[a]	0.57[a]	0.45[a]	0.74[a]
24M_66% Tibia SSI	-0.09	-0.02	0.71[a]	0.71[a]	0.73[a]	0.48[a]	0.77[a]
24M_ Femur Tot vBMD	-0.03	0.04	0.26[a]	0.12	0.15[b]	0.19[a]	0.28[a]
24M_20% Femur Cort vBMD	0.08	0.03	0.56[a]	0.44[a]	0.393[a]	0.21[a]	0.50[a]
24M_4% Femur Trab vBMD	-0.06	-0.00	0.10	-0.02	0.04	0.20[a]	0.15[b]
24M_Tibia Tot vBMD	-0.08	0.03	0.37[a]	0.25[a]	0.23[a]	0.21[a]	0.39[a]
24M_66% Tibia Cort vBMD	0.02	0.044	0.61[a]	0.53[a]	0.51[a]	0.19[a]	0.54[a]
24M_4% Tibia Trab vBMD	-0.07	0.02	0.19[a]	0.15[b]	0.16[b]	0.22[a]	0.32[a]

TBFM= total body fat mass; TBLM= total body lean mass; Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); BSI = bone strength index; SSI = Strength-strain index.
[a] $P < 0.001$; Pearson's r for continuous variables
[b] $P < 0.05$; Pearson's r for continuous variables

Table 4: Bivariate Correlations between baseline dietary fatty acids and 24-month pQCT bone measurements

Bone Variable	ALA (g)	LA (g)	PUFA (g)	AA (g)	EPA (g)	DHA (g)	OMEGA-3 (g)	Total PUFA (g)	AA/ omega-3 (g)	LA/ ALA (g)	total n-6 (g)	total n-3 (g)	total n-6/total n-3
24M_4% Femur BSI	-0.03	-0.10	-0.10	-0.05	-0.02	-0.05	-0.01	-0.04	-0.02	-0.14[b]	-0.10	-0.03	-0.15[b]
24M_20% Femur SSI	-0.00	-0.10	-0.11	-0.08	-0.10	-0.08	-0.09	-0.10	0.04	-0.20[a]	-0.10	-0.02	-0.18[a]
24M_4% Tibia BSI	-0.02	-0.10	-0.10	-0.05	-0.05	-0.06	-0.03	-0.06	-0.00	-0.17 [a]	-0.10	-0.02	-0.17[a]
24M_66% Tibia SSI	-0.05	-0.12	-0.12	-0.08	-0.06	-0.05	-0.05	-0.07	-0.01	-0.14[b]	-0.12	-0.05	-0.14[b]
Femur Tot vBMD	0.02	-0.01	-0.02	0.01	0.05	0.04	0.08	0.05	-0.08	-0.07	-0.01	0.02	-0.09
24M_20% Femur Cort vBMD	0.08	0.06	0.06	-0.04	-0.05	-0.04	-0.05	-0.05	0.02	-0.06	0.06	0.08	-0.04
24M_4% Femur Trab vBMD	-0.00	-0.03	-0.03	0.02	0.08	0.04	0.09	0.06	-0.08	-0.07	-0.03	0.01	-0.09
24M_ Tibia Tot vBMD	-0.05	-0.08	-0.09	-0.09	-0.06	-0.06	-0.03	-0.07	-0.02	-0.06	-0.08	-0.06	-0.05
24M_66% Tibia Cort vBMD	0.05	-0.02	-0.02	-0.06	-0.07	-0.05	-0.06	-0.07	0.02	-0.15[b]	-0.02	0.04	-0.14[b]
24M_4% Tibia Trab vBMD	-0.03	-0.07	-0.07	-0.01	0.05	0.05	0.09	0.04	-0.10	-0.07	-0.07	-0.02	-0.10

All diet variables were normalized using log transformed, except for EPA and DHA, in which a square-root transformation was applied. Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); Omega-3: EPA+DHA, total n-3 LCPUFA: EPA+DHA+ALA; total n-6 LCPUFA: LA+ AA; sum LCPUFA: AA+EPA+DHA; ratio total n-6/total n-3: LA+AA/EPA+DHA+ALA; LCPUFA, long-chain poly unsaturated fatty acid; ALA, alpha-Linolenic acid (18:3n-3); EPA, Eicosapentaenoic acid (20:5n-3); DHA, Docosahexaenoic acid (22:6n-3); PUFA, poly-unsaturated fatty acid; LA, Linoleic acid (18:2n-6); AA, Arachidonic acid (20:4n-6); BSI = bone strength index; SSI = Strength-strain index.

[a] $P < 0.001$; Pearson's r for continuous variables

[b] $P < 0.05$; Pearson's r for continuous variables

Table 5: Regression coefficients for *select* baseline dietary fats regressed on 24-month bone density parameters

	AA		ratio LA/ALA		ratio total n6/total n3	
Bone Density	**Partial r**	**Model R^2_{adj}**	**Partial r**	**Model R^2_{adj}**	**Partial r**	**Model R^2_{adj}**
Femur Tot vBMD	0.02	0.78	0.01	0.78	0.00	0.78
20% Femur Cort vBMD	-0.13[b]	0.54	0.04	0.53	0.04	0.53
4% Femur Trab vBMD	0.07	0.76	0.00	0.76	0.00	0.76
Tibia Tot vBMD	-0.08	0.76	0.15[b]	0.76	0.17[a]	0.76
66% Tibia Cort vBMD	-0.03	0.62	-0.04	0.63	-0.04	0.62
4% Tibia Trab vBMD	-0.01	0.77	0.04	0.77	0.03	0.77

All diet variables were normalized using log transformed, except for EPA and DHA, in which a square-root transformation was applied. Model covariates: baseline calorie intake (kcal), calcium intake (mg/day), maturation status, total body fat (kg), total body lean mass (kg), bone length (femur; tibia), and the respected estimated of the baseline bone outcome. Trab vBMD= Trabecular volumetric bone density (mg/cm^3); Cort vBMD=cortical volumetric bone mineral density (mg/cm^3); AA, Arachidonic acid (20:4n-6); ALA, alpha-Linolenic acid (18:3n-3); LA, Linoleic acid (18:2n-6); ratio total n-6/total n-3: LA+AA/EPA+DHA+ALA; EPA, Eicosapentaenoic acid (20:5n-3); DHA, Docosahexaenoic acid (22:6n-3);

[a] P<0.01, Pearson's r for continuous variables

[b] P<0.05, Pearson's r for continuous variables

Table 6: Adjusted means of 24-month bone strength and bone density parameters by groups of select baseline dietary FAs.

0 vs. 1	**OMEGA3**	**ALA**	**LA**	**total n-6/n-3**
Bone Strength Variables				
4% Femur BSI	0.73	0.54	0.72	0.46
20% Femur SSI	0.10	0.85	0.38	0.25
4% Tibia BSI	0.45	0.14	0.83	0.57
66% Tibia SSI	0.23	0.60	0.65	0.54
Bone Density Variables				
Femur Tot vBMD	0.17	0.75	0.17	0.27
20% Femur Cort vBMD	0.46	0.28	0.32	0.79
4% Femur Trab vBMD	0.84	0.78	0.57	0.44
Tibia Tot vBMD	0.02[a]	0.31	0.11	0.52
66% Tibia Cort vBMD	0.80	0.75	0.77	0.38
4% Tibia Trab vBMD	0.59	0.12	0.94	0.18

Estimated means ±SE for 24M femur and tibia bone strength and density indices across groups of select dietary FAs
All diet variables were normalized using log transformed, except for EPA and DHA, in which a square-root transformation was applied. Model covariates: baseline calorie intake (kcal), calcium intake (mg/day), maturation status, total body fat (kg), total body lean mass (kg), bone length (femur; tibia), and the respected estimated of the baseline bone outcome.Omega-3: EPA+DHA, ALA, alpha-Linolenic acid (18:3n-3); LA, Linoleic acid (18:2n-6); ratio total n-6/total n-3: LA+AA/EPA+DHA+ALA; EPA, Eicosapentaenoic acid (20:5n-3); DHA, Docosahexaenoic acid (22:6n-3); BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3);Tot vBMD= total (average) volumetric bone mineral density (mg/cm3); Cort vBMD= cortical volumetric bone mineral density (mg/cm3); Trab vBMD=trabecular volumetric bone mineral density (mg/cm^3);

[a]Significant at p<0.05; ANCOVA

APPENDIX E: LADDU DR, LEE VR, BLEW RM, SATO T, LOHMAN TG, GOING, SB. PREDICTING VISCERAL ADIPOSE TISSUE BY MRI USING DXA AND ANTHROPOMETRY IN ADOLESCENTS AND YOUNG ADULTS. IJBCR 2012; 10(4): 93-100.

