The Link Between Dietary Protein, Egg Intake, and Inflammation on Pediatric Bone Development

by

LAUREN MICHELLE COHELEY

(Under the Direction of RICHARD D. LEWIS)

ABSTRACT

Nutrition plays an important role in pediatric bone development. Research into dietary factors that influence pediatric bone have focused primarily on vitamin D and calcium; however, recently other nutrients and foods, specifically protein and eggs, have been highlighted for their potential role in bone health. Additionally, there is interest in the inflammatory nature of the diet, as measured by the dietary inflammation index (DII), to impact bone health. Pro-inflammatory diets have been associated with poorer bone mass in adults. It is unknown whether these associations are evident in children. The objective of this dissertation is to utilize existing data to examine the relationships between egg intake, dietary inflammation, and protein consumption on pediatric bone health. Chapter 3 examined the relationship between egg intake and bone outcomes, measured by peripheral quantitative computed tomography (pQCT) in 290 children ages 9-13 years. Egg intake was a positive predictor of mid-radius cortical bone mineral content (CtBMC) and the association between egg and CtBMC was mediated through fat-free soft tissue. Additionally, egg intake was positively correlated with the biomarker of bone turnover, osteocalcin. Chapter 4 addressed the relationship between DII-scores and bone outcomes,

measured by pQCT in 290 boys and girls ages 9-13 years. There were no relationships between DII-scores and cortical bone outcomes or serum inflammation; however, the biomarker of inflammation, monocyte chemoattractant protein-1 was negatively associated with tibia cortical thickness. Chapter 5 is a systematic review investigating the role of protein consumption on bone in 5,620 boys and girls. There was only one randomized controlled trial included in the systematic review; therefore, the effect of protein on pediatric bone was given a limited grade (grade C). These data are the first to investigate the role of both egg and the DII-scores on childhood bone and to systematically review the association between protein intake and pediatric bone. Randomized controlled trials and prospective studies are needed to determine whether egg and protein intake and elevated serum inflammation influence long-term bone health. Findings from this dissertation provided preliminary evidence to support the funding of the SCENE (Skeletal and Cognitive Effects of Nutrition from Eggs) intervention study.

INDEX WORDS: EGG, PROTEIN, INFLAMMATION, BODY COMPOSITION, INFLAMMATORY INDEX, BONE, PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY, CHILDREN, DUAL ENERGY X-RAY ABSORPTIOMETRY

The Link Between Dietary Protein, Egg Intake, and Inflammation on Pediatric Bone Development

BY

LAUREN MICHELLE COHELEY

B.S.F.C.S., The University of Georgia, 2012

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2020

The Link Between Dietary Protein, Egg Intake, and Inflammation on

Pediatric Bone Development

By

LAUREN MICHELLE COHELEY

Major Professor: RICHARD D. LEWIS
Committee: LYNN B. BAILEY
ELLEN M. EVANS
EMMA M. LAING
ASSAF OSHRI

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
May 2020

DEDICATION

This work is dedicated to my parents, Debra and Melvin Coheley. Thank you for always believing in me. Also, I would like to dedicate this dissertation to Grandma Sue and my family members that are no longer with us, my godmother, Aunt Chris, and my paternal Grandfather, Grandpa James and my maternal Grandparents, Grandma Lois and Grandpa George.

ACKNOWLEDGEMENTS

This PhD experience would not be possible without my village. First, I would like to thank my amazing parents, Debra and Melvin Coheley. You taught me the value of hard work, integrity, the importance of family, and so much more. Thank you for always believing in me, even when I did not believe in myself. To my brother and sister, Chris and Jillian, thank you for encouraging me, making me laugh, and making me a proud big sister. I love you all! And of course, none of this would be possible with my amazing dog, Sadie Mae. Sadie has listed to countless practice lectures and didn't complain once!

In 2015, I became a part of the Lewis Lab family. Thank you, Dr. Lewis, for mentoring me to become a confident scientist. I would also like to thank my amazing committee, Dr. Bailey, Dr. Oshri, Dr. Evans, and Dr. Laing. I feel so grateful for being able to learn from the best and the brightest in their respective fields. Thank you to my FACS family! I feel truly blessed to be a double dawg in the College of Family and Consumer Sciences.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Childhood and adolescence are periods of rapid bone growth, areal bone expansion and bone mineral accrual. Indeed, 90% of adult bone mineral content is achieved by 18 years of age (1). This rapid growth period is important given bone mineral density tracks through childhood into adulthood (2). Given lifestyle choices during youth influence 20-40% of adult bone mass, optimizing factors known to influence peak bone mass and strength are important strategies aimed at reducing osteoporosis or low bone mass later in life (3). This is especially important given osteoporosis affects 44 million men and women aged 50 years and older in the United States and incurs an estimated 16 billion dollars in medical expenses each year (4).

Modifiable lifestyle factors such as the diet have been associated with bone health in youth and adulthood. In children, the assessment of dietary factors thought to influence bone mass has focused primarily on intakes of calcium and vitamin D. However, skeletal health appears also to also be influenced by nutrients other than calcium and vitamin D, specifically protein (5, 6). The rationale for examining the role of dietary protein on bone health is related to the fact that protein comprises approximately 50% of bone volume and approximately 33% of its mass. Protein intake stimulates the activity of anabolic hormones and growth factors including insulin-like growth factor-1 (IGF-1). IGF-1 is a major component of the organic skeletal matrix, a strong predictor of skeletal muscle mass, and is the most important differentiation factor for the bone building cells,

osteoblasts (7). Skeletal muscle development precedes bone mass accrual during youth, and it is well documented that fat-free soft tissue mass is the most important determinant of bone strength during childhood (7). Low protein intakes (<0.80 grams per kilogram of body weight per day) reduces intestinal calcium absorption and increases levels of circulating parathyroid hormone, resulting in the release of calcium from the bone which can lead to reduced bone mass (8). Thus, it seems that adequate dietary protein intake is essential for optimal bone growth during childhood. Future intervention studies are needed to better understand the role of dietary protein in pediatric bone development.

The osteogenic effect of protein intake may be improved when packaged together and consumed contemporaneously (3, 9, 10) or as whole foods (11, 12). However, with the exception of dairy foods, little is known about the effects of whole foods on bone health. Eggs contain high-quality protein, the bone nutrients vitamin D and zinc, and the osteogenic bioactive components lutein and zeaxanthin. Whole eggs have been examined in prior adult studies concerning their beneficial effects on bone and fracture outcomes, but egg intake was only considered as part of larger food groupings and not examined independently. Whole egg protein and lecithin, a primary amino acid in whole eggs, have been demonstrated to stimulate muscle protein synthesis in adults (13, 14). Thus, it is plausible that the amino acid and protein components of eggs may positively influence bone strength through increased muscle mass.

Eggs contain a variety of essential nutrients and bioactive compounds that possess anti-inflammatory properties. Recently, dietary inflammation has been highlighted for its potential detrimental effects on bone. For example, systemic, low-grade inflammation has been associated with osteoporosis in adults (15, 16) and reduced bone mass in children

with inflammatory diseases (e.g., ulcerative colitis, cystic fibrosis, and systemic lupus erythematous) (17). The primary research approach to determine whether nutrition influences serum inflammation is to target individual nutrients such as omega-3 fatty acids and antioxidant vitamins such as vitamin E and vitamin C (18, 19). However, there are published reports that dietary patterns or whole foods may be more robust than individual nutrients in producing pro- or anti- inflammatory affects (20). One diet that has been highlighted for its role in decreasing serum inflammation and reducing osteoporosis risk is the Mediterranean diet (21). The Dietary Inflammatory Index (DII), a relatively new method of evaluating the diet, was developed by Cavicchia et al (22) and updated by Shivappa et al (23) to assess the overall quality of the diet on a continuum from maximally anti-inflammatory to maximally pro-inflammatory. In adults, a pro-inflammatory diet as measured by a high DII score has been associated with decreased lumbar spine bone mineral density (BMD) in women (24) and decreased total femur, femoral neck, trochanter, and intertrochanter BMD in U.S. adults (25). Although there are few studies in adults investigating the role of the DII on bone health, the limited published studies suggest that dietary inflammation, as measured by the DII, may negatively influence the adult skeleton. To our knowledge, there are no published studies examining the effects of a pro-inflammatory diet, as measured by high DII scores on pediatric bone health.

The literature review (Chapter 2) provides a summary of the current body of evidence relating to the following topics: 1) bone, 2) pediatric bone development, 3) measuring properties of bone, 4) protein and eggs, 5) protein and bone, 6) eggs and bone, 7) inflammation, 8) inflammation and bone, 9) inflammation and diet. Although eggs

contain high quality protein and bioactive components that are thought to influence bone health, no studies have investigated the independent effect of egg intake on bone. Chapter 3 presents the first manuscript of this dissertation, which is a cross-sectional ancillary study of a previously conducted randomized controlled trial aimed at investigating the role of egg intake on cortical bone outcomes and body composition in healthy boys and girls ages 9-13 years. Recently, dietary inflammation has been highlighted for its potential negative effects on bone. Whether a pro-inflammatory diet as determined by high DII scores influences pediatric bone is unknown. Chapter 4 is a cross-sectional ancillary study of a previously conducted randomized controlled trial investigating the influence of the DII-scores on bone outcomes and serum inflammation in healthy boys and girls ages 9-13 years. To our knowledge, there is only one systematic review and meta-analysis investigating the role of dietary protein intake in children and adolescents, but this study focused primarily on adults and assessed the evidence published over the past 40 years. Therefore, Chapter 5 presents a systematic review of seventeen articles examining the relationships between dietary protein intake and pediatric bone health outcomes as measured by dual-energy x-ray absorptiometry, peripheral quantitative computed tomography and high-resolution peripheral quantitative computed tomography.

References

1. Bailey DA, Martin AD, McKay HA, Whiting S, Mirwald R. Calcium accretion in girls and boys during puberty: a longitudinal analysis. J Bone Miner Res 2000;15(11):2245-50. doi: 10.1359/jbmr.2000.15.11.2245.

2. Kalkwarf HJ, Gilsanz V, Lappe JM, Oberfield S, Shepherd JA, Hangartner TN, Huang X, Frederick MM, Winer KK, Zemel BS. Tracking of bone mass and density during childhood and adolescence. J Clin Endocrinol Metab 2010;95(4):1690-8. doi: 10.1210/jc.2009-2319.

3. Weaver CM, Gordon CM, Janz KF, Kalkwarf HJ, Lappe JM, Lewis R, O'Karma M, Wallace TC, Zemel BS. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. Osteoporos Int 2016;27(4):1281-386. doi: 10.1007/s00198-015-3440-3.

4. Blume SW, Curtis JR. Medical costs of osteoporosis in the elderly Medicare population. Osteoporos Int 2011;22(6):1835-44. doi: 10.1007/s00198-010-1419-7.

5. Tucker KL, Hannan MT, Chen H, Cupples LA, Wilson PW, Kiel DP. Potassium, magnesium, and fruit and vegetable intakes are associated with greater bone mineral density in elderly men and women. Am J Clin Nutr 1999;69(4):727-36. doi: 10.1093/ajcn/69.4.727.

6. New SA, MacDonald HM, Campbell MK, Martin JC, Garton MJ, Robins SP, Reid DM. Lower estimates of net endogenous non-carbonic acid production are positively associated with indexes of bone health in premenopausal and perimenopausal women. Am J Clin Nutr 2004;79(1):131-8. doi: 10.1093/ajcn/79.1.131.

7. Ekbote VH, Khadilkar VV, Khadilkar AV, Mughal Z, Chiplonkar SA, Palande SA, Phanse-Gupte SS, Patwardhan VG, Shilvant DS. Relationship of insulin-like growth factor 1 and bone parameters in 7-15 years old apparently, healthy Indian children. Indian J Endocrinol Metab 2015;19(6):770-4. doi: 10.4103/2230-8210.167549.

8. Kerstetter JE, O'Brien KO, Insogna KL. Dietary protein, calcium metabolism, and skeletal homeostasis revisited. Am J Clin Nutr 2003;78(3 Suppl):584S-92S. doi: 10.1093/ajcn/78.3.584S.

9. Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. Nutrition 2009;25(2):165-71. doi: 10.1016/j.nut.2008.07.019.

10. Okubo H, Sasaki S, Horiguchi H, Oguma E, Miyamoto K, Hosoi Y, Kim MK, Kayama F. Dietary patterns associated with bone mineral density in premenopausal Japanese farmwomen. Am J Clin Nutr 2006;83(5):1185-92. doi: 10.1093/ajcn/83.5.1185.