Author contributions:

Author contributions: DRL analyzed data, interpreted results of experiments, and drafted the manuscript prepared figures. VRL and RMB were project coordinators, and collected data. TS collected and analyzed MRI-VAT data. DRL and SBG edited and revised manuscript. TGL and SBG were the Project Investigators and SBG approved final version of the manuscript.

[1] Department of Nutritional Sciences, University of Arizona, Tucson, AZ, USA
[2] Department of Physiological Sciences, University of Arizona, Tucson, AZ, USA
[3] Research and Development Department Technological strategy Group, OMRON HEALTHCARE Co., Ltd., Kyoto, Japan

Running Title: Abdominal visceral adiposity by DXA

Author emails:

Deepika Laddu (dladdu@email.arizona.edu)
Vinson Lee (vinsonl@email.arizona.edu)
Robert Blew (rblew@email.arizona.edu)
Tetsuya Sato (tetsuya_sato@ohq.omron.co.jp)
Timothy G. Lohman (lohman@email.arizona.edu)
Scott B. Going (going@email.arizona.edu)

Corresponding author:

Deepika Laddu
Department of Nutritional Sciences
The University of Arizona
1713 E. University Blvd. #93
Tucson, AZ, USA 85721-0093
Phone: 602-615-7338
Fax: 520-621-8170
E-mail: dladdu@gmail.com

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Abreviations:
VAT- visceral adipose tissue (*adiposity*)
MRI - magnetic resonance imaging
DXA- dual-energy X-ray absorptiometry
AFM- Android fat mass
ROI- region of interest
CT: Computed tomography
IAAT- Intra-abdominal adipose tissue
SAT: Subcutaneous adipose tissue
WC- waist circumference
HC - hip circumference
WHR- waist-to-hip ratio
BMI- body mass index
SEE- standard error estimate

ABSTRACT

Objective: Accumulation of intra-abdominal (visceral) adipose tissue, independent of total adiposity, is associated with development of metabolic abnormalities such as insulin resistance and type-2 diabetes in children and adults. The **objective** of this study was to develop prediction equations for estimating visceral adiposity (VAT) measured by magnetic resonance imaging (MRI) using anthropometric variables and measures of abdominal fat mass from DXA in adolescents and young adults.

Methods: Cross-sectional data was collected from a multiethnic population of seventy males and females, aged 12-25 years, with BMI ranging from 14.5-38.1 kg/m^2. Android (AFM; android region as defined by manufacturers instruction) and lumbar L1-L4 regional fat masses were assessed using DXA (GE Lunar Prodigy; GE Lunar Corp, Madison, WI, USA). Criterion measures of intra-abdominal visceral fat were obtained using single-slice MRI (General Electric Signa Model 5x 1.5T) and VAT area was analyzed at the level OF L4–L5. Image analysis was carried out using ZedView 3.1.

Results: DXA measures of AFM (r=0.76) and L1-L4 (r=0.71) were significantly ($p<0.0001$) correlated with MRI-measured VAT. DXA AFM, together with gender and weight, explained 62% of the variance in VAT (SEE=10.06 cm^2). DXA L1-L4 fat mass with gender explained 54% of the variance in VAT (SEE=11.08 cm^2). Addition of the significant interaction, gender x DXA fat mass, improved prediction of VAT from AFM (R_{adj}^2=0.61, SEE=10.10cm^2) and L1-L4 (R_{adj}^2=0.59, SEE=10.39cm^2).

Conclusion: These results demonstrate that VAT is accurately estimated from regional fat masses measured by DXA in adolescents and young adults.

INTRODUCTION

Excess intra-abdominal (visceral) adipose tissue, independent of total adiposity, is a major determinant of the metabolic syndrome [165, 292, 327], insulin resistance [165, 274, 328], cardiovascular disease (CVD) [165, 329, 330], and type-2 diabetes [165] in children, adolescents [38, 164] and adults. At present, reliable imaging techniques for measuring visceral abdominal adiposity include magnetic resonance imaging (MRI) and computed tomography (CT), which directly measure intra-abdominal adipose tissue (IAAT), allowing for quantification of several fat depots with a degree of accuracy comparable to chemical analysis [292-296]. However, both are expensive, and access is often limited. Also, the radiation exposure from CT is high, limiting its use in children and longitudinal designs [331]. Lower cost, accessible methods for safely estimating IAAT, especially in children and youth, are needed.

Indirect methods, including the use of a variety of anthropometric measures, e.g., waist circumference (WC), hip circumference (HC), waist-to-hip ratios (WHR) and skin folds [292, 294, 332-334], have also been used to estimate visceral adiposity (VAT). These methods are the most common because they are practical, portable, noninvasive, and inexpensive, and they can be used to monitor changes in diverse clinical settings. Nevertheless, the accuracy of anthropometric variables is limited as variations in body build and the percentage and distribution of adipose tissue, which varies with age, sex, and ethnicity [335], confounds the relationship with VAT [294, 336]. While WC and WHR have been used as convenient surrogates for central adiposity [274, 294, 333, 337, 338] and are useful for characterizing fat distribution, they do not accurately detect small

changes in VAT that can occur over time. Moreover, because a standard measurement site has not been adopted, measurements of WC or WHR are often not comparable [293].

Dual-energy X-ray absorptiometry (DXA), which has emerged as a criterion method for assessing regional and whole body soft tissue composition [293, 294, 331, 339], is less invasive, less expensive and more accessible than CT, and involves only minimal exposure to ionizing radiation [292, 293]. Previous studies have demonstrated that DXA-derived trunk fat mass [294] and abdominal tissue mass in the L1–L4 area are associated with abdominal fat mass and VAT in adults [292, 340]. When compared to WC, DXA can predict fat mass with greater accuracy and reproducibility and may potentially serve as a useful tool for tracking small changes in abdominal fat during weight loss and maintenance therapies [292, 293]. Past studies to predict CT-measured VAT using DXA measures of trunk [294] or regional abdominal fat [292, 341, 342], have focused primarily on adults and its utility for estimating VAT in adolescents has not been established. Thus, the primary aim of this study was to develop an algorithm for accurately estimating VAT from DXA using MRI to obtain criterion measures of VAT. Prediction of VAT from a manually drawn region of interest (ROI) spanning the abdomen (L1-L4) was compared to manufacturer's default regions (trunk and android regions) to assess whether prediction for the default ROI was as good as a manual ROI. Anthropometric variables were included in the analysis to determine whether prediction improved when anthropometry and DXA were combined.

METHODS

Participants

Anthropometric characteristics and body composition assessments were

completed on 70 males and females, 12-25 years of age, following the procedures described below. The protocol was approved by the University of Arizona Human Subjects Protection Committee, and the study was conducted in accordance with the Helsinki Declaration. Written informed consent was received from all subjects and the guardians of participants under the age of 18 years. Volunteers were ***excluded*** if they had a history of chronic disease (e.g., HIV/AIDS, congestive heart failure, unstable angina), or cancer; any implanted electronic medical equipment or external life support equipment; metal implants; jewelry that could not be removed; had taken medications that may affect body composition, fat distribution, or physical activity (i.e. growth hormone); had been diagnosed with a disease or condition that may affect body composition (i.e., Cushing's Syndrome, Type 1 or Type 2 diabetes, thyroid disorder) [334] or had learning disabilities that made it difficult to complete questionnaires, were unable to comply with assessment protocols, or unable to read and understand English. Females who were pregnant or nursing were also excluded. Individuals were also excluded if they had a fear of small-enclosed spaces or were unable to remain in a lying or sitting position for an extended period (≥30minutes) of time, as required by the MRI procedures.

At the initial visit, demographic data (i.e., age, gender, race/ethnicity) were obtained through questionnaires; anthropometric measurements, including weight, height, waist, and hip circumferences were also taken. Subjects were then scheduled for a whole body DXA scan and total body MRI scan. All measurements were completed within a 7-day period.

Anthropometry

Measures of body weight, standing height, and waist and hip circumference were obtained by a trained anthropometrist. Body weight was measured to the nearest 0.1 kg, using a calibrated digital weighing scale (Seca Model 770 scale, Hamburg, Germany), with subjects minimally clothed in light-weight swimwear or underwear. Standing height (stature) was measured to the nearest 0.1 cm with the shoes removed and the head in the Frankfort plane using a standard stadiometer (Shorr Height Measuring Board, Olney, MD). Waist circumference was measured to the nearest 0.1 cm with anthropometric tape placed at the midpoint between the lower rib margin and the iliac crest, and hip circumference was measured to the nearest 0.1 cm at the widest point over the greater trochanters. Each anthropometric variable was measured three times and the respective averages were used in the analysis.

Dual energy x-ray absorptiometry (DXA)

Soft tissue mass and composition, including total-body mass, total-body fat mass, and region specific abdominal fat mass, were assessed using dual-energy X-ray absorptiometry (DXA) using the GE Lunar Prodigy (software Version 5.60.003) densitometer (GE Lunar Corp, Madison, WI, USA). Subjects were positioned following the standard manufacturer protocols. Participants were asked to lay supine with their arms resting by their sides (not touching the body), wrists pronated, and hands flat [339]. Subjects were scanned in light clothing or hospital gowns, with all artifacts removed from the scan area [293, 343]. All participants were scanned on the same machine, and DXA scan acquisition and review were performed by one of two certified technicians. The

Lunar Prodigy was calibrated daily according to the standard procedures for maintenance and use as recommended by the manufacturer. DXA CVs for precision of whole body and regional soft tissue composition in our laboratory are <1 to 3% (22), similar to estimates reported from other laboratories [262, 344].

DXA *Regional* Analysis

Abdominal area regions of interest (ROIs) included android and L1-L4 regions. Android fat mass (AFM), available from the manufacturer's automated ROIs, is defined as the area enclosed between a demarcation above the iliac crest to the 20% mark of the total distance between the iliac crest and the base of the skull. The manually drawn L1-L4 ROI was chosen based on previous studies in adults [292, 343] and adolescents [339]. L1-L4 was defined via delineation of the lumbar spine region including the area bounded by the upper most border of the L1 to the lower most border of L4.

Magnetic Resonance Imaging

Abdominal VAT was estimated using whole-body magnetic resonance imaging (MRI). MRI was performed by an experienced technician using the General Electric Signa Model 5x 1.5T MRI scanner. Subjects lay supine on the scanner bed, with their arms extended above their heads. Images in abdominal and thoracic regions were obtained with the subjects holding their breath. The scanning process was divided into two parts with the ischial tuberosity as the point of origin to divide the body into upper and lower sections. The lower body was scanned first, followed by the upper body. Total test time was approximately one hour. The total number of axial images taken across the abdominal area was determined relative to the participant's height (height/50mm; spacing between slice=50mm; field of view 480 mm (1.875 mm * 256 pixel); slice

thickness=10.0 mm thickness). The single slice method was used to estimate the intra-abdominal visceral fat area (cm^2). Images were analyzed using ZedView 3.1 (LEXI Corporation, Ltd., Tokyo, Japan (http://www.lexi.co.jp/e_zedview.html). Protocol details have been published elsewhere [345]. Briefly, the software employed knowledge-based image processing to label pixels as fat and nonfat components using on the basis of the gray-level histograms of the images. Each slice was manually reviewed and VF area was analyzed at the level L4–L5. Voxels arising from fatty bowel content were deleted. VF in cm^2 was divided by 10 and rounded to derive VF_{level} [345]. The MRI scanner was calibrated daily according to the manufacturer instructions for maintenance and use.

Statistical analysis

Scatter plots were examined for outliers and skewness and kurtosis were calculated for all variables. Descriptive statistics were calculated for the entire sample. Bivariate relationships were estimated using Pearson's correlation coefficients (r) for continuous variables and Spearman's rank order correlation coefficients (rho) for categorical variables. Fischer's Z-transformation test (FZT) was used to test correlation coefficients for differences between males and females. Stepwise multiple regression analyses were run to derive prediction equations for estimating MRI total VAT mass from DXA derived android or L1-L4 abdominal fat in combination with anthropometric measures. Other independent variables *considered* in the models included body weight, height, body mass index (BMI), waist circumference, age, gender (male= -1, female = 1), and ethnicity (Asian=1, African American=2, Hispanic=3, White=4), and the interaction of gender with android and L1-L4 fat. The level of significance was set at $P<0.05$ (two-tailed). All analyses were performed using the Statistical Package for the Social Sciences

for Windows, Version 18.0 (SPSS, Chicago, IL, USA).

RESULTS

Descriptive Characteristics

Descriptive characteristics for the entire sample and by sex are shown in **Table 1**. The sample was comprised of 35 males and 35 females (n=70) and included 2 Asians, 7 African Americans, 15 Hispanic and 46 non- Hispanic white subjects. The sample was a mixture of underweight (n=10), normal weight (n=43), overweight (n=15) and obese (n=2) individuals based on BMI. The mean weight for the entire sample was 64.4kg. Bivariate correlations between potential model covariates and fat masses are shown in table 2. The coefficients did not differ ($p>0.05$) between males and females, thus only results for the total sample are reported (table 2). Age ($r=0.25$; $p<0.04$), waist ($r=0.58$; $p<0.0001$), BMI ($r=0.56$; $p<0.0001$), and weight ($r=0.42$; $p<0.0001$) were significantly correlated with MRI estimates of VAT. Additionally, DXA measures of android fat mass (AFM) ($r=0.76$, $p<0.0001$) and L1-L4 ($r=0.71$, $p<0.0001$) were positively correlated to MRI-measured VAT; the relationship was slightly stronger with android fat mass than with L1-L4 ROI.

Development of prediction equations by subsection

Results for models predicting MRI VAT from DXA measures of fat mass are reported in **Tables 3 and 4.** Because AFM and L1-L4 are highly inter-correlated ($r=0.982$), they were tested in separate models for estimating VAT. DXA measures of anthropometric covariates (weight, height, BMI, waist) and demographic covariates (e.g., age, race, and gender) were then added (stepwise) to test their additional contributions (**Tables 3 and 4**).

The model using only DXA-derived AFM ($p<0.0001$) explained 57% of the variance in VAT by MRI. Results from stepwise regression showed that only gender ($p<0.013$) was a significant predictor of VAT and inclusion of gender in the AFM model increased the R_{adj}^2 from 0.57 to 0.60 and reduced the SEE (cm^2) from 10.70 to 10.30 cm^2. Addition of the anthropometric covariate, weight, ($p<0.046$) resulted in a further improvement in the prediction of VAT, increasing the R_{adj}^2=0.62 (SEE=10.06cm^2) (**Table 3**). Further analysis suggested an interaction between gender and AFM ($p<0.062$). The final model to predict VAT using AFM, gender and the interaction term explained 61% of the variance in VAT (SEE=10.10cm^2) (Table 3).

The model using only DXA-derived L1-L4 ROI ($p<0.0001$) explained 50% of the variance in VAT. Inclusion of gender ($p<0.014$) to the L1-L4 model increased the variance explained by the model, increasing the R_{adj}^2 = 0.54, and reducing the SEE from 11.51cm^2 to 11.08cm^2 (**Table 4**); however, in L1-L4 models, no other anthropometric or demographic variables were significant predictors of VAT. Further analysis revealed a significant interaction between gender and L1-L4 (gender*L1-L4). The final model to predict VAT using DXA L1-L4, gender and the interaction term explained 59% of the variance, and reduced SEE to 10.39cm^2.

Models including AFM were, overall, better at predicting VAT than L1-L4 ROI, and for any given model, AFM explained 2% to 7% more variance in VAT compared to L1-L4 ROI. The prediction of VAT was further improved when the interaction between gender and AFM or L1-L4 was included in the model.

DISCUSSION

DXA-derived L1-L4 and android fat mass (AFM) were evaluated separately and

with anthropometric variables to predict VAT. AFM and L1-L4 ROI had similar, significant correlations with VAT (R=0.76; R=0.71, $p<0.0001$), and both provided more accurate prediction of VAT than anthropometry alone and anthropometry combined with demographic covariates (data not shown). Thus, DXA measures of AFM and L1-L4 have a clear advantage over anthropometry alone for predicting VAT.-Demographic variables were also evaluated in models with AFM and L1-L4 fat mass as previous studies have shown that age, race and gender differences in fat distribution [346-348] might affect the relationship between AFM and L1-L4 VAT. In this sample, only gender was a significant predictor of VAT in models with AFM and L1-L4, and addition of body weight to models including gender and AFM (but not L1-L4 fat mass) reduced error and increased the variance explained in VAT. Inclusion of the interaction of gender with AFM or L1-L4 further improved the prediction of VAT.