11. Moore LL, Bradlee ML, Gao D, Singer MR. Effects of average childhood dairy intake on adolescent bone health. J Pediatr 2008;153(5):667-73. doi: 10.1016/j.jpeds.2008.05.016.

12. Laird E, Molloy AM, McNulty H, Ward M, McCarroll K, Hoey L, Hughes CF, Cunningham C, Strain JJ, Casey MC. Greater yogurt consumption is associated with increased bone mineral density and physical function in older adults. Osteoporos Int 2017;28(8):2409-19. doi: 10.1007/s00198-017-4049-5.

13. Glynn EL, Fry CS, Drummond MJ, Timmerman KL, Dhanani S, Volpi E, Rasmussen BB. Excess leucine intake enhances muscle anabolic signaling but not net protein anabolism in young men and women. J Nutr 2010;140(11):1970-6. doi: 10.3945/jn.110.127647.

14. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. Am J Clin Nutr 2009;89(1):161-8. doi: 10.3945/ajcn.2008.26401.

15. Ginaldi L, Di Benedetto MC, De Martinis M. Osteoporosis, inflammation and ageing. Immun Ageing 2005;2:14. doi: 10.1186/1742-4933-2-14.

16. Ganesan K, Teklehaimanot S, Tran TH, Asuncion M, Norris K. Relationship of C-reactive protein and bone mineral density in community-dwelling elderly females. J Natl Med Assoc 2005;97(3):329-33.

17. Calder PC, Ahluwalia N, Albers R, Bosco N, Bourdet-Sicard R, Haller D, Holgate ST, Jonsson LS, Latulippe ME, Marcos A, et al. A consideration of biomarkers to be used for evaluation of inflammation in human nutritional studies. Br J Nutr 2013;109 Suppl 1:S1-34. doi: 10.1017/S0007114512005119.

18. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, Teeling JL, Blaak EE, Fenech M, Vauzour D, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr 2015;114(7):999-1012. doi: 10.1017/S0007114515002093.

19. Sears B, Ricordi C. Anti-inflammatory nutrition as a pharmacological approach to treat obesity. J Obes 2011;2011. doi: 10.1155/2011/431985.

20. Nicklas TA, Baranowski T, Cullen KW, Berenson G. Eating patterns, dietary quality and obesity. J Am Coll Nutr 2001;20(6):599-608.

21. Jennings A, Cashman KD, Gillings R, Cassidy A, Tang J, Fraser W, Dowling KG, Hull GLJ, Berendsen AAM, de Groot L, et al. A Mediterranean-like dietary pattern with vitamin D3 (10 microg/d) supplements reduced the rate of bone loss in older Europeans with osteoporosis at baseline: results of a 1-y randomized controlled trial. Am J Clin Nutr 2018;108(3):633-40. doi: 10.1093/ajcn/nqy122.

22. Cavicchia PP, Steck SE, Hurley TG, Hussey JR, Ma Y, Ockene IS, Hebert JR. A new dietary inflammatory index predicts interval changes in serum high-sensitivity C-reactive protein. J Nutr 2009;139(12):2365-72. doi: 10.3945/jn.109.114025.

23. Shivappa N, Steck SE, Hurley TG, Hussey JR, Hebert JR. Designing and developing a literature-derived, population-based dietary inflammatory index. Public Health Nutr 2014;17(8):1689-96. doi: 10.1017/S1368980013002115.

24. Shivappa N, Hebert JR, Askari F, Kardoust Parizi M, Rashidkhani B. Increased Inflammatory Potential of Diet is Associated with Increased Risk of Prostate Cancer in Iranian Men. Int J Vitam Nutr Res 2016;86(3-4):161-8. doi: 10.1024/0300-9831/a000395.

25. Mazidi M, Shivappa N, Wirth MD, Hebert JR, Vatanparast H, Kengne AP. The association between dietary inflammatory properties and bone mineral density and risk of fracture in US adults. Eur J Clin Nutr 2017;71(11):1273-7. doi: 10.1038/ejcn.2017.133.

CHAPTER 2

REVIEW OF THE LITERATURE

Bone

There are two major types of bone that comprise the human skeleton: cortical (80% of total body skeleton) and trabecular (20% of skeletal tissue) (1). Cortical bone is located primarily within the mid-regions (diaphysis) of the long bones (i.e., tibia, radius, and femur). The cortical bone compartment is separated into two distinct regions, the endosteum and periosteum. The endosteum is a thin vascular membrane of connective tissue that lines the inner surface of the bony tissue that forms the medullary cavity/marrow cavity. The periosteum is a connective sheath covering the outer surfaces of the bone (2, 3) **(Figure 1).** The epiphysis and metaphysis are located distally from the diaphysis of long bones. The epiphysis is the rounded end of long bones and the metaphysis is the region between the diaphysis and epiphysis **(Figure 2).** The metaphasis contains the growth plate and overtime fuses with the diaphysis and epiphysis. At the epiphysis and metaphasis, the cortical bone becomes thinner and the medullary cavity/marrow cavity is replaced by trabecular/cancellous bone, often referred to as “spongy bone”. Trabecular bone is characterized by an inner network of calcified trabeculae and is also found in the spine, femoral neck, ilium, and skull (2). Cortical and trabecular bone are designed to perform different functions with cortical bone offering structure and protection while trabecular bone playing a more active metabolic role.

Bone is a unique tissue that is responsible for supporting mechanical loads, metabolic demands, and mineral homeostasis. Material properties determine bone mass and structural properties dictate bone strength. The material properties of bone are composed of an organic component (20-25% by weight), an inorganic component (70% by weight), and a water component (5% by weight) (4). Type I collagen, bone cells, and a small amount of noncollagenous protein comprise the organic component, while the inorganic component primarily consists of crystalline calcium hydroxyapatite (4). The major structural property of bone is collagen, a protein that consists of three polypeptide chains and over 1,000 amino acids. These molecules align in a parallel fashion to form a collagen fibril. These collagen fibrils are grouped together to create collagen fibers. Hydroxyapatite, the major inorganic component of bone is located in and around the collagen fibers.

Bone cells that comprise the organic component of bone, also play an important role in bone metabolism and maintenance of skeletal tissue by responding to various environmental signals such as chemical, mechanical, electrical and magnetic stimuli. These bone cells include osteoblasts, osteocytes, and osteoclasts. Osteoblasts, or bone forming cells, are located on the surface of bone and are derived from mesenchymal stem cells. Osteoblasts mature into osteocytes which embedded deep within osteocytic lacunae and form a network throughout the bone matrix. This network allows for the communication required for mechanotransduction or the process by which physical forces are converted into biological signals that are then integrated into cellular responses (5). Bone lining cells provide a protective layer on inactive bone surfaces (6). Osteoclasts or bone resorption cells are derived from hematopoietic stem cells and are

responsible for maintaining calcium homeostasis. Osteoblasts signal osteoclasts to resorb bone into circulation. A concerted effort between osteoblasts and osteoclasts leads to bone modeling and remodeling. Bone modeling refers to the process in which new bone is formed at the bone surface resulting in a change in bone size and shape (7). Bone remodeling refers to the coupled process in which osteoclasts first resorb bone followed by osteoblasts adding new bone in the resorbed bone surface **(Figure 3).** The bone remodeling process serves to modify bone architecture to meet changing mechanical needs and to assist in repairing micro-damages in the bone matrix which prevents an over accumulation of bone (8). Additionally, bone remodeling plays a vital role in maintaining plasma calcium homeostasis which is important because a variety of bodily functions depend on calcium including nerve transmission, nerve conduction and muscle contraction (8).

During growth bone modeling dominants. Through the first three decades of life, bone turnover is tightly coupled to maintain a steady state between bone formation and bone resorption. Later in life, there is a significant increase in bone resorption over formation leading to accelerated bone loss and increased risk of falls, fractures, and osteoporosis (9).

Pediatric Bone Development

Establishing healthy bones during childhood serves as a blueprint for skeletal health in adulthood. The main causes of osteoporotic fractures is reduced bone mass and this reduction in bone mass can be attributed to age-related bone loss and/or failure to achieve optimal peak bone mass during childhood (10). Therefore, peak bone mass accrual during childhood is a key determinant of skeletal health throughout life.

Peak height velocity (PHV) precedes peak bone mass accrual and is achieved in girls and boys around the ages of 12 and 13.5 years, respectively (11-14) **(Figure 4).** After PHV is achieved, peak lean mass velocity is achieved, and is followed months later by peak bone mineral content accrual. Peak lean mass accrual plateaus around the age of 18 years and precedes peak bone mass accrual by months or even years depending on the skeletal site (11, 12) **(Figures 4 and 5).** The period between PHV and peak bone mineral content accrual is a time when children are at an increased risk for sustaining a skeletal fracture, primarily at bone loading sites including the distal tibia and radius (14). For approximately two years following PHV, roughly one-fourth of adult bone mass is accrued; therefore, this is a desirable time for dietary and physical activity interventions to promote bone strength.

During growth both cortical and trabecular bone adapt to withstand stresses from vertical growth and increased muscle force. Trabecular parameters (e.g., trabecular thickness and trabecular separation) increase during development while trabecular number decreases, leading to a consistent bone volume to tissue volume fraction. During this period of growth, boys have a more optimal trabecular microarchitectural framework; however, in both boys and girls, cortical bone size, volumetric density, and strength increase significantly and lag behind the growth trajectory of muscle cross-sectional area (13, 15-17). Boys tend to have larger, thicker, and stronger bones while girls tend to have denser and less porous cortical bones (15, 16). These sex-related differences in cortical bone geometric and material properties are in part attributed to differences in skeletal muscle mass (13, 15). However, sex hormones, specifically estrogen, are believed to

contribute to the stronger muscle-bone relationship between girls and boys despite higher lean body mass in boys (18).

Measuring Properties of Bone

The measurement of bone quality is employed in both clinical and research settings by non-invasive imaging technologies. Dual-energy X-ray absorptiometry (DXA) is a three-component imaging technique that is able to perform site-specific bone scans to measure bone mineral content (BMC) and areal bone mineral density (aBMD). DXA is used to diagnose osteoporosis and is the gold standard for measuring body composition, as it provides a measurement of fat mass and fat-free soft tissue (FFST) mass, which are also determinants of bone material properties (19). Advantages of DXA include the low level of radiation exposure (equivalent to ~0.03% of the natural annual dose of radiation), accuracy and precision, ability to measure bone mineral at axial and appendicular sites, and its ability to measure both bone mineral and soft tissue composition (20). Although DXA provides good insight into bone quality, it does not provide a measure of the geometrical properties of bone or the bone microarchitecture (e.g., trabecular number, connectivity and orientation) (21) and osteoporosis involves changes in aBMD bone dimensions and microarchitecture (22). Moreover, although DXA measures of aBMD incorporate bone size and are less influenced by bone size than BMC, the bone size adjustment is incomplete. DXA provides a two-dimensional image and does not provide information about the depth of bone, thus smaller bones of comparable volumetric BMD appear to have lower aBMD (23).

Over the past decade, there has been an increase in the use of peripheral quantitative computed tomography (pQCT) for the assessment of skeletal health. Using

pQCT, trabecular and cortical bone parameters can be analyzed separately, thus allowing the examination of bone distribution, architecture and geometry at fracture-prone sites. Additionally, pQCT provides measurements that may be used to calculate muscle strength. For example, the strength strain index is an estimate of bone bending-strength and is derived from material properties of bone. The pQCT scanner must be positioned at pre-measured distances from a reference point. This reference point is at either the growth plate (children) or endplate (adults) in order to capture compartments of the peripheral bones (i.e., radius, tibia, or femur) that are predominantly composed of trabecular bone (metaphyseal site, 4%) or cortical bone (diaphyseal site, 33%, 38%, 65%, or 66%), or to allow for determination of muscle density (diaphyseal site, 65% or 66%) (24-26). Radiation exposure from pQCT is low (0.01 mSV), although slightly higher if compared with DXA (0.004-0.005 mSV) (27).

Protein and Eggs

Modifiable factors such as the diet are thought to positively influence pediatric bone growth. The majority of evidence supporting the role of nutrition on bone health has focused on individual nutrients such as calcium and vitamin D. Recently there is increasing interest in the role of protein intake on bone and muscle development in children. Protein, an essential nutrient in every cell in the body, is composed of amino acids, which serve as building blocks to help grow and maintain the body's tissues (e.g., muscles, blood, vessels, skin, hair, and nails).

The Dietary Guidelines for Americans (DGAs) provide recommendations for diet and lifestyle choices. One of the key recommendations from the 2015-2020 DGAs is to support healthy eating patterns for all (28). This healthy eating pattern includes

consuming a variety of protein foods including seafood, lean meats and poultry, eggs, legumes (e.g., beans and peas), and nuts, seeds, and soy products.

The recommended dietary allowance (RDA) for protein and the average protein intakes for children ages 1-18 years are provided in **Table 1.** In the United States, both boys and girls are exceeding the RDA for protein in all age categories. For boys and girls ages 2-18, milk provides the greatest percentage of protein in the diet at 19.1% followed by beef at 15.0% and poultry at 10.0%. Pasta and eggs provided the least amount of protein in the diet at 2.3% and 2.4%, respectively (29).

Eggs contain high-quality protein and are a functional food, containing essential nutrients that may benefit bone health including vitamin D and zinc, as well as the bioactive compounds, lutein, zeaxanthin, lecithin, and zeaxanthin **(Table 2).** Lutein and zeaxanthin are carotenoids best known for their function in the neural retina where they are found in high concentrations. Additionally, lutein and zeaxanthin are known for their antioxidant and anti-inflammatory functions and therefore are thought to play a role in reducing immune-mediated macular degeneration and age-related cataract formation (30). Lecithin is a functional and structural component of all biological membranes and acts in the rate-limiting step in the activation of membrane enzymes including superoxide dismutase. Eggs are one of the only foods that contain high concentrations of choline. Choline has several important physiologic functions including the synthesis of phospholipids, the metabolism of methyl and cholinergic neurotransmission, and is essential for normal brain development (31).

The composition of eggs can be affected by several factors including hen diet, age, strain, as well as environmental factors. Despite the high nutritional value, culinary

versatility, and low costs, eggs have been considered a controversial food for decades because of the saturated fat content (~3 g/100g) and cholesterol content (200-300 mg/100g) (32). Over the past 40 years, the public has been warned about frequent egg consumption due to a supposed increase in cardiovascular disease risk. This risk was based on the assumption that increased dietary cholesterol consumption is associated with increased serum cholesterol levels and thus cardiovascular disease risk. Reviews conducted by Shin et al. (33) and Griffin et al. (34) evaluated results from 16 and 12 studies, respectively. These studies showed that egg consumption was not associated with hyperlipidemia, risk of cardiovascular disease, or cardiac mortality in the general population. These reviews provided the basis for the 2015 DGAs which de-emphasized the recommendation to limit cholesterol rich foods, including eggs (28).

Protein and Bone

Protein is a major component of bone, making up approximately 50% of bone volume and approximately 33% of its mass (35). Additionally, it provides the structural matrix of the bone (calcium is the prevailing mineral within the matrix). Protein intake stimulates the activity of anabolic hormones and growth factors such as insulin-like growth factor-1 (IGF-1). During pubertal growth, bone mineral content accrual is markedly influenced by increasing IGF-1 (36). Thus, adequate dietary protein intake appears to be essential for optimal bone growth during childhood. Lastly, dietary protein intake may increase calcium absorption from the gut (37), which is likely to be beneficial for bone mineralization.

Studies examining the association between protein intake and bone health are limited and focus primarily on adult populations. A meta-analysis published in 2009

found no effects of protein consumption on fracture risk in healthy adults (38), while two more recent meta-analyses demonstrated a slight reduction in hip fractures with increased protein intake (39) and protective effects on lumbar spine bone mineral density with increased protein intakes (40). Together these studies suggest there is a slight benefit of increasing protein intake for bone health in adults and no detrimental effects of protein intake.

Although it is suspected that protein intake is important for improving and maintaining bone health, there is some concern that higher intakes of sulfur containing amino acids (primarily from red meats) increases net physiological acid production. It is hypothesized that the acidic environment increases release of calcium from bone and increases osteoclast activity (41). An established method of estimating the acid loads of foods or diets is to calculate the potential renal acid load (PRAL) (42). The PRAL provides an estimate of the production of endogenous acid that exceeds the level of alkali produced for a given amount of food ingested daily. The PRAL considers different intestinal absorption rates of individual minerals, sulfur-containing protein, and the amount of sulfate produced from metabolized proteins (43). Elevated PRAL levels have been demonstrated to be detrimental to bone health outcomes in children, adolescents, and adults (44). Consuming protein with other components of the diet may modify the overall net acid concentration and decrease the dietary PRAL. For example, fruits, vegetables, and dairy products have an alkalizing effect and soy isoflavones have estrogenic effects (44, 45). Future research needs to include larger-scale intervention studies to determine the effect of increased protein intake on bone outcomes and fracture risk.

Protein and Pediatric Bone

Given establishing healthy bones during childhood serves as a blueprint for bone health in adulthood, it is of interest to determine whether nonpharmalogic dietary approaches, such as increasing dietary protein intake, can improve peak bone mass gains during childhood. To our knowledge there is only one systematic review and meta-analysis examining the role of protein intake in children and adolescents, but this study focused primarily on adult populations and assessed evidence published over the last 40 years (46). They did, however, find that protein intake accounted for 0-14% of areal BMC variance in children and adolescents. In young adults ages 18-25 years, there was no effect of protein supplementation in conjunction with a strength and conditioning training program for 6 months on trabecular or cortical bone at the 4% and 20% sites of the distal tibia, respectively (47). Prospectively, Alexy et al., demonstrated in a cohort of German children that protein intakes over 4 years was positively associated with and was a predictor of, forearm periosteal circumference (PC), cortical area (CA), BMC, and stress strain index (SSI). These investigators also showed that long-term dietary PRAL was negatively associated with forearm BMC and CA (48). In the same cohort, Remer et al. demonstrated that urinary nitrogen was positively associated with forearm PC, CA, BMC, SSI, and urinary PRAL was negatively associated with forearm BMC and CA (49). The authors did not specify what types of protein the children were consuming in these prospective studies. Budek et al., investigated the relationships between total protein but also, milk, dairy, and meat protein on total body BMC. After adjusting for BA, weight, height, and sex, only total protein and milk protein were associated with total body BMC. Additionally, when calcium, energy intake, and physical activity were added

to the model, only the relationship between milk protein and total body BMC persisted (50).

Eggs and Bone

The osteogenic effect of individual nutrients may be improved when packaged together and consumed contemporaneously (51, 52) or as whole foods (53, 54). With the exception of dairy foods (51) little data exists on the effects of whole foods on bone health in children.

Whole eggs have been examined in prior adult studies with respect to their beneficial effects on bone and fracture outcomes, but egg intake was only considered in food groupings and not independently. For example, Inose et al., showed greater Z-scores for tibia cortical speed of sound, a measure associated with cortical strength, in mothers who consumed more milk, dairy products, and eggs (55). In older adults, intakes of red meat, but not poultry or eggs, was associated with greater risk for skeletal fractures (56). While these two adult studies suggest the bone augmenting potential of eggs, it is difficult to ascertain whether and to what degree eggs independently contributed to these positive relationships given they were not studied in isolation.

The ability of whole eggs to positively impact cortical bone may occur indirectly through effects on skeletal muscle. Skeletal muscle development precedes bone mass accrual during pubertal growth, and it is well documented that FFST mass is the most important determinant of bone growth in youth (57, 58). In young men, consuming 20 grams of whole egg protein stimulated greater muscle protein synthesis following resistance exercise than in those not consuming whole egg protein (59). Moreover, leucine, a primary amino acid in whole eggs, improved skeletal muscle synthesis in

young adults (60). Thus, it is possible that the amino acid and protein components of eggs may indirectly enhance bone strength through increased muscle mass. Although eggs contain protein and bioactive nutrients that are thought to improve pediatric bone health, whether egg consumption benefits bone health growing children remains to be elucidated.

Inflammation

Acute inflammation is a normal, adaptive physiological response of the immune system to pathogens and tissue injury. Mediated by immune cells, acute inflammation typically lasts only a few days. During acute inflammation, immune cells function rapidly and efficiently to eliminate pathogenic stimuli and return the affected tissue to its normal homeostatic state through a coordinated activation and resolution of pro-inflammatory leukocyte activity (31). Classic signs of acute inflammation include heat, redness, swelling, pain and loss of function.

If the body fails to appropriately execute and resolve acute inflammatory responses this can lead to a detrimental chronic inflammatory tissue state. Chronic inflammation is characterized by pathological tissue remodeling, fibrosis, and impaired functioning due to persistent inflammatory cell infiltration, activation, and leukocyte-mediated tissue damage (61, 62). These detrimental effects are observed in cases of inappropriate activation of the immune system such as autoimmune conditions (e.g., rheumatoid arthritis and irritable bowel disease) and allergic reactions (63-65). Additionally, systematic, low-grade inflammation is associated with several other chronic diseases including cardiovascular disease (66, 67), type 2 diabetes mellitus (68), obesity (69), non-alcoholic fatty liver disease (70) and osteoporosis (71, 72).

Inflammation and Bone

Osteoporosis is a heterogeneous condition occurring during many life stages and its etiology is attributed to various endocrine, metabolic, and mechanical factors (e.g., vitamin D and calcium deficiency, postmenopausal hormonal changes, etc.) (73). The heightened interest in the link between inflammation and bone is derived primarily from observational studies of low BMD in children and adults with inflammatory conditions (e.g., Crohn's Disease, Ulcerative Colitis, Cystic Fibrosis and Rheumatoid Arthritis) (74). Potential side effects of these inflammatory diseases are thought to indirectly be detrimental to bone **(Figure 6).** For example, most chronic inflammatory diseases are associated with a catabolic state that favors the loss of lean body mass which can be detrimental to bone. Systemic inflammation can impact the secretion and action of parathyroid hormone which can increase the activity of osteoclasts thus increasing bone resorption. Steroids such as glucocorticoids are commonly used to treat inflammatory diseases and these drugs frequently have major adverse effects on bone health. Lastly, malnutrition is common in chronic inflammatory diseases which may be indirectly detrimental to bone.

Elevated levels of pro-inflammatory cytokines seen in chronic inflammatory diseases can directly affect bone resorption and/or bone formation, leading to increased bone loss. For example, studies in untreated celiac disease patients found that serum interleukin-6 (IL-6) was negatively associated with total body BMD and positively associated with carboxyterminal telopeptide region of type 1 collagen (marker of bone resorption) (75, 76). In a cross-sectional study in otherwise healthy adolescent boys and girls, monocyte chemoattractant protein-1(MCP-1) was negatively associated with tibia

cortical thickness after adjusting for the covariates, stage of sexual maturation, sex, race, muscle cross-sectional area, and height (77). Similarly, a systematic review in individuals with rheumatoid arthritis demonstrated that following treatment with a tumor necrosis factor alpha (TNF-α) antagonists, patients had an increase in total body BMD (78). This suggests that by decreasing inflammation, individuals with chronic inflammatory diseases may have stronger bones and thus decreased risk for osteoporosis and fractures later in life.

Of the inflammatory diseases that may influence bone, obesity affects 18.5% of children and adolescents in the United States and may therefore provide the greatest insight into the inflammation-bone relationship in otherwise healthy youth (79). With obesity, there is an increase in the amount of adipose tissue, leading to increased macrophage infiltration into the adipose tissue. As a result of this macrophage infiltration, pro-inflammatory cytokines, chemokines, and growth factors are released into circulation. Russell et al., conducted a study in 12 to 18-year-old obese and non-obese females to determine whether associations between visceral and subcutaneous adipose tissue (VAT and SAT) and bone density measures were influenced by the pro-inflammatory biomarkers TNF-α, IL-6, and the adhesion molecule E-selectin. Surprisingly there were no relationships between TNF-α and IL-6 with VAT or SAT. However, in a regression model that included VAT/SAT, adipokines, and cytokines, E-selectin was a negative predictor of spine and whole-body BMD (80).

Although data have shown that individuals with chronic inflammatory diseases are at risk for bone loss and osteoporosis, findings in otherwise healthy adults at risk for age-related bone loss may be more applicable to the general population. Lim et al.

demonstrated that elevated C-reactive protein (CRP) levels were associated with decreased lumbar spine and femoral neck BMD in healthy adults (81). Additionally, in a study in older women, those with elevated biomarkers of inflammation (IL-6 and TNF-α) had a 1.5-fold increased risk for hip fracture in comparison to those with lower levels of inflammation (82). Lastly, a prospective study by Cauley et al. (83) demonstrated that higher serum concentrations of inflammatory-related biomarkers, TNF-α, and IL-6 predicts a higher incidence of fracture over 6 years in 70 to 79 year-old adults (83). These findings support that chronic, systemic inflammation is not only a risk factor for bone loss in individuals with chronic diseases but also potentially in otherwise healthy adults. Whether elevated levels of pro-inflammatory cytokines in otherwise healthy children negatively influences bone health outcomes remains unknown.