The findings of this study agree with earlier studies in both adults and children which have investigated the combination of anthropometric measures and DXA estimates of regional adipose tissue distribution as potential correlates of VAT and intra-abdominal adiposity. While some studies have focused on standardized regions included in DXA software, others have delineated custom regions of interest and examined their relationship with VAT [331, 339]. Conventional assessment of trunk fat by DXA, for example, has been used to predict intra-abdominal adiposity. In adult women [349] and pre-pubescent children [334], DXA derived trunk fat combined with anthropometric variables explained 81% (SEE=24.6cm^2) [349] to 85% (SEE=8.9cm^2) [334] of the variance, respectively, in CT measures of IAAT. However, the accuracy of these DXA prediction equations is limited, because trunk includes the entire thoracic and abdominal

areas rather than an anatomical region more closely aligned with VAT [334]. Hill and colleagues (2006) found that in their sample of overweight or obese women, DXA fat mass from manually drawn abdominal ROIs at 5-cm (r=0.70) and 10-cm (r=0.78) regions (above iliac crest), which were closer in proximity to IAAT measures from CT, were moderately correlated with IAAT (27). Inclusion of abdominal skinfolds with the DXA 10-cm ROI improved the amount of variance in IAAT (R=0.82), that could be explained. Interestingly, Bertin and colleagues (2000) found that DXA abdominal fat estimates using a specially designed version of the software that accounted for intraabdominal cavity thickness (e.g. transverse internal diameter and transverse external diameter), combined with abdominal sagittal diameter, age, and waist circumference, resulted in strong correlations with CT-measured IAAT in obese men (r=0.88) and obese women (r=0.94) with an estimated error for the combined sample of men and women of 38.2cm^2 [350]. Other studies in adults, examining ROIs at the L2 – L4 area combined with waist circumference, have shown similar correlations with IAAT (r = 0.74-0.75) in obese women. The relationship was weaker (r=0.46) in obese men for whom waist circumference was not significant [331, 341, 351], suggesting that the predictive power of DXA combined with anthropometry to estimate IAF may be dependent on sex, and the degree of obesity in adult populations [351]. In older adults, both regional (trunk and manually defined ROI) and total abdominal fat masses from DXA were significantly correlated with VAT [350]. However, neither of these DXA measurements was superior to anthropometric measurements (waist circumference, sagittal diameter; r<0.74) [350], and models improved only slightly when combinations of DXA with anthropometry were examined.

To our knowledge, this is the first study to examine the associations of DXA android fat mass and anthropometry with VAT in adolescents and young adults. Despite the high inter-correlation between DXA trunk fat and android and L1-L4 regions (all r=0.97) in our study, trunk fat showed lower correlations with VAT (r=0.68) compared to AFM (r=0.76) and L1-L4 (r=0.71). Thus, android and regional L1-L4 fat masses were used as predictors because they are more anatomically associated with VAT. Importantly, L1-L4 has been validated in adults (20, 33, 53), to accurately estimate IAAT by CT, another reference standard for measuring visceral adiposity, and in adolescents (46) to predict metabolic risk factors associated with the accumulation of VAT.

Differences in the distribution of adipose tissue by gender are apparent as early as pre-puberty, and the magnitude of the sex difference increases with maturation, with young adult males displaying higher relative central fat deposition and young adult females displaying more peripheral fat distribution compared with those in late adolescence [347]. Ethnic differences in abdominal fat distribution are also evident in young adults [348] and children [346], especially between Asian, Caucasian and African-American children [334, 352]. Ethnicity was not a significant predictor of VAT in this study, most likely because the number of subjects in each ethnic group was limited. Although DXA is considered a criterion method for assessing body composition, limitations in the use of DXA to predict VAT are evident from reports indicating that DXA significantly underestimates abdominal adiposity in individuals with less abdominal fat [353, 354] and overestimates this measure in individuals who are more obese or who have larger amounts of abdominal fat mass [296, 354-358]. Earlier studies that investigated this issue showed that errors in estimates of body fat were positively

correlated to tissue thickness [356]. Typically, the thicker the tissue under analysis, the more difficult it is for DXA to accommodate beam hardening at a preferential energy value and differentiate soft tissue composition. Tissue thickness >20cm is projected to result in DXA overestimations of tissue fat [356, 359]. Because estimations in heavier individuals are subject to greater error that may introduce bias into regression equations that predict VAT, population- specific (i.e., fatness groups) equations may be necessary to accurately predict VAT in overweight and obese individuals.

By its design, DXA cannot distinguish between intra-abdominal (IAAT) and subcutaneous (SAT) fat depots [352]. Several studies have investigated the utility of anthropometric variables (e.g., skinfold, abdominal thickness) to quantify regional adiposity (i.e., trunk, abdominal, gynoid) because of their practicality, accessibility, low cost, and reproducibility in the clinical setting [360]. Because android fat and the L1-L4 fat mass regions include VAT and SAT depots, using skinfold measurements to act as a surrogate of abdominal SAT [361] in combination with DXA abdominal fat measures may be beneficial in improving the accuracy of predicting abdominal VAT. Indeed previous studies have shown the use of skinfolds do improve the explained variance in models predicting IAAT by CT [292, 334].

Notably, because manually drawn ROIs are necessary to analyze L1-L4 regions, the potential for human error increases. Also, the anatomic arrangement of the ribs and spine may limit the area (number of pixels) for estimating bone-free soft tissue by DXA and consequently lead to underestimations of the total fat mass within the abdominal and thoracic area [293, 356]. Identification of the L1-L4 ROI maybe confounded by the degree of adiposity in this area, thereby reducing the clarity of the images and increasing

the potential for observer error in delineating specific regions, for example, the respective inter-vertebral spaces [293, 352], an observation that was noted in our study when evaluating obese and overweight subjects. In fact, incorrect placement of the intervertebral disk spaces on the image for ROI placement is reported as the most common operator-dependent error when taking measurements in the spinal or thoracic cavity [362]. Thus, for DXA ROIs to be used as predictors of VAT, correct numbering of lumbar vertebral levels and correct ROI placement is imperative when analyzing abdominal adiposity [352, 356]. Use of standard, validated equations employed in the manufacturers' automated protocol for estimating AFM may help explain why AFM predicted VAT better than the manually drawn L1-L4 ROI. Additional factors that can influence DXA ROI placement include incorrect posture, overlapping of upper limbs or placement of upper limbs behind the trunk, vertebral conditions (i.e., floating ribs), and technical skill.

In summary, DXA AFM and L1-L4 ROI provide acceptable estimates of VAT in adolescents and young adults. Estimation was improved with the inclusion of gender and weight in models with AFM. Because gender appeared to be a moderator in the prediction of VAT, particularly with DXA L1-L4, the utility of different DXA ROIs to predict VAT may be dependent on gender, an issue that needs investigation. AFM was a better predictor of VAT than the manually drawn L1-L4 ROI, although the difference was not large. We conclude the combination of DXA-derived fat mass in the L1-L4 or android regions of interest with anthropometric measures (i.e., weight) can provide researchers and clinicians with a feasible, cost-effective and accurate method of estimating visceral adipose tissue.

TABLES:

Table 1: Descriptive characteristics by Gender and of the Total Sample

	Total Sample (n=70)			Males (n=35)			Females (n=35)		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Age (years)	19.19	3.67	11.0-25.0	19.54	3.93	12.0-25.0	18.84	3.41	11.0-24.80
Weight (kg)	64.43	14.96	28.9-119.7	70.10	17.26	28.9-119.70	58.77	9.49	39.2-78.20
Height (cm)	169.43	10.91	138.9-198.6	174.73	11.21	138.9-198.6	164.12	7.62	146.4-177.3
BMI (kg/m^2)	22.28	3.94	14.5-38.1	22.76	4.58	14.5-38.10	21.80	3.18	16.5-28.0
Waist (cm)	75.60	10.14	46.3-114.5	77.93	11.35	46.3-114.5	73.27	8.30	50.4-93.10
Android fat mass (kg)[1]	0.99	0.79	0.16-5.10	0.87	921.18	0.16-5.10	1.10	622.12	0.26-2.56
L1toL4 fat mass (kg)[1]	1.43	1.14	0.21-6.72	1.24	1215.52	0.21-6.72	1.62	1033.67	0.33-4.60
DXA percent fat[1]	22.10	11.05	4.8-47.4	15.97	8.48	4.8-41.0	28.23	9.93	11.6-47.40
VFA (cm^2)	27.18	16.24	6.0-111.6	28.53	19.99	6.0-111.6	25.83	11.49	8.4-63.50

[1]Measured by DXA

[2]Measured by MRI

Table 2: Bivariate Correlations between Covariates and Fat Masses

Variable	**Age**	**gender**	**race**	**Weight**	**Height**	**BMI**	**Waist**
Android Fat mass (g)	0.272*	-0.143	-0.09	0.573*	-0.055	0.788*	0.751*
L1-L4 fat mass (g)	0.279*	-0.17	-0.10	0.558*	-0.071	0.785*	0.754*
MRI VFA (cm^2)	0.252*	0.083	-0.124	0.422*	-0.045	0.563*	0.579*

Spearman's correlation coefficients for potential model covariates and fat masses
* significant at $p \leq 0.05$; Pearson's r for continuous and Spearman's rho for categorical variables[1]

[1] Results from bivariate correlations Fischer's transformation z-test concluded no significant differences in pearson's correlation coefficients among male and females; thus, bivariate correlations are reported for the total sample.

Table 3: Multiple Regression Equations for Estimating VAT from AFM

	Regression Equation	R_{adj}^2	SEE (cm^2)
AFM	VAT=0.016AFM* + 11.815	0.57	10.70
AFM+gender	VAT=0.016AFM + 6.360Gen* + 1.765	0.60	10.30
AFM+Gen+WT	VAT=0.019AFM - 4.880Gen* + - 0.245WT* + 24.08	0.63	10.06
AFM + GENDER + (GENDER x AFM)	VAT=0.015AFM* + 0.113Gen - 0.003(G x AFM)* + 12.78	0.61	10.10

VAT, visceral adipose tissue; AFM, android fat mass; Gen, gender; WT, weight

* significant at $p \leq 0.05$

Table 4: Multiple Regression Equations for Estimating VAT from L1-L4 ROI

	Regression Equation	R_{adj}^2	SEE (cm^2)
L1_L4 fat mass	VAT=0.010 $L_{1\text{-}4}$FM* + 12.653	0.50	11.51
L1_L4 fat mass+Gen	VAT=0.011 L1-4FM* - 3.40Gen* + 11.921	0.54	11.08
L1_L4 + GENDER + (GENDER x L1_L4)	VAT= 0.010 $L_{1\text{-}4}$ FM* + 1.883Gen - 0.004(Gen x L1-4 FM)* + 13.45	0.59	10.39

$L_{1\text{-}4}$FM, L1-L4 fat mass; Gen, gender

* significant at $p \leq 0.05$

SUPPLEMENTARY MATERIALS

TABLE OF RECOMMENDED INTAKES OF COMMON DIETARY FATTY ACIDS ESTABLISHED FOR HEALTHY YOUNG AMERICANS: SPECIFIC AIM 3

Table 22: Comparison between FA recommended intakes and Average FA consumed by Sample

Fatty acid [a]	**DRI (g/d)**	**Sample Intake (Mean±SD)**
ALA	1.0-1.2 g/d	1.0 g/d±0.44
LA	10g/d	10.15g/d±4.23
Omega-3	0.2-0.25 g/d	0.07g/d±0.05
PUFA	0.5g/d	11.55g/d ±4.8
n-6/n-3 FA ratio	5:1 -10:1g/d	9.8:1g/d ±1.7

[a] Values include sources from diet and supplements

Dietary Recommended Intakes (DRI) established by the IOM **[267]** and DGA [234].

ALA, alpha-Linolenic acid (18:3n-3); PUFA, poly-unsaturated fatty acid; LA, Linoleic acid (18:2n-6); AA, Arachidonic acid (20:4n-6); omega-3 (EPA+DHA); total n-6/totaln-3, AA+LA/ALA+EPA+DHA

TABLE SHOWING THE EFFECT OF FATTY ACID INTAKES ON 2-YEAR OUTCOMES IN YOUNG GIRLS: SPECIFIC AIM 3

Table 23: Adjusted means of 24-month bone strength and bone density parameters by groups of select baseline dietary FAs.

	Below Recommendations	Met Recommendations
OMEGA 3		
24M_4% Femur BSI	124.7±1.2	125.7±2.3
24M_20% Femur SSI	1883.08±14.0	1933.4±26.4
24M_4% Tibia BSI	68.67±0.6	67.7±1.1
24M_66% Tibia SSI	1597.3±11.3	1626.9±21.1
24M_Femur total vBMD (ave)	289.7±1.4	293.8±2.6
24M_20% Femur Cort vBMD	1099±1.6	1065.6±3.0
24M_4% Femur Trab vBMD	247.6±1.3	248.2±2.5
24M_Tibia total vBMD	324.7±1.7[a]	316.1±3.2
24M_66% Tibia Cort. vBMD	1057.5±1.7	1056.5±3.1
24M_4% Tibia Trab vBMD	230.6±1.1	229.4±2.0
ALA		
24M_4% Femur BSI	125.7±1.6	124.0±1.8
24M_20% Femur SSI	1897.4±17.8	1891.9±20.5
24M_4% Tibia BSI	69.28±0.8	67.4±0.9
24M_66% Tibia SSI	1610.0±14.4	1597.2±16.6
24M_Femur total vBMD (ave)	290.2±1.8	291.2±2.0
24M_20% Femur Cort vBMD	1069.1±2.0	1065.4±2.4
24M_4% Femur Trab vBMD	248.1±1.7	247.3±1.9
24M_Tibia total vBMD	324.3±2.2	320.6±2.5
24M_66% Tibia Cort. vBMD	1057.7±2.1	1056.6±2.5
24M_4% Tibia Trab vBMD	231.9±1.4	228.3±1.6
LA		
24M_4% Femur BSI	125.4±1.7	124.4±1.9
24M_20% Femur SSI	1908.2±19.2	1879.8±20.9
24M_4% Tibia BSI	68.3±0.8	68.6±0.9
24M_66% Tibia SSI	1609.9±15.5	1597.9±17.0
24M_Femur total vBMD (ave)	288.6±1.9	293.0±2.1
24M_20% Femur Cort vBMD	1065.8±2.2	1069.5±2.4
24M_4% Femur Trab vBMD	248.6±1.8	246.8±2.0
24M_Tibia total vBMD	319.7±2.4	326.1±2.6
24M_66% Tibia Cort. vBMD	1056.7±2.3	1057.8±2.5
24M_4% Tibia Trab vBMD	230.2±1.5	230.4±1.6
total n-6/n-3		
24M_4% Femur BSI	125.8±1.5	124.1±1.5
24M_20% Femur SSI	1909.3±17.2	1881±17.0
24M_4% Tibia BSI	68.7±0.7	68.1±0.7
24M_66% Tibia SSI	1610.4±14.0	1598.4±13.8
24M_Femur total vBMD (ave)	292±1.7	289.3±1.7
24M_20% Femur Cort vBMD	1067.9±2.0	1067.1±2.0
24M_4% Femur Trab vBMD	248.7±1.6	246.9±1.6
24M_Tibia total vBMD	321.6±2.1	323.6±2.1
24M_66% Tibia Cort. vBMD	1058.5±2.1	1056±2.0
24M_4% Tibia Trab vBMD	231.7±1.3	229.1±1.3

Estimated means ±SE for 24M femur and tibia bone strength and density indices across groups of select dietary FAs Omega-3: EPA+DHA, ALA, alpha-Linolenic acid (18:3n-3); LA, Linoleic acid (18:2n-6);. BSI=bone strength index (mg^2/mm^4); SSI=strength–strain index (mm^3); ratio total n-6/total n-3: LA+AA/EPA+DHA+ALA; EPA, Eicosapentaenoic acid (20:5n-3); DHA, Docosahexaenoic acid (22:6n-3); PUFA, poly-unsaturated fatty acid; LA, Linoleic acid (18:2n-6); AA, Arachidonic acid (20:4n-6); Tot.vBMD= total (average) volumetric bone mineral density (mg/cm^3); Cort.vBMD= cortical volumetric bone mineral density (mg/cm^3); Trab vBMD=trabecular volumetric bone mineral density (mg/cm^3);

[a]Significant at $p<0.05$

TABLES OF PREDICTION EQUATIONS FOR VAT USING DXA MEASURES OF FAT MASS AND ANTHROPOMETRIC AND DEMOGRAPHIC COVARIATES IN ADOLESCENTS AND YOUNG ADULTS: ANCILLARY AIM

Table 24: Multiple Regression Equations for Estimating VAT from AFM

	Regression Equation	R_{adj}^2	SEE (cm^2)
Base Model covariates	VAT=1.04A* - 1.59R + 1.457Gen + 10.55	0.03	15.99
Base+W_C+WT+HT	VAT=0.36A+0.78R+3.59 Gen +0.95Wc*+0.13WT-0.64HT*+40.63	0.38	12.76
AFM	VAT=0.016AFM* + 11.82	0.57	10.70
AFM + base	VAT=0.016AFM* + 0.09A - 0.26R + 6.14Gen + 1.38	0.59	10.45
AFM + base + W_C	VAT=0.019AFM* + 0.24A - 0.19R + 8.25Gen - 0.32Wc + 16.36	0.59	10.37
AFM + base + WT	VAT=0.019AFM* + 0.46A + 0.24R + 10.29Gen* - 0.310WT* + 3.01	0.61	10.11
AFM + base + WT + HT	VAT=0.019AFM* + 0.46A + 0.28R + 10.29Gen - 0.31WT + 0.002HT + 2.72	0.61	10.19
AFM + base + WT+ HT + W_C	VAT=0.019AFM* + 0.46A + 0.24R + 10.27Gen -0.32WT + 0.002HT + 0.01Wc + 2.36	0.60	10.27
AFM + base + BMI	VAT=0.021AFM* + 0.35A - 0.57R + 8.09Gen* - 1.23BMI* + 17.31	0.61	10.18
AFM + base + BMI + W_C	VAT=0.021AFM* + 0.37A - 0.51R + 8.58Gen* - 1.08BMI - 0.110 Wc + 20.53	0.60	10.25
AFM + Gen + (Gen x AFM)	VAT=0.015AFM* + 0.11Gen - 0.003(Gen x AFM)* + 12.78	0.61	10.10