Inflammation and Diet

There is a substantial amount of evidence suggesting that many foods, nutrients, and non-nutrient food components influence the inflammatory response process (84). The primary research approaches to test whether nutrition modifies inflammation is to target individual nutrients (85, 86). For example, consuming omega-6 fatty acids and antioxidant vitamins (e.g., vitamin E, lutein and vitamin C) have been associated with decreased serum inflammation (86), while consuming saturated fatty acids and excessive refined grains have been correlated with higher levels of pro-inflammatory cytokines (85).

There are published reports that dietary patterns or whole foods may be more robust than individual nutrients in producing pro- or anti- inflammatory affects (87). For example, in healthy adults, adherence to the traditional Mediterranean diet was associated

with a reduction in the concentrations of the inflammatory biomarkers, IL-6, TNF-α, and CRP. In a cross-sectional study of 732 women who participated in the Nurses' Health Study, researchers investigated the relationship between dietary patterns and inflammatory biomarkers. Using food-frequency questionnaires, the researchers established two common dietary patterns, the prudent pattern diet and the Western diet. The prudent pattern diet is similar to the Mediterranean diet with an emphasis on vegetables, fruits, legumes, whole grains, fish and poultry. The Western dietary pattern was characterized by increased consumption of red meat, processed meats, refined grains, sweets, and fried foods. After adjusting for the covariates, age, body mass index (BMI), physical activity, smoking status and alcohol consumption, the prudent pattern was inversely associated with plasma concentrations of CRP and E-selectin. The Western dietary pattern was positively associated with increased levels of CRP, IL-6, E-selectin, soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1 after adjusting for the all covariates except BMI. When BMI was added to the model, the relationships between the western dietary pattern and IL-6 were nullified (88).

An increased understanding of how diet influences inflammation led to the development of the Dietary Inflammatory Index (DII) by Cavicchia et al (89) to assess the diet on a continuum from maximally anti-inflammatory to maximally pro-inflammatory. Utilizing the first version of the DII, Cavicchia et al. demonstrated that the DII scores correlate with the inflammatory biomarker CRP in a longitudinal study of apparently healthy adults (89). These analyses were repeated for the next generation DII which was developed to include peer-reviewed articles published from 2007 to 2010 and to refine the scoring algorithm (90). Several cross-sectional studies have demonstrated

significant relationships between the updated DII score and CRP in adults (91), as well as, IL-6, TNF-α and a combined inflammatory biomarker score in postmenopausal women (92). Among children and adolescents, whether a pro-inflammatory diet (indicated by higher DII scores) is associated with serum biomarkers of inflammation is unknown.

Eggs and Inflammation

Eggs contain a variety of essential nutrients and bioactive compounds that contain anti-inflammatory properties. Examples include phospholipids, cholesterol, high-quality protein, and the carotenoids lutein and zeaxanthin.

Egg yolks are one of the richest dietary sources of phospholipids. The predominant phospholipid class found in eggs is glycerophospholipid phosphatidylcholine. Egg derived phospholipids are highly bioavailable, with glycerophospholipid phosphatidylcholine being absorbed at >90% efficiency (93, 94). Phospholipids are known to influence plasma lipids by increasing high-density lipoprotein cholesterol (HDL-C) concentrations (95, 96). HDL-C is thought to be anti-inflammatory due to its ability to selectively decrease endothelial cell adhesion molecules which facilitate the binding of mononuclear cells to vessel walls (97). Moreover, HDL-C limits the expression of the pro-inflammatory cytokines, TNF-α and interleukin-1 (98).

In addition to phospholipids, egg yolks contain a variety of antioxidant carotenoids (99, 100). The carotenoid composition of egg yolks is reflective of the hen's diet, with greater intake of carotenoid-rich foods conferring greater yolk carotenoid enrichment (101). The two predominant carotenoids found in egg yolks are lutein and zeaxanthin, although beta-carotene, alpha carotene, and beta-cryptoxanthin are also found

in lower levels (99). Both lutein and zeaxanthin function as antioxidants and anti-inflammatory agents (102, 103). For example, lutein and zeaxanthin are efficient physical quenchers of singlet oxygen and scavengers of other reactive oxygen species (ROS) (104). Additionally, they can act as chemical quenchers, undergoing irreversible oxygenation (104). The antioxidant potential of the carotenoids lutein and zeaxanthin are of particular significant to human health because reducing ROS, results in decreased oxidative stress and damage which is a critical factor in the pathogenic processes of several chronic diseases such as cardiovascular disease and cancer (104).

Lastly, eggs contain a variety of bioactive proteins in the egg white fraction, including ovalbumin, ovotransferrin, ovomucin, lysozyme and avidin (105). For example, the egg-white derived lysozyme naturally exerts antimicrobial effects against Gram-positive and Gram-negative bacterial through hydrolysis of structural peptidogylcans in the bacterial cell walls (106). Moreover, in a porcine model of dextran sodium sulfate induced colitis, egg protein supplementation decreased intestinal gene expression of the pro-inflammatory cytokines, TNF-α, IL-6, interferon-gamma, interleukin-8, and interleukin-17, while increasing the expression of anti-inflammatory interleukin-4 and transforming growth factor beta (107).

Although eggs contain a variety of bioactive compounds associated with anti-inflammatory effects, there are a limited number of intervention trials examining the effects of egg intake on inflammation, and all of these studies have been conducted in adults with obesity, metabolic disorder or type 2 diabetes (31). For example, in overweight men following a carbohydrate-restricted diet (% energy from carbohydrate:fat:protein = 17:57:26) for 12 weeks, the men who consumed eggs had

decreased plasma CRP compared to control (108). Moreover, in men and women with metabolic syndrome, whole egg intake during moderate carbohydrate restriction (25-30% of energy) improved peripheral blood mononuclear cell inflammation, highlighting the anti-inflammatory effect of this dietary intervention (109). Lastly, in comparison to an oatmeal-based breakfast, egg consumption (one per day) for five weeks resulted in decreased TNF-α concentrations in individuals with type 2 diabetes (110). Collectively, these studies provide support for eggs to mitigate inflammation under specific health conditions; however, future research needs to be conducted to determine the anti-inflammatory effects of egg consumption in healthy individuals. To my knowledge there are no studies investigating the anti-inflammatory potential of eggs in healthy children and adolescents.

Summary

Adolescence is a period of rapid and significant longitudinal bone growth, areal bone mineral accrual, and bone expansion (12, 13, 16, 17). Thus, achieving optimal peak bone mass gains during adolescence is important to prevent low bone mass and osteoporosis later in life. Recently, dietary protein has been highlighted for its potential benefit on adult bone health outcomes; however, limited research has investigated the role of dietary protein on pediatric bone outcomes. It has been postulated that whole foods or dietary compounds may be more robust in influencing not only pediatric bone growth but also inflammatory response patterns. Eggs are a high-quality protein providing 6 grams per egg and contain bioactive components that are thought to influence pediatric bone growth and inflammation. Whether whole egg consumption during childhood influences bone mass and reduces inflammation remains to be elucidated.

Moreover, whether and how inflammation influences bone mass in healthy children is unknown. Therefore, this dissertation seeks to address two specific gaps in the current body of evidence: 1) the relationships between egg intake and protein intake and cortical bone endpoints in apparently healthy children, and 2) the influence of dietary and serum inflammation on cortical bone outcomes in apparently healthy children.

Chapter 3 of this dissertation found a positive link between whole egg consumption and radius cortical bone in healthy, black and white children in the early stages of puberty and showed for the first time that FFST mediated these relationships. Additionally, egg intake was positively correlated with the biomarker of bone turnover, osteocalcin. Chapter 4 of this dissertation found that the pro-inflammatory biomarker, MCP-1 was inversely related to tibia cortical thickness in healthy children and adolescents. Chapter 5 of this dissertation found limited evidence (Grade C) for a positive impact of protein intake on pediatric bone health. The limited grade was given was due to there being only one dietary protein intervention in children and adolescents and this intervention was in conjunction with an exercise intervention. Currently there are no intervention studies independently assessing the influence of dietary protein on pediatric bone health.

The findings from this dissertation led to the funding of the Skeletal and Cognitive Effects of Nutrition from Eggs (SCENE study) **(Figure 7).** The SCENE study is a 9-month randomized controlled feeding trial in healthy and obese boys and girls ages 9-13 years. The SCENE study is a 2-phase study. SCENE phase 1 was a pilot study aimed at determining the feasibility of consuming egg products and control products (i.e., whole milk protein and gelatin) in children ages 9-13 years. All products were tested in a

minimum of 75 children to determine overall acceptability. After being tested, all products were judged as acceptable for inclusion in the randomized controlled trial (SCENE phase 2). The SCENE study central hypothesis is that the consumption of whole egg products will enhance skeletal strength in children to a greater degree that controls. The secondary hypothesis is that biomarkers of inflammation will partially mediate the positive effects of eggs on skeletal strength. The tertiary hypothesis is that egg intake will positively influence cognitive function in children.

References

1. Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simoes MJ, Cerri PS. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. Biomed Res Int 2015;2015:421746. doi: 10.1155/2015/421746.

2. Ammann P, Rizzoli R. Bone strength and its determinants. Osteoporos Int 2003;14 Suppl 3:S13-8. doi: 10.1007/s00198-002-1345-4.

3. Augat P, Schorlemmer S. The role of cortical bone and its microstructure in bone strength. Age Ageing 2006;35 Suppl 2:ii27-ii31. doi: 10.1093/ageing/afl081.

4. Khan K MH KP, Bailey D, Wark J, Bennell K. Physical acitivity and bone health. Human Kinetics 2001:.

5. Huang C, Ogawa R. Mechanotransduction in bone repair and regeneration. FASEB J 2010;24(10):3625-32. doi: 10.1096/fj.10-157370.

6. Miller SC, de Saint-Georges L, Bowman BM, Jee WS. Bone lining cells: structure and function. Scanning Microsc 1989;3(3):953-60; discussion 60-1.

7. Seeman E. Bone modeling and remodeling. Crit Rev Eukaryot Gene Expr 2009;19(3):219-33.

8. Hadjidakis DJ, Androulakis, II. Bone remodeling. Ann N Y Acad Sci 2006;1092:385-96. doi: 10.1196/annals.1365.035.

9. Demontiero O, Vidal C, Duque G. Aging and bone loss: new insights for the clinician. Ther Adv Musculoskelet Dis 2012;4(2):61-76. doi: 10.1177/1759720X11430858.

10. Ferrari S, Rizzoli R, Slosman D, Bonjour JP. Familial resemblance for bone mineral mass is expressed before puberty. J Clin Endocrinol Metab 1998;83(2):358-61. doi: 10.1210/jcem.83.2.4583.

11. Baxter-Jones AD, Faulkner RA, Forwood MR, Mirwald RL, Bailey DA. Bone mineral accrual from 8 to 30 years of age: an estimation of peak bone mass. J Bone Miner Res 2011;26(8):1729-39. doi: 10.1002/jbmr.412.

12. Boot AM, de Ridder MA, van der Sluis IM, van Slobbe I, Krenning EP, Keizer-Schrama SM. Peak bone mineral density, lean body mass and fractures. Bone 2010;46(2):336-41. doi: 10.1016/j.bone.2009.10.003.

13. Jackowski SA, Faulkner RA, Farthing JP, Kontulainen SA, Beck TJ, Baxter-Jones AD. Peak lean tissue mass accrual precedes changes in bone strength indices at

the proximal femur during the pubertal growth spurt. Bone 2009;44(6):1186-90. doi: 10.1016/j.bone.2009.02.008.

14. Rauch F, Neu C, Manz F, Schoenau E. The development of metaphyseal cortex--implications for distal radius fractures during growth. J Bone Miner Res 2001;16(8):1547-55. doi: 10.1359/jbmr.2001.16.8.1547.

15. Macdonald HM, Kontulainen SA, Mackelvie-O'Brien KJ, Petit MA, Janssen P, Khan KM, McKay HA. Maturity- and sex-related changes in tibial bone geometry, strength and bone-muscle strength indices during growth: a 20-month pQCT study. Bone 2005;36(6):1003-11. doi: 10.1016/j.bone.2004.12.007.