VAT, visceral adipose tissue; AFM, android fat mass; Wc, waist; Gen, gender; R, race; WT, weight; HT, height; BMI, body mass index; Base model: AFM, age, race, gender, * significant at $p \leq 0.05$

Table 25: Multiple Regression Equations for Estimating VAT from L1-L4 ROI

	Regression Equation	R_{adj}^2	SEE (cm^2)
Base Model covariates	VAT=1.04A - 1.59R + 1.457Gen + 10.55	0.03	15.99
Base+WC+WT+HT	VAT=0.36A+0.78R+3.59Gen+0.95Wc*+0.13WT-0.64HT*+40.63	0.38	12.76
L1_L4 fat mass	VAT=0.01 L_{1-4}FM* + 12.65	0.50	11.51
L1_L4 + base	VAT=0.011 L_{1-4}FM* + 0.11A + 6.63Gen* -0.111R +0.41	0.52	11.24
L1_L4 + base + Wc	VAT= 0.012 L_{1-4} FM* + 0.20A-0.032R + 8.13Gen* - 0.210Wc +9.96	0.52	11.27
L1_L4 + base +WT	VAT=0.012 L_{1-4} FM* +0.38A + 0.30R + 9.837Gen* - 0.23WT + 1.38	0.53	11.11
L1_L4 + base +WT+ HT	VAT=0.012 L_{1-4} FM* +0.39A + 0.49R + 9.98Gen* - 0.15WT - 0.117HT + 15.92	0.53	11.17
L1_L4 + base WT+HT+ Wc	VAT=0.011 L_{1-4} FM* +0.39A + 0.53R + 9.799Gen* - 0.18WT-0.124HT +0.10Wc + 12.56	0.52	11.26
L1_L4 + base +BMI	VAT= 0.013 L_{1-4} FM* + 0.27A - 0.26R + 8.04Gen* - 0.79BMI + 10.32	0.52	11.20
L1_L4 + base +BMI+ Wc	VAT= 0.013 L_{1-4} FM* + 0.29A - 0.217R + 8.40Gen* - 0.695BMI - 0.075Wc + 12.54	0.52	11.28
L1_L4 + Gen + (Gen x L1_L4)	VAT= 0.010 L_{1-4} FM* + 1.88Gen - 0.004(Gen x L1-4 FM)* + 13.45	0.59	10.39

L_{1-4}FM, L1-L4 fat mass; Wc, waist; Gen, gender; R, race; WT, weight; HT, height; BMI, body mass index
Base model: L1-4FM, age, race, gender, * significant at $p \leq 0.05$

MINOR ASSESST FORM

APPROVED BY UNIVERSITY OF AZ
THIS STAMP MUST APPEAR ON
DOCUMENTS USED TO CONSENT SUBJE
DATE: 1/28/08 EXPIRATION: 1/28/0

MINOR'S ASSENT FORM

Jump-In – Building Better Bones

Your mother/father/guardian has told us it was okay for you to take part in this study measuring how physical activity affects bone in girls. If you agree to take part, you will be asked about your eating habits, smoking, physical activity, and physical development five different times over the next four years.
All five times, you will:

1. Have measurements taken of your height, weight, body composition (muscle and body fat), physical activity, and leg bone strength.

2. Look at outlines of girls in different stages of physical maturity (such as different stages of breast development, body shape, body hair, etc.) and mark the drawings that look most like you.

3. Wear a small machine like a pedometer on your waist for seven days in a row to measure physical activity.

4. Answer questions about your eating habits, including if you ever drink alcohol (such as beer and wine), and questions about if you smoke or relatives and friends smoke around you. No one will see your answers, except the researchers, not even your parents.

5. Have your muscle, body fat and bone strength measured using two special scanning machines. For each scan, you will have to lie quietly on a table for a few minutes while the machine measures you. The tests do not hurt. Both scanning machines will expose you to a very small amount of radiation (like x-rays) that will not harm you. Girls who have started their periods will need to have a urine pregnancy test before having the scans. If a girl's urine pregnancy test shows that she might be pregnant or another test shows something a doctor needs to see, the girl will be told in private and her parents or guardians will also be told.

To thank you for being in the study, you will get $10 after the first measurements, $15 after the second, $20 after the third, $25 after the fourth, and $30 after the fifth and last measurements. If you complete all five measurement sessions over the four years of the study, you will receive a total of $100.

You do not have to be in this research study. If you do take part, you can stop at any time. Your grades and your treatment in school will be the same whether or not you decide to take part.

Do you have any questions? Would you like to take part?

_____
Child's Name

_____ _____
Child's Signature Date

_____ _____
Presenter's Signature Date

_____ _____
Investigator's Signature Date

APPROVED PARENT CONSENT FORM

DOCUMENTS USED TO CONSENT SUBJEC
DATE: 1/28/08 EXPIRATION: 1/28/0

Parent/Guardian Informed Consent

EXERCISE AND BONE DEVELOPMENT IN YOUNG GIRLS

Introduction

Your daughter is being invited to take part in a research study. The information in this form is provided to help you decide whether or not she can take part. Study personnel will be available to answer your questions and provide additional information. If you decide to allow your child to take part in the study, you will be asked to sign this consent form. A copy of this form will be given to you.

What is the purpose of this research study?

Developing strong bones as a girl lowers the risk of developing too soft bones (osteoporosis) as a woman. Osteoporosis is a common problem affecting older women in the United States. In this study, we intend to measure how different types of physical activity, taught at school during PE class or recess, affect bone development in elementary and middle school girls.

Why is your daughter being asked to participate?

Your daughter is being asked to participate because she is a healthy girl in 4th or 6th grade.

How many people will be asked to participate in this study?

Approximately 560 girls from eight schools will be asked to take part in the measurement activities. About 64 girls from your daughter's school will be asked to take part.

What will happen during this study?

Your daughter will answer questions about her eating habits, level of physical activity, smoking and exposure to cigarette smoke (e.g., due to relatives or friends who smoke) and stage of physical development, and have measurements of her height, weight, physical activity, and bone strength – five times over the next four years, in the fall and spring of her first year in the study and every spring for three more years. The eating habits questionnaire includes questions about alcohol consumption. Her physical activity will be measured using a simple measuring device about the size of a pack of cards called an accelerometer that she will be asked to wear on her waist while awake for seven days in a row. Her bone strength will be measured with dual-energy x-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT) scanning machines. We will be looking at the strength of your daughter's bones in her spine and hip with the DXA scan and in her upper and lower leg with the pQCT scan. For each test, she will have to lie quietly on a table for a few

APPROVED PARENT CONSENT-*continued*

minutes while the machine makes its measurements. She will not feel any pain or discomfort from the test.

How much of your daughter's time will it take to be in this study?
It will take about two hours each time for your daughter to complete the questionnaires and have the other measurements performed. It will take about 10 hours of her time spread out over four years to complete the study.

Are there any risks to your daughter?
Both DXA & pQCT measurements will expose your daughter to very low doses of radiation – about 3-10 mrem for DXA and 6-10 mrem for pQCT measurements. These are very small amounts compared to the 300 or more mrem that all of us are naturally exposed to every year from the environment. The health risks of exposure to small amounts of radiation below 5-10 mrem from medical testing are thought to be too small to be determined or none.
Just to be sure that we are not accidentally exposing an unborn child to even a small amount of radiation; all menstruating girls (girls who have started their periods) will have a urine pregnancy test before the DXA and pQCT measurements. Any girl with a positive pregnancy test will be excused from testing and from further participation in the study. She will also be advised to talk about the result with her parent(s)/guardian(s). If she requests, she will be referred to the school guidance counselor. As required by Arizona law in girls under 18 years old, positive pregnancy test results will be reported to a parent or legal guardian and, if indicated, to the appropriate State or local government agency.

Your daughter might be embarrassed about her weight and about having it known by others. To avoid this, your daughter's height and weight will be measured in a private setting and not be announced for others to hear.

Your daughter might be embarrassed by the questionnaire used to measure her physical maturity. It shows outlines of girls in different stages of physical maturity (such as different stages of breast development, body shape, body hair, etc) and asks your daughter to identify the outlines that are most like her body. The results of these questionnaires will be kept strictly private.

Are there any benefits to your daughter?
Your daughter may have increased bone density as a result participating in the study. Increased bone density at young ages may provide some protection against broken bones later in life. If the study shows that some activities are better at strengthening girls' bones, we will propose that those activities be taught to more girls in more schools in the future.

APPROVED PARENT CONSENT-*continued*

Will there be any costs to your daughter?
Aside from your daughter's time, there are no costs from taking part in the study.

Will your daughter be paid to participate in the study?
Your daughter will receive payment after completing each of the five measurement sessions – $10 for the first measurements, $15 for the second, $20 for third, $25 for the fourth, and $30 for the fifth. If she completes all five measurement sessions over the four years of the study, she will receive a total of $100.

Will video or audio recordings be made of your daughter during the study?
No.

Will the information that is obtained from your daughter be kept confidential?
Your daughter's records will be kept confidential. The only persons who will have access to her measurements are the principal investigator and his research team. She will not be named or identified in any reports or publications resulting from the study. Your daughter's responses to questions about smoking and alcohol use will be confidential and will not be shared with you. A positive pregnancy test will be reported to you. It is possible that representatives from the National Institutes of Health (which supports this study) or the University of Arizona Human Subjects Protection Program will want to review your daughter's information. If that occurs, a copy of the information may be provided to them but your daughter's name will be removed before the information is released.

What if you believe that your daughter has been harmed by the study procedures?
Side effects or harm are possible in any research program and could occur through no fault of yours, your daughter, or the investigator involved. You do not give up any of your, or your daughter's, legal rights by signing this form. In the event that your daughter requires, or you are billed for, medical care that you feel has been caused by her participation in this study, you should contact the principal investigator, Scott Going, Ph.D. at (520) 621-4705. If you have questions concerning your daughter's rights as a research subject, you can call the University of Arizona Human Subjects Protection Program office at (520) 626-6721. (If you are out of state, you can use the toll-free number: 1-866-278-1455.)

Can you change my mind about your daughter participating?
Your daughter's participation in the study is voluntary. You (or she) may decide at any time that she not start the study or that she stop her participation in the study. Refusing to allow your daughter to participate will have no effect on how she is treated at school or on her grades. Stopping your daughter's participation will have no effect on how she is treated at school or on her grades. If any new information

APPROVED PARENT CONSENT-*continued*

becomes available about the study that could affect your willingness to allow your daughter to participate, it will be shared with you.

Whom may I contact for additional information?
You can obtain further information about the research or voice concerns or complaints about the research by calling the Principal Investigator, Scott Going, Ph.D. at (520) 621-4705. If you: (1) have questions concerning your daughter's rights as a research participant; (2) have general questions, concerns or complaints; (3) would like to give input about the research and can't reach the research team; or (4) want to talk to someone other than the research team – you may call the University of Arizona Human Subjects Protection Program office at (520) 626-6721. (If out of state, use the toll-free number 1-866-278-1455.) If you would like to contact the Human Subjects Protection Program via the web, please visit the following website: http://www.irb.arizona.edu/contact.

Your Signature
By signing this form, I affirm that I have read the information contained in the form, that the study has been explained to me, that my questions have been answered. I do not give up any of my or my daughter's legal rights by signing this form.
Please mark one of the choices below:
_____YES, my daughter may take part in the measurements.
_____NO, my daughter may **not** take part in the measurements.

_____
Name (Printed)

_____ _____
Parent's/Legal Guardian's Signature Date signed

_____
Student Name

Statement by person obtaining consent
I certify that I have explained the research study to the parent/guardian and that he or she has been informed of the purpose, the procedures, the possible risks and potential benefits associated with her daughter's participation in this study. Any questions raised have been answered to the parent's/guardian's satisfaction.

_____
Name of study personnel

_____ _____
Study personnel Signature Date signed

HEALTH HISTORY QUESTIONNAIRE

1. Has **your daughter** ever been diagnosed by a medical professional (doctor, nurse-practitioner, physician's assistant), psychologist, or psychiatrist as having any of the following illnesses?

Does your child have any of the following diseases or conditions? (CIRCLE ONE)	Disease/Condition	Does your child take medication for this disease or condition? (CIRCLE ONE)	Name of medication	Amount	How often	How long
Yes or No or Don't Know	Example: Asthma	Y	Advair	50 mg	3 x's a day	2 years
Y or N or Don't Know	Anorexia	Y or N				
Y or N or Don't Know	Bulimia	Y or N				
Y or N or Don't Know	Allergies	Y or N				
Y or N or Don't Know	Asthma	Y or N				
Y or N or Don't Know	Autoimmune disorder (i.e. AIDS, Lupus, etc.)	Y or N				
Y or N or Don't Know	Bipolar (Manic-depressive) disorder	Y or N				
Y or N or Don't Know	Depression	Y or N				
Y or N or Don't Know	Brittle bones (osteogenesis imperfecta)	Y or N				
Y or N or Don't Know	Cancer	Y or N				
Y or N or Don't Know	Crohn's disease	Y or N				
Y or N or Don't Know	Cushing's Syndrome	Y or N				
Y or N or Don't Know	Heart disease	Y or N				
Y or N or Don't Know	Hypertension					
Y or N or Don't Know	Hodgkin's disease	Y or N				
Y or N or Don't Know	Hyperparathyroidism	Y or N				
Y or N or Don't Know	Kidney disease	Y or N				
Y or N or Don't Know	Leukemia	Y or N				
Y or N or Don't Know	Lymphoma	Y or N				
Y or N or Don't Know	Rheumatoid arthritis	Y or N				
Y or N or Don't Know	Seizure disorder	Y or N				
Y or N or Don't Know	Thyroid disease	Y or N				
Y or N or Don't Know	Ulcerative colitis	Y or N				
Y or N or Don't Know	Blood Disorder (i.e. hemophelia, sickle cell trait or disease)	Y or N				
Y or N or Don't Know	Other?	Y or N				
Y or N or Don't Know	Other?	Y or N				

2. Has **your daughter** ever had any broken bones? Yes ____No ____If yes, please describe below:

Name	Date	Surgery needed?
Example: Right lower leg	May 1990	No

If your daughter has had any broken bones, has she been cleared for full activity by her physician? Yes ____ No ____

3. Has your daughter ever had any surgery? Yes _____ No _____ If yes, please describe below:

Type of Surgery	Date	Why
Example: Tonsillectomy	July 1993	Frequent infections

If your daughter has had any surgery, has she been cleared for full activity by her physician? Yes ____No ____

4. Has a doctor ever told you that **your daughter** has a heart problem, such as congenital heart disease, or any other heart related problem? Yes _____No ____

   If yes, please describe below:

_____

_____

5. Does **your daughter** have any condition that interferes with her ability to run, play, exercise, or participate in PE? Yes ____No ____

   If yes, please describe below:

_____

_____

6. Does **your daughter** have to sit out during PE or other organized exercise program for any specific activities? Yes ____No ____

   If yes, please describe below:

_____

_____

7. Is **your daughter** able to read and understand English? Yes

   ____No ____

   If not, please explain below:

_____

_____

Health Update

1. **Please list any CURRENT medications your daughter is using.**

***<u>Prescription</u> medications**:*

NAME/BRAND	DOSAGE (i.e. 10 mg.)	REGIMEN (i.e. once/twice daily)

<u>Non</u>-prescription medications:

NAME/BRAND	DOSAGE (i.e. 10 mg.)	REGIMEN (i.e. once/twice daily)

2. **Please list <u>any</u> medications your daughter WAS USING in the past 6-9 MONTHS and IS NO LONGER USING:**

NAME/BRAND	DOSAGE (i.e. 325 mg.)	REGIMEN (i.e. once/twice daily)

3. **Please list <u>any</u> vitamins/minerals or other supplements that your daughter takes on a regular basis:**

NAME/BRAND	DOSAGE (i.e. 325 mg.)	REGIMEN (i.e. once/twice daily)

Has your daughter had any of the following in the last 6-9 months?

- **Injuries (**requiring medical attention) ☐ Yes ☐ No

  *(this includes **broken bones/fractures and injuries to muscles and joints**)*

  Please describe: _____

  _____

- **Surgeries** ☐ Yes ☐ No

  Type/reason for surgery: _____

- **Hospitalization(s)** ☐ Yes ☐ No

1. Diagnosis: _____

   Length of stay: _____

   Treatment: _____

2. Diagnosis: _____

   Length of stay: _____

   Treatment: _____

- **Additional medical concerns** ☐ Yes ☐ No

  Please describe: _____

TANNER STAGES OF DEVELOPMENT

Jump In

Building Better Bones

Stages of Physical Development

Between the ages of 8 and 18 your body goes through many physical changes. The following questions will help us understand your current state of physical development.