16. Nishiyama KK, Macdonald HM, Moore SA, Fung T, Boyd SK, McKay HA. Cortical porosity is higher in boys compared with girls at the distal radius and distal tibia during pubertal growth: an HR-pQCT study. J Bone Miner Res 2012;27(2):273-82. doi: 10.1002/jbmr.552.

17. Xu L, Nicholson P, Wang Q, Alen M, Cheng S. Bone and muscle development during puberty in girls: a seven-year longitudinal study. J Bone Miner Res 2009;24(10):1693-8. doi: 10.1359/jbmr.090405.

18. Schiessl H, Frost HM, Jee WS. Estrogen and bone-muscle strength and mass relationships. Bone 1998;22(1):1-6.

19. Blake GM, Fogelman I. Technical principles of dual energy x-ray absorptiometry. Semin Nucl Med 1997;27(3):210-28.

20. Genant HK, Faulkner KG, Gluer CC. Measurement of bone mineral density: current status. Am J Med 1991;91(5B):49S-53S.

21. Genant HK, Engelke K, Fuerst T, Gluer CC, Grampp S, Harris ST, Jergas M, Lang T, Lu Y, Majumdar S, et al. Noninvasive assessment of bone mineral and structure: state of the art. J Bone Miner Res 1996;11(6):707-30. doi: 10.1002/jbmr.5650110602.

22. Parfitt AM, Mathews CH, Villanueva AR, Kleerekoper M, Frame B, Rao DS. Relationships between surface, volume, and thickness of iliac trabecular bone in aging and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss. J Clin Invest 1983;72(4):1396-409. doi: 10.1172/JCI111096.

23. Zemel BS, Leonard MB, Kelly A, Lappe JM, Gilsanz V, Oberfield S, Mahboubi S, Shepherd JA, Hangartner TN, Frederick MM, et al. Height adjustment in assessing dual energy x-ray absorptiometry measurements of bone mass and density in children. J Clin Endocrinol Metab 2010;95(3):1265-73. doi: 10.1210/jc.2009-2057.

24. Zemel B, Bass S, Binkley T, Ducher G, Macdonald H, McKay H, Moyer-Mileur L, Shepherd J, Specker B, Ward K, et al. Peripheral quantitative computed tomography in children and adolescents: the 2007 ISCD Pediatric Official Positions. J Clin Densitom 2008;11(1):59-74. doi: 10.1016/j.jocd.2007.12.006.

25. Felin EM, Prahalad S, Askew EW, Moyer-Mileur LJ. Musculoskeletal abnormalities of the tibia in juvenile rheumatoid arthritis. Arthritis Rheum 2007;56(3):984-94. doi: 10.1002/art.22420.

26. Qin L, Au SK, Chan KM, Lau MC, Woo J, Dambacher MA, Leung PC. Peripheral volumetric bone mineral density in pre- and postmenopausal Chinese women in Hong Kong. Calcif Tissue Int 2000;67(1):29-36.

27. Damilakis J, Adams JE, Guglielmi G, Link TM. Radiation exposure in X-ray-based imaging techniques used in osteoporosis. Eur Radiol 2010;20(11):2707-14. doi: 10.1007/s00330-010-1845-0.

28. DeSalvo KB, Olson R, Casavale KO. Dietary Guidelines for Americans. JAMA 2016;315(5):457-8. doi: 10.1001/jama.2015.18396.

29. Subar AF, Krebs-Smith SM, Cook A, Kahle LL. Dietary sources of nutrients among US children, 1989-1991. Pediatrics 1998;102(4 Pt 1):913-23.

30. Herron KL, Fernandez ML. Are the current dietary guidelines regarding egg consumption appropriate? J Nutr 2004;134(1):187-90. doi: 10.1093/jn/134.1.187.

31. Andersen CJ. Bioactive Egg Components and Inflammation. Nutrients 2015;7(9):7889-913. doi: 10.3390/nu7095372.

32. Li Y, Zhou C, Zhou X, Li L. Egg consumption and risk of cardiovascular diseases and diabetes: a meta-analysis. Atherosclerosis 2013;229(2):524-30. doi: 10.1016/j.atherosclerosis.2013.04.003.

33. Shin JY, Xun P, Nakamura Y, He K. Egg consumption in relation to risk of cardiovascular disease and diabetes: a systematic review and meta-analysis. Am J Clin Nutr 2013;98(1):146-59. doi: 10.3945/ajcn.112.051318.

34. Griffin JD, Lichtenstein AH. Dietary Cholesterol and Plasma Lipoprotein Profiles: Randomized-Controlled Trials. Curr Nutr Rep 2013;2(4):274-82. doi: 10.1007/s13668-013-0064-0.

35. Heaney RP. Protein intake and the calcium economy. J Am Diet Assoc 1993;93(11):1259-60.

36. Breen ME, Laing EM, Hall DB, Hausman DB, Taylor RG, Isales CM, Ding KH, Pollock NK, Hamrick MW, Baile CA, et al. 25-hydroxyvitamin D, insulin-like growth factor-I, and bone mineral accrual during growth. J Clin Endocrinol Metab 2011;96(1):E89-98. doi: 10.1210/jc.2010-0595.

37. Cao JJ, Johnson LK, Hunt JR. A diet high in meat protein and potential renal acid load increases fractional calcium absorption and urinary calcium excretion without affecting markers of bone resorption or formation in postmenopausal women. J Nutr 2011;141(3):391-7. doi: 10.3945/jn.110.129361.

38. Darling AL, Millward DJ, Torgerson DJ, Hewitt CE, Lanham-New SA. Dietary protein and bone health: a systematic review and meta-analysis. Am J Clin Nutr 2009;90(6):1674-92. doi: 10.3945/ajcn.2009.27799.

39. Wu AM, Sun XL, Lv QB, Zhou Y, Xia DD, Xu HZ, Huang QS, Chi YL. The relationship between dietary protein consumption and risk of fracture: a subgroup and dose-response meta-analysis of prospective cohort studies. Sci Rep 2015;5:9151. doi: 10.1038/srep09151.

40. Shams-White MM, Chung M, Du M, Fu Z, Insogna KL, Karlsen MC, LeBoff MS, Shapses SA, Sackey J, Wallace TC, et al. Dietary protein and bone health: a systematic review and meta-analysis from the National Osteoporosis Foundation. Am J Clin Nutr 2017;105(6):1528-43. doi: 10.3945/ajcn.116.145110.

41. Arnett TR, Dempster DW. Effect of pH on bone resorption by rat osteoclasts in vitro. Endocrinology 1986;119(1):119-24. doi: 10.1210/endo-119-1-119.

42. Remer T, Manz F. Potential renal acid load of foods and its influence on urine pH. J Am Diet Assoc 1995;95(7):791-7. doi: 10.1016/S0002-8223(95)00219-7.

43. Remer T, Dimitriou T, Manz F. Dietary potential renal acid load and renal net acid excretion in healthy, free-living children and adolescents. Am J Clin Nutr 2003;77(5):1255-60. doi: 10.1093/ajcn/77.5.1255.

44. Mangano KM, Walsh SJ, Kenny AM, Insogna KL, Kerstetter JE. Dietary acid load is associated with lower bone mineral density in men with low intake of dietary calcium. J Bone Miner Res 2014;29(2):500-6. doi: 10.1002/jbmr.2053.

45. Massey LK. Dietary animal and plant protein and human bone health: a whole foods approach. J Nutr 2003;133(3):862S-5S. doi: 10.1093/jn/133.3.862S.

46. Darling AL, Manders RJF, Sahni S, Zhu K, Hewitt CE, Prince RL, Millward DJ, Lanham-New SA. Dietary protein and bone health across the life-course: an updated systematic review and meta-analysis over 40 years. Osteoporos Int 2019;30(4):741-61. doi: 10.1007/s00198-019-04933-8.

47. Ballard TL, Specker BL, Binkley TL, Vukovich MD. Effect of protein supplementation during a 6-month strength and conditioning program on areal

and volumetric bone parameters. Bone 2006;38(6):898-904. doi: 10.1016/j.bone.2005.10.020.

48. Alexy U, Remer T, Manz F, Neu CM, Schoenau E. Long-term protein intake and dietary potential renal acid load are associated with bone modeling and remodeling at the proximal radius in healthy children. Am J Clin Nutr 2005;82(5):1107-14. doi: 10.1093/ajcn/82.5.1107.

49. Remer T, Manz F, Alexy U, Schoenau E, Wudy SA, Shi L. Long-term high urinary potential renal acid load and low nitrogen excretion predict reduced diaphyseal bone mass and bone size in children. J Clin Endocrinol Metab 2011;96(9):2861-8. doi: 10.1210/jc.2011-1005.

50. Budek AZ, Hoppe C, Ingstrup H, Michaelsen KF, Bugel S, Molgaard C. Dietary protein intake and bone mineral content in adolescents-The Copenhagen Cohort Study. Osteoporos Int 2007;18(12):1661-7. doi: 10.1007/s00198-007-0422-0.

51. Weaver CM, Gordon CM, Janz KF, Kalkwarf HJ, Lappe JM, Lewis R, O'Karma M, Wallace TC, Zemel BS. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. Osteoporos Int 2016;27(4):1281-386. doi: 10.1007/s00198-015-3440-3.

52. Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. Nutrition 2009;25(2):165-71. doi: 10.1016/j.nut.2008.07.019.

53. Moore LL, Bradlee ML, Gao D, Singer MR. Effects of average childhood dairy intake on adolescent bone health. J Pediatr 2008;153(5):667-73. doi: 10.1016/j.jpeds.2008.05.016.

54. Laird E, Molloy AM, McNulty H, Ward M, McCarroll K, Hoey L, Hughes CF, Cunningham C, Strain JJ, Casey MC. Greater yogurt consumption is associated with increased bone mineral density and physical function in older adults. Osteoporos Int 2017;28(8):2409-19. doi: 10.1007/s00198-017-4049-5.

55. Inose T, Takano T, Nakamura K, Kizuki M, Seino K. Tibial cortical bone properties of preadolescents and their mothers in an urban area associated with lifestyle: a longitudinal study. Acta Paediatr 2006;95(3):276-82. doi: 10.1080/08035250500352169.

56. Zeng FF, Fan F, Xue WQ, Xie HL, Wu BH, Tu SL, Ouyang WF, Chen YM. The association of red meat, poultry, and egg consumption with risk of hip fractures in elderly Chinese: a case-control study. Bone 2013;56(2):242-8. doi: 10.1016/j.bone.2013.06.023.

57. Baptista F, Barrigas C, Vieira F, Santa-Clara H, Homens PM, Fragoso I, Teixeira PJ, Sardinha LB. The role of lean body mass and physical activity in bone health in children. J Bone Miner Metab 2012;30(1):100-8. doi: 10.1007/s00774-011-0294-4.

58. Hamrick MW. The skeletal muscle secretome: an emerging player in muscle-bone crosstalk. Bonekey Rep 2012;1:60. doi: 10.1038/bonekey.2012.60.

59. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. Am J Clin Nutr 2009;89(1):161-8. doi: 10.3945/ajcn.2008.26401.

60. Glynn EL, Fry CS, Drummond MJ, Timmerman KL, Dhanani S, Volpi E, Rasmussen BB. Excess leucine intake enhances muscle anabolic signaling but not net protein anabolism in young men and women. J Nutr 2010;140(11):1970-6. doi: 10.3945/jn.110.127647.

61. Huang W, Glass CK. Nuclear receptors and inflammation control: molecular mechanisms and pathophysiological relevance. Arterioscler Thromb Vasc Biol 2010;30(8):1542-9. doi: 10.1161/ATVBAHA.109.191189.

62. Bannenberg G, Serhan CN. Specialized pro-resolving lipid mediators in the inflammatory response: An update. Biochim Biophys Acta 2010;1801(12):1260-73. doi: 10.1016/j.bbalip.2010.08.002.

63. Amin K. The role of mast cells in allergic inflammation. Respir Med 2012;106(1):9-14. doi: 10.1016/j.rmed.2011.09.007.

64. Todd DJ, Lee AH, Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. Nat Rev Immunol 2008;8(9):663-74. doi: 10.1038/nri2359.

65. Rodriguez-Reyna TS, Alarcon-Segovia D. The different faces of shared autoimmunity. Autoimmun Rev 2006;5(2):86-8. doi: 10.1016/j.autrev.2005.09.005.

66. Libby P. Inflammation and cardiovascular disease mechanisms. Am J Clin Nutr 2006;83(2):456S-60S. doi: 10.1093/ajcn/83.2.456S.

67. Martinez BK, White CM. The Emerging Role of Inflammation in Cardiovascular Disease. Ann Pharmacother 2018;52(8):801-9. doi: 10.1177/1060028018765939.

68. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 2011;11(2):98-107. doi: 10.1038/nri2925.

69. Skalicky J, Muzakova V, Kandar R, Meloun M, Rousar T, Palicka V. Evaluation of oxidative stress and inflammation in obese adults with metabolic syndrome. Clin Chem Lab Med 2008;46(4):499-505. doi: 10.1515/CCLM.2008.096.

70. Farrell GC, van Rooyen D, Gan L, Chitturi S. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. Gut Liver 2012;6(2):149-71. doi: 10.5009/gnl.2012.6.2.149.

71. Ginaldi L, Di Benedetto MC, De Martinis M. Osteoporosis, inflammation and ageing. Immun Ageing 2005;2:14. doi: 10.1186/1742-4933-2-14.

72. Ganesan K, Teklehaimanot S, Tran TH, Asuncion M, Norris K. Relationship of C-reactive protein and bone mineral density in community-dwelling elderly females. J Natl Med Assoc 2005;97(3):329-33.

73. Yun AJ, Lee PY. Maldaptation of the link between inflammation and bone turnover may be a key determinant of osteoporosis. Med Hypotheses 2004;63(3):532-7. doi: 10.1016/S0306-9877(03)00326-8.

74. Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol 2009;201(3):309-20. doi: 10.1677/JOE-08-0568.

75. Di Stefano M, Mengoli C, Bergonzi M, Corazza GR. Bone mass and mineral metabolism alterations in adult celiac disease: pathophysiology and clinical approach. Nutrients 2013;5(11):4786-99. doi: 10.3390/nu5114786.

76. Fornari MC, Pedreira S, Niveloni S, Gonzalez D, Diez RA, Vazquez H, Mazure R, Sugai E, Smecuol E, Boerr L, et al. Pre- and post-treatment serum levels of cytokines IL-1beta, IL-6, and IL-1 receptor antagonist in celiac disease. Are they related to the associated osteopenia? Am J Gastroenterol 1998;93(3):413-8. doi: 10.1111/j.1572-0241.1998.00413.x.

77. Coheley LM, Shivappa N, Hebert JR, Lewis RD. Dietary inflammatory index(R) and cortical bone outcomes in healthy adolescent children. Osteoporos Int 2019. doi: 10.1007/s00198-019-04946-3.

78. Sakthiswary R, Das S. The effects of TNF alpha antagonist therapy on bone metabolism in rheumatoid arthritis: a systematic review. Curr Drug Targets 2013;14(13):1552-7.

79. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. JAMA 2012;307(5):483-90. doi: 10.1001/jama.2012.40.

80. Russell M, Mendes N, Miller KK, Rosen CJ, Lee H, Klibanski A, Misra M. Visceral fat is a negative predictor of bone density measures in obese adolescent girls. J Clin Endocrinol Metab 2010;95(3):1247-55. doi: 10.1210/jc.2009-1475.

81. Lim HS, Park YH, Kim SK. Relationship between Serum Inflammatory Marker and Bone Mineral Density in Healthy Adults. J Bone Metab 2016;23(1):27-33. doi: 10.11005/jbm.2016.23.1.27.

82. Barbour KE, Lui LY, Ensrud KE, Hillier TA, LeBlanc ES, Ing SW, Hochberg MC, Cauley JA, Study of Osteoporotic Fractures Research G. Inflammatory markers and risk of hip fracture in older white women: the study of osteoporotic fractures. J Bone Miner Res 2014;29(9):2057-64. doi: 10.1002/jbmr.2245.

83. Cauley JA, Danielson ME, Boudreau RM, Forrest KY, Zmuda JM, Pahor M, Tylavsky FA, Cummings SR, Harris TB, Newman AB, et al. Inflammatory markers and incident fracture risk in older men and women: the Health Aging and Body Composition Study. J Bone Miner Res 2007;22(7):1088-95. doi: 10.1359/jbmr.070409.

84. Calder PC, Ahluwalia N, Albers R, Bosco N, Bourdet-Sicard R, Haller D, Holgate ST, Jonsson LS, Latulippe ME, Marcos A, et al. A consideration of biomarkers to be used for evaluation of inflammation in human nutritional studies. Br J Nutr 2013;109 Suppl 1:S1-34. doi: 10.1017/S0007114512005119.

85. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, Teeling JL, Blaak EE, Fenech M, Vauzour D, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr 2015;114(7):999-1012. doi: 10.1017/S0007114515002093.

86. Sears B, Ricordi C. Anti-inflammatory nutrition as a pharmacological approach to treat obesity. J Obes 2011;2011. doi: 10.1155/2011/431985.

87. Nicklas TA, Baranowski T, Cullen KW, Berenson G. Eating patterns, dietary quality and obesity. J Am Coll Nutr 2001;20(6):599-608.

88. Lopez-Garcia E, Schulze MB, Fung TT, Meigs JB, Rifai N, Manson JE, Hu FB. Major dietary patterns are related to plasma concentrations of markers of inflammation and endothelial dysfunction. Am J Clin Nutr 2004;80(4):1029-35. doi: 10.1093/ajcn/80.4.1029.

89. Cavicchia PP, Steck SE, Hurley TG, Hussey JR, Ma Y, Ockene IS, Hebert JR. A new dietary inflammatory index predicts interval changes in serum high-sensitivity C-reactive protein. J Nutr 2009;139(12):2365-72. doi: 10.3945/jn.109.114025.

90. Shivappa N, Steck SE, Hurley TG, Hussey JR, Hebert JR. Designing and developing a literature-derived, population-based dietary inflammatory index. Public Health Nutr 2014;17(8):1689-96. doi: 10.1017/S1368980013002115.

91. Shivappa N, Steck SE, Hurley TG, Hussey JR, Ma Y, Ockene IS, Tabung F, Hebert JR. A population-based dietary inflammatory index predicts levels of C-reactive protein in the Seasonal Variation of Blood Cholesterol Study (SEASONS). Public Health Nutr 2014;17(8):1825-33. doi: 10.1017/S1368980013002565.

92. Tabung FK, Steck SE, Zhang J, Ma Y, Liese AD, Agalliu I, Hingle M, Hou L, Hurley TG, Jiao L, et al. Construct validation of the dietary inflammatory index among postmenopausal women. Ann Epidemiol 2015;25(6):398-405. doi: 10.1016/j.annepidem.2015.03.009.

93. Kullenberg D, Taylor LA, Schneider M, Massing U. Health effects of dietary phospholipids. Lipids Health Dis 2012;11:3. doi: 10.1186/1476-511X-11-3.

94. Zierenberg O, Grundy SM. Intestinal absorption of polyenephosphatidylcholine in man. J Lipid Res 1982;23(8):1136-42.

95. Klimov AN, Konstantinov VO, Lipovetsky BM, Kuznetsov AS, Lozovsky VT, Trufanov VF, Plavinsky SL, Gundermann KJ, Schumacher R. "Essential" phospholipids versus nicotinic acid in the treatment of patients with type IIb hyperlipoproteinemia and ischemic heart disease. Cardiovasc Drugs Ther 1995;9(6):779-84.

96. Bunea R, El Farrah K, Deutsch L. Evaluation of the effects of Neptune Krill Oil on the clinical course of hyperlipidemia. Altern Med Rev 2004;9(4):420-8.

97. Fogelman AM. When good cholesterol goes bad. Nat Med 2004;10(9):902-3. doi: 10.1038/nm0904-902.

98. Baker PW, Rye KA, Gamble JR, Vadas MA, Barter PJ. Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells. J Lipid Res 1999;40(2):345-53.

99. Blesso CN, Andersen CJ, Bolling BW, Fernandez ML. Egg intake improves carotenoid status by increasing plasma HDL cholesterol in adults with metabolic syndrome. Food Funct 2013;4(2):213-21. doi: 10.1039/c2fo30154g.

100. Johnson EJ. The role of carotenoids in human health. Nutr Clin Care 2002;5(2):56-65.

101. Karadas F, Pappas AC, Surai PF, Speake BK. Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. Comp Biochem Physiol B Biochem Mol Biol 2005;141(2):244-51. doi: 10.1016/j.cbpc.2005.04.001.

102. Krinsky NI. Possible biologic mechanisms for a protective role of xanthophylls. J Nutr 2002;132(3):540S-2S. doi: 10.1093/jn/132.3.540S.

103. Sasaki M, Ozawa Y, Kurihara T, Noda K, Imamura Y, Kobayashi S, Ishida S, Tsubota K. Neuroprotective effect of an antioxidant, lutein, during retinal inflammation. Invest Ophthalmol Vis Sci 2009;50(3):1433-9. doi: 10.1167/iovs.08-2493.

104. Fiedor J, Burda K. Potential role of carotenoids as antioxidants in human health and disease. Nutrients 2014;6(2):466-88. doi: 10.3390/nu6020466.

105. Kovacs-Nolan J, Phillips M, Mine Y. Advances in the value of eggs and egg components for human health. J Agric Food Chem 2005;53(22):8421-31. doi: 10.1021/jf050964f.

106. Ibrahim HR, Aoki T, Pellegrini A. Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. Curr Pharm Des 2002;8(9):671-93.

107. Lee M, Kovacs-Nolan J, Yang C, Archbold T, Fan MZ, Mine Y. Hen egg lysozyme attenuates inflammation and modulates local gene expression in a porcine model of dextran sodium sulfate (DSS)-induced colitis. J Agric Food Chem 2009;57(6):2233-40. doi: 10.1021/jf803133b.

108. Wong WW, Lewis RD, Steinberg FM, Murray MJ, Cramer MA, Amato P, Young RL, Barnes S, Ellis KJ, Shypailo RJ, et al. Soy isoflavone supplementation and bone mineral density in menopausal women: a 2-y multicenter clinical trial. Am J Clin Nutr 2009;90(5):1433-9. doi: 10.3945/ajcn.2009.28001.

109. Andersen CJ, Lee JY, Blesso CN, Carr TP, Fernandez ML. Egg intake during carbohydrate restriction alters peripheral blood mononuclear cell inflammation and cholesterol homeostasis in metabolic syndrome. Nutrients 2014;6(7):2650-67. doi: 10.3390/nu6072650.

110. Ballesteros MN, Valenzuela F, Robles AE, Artalejo E, Aguilar D, Andersen CJ, Valdez H, Fernandez ML. One Egg per Day Improves Inflammation when Compared to an Oatmeal-Based Breakfast without Increasing Other Cardiometabolic Risk Factors in Diabetic Patients. Nutrients 2015;7(5):3449-63. doi: 10.3390/nu7053449.