1. Have you started your period?

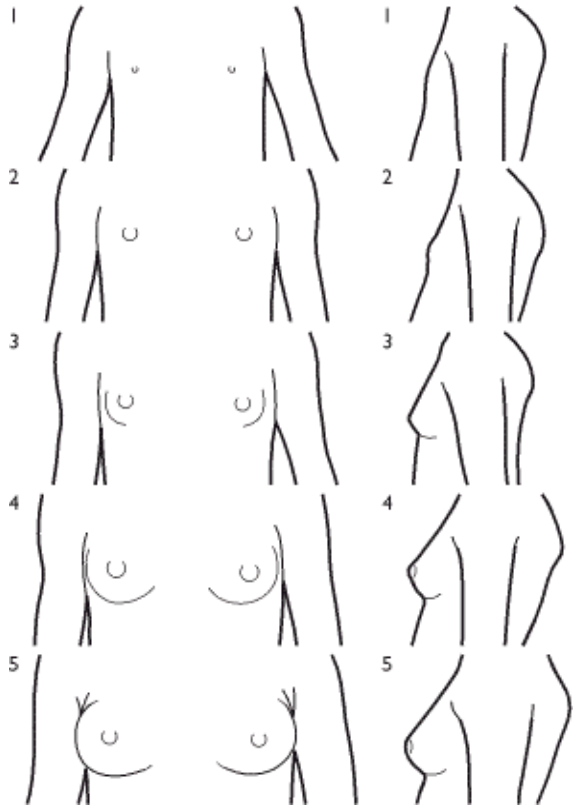
☐ No (Please go to question 2A).

☐ Yes (Please answer 1a and 1b below)

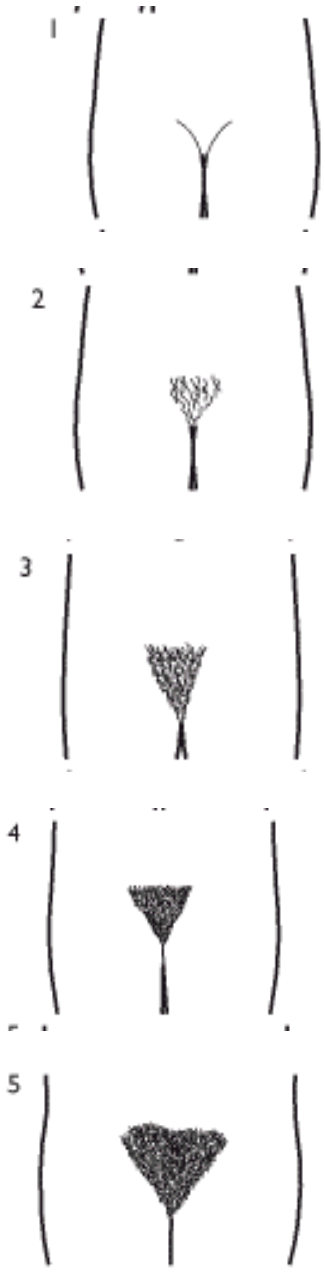
1a. When was your first period? _____ / _____
Month Year

1b. How old were you? _____ (age in years)

2a. Look at the pictures below of the upper body and circle the number next to the picture that most resembles you. You only need to circle one number (the one on the left).



Look at the pictures below of the lower body and circle the number next to the picture that most resembles you.



If you answered "No" to Question 1, please read the following:

When your first period does come someday in the future, please record the date. We would appreciate it if you would call us any time to report your first period (626-2639).

PAST YEAR PHYSICAL ACTIVITY QUESTIONNAIRE

Page 1: Instructions: (please read carefully)

The Past Year Physical Activity Questionnaire is to be filled out during an interview with the subject by Jump-In research staff or by a parent/guardian. The purpose of this questionnaire is to assess the physical activities that the subject has engaged at various times throughout the past year along with the frequency and duration of those activities.

Page 2: The subject is asked 4 multiple choice questions that assess "hard and light exercise" over the past two weeks, daily television watching, and competitive athletic participation. This is followed by listing the specific competitive/organized athletics in which the subject regularly engages.

Page 3: The subject is instructed to identify all leisure activities from a list in which she participated in at least 10 times in the past year (as the interviewer checks all positive responses). Record the total number of activities at the bottom of this page.

Page 4: After the list has been read and all of the positive responses have been checked, the interviewer writes down each activity that was identified on page 3 in the "Activity" column provided. Estimates of frequency and duration are then obtained for each of these activities. Specifically, for each activity, each month that the activity was performed over the past year (past 12 months) is checked and the total is recorded, followed by the average number of days per week, and the average minutes per day which are all entered in the appropriate columns.

1. How many times in the past 14 days have you done at least 20 minutes of exercise hard enough to make you breathe heavily and make your heart beat fast? (Hard exercises include, for example, playing basketball, running, or fast bicycling; please **do not** include time in physical education class)

- None
- 1 to 2 days
- 3 to 5 days
- 6 to 8 days
- 9 or more days

2. How many times in the past 14 days have you done at least 20 minutes of light exercise that was not hard enough to make you breathe heavily and make your heart beat fast? (Light exercises include, for example, household chores, walking, or slow bicycling; please **do not** include time in physical education class).

- None
- 1 to 2 days
- 3 to 5 days
- 6 to 8 days
- 9 or more days

3. During a normal week, how many hours a day do you watch television and movies, or play computer or video games before or after school?

- None
- 1 hour or less
- 2 to 3 hours
- 4 to 5 hours
- 6 or more hours

4. During the past year, how many team or individual sports or activities did you participate in on a competitive level, such as school-based team sports, intramurals, or other organized teams, clubs or programs?

- None
- 1 activity
- 2 activities
- 3 activities
- 4 or more activities

Please list all of the competitive activities that you competed in?

1. _____ 4. _____

2. _____ 5. _____

3. _____ 6. _____

Check all activities (**do not** include activities during physical education classes) from the list below in which the subject participated in **at least 10 times in the past year (past 12 months)** by checking the appropriate circles.

- ○ Aerobics, Jazzercise, Taebo
- ○ Basketball
- ○ Bicycling, Mountain Biking
- ○ Bowling
- ○ Calisthenics / Exercises (push-ups, sit-ups, jumping jacks)
- ○ Cheerleading, Drill Team
- ○ Dance (At home, at a class, in school, at a party, at a place of worship)
- ○ Exercise Machine (Cycle, treadmill, stair master, rowing)
- ○ Football
- ○ Frisbee
- ○ Garden / Yard Work
- ○ Golf / Mini-golf
- ○ Gymnastics / Tumbling
- ○ Hiking
- ○ Hockey (Ice, field, street, or floor)
- ○ Horseback Riding
- ○ Jumping Rope
- ○ Kick Boxing
- ○ Lacrosse
- ○ Martial Arts (Karate, judo, boxing, tai kwan do, tai chi)
- ○ Playing Games (Tether ball, four square, dodge ball, kick ball)
- ○ Rock Climbing
- ○ Roller Blading / Skating
- ○ Riding Scooters
- ○ Running / Jogging for Exercise
- ○ Skateboarding
- ○ Snow Skiing / Boarding
- ○ Soccer
- ○ Softball / Baseball
- ○ Surfing / Body Boarding
- ○ Swimming (Laps)
- ○ Swimming (Play, pool games, water volleyball, snorkeling)
- ○ Tennis, Racquetball, Badminton
- ○ Trampolining
- ○ Track & Field
- ○ Volleyball
- ○ Walking for Exercise
- ○ Water Skiing
- ○ Weightlifting
- ○ Wrestling
- ○ Yoga / Stretching

Others:

- ○ _____
- ○ _____
- ○ _____

Total # of Activities = _____

Please read all instructions carefully before filling out the table.

1. List each activity that the subject engaged according to the previous page in **Column A** of the table below.

2. Check the months within the past year that the subject regularly participated in each activity.

3. Add the number of months for each activity and record the total number of months per year in **Column B**.

4. Record the average number of days per week in which the subject regularly participated in each activity in **Column C**.

5. Record the average number of minutes per day in which the subject regularly participated in each activity in **Column D**.

A:													**B:**	**C:**	**D:**
Activity	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mon/ Year	Days /wk	Min/ day
1.															
2.															
3.															
4.															
5.															
6.															
7.															
8.															
9.															
10															
11.															
12.															
13															
14.															
15.															

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