111. National Cancer Institute SEER Training Modules. Classification of Bones. Version current 1. Internet: https://training.seer.cancer.gov/anatomy/skeletal/classification.html

112. Medical Gallery of Blausen Medical. Version current 1 August 2014. Internet: https://en.wikiversity.org/wiki/WikiJournal_of_Medicine/Medical_gallery_of_Blausen_Medical_2014

113. Describe the Process of Bone Remodeling. Version current 1 January 2014. Internet:https://www.orthopaedicsone.com/display/Clerkship/Describe+the+process+of+bone+remodeling

Table 2.1: Average protein intakes versus RDA

Average Protein Intakes (grams)			RDA (grams/day)		
Age (years)	**Males**	**Females**	**Age (years)**	**Males**	**Females**
2-4	55	51	1-3	13	
6-11	69	65	4-8	19	
12-19	85	63	9-13	34	34
			14-18	52	46

RDA, recommended dietary allowances

Table 2.2: Nutritional composition of hen eggs

Component (Unit)	Amount	Component (Unit)	Amount
Egg shell (%)	10.5	Calcium (mg)	56.0
Egg yolk (%)	31	Magnesium (mg)	12.0
Egg white (%)	58.5	Iron (mg)	2.1
Water (g)	74.5	Phosphorus (μg)	180.0
Energy (Kcal)	162	Zing (mg)	1.44
Protein (g)	12.1	Thiamine (mg)	0.09
Carbohydrates (g)	0.68	Riboflavin (mg)	0.3
Lipids (g)	12.1	Niacin (mg)	0.1
Saturated fatty acids (g)	3.3	Folic acid (μg)	65.0
Monounsaturated fatty acids (g)	4.9	Cyanocobalamin (μg)	66.0
Polyunsaturated fatty acids (g)	1.8	Pyridoxine (mg)	0.12
Cholesterol (mg)	410	Retinol equivalents (μg)	227.0
Iodine (μg)	12.7	Potassium (mg)	147
Tocopherol (μg)	1.93	Carotenoids (μg)	10
Selenium (μg)	10	Cholecalciferol (μg)	1.8

Quantities represent an edible portion of about 100 g
g, grams; mg, miligrams; μg, micrograms

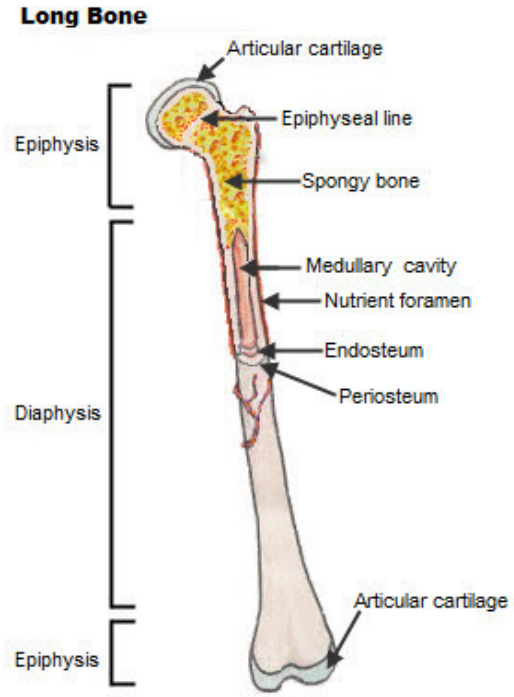


Figure 2.1. Bone anatomy (111)

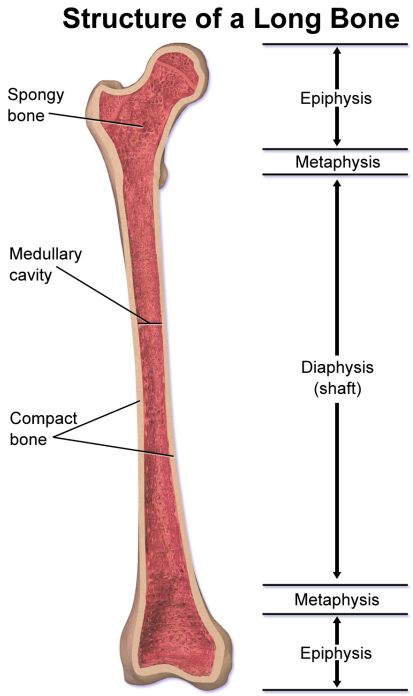


Figure 2.2. Structure of a long bone (112)

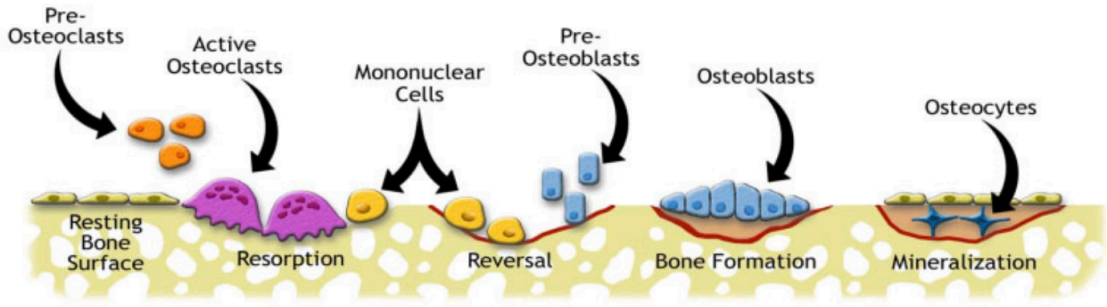


Figure 2.3. Bone remodeling process (113)

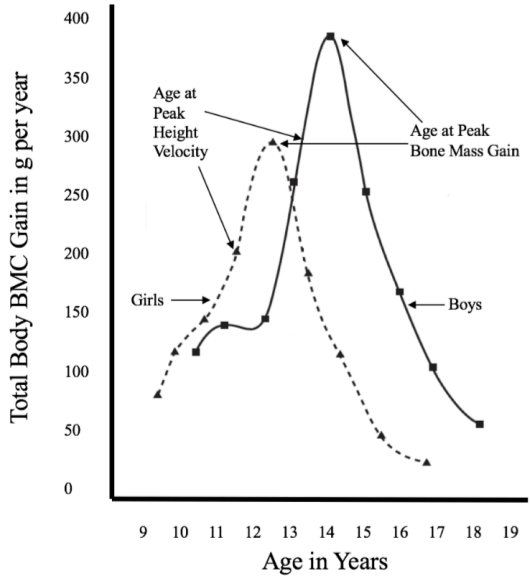


Figure 2.4. Peak bone mineral content (BMC) gains and peak height velocity (PHV) in boys and girls (51)

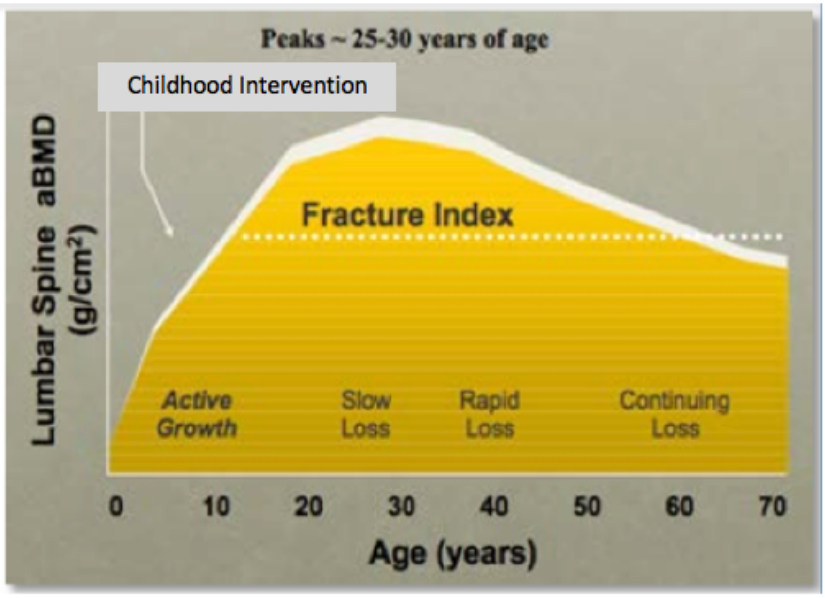


Figure 2.5. Peak bone mineral density (BMD) at the lumbar spine over time

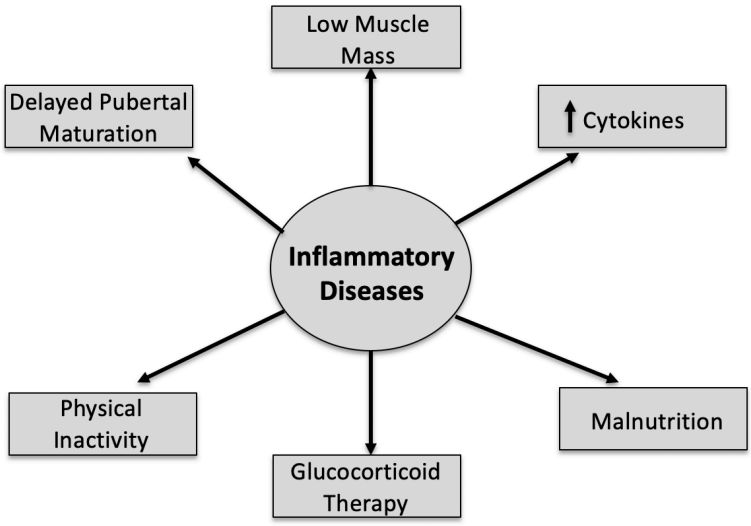
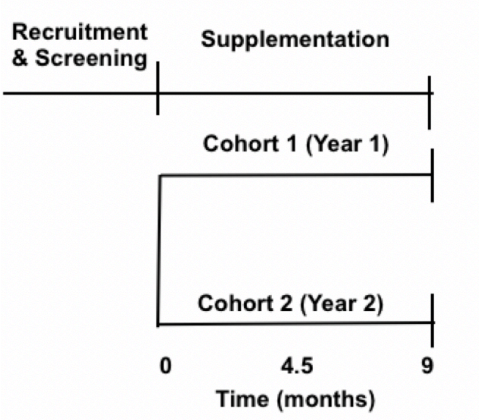


Figure 2.6. Inflammatory disease states



Independent Variables:

Treatment: Placebo, EGG, MILK
Sex: Male, Female
Race: White, Black or African American, Hispanic or Latino
Location: University Georgia in Athens, GA

Potential Covariates:

Maturation: Pubertal Stage
Anthropometry: Height, Weight, BMI, Waist Circumference
Body Composition: % Fat, Fat Mass, FFST, MCSA
Dietary Intake: Energy, Protein, Egg Intake
Physical Activity: Energy Expenditure
Variables related to IR: Fasting insulin and fasting glucose

Dependent Variables:

Inflammatory Markets: MCP-1, TNF-alpha, IL-6, IL-1B, CRP
Bone Outcomes (pQCT): Total BMD, Trab BMD, Total Area, Ct.BMD, Ct.BMC, Tt.Ar, Ct.Ar, Ct.Th, PC, EC, SSI
Bone Outcomes (DXA): aBMD, aBMC, BA
Cognitive Outcomes as measured by the NIH toolbox

Figure 2.7. SCENE study timeline

CHAPTER 3[3]

WHOLE EGG CONSUMPTION AND CORTICAL BONE IN HEALTHY CHILDREN

[3] Coheley LM, Kindler JM, Laing EM, Oshri A, Hill-Gallant KM, Warden SJ, Peacock M, Weaver CM, Lewis RD. Accepted by Osteoporosis International. Reprinted here with permission of the publisher.

Abstract

Purpose: This study examined the relationships between egg consumption and cortical bone in children.

Methods: The cross-sectional study design included 294 9-13-year-old black and white males and females. Three-day diet records determined daily egg consumption. Peripheral quantitative computed tomography measured radius and tibia cortical bone. Body composition and biomarkers of bone turnover were assessed using dual energy X-ray absorptiometry and ELISA, respectively.

Results: Egg intake was positively correlated with radius and tibia cortical bone mineral content (Ct.BMC), total bone area, cortical area, cortical thickness, periosteal circumference, and polar strength strain index in unadjusted models (r=0.144-0.224, all $P<.050$). After adjusting for differences in race, sex, stage of sexual maturation, fat-free soft tissue mass (FFST), and protein intakes, tibia relationships were nullified; however, egg intake remained positively correlated with radius Ct.BMC (r=0.138, $P=.031$). Egg intake positively correlated with total body bone mineral density, bone mineral content, and bone area in the unadjusted models only (r=0.119-0.224; all $P<.050$). After adjusting for covariates, egg intake was a positive predictor of radius FFST (β=0.113, $P<.050$) and FFST was a positive predictor of radius Ct.BMC (β=0.556, $P<.050$) in path analyses. There was a direct influence of egg on radius Ct.BMC (β=0.099, $P=.035$), even after adjusting for the mediator, FFST (β=0.137, $P=.020$). Egg intake was positively correlated with osteocalcin in both the unadjusted ($P=.005$) and adjusted ($P=.049$) models.

Conclusion: If the positive influence of eggs on Ct.BMC observed in this study are

confirmed through future randomized controlled trials, whole eggs may represent a viable strategy to promote pediatric bone development and prevent fractures.

Key Words: egg, cortical bone, fat-free soft tissue, pQCT

Mini Abstract:

Eggs contain bioactive compounds thought to benefit pediatric bone. This cross-sectional study shows a positive link between childhood egg intake and radius cortical bone. If randomized trials confirm our findings, incorporating eggs into children's diets could have a significant impact in preventing childhood fractures and reducing the risk of osteoporosis.

Introduction

The majority of evidence supporting the role of nutrition on bone health has focused on individual nutrients, particularly calcium, vitamin D, and protein (1-7). There is strong evidence for supplemental calcium (7) to increase bone mineral accrual in children, whereas findings are less consistent with respect to vitamin D (5, 6) or protein (8,9) supplementation and their effects on childhood bone accrual. The osteogenic effects of these individual nutrients may be improved when packaged together and consumed contemporaneously (7-9) or as whole foods (10, 11). For example, in a prospective study of children ages 15-17 years, consuming at least two servings of dairy per day, in comparison to fewer than two servings per day, was associated with significantly higher mean bone mineral content (BMC) and bone area (10). In older adults, greater yogurt consumption is related to higher bone mineral density and physical function scores (11). With the exception of dairy foods (9) little data exist on the effects of whole foods on bone health.

Eggs contain nutrients that may benefit bone health including vitamin D and zinc and osteogenic bioactive components, lutein and zeaxanthin. Whole eggs have been examined in prior studies with respect to their beneficial effects on bone and fracture outcomes, but egg intake was only considered in food groupings and not independently. For example, Inose et al. showed greater Z-scores for tibia cortical speed of sound, a measure associated with cortical strength, in mothers who consumed more milk, dairy products, and eggs (12). In older adults, intakes of red meat, but not poultry or eggs, was associated with greater risk for skeletal fractures (13). While these two adult studies suggest the bone augmenting potential of eggs, it is difficult to ascertain whether and to

what degree eggs contributed to these positive relationships given they were not studied in isolation.

The ability of whole eggs to positively impact cortical bone may occur indirectly through effects on skeletal muscle mass. Skeletal muscle development precedes bone mass accrual during pubertal growth, and it is well documented that fat-free soft tissue (FFST) mass is the most important determinant of bone strength in youth (14, 15). In young men, consumption of 20 grams of whole egg protein was shown to stimulate greater muscle protein synthesis following resistance exercise than in those not consuming whole egg protein (16). Moreover, leucine, a primary amino acid in whole eggs, improved skeletal muscle synthesis in young adults (17). Thus, it is possible that the amino acid and protein components of eggs may indirectly enhance bone strength through increased muscle mass.

The 2015 Dietary Guidelines for Americans de-emphasized the recommendation to limit cholesterol-rich foods, including eggs, which had been restricted in years past due to their perceived risk on cardiovascular health (18). Reviews conducted by Shin et al. (19) and Griffin et al. (20) evaluated results from 16 and 12 studies, respectively, and provided the basis for the 2015 guidelines. These studies showed that egg consumption was not associated with hyperlipidemia, risk of cardiovascular disease, and cardiac mortality in the general population. Given that eggs are the least expensive source of high-quality protein per standard United States Department of Agriculture serving, incorporating eggs into everyday diets may be a cost-effective strategy to benefit skeletal health in growing youth.

To our knowledge, studies investigating the associations between egg consumption and bone outcomes during the critical period of rapid pubertal growth have not been conducted. Therefore, the aim of this study was to cross-sectionally explore the relationships between whole egg intake and cortical bone parameters, total body bone outcomes, and biomarkers of bone turnover in children entering the early stages of puberty. A secondary aim was to determine if FFST has a mediating role on the relationship between egg intake and cortical bone.

Subjects and Methods

Study design and participant characteristics

This study was a secondary analysis of baseline data obtained from a multi-site randomized controlled vitamin D clinical trial (21). Study sites were in Georgia (University of Georgia [UGA]) and Indiana (Purdue University [PU] and Indiana University [IU]). Reported healthy participants who were free from chronic diseases (N=323) were included in the parent study if they were between ages 9-13 years, in the early stages of puberty with self-reported sexual maturation ratings of 2 or 3 for breast development in females and genital development in males as described by Tanner (22), and self-reported non-Hispanic white or black race, with both sets of biological parents and grandparents all identifying as the same race. Exclusion criteria included menarche (for females), the presence of growth disorders/chronic disease (e.g., cerebral palsy) or the use of medications (e.g., corticosteroids) known to influence bone metabolism. A power analysis was conducted on the outcome variable tibia strength strain index using the existing sample size from the original study of 323 With a medium effect size of .30 and an alpha of .05, the expected statistical power for this cross-sectional study is greater

than .99, which indicates a high probability to detect an existing effect in our data. Twenty-nine participants were excluded from this ancillary study due to incomplete diet records, as described below. The Institutional Review Boards for Human Subjects at all study sites approved the study procedures. Informed assent and permission were obtained from each participant and their parent/guardian, respectively.

Demographic and dietary assessment

With assistance from parents or guardians, three-day diet records, a valid and reliable method for estimating energy and nutrient intakes in children (23-25), were completed at home on two weekdays and one weekend day. Records were analyzed by one trained registered dietitian nutritionist using Food Processor SQL version 9.7.3 (ESHA Research). Three-day diet records were used to estimate total whole egg intake (e.g., scrambled eggs, hard-boiled eggs, fried eggs) over three days, as well as mean daily energy (kcal) and mean daily protein (g) intakes. Egg consumption from mixed dishes was not quantified and therefore was not included in these analyses. Average measure (three-day) intraclass correlation coefficients (ICCs) were calculated in girls ages 6-10 years (N=10), whose three-day diet records were completed twice over two weeks and calculated for vitamin D, calcium, and energy (≥0.86).

Anthropometry

Height (to the nearest 0.1 cm) and body mass (to nearest 0.1 kg) were measured using a wall-mounted stadiometer and electronic scale, respectively. Body mass index (BMI, in kilograms per meter squared) was calculated, and sex- and age-specific percentiles derived using a BMI percentile calculator (26). Single-measure ICCs and test-retest coefficients of variation (CV) were determined previously in our lab for standing

height (0.99 and 0.4%) and body weight (0.99 and 1.4%) in females ages 6-10 years (N=10) who were measured twice over a two-week period by the same researcher.

Body composition and whole body bone measurements

Fat mass, percent fat, and FFST were assessed using dual energy X-ray absorptiometry (DXA; Delphi-A, Hologic Inc. [UGA]; Lunar iDXA, GE Medical Instruments [PU]; and Hologic Discovery-W [IU]). Whole body bone mineral density (BMD), BMC, and bone area were also assessed with DXA. At each study site, the same technician completed scans and performed analyses using instrument-specific software and protocols. ICCs were calculated for body composition in females, ages 5-8 years (N=10), scanned twice at UGA over 7 days (all ≥ 0.98). As previously reported, DXA scanners at each testing site were cross-calibrated, and regression formulae were determined to the adjust data (21, 27, 28).

Cortical bone measurements

Cortical bone was assessed using peripheral quantitative computed tomography (pQCT) at each study site using Stratec XCT 2000 machines (Stratec Medizintechnik GmbH, Pforzheim, Germany), as reported previously (27). A single tomographic slice was taken at the tibia and radius 66% site relative to the distal growth plate. Subjects were positioned supine with their self-reported non-dominant leg and arm in the center of the gantry of the pQCT machine. A cortical bone phantom specific to the pQCT machine with known properties was scanned a minimum of 20 times on each scanner to ensure comparability of machines between each testing site. The variation in phantom measures differed by <1% (27).

Cort mode 1 (threshold, 710 mg/cm^3) was used to obtain cortical volumetric bone mineral density (Ct.vBMD mg/cm^3), cortical bone mineral content (Ct.BMC; mg/mm), and cortical area (Ct.Ar; cm^2) and to define the outermost edge of the bone. Peel mode 2 (threshold, 400 mg/cm^3) was used to separate the cancellous and cortical bone compartments. Total bone area (Tt.Ar, mm^2), cortical thickness (Ct.Th, mm), periosteal perimeter (Ps.Pm, mm), and endosteal perimeter (Es.Pm, mm) were also measured. This same threshold was used to calculate polar-strength strain index (pSSI, mm^3), which is derived from Ct.vBMD, section modulus, and normal physiological bone density that is estimated at 12,000 mg/mm^3 (27, 29, 30). Using a F03F05 filer (contour mode 3 [threshold of -100 mg/cm^3] and peel mode 2 [threshold of 40 mg/cm^3]), muscle cross-sectional area (MCSA) was measured. Five healthy females (ages 18-24 years) were scanned at the UGA site to determine test-retest reliability (31). One-way random effects model and single measure ICCs for all pQCT variables were $R \geq 0.97$. At the IU site, short-term precision for the pQCT scanning procedure on 30 healthy individuals scanned six times with interim repositioning showed root mean square coefficients of variation (RMS-CVs) of <1% for bone density, mass, structure, and estimated strength measures, and <1.5% for mCSA (32).

Biochemical analyses

Blood and urine samples were collected in the morning following an overnight fast. All samples were prepared for storage and frozen in a <-80°C freezer until analyses. Reference controls (kits) and internal controls (in-house pooled samples) were included with each assay run for quality control. Repeat analyses were conducted when duplicate samples differed by ≥10%. Serum osteocalcin (OC) and bone-specific alkaline

phosphatase (BSAP) were assessed as measures of bone formation, and were measured by ELISA (Quidel Corp., San Diego, CA). Urine N-terminal telopeptide (NTX) was assessed as a measure of bone resorption, and was measured by ELISA (Ostex International, Seattle, WA). Mean interassay CVs for the bone turnover markers ranged from 4.1%-8.0%. Serum 25(OH)D was assessed using a 2-step RIA (Diasorin). The inter- and intra-assay CV were 5.6% to 8.4% and 5.5% to 7.0%, respectively. Analytical reliability of 25(OH)D assays was further monitored through DEQAS (the Vitamin D External Quality Assessment Scheme).

Statistical analyses

Data were analyzed using SPSS version 21 (SPSS, Inc.) for the Mac OS X. Histograms were visually inspected for outliers and normal distribution. Distributions were classified as skewed or kurtotic if > 2.0 standard deviations (SDs). An outlier was detected when visually inspecting the histogram of three-day egg consumption. Given that one participant reported consumption of 12 whole eggs during the three-day dietary recall period, this participant was removed from our data. Because three-day egg consumption, urine NTX, and serum OC each had positive skewed distributions, they were log-transformed (i.e., NTX) or square-root-transformed (i.e., three-day egg consumption and OC) prior to analyses. Pearson's bivariate and partial correlations were conducted to determine the association between egg intake and whole body bone outcomes and cortical bone outcomes while adjusting for stage of sexual maturation, sex, race, FFST, and average three-day total protein intake. Using Mplus software (version 7.31), path analysis was performed to examine the FFST-mediated relationship between egg intake and Ct.BMC. Indirect effects tests were conducted using the product

coefficient method (33). Each of the above-mentioned path models was identified and included sexual maturation, race, and sex as covariates. A *P*-value < .05 was considered statistically significant for all analyses.

Results

Participant characteristics

Descriptive participant characteristics are presented in **Table 1**. Approximately 30% of study participants consumed eggs during the three-day dietary recording period (n=87). Between-group differences were assessed by independent samples *t*-tests. There were statistically significant differences in stages of sexual maturation between egg consumers and non-egg consumers **(Table 1)**. Moreover, egg consumers were significantly taller, heavier, and had greater FFST in comparison to the non-consumers. Total protein intake was higher in egg consumers; however, energy intake did not differ between the two groups.

Egg consumption and cortical bone outcomes

Egg consumption was positively associated with mid-tibia and mid-radius Ct.BMC, Tt.Ar, Ct.Ar, Ct.Th, PC, and pSSI **(Table 2)**. After adjustments for stage of sexual maturation, sex, race, FFST, and average total protein intake, the relationships between egg consumption and mid-tibia cortical bone outcomes were nullified, but egg intake remained positively correlated with mid-radius Ct.BMC (*P*=.031, **Table 2**).

Egg consumption and total body outcomes

Egg consumption was positively associated with total body FFST in the unadjusted model (r=.185, *P*=.001) and remained positively associated with total body FFST when adjusting for the mediators, stage of sexual maturation, sex, race, and

average total protein intake (r=.126, *P*=.033). Egg intake was positively associated with total body bone mineral density (BMD; r=.181, *P*=.002), bone mineral content (BMC; r=.224, *P*<.001), and bone area (BA; r=.119, *P*=.043) in the unadjusted model. After controlling for the mediators, all total body relationships were nullified.

Mediating role of FFST in the association between egg intake and Ct. BMC at the mid-radius

The path models presented in **Figure 1** represent the FFST-dependent relationship between egg intake and mid radius Ct.BMC while controlling for race, sex, and stage of sexual maturation. Three-day egg intake was a positive predictor of FFST (*P*=.027) and FFST was a positive predictor of Ct.BMC (*P*<.001). After adjusting for the mediator, FFST, egg intake remained a positive predictor of Ct.BMC (*P*<.050). The test for an indirect effect was statistically significant (*P*=.020).

Egg consumption and bone biomarkers

Egg consumption was positively correlated with OC (r=0.170, *P*=.005) and negatively correlated with both BSAP (r=-0.084, *P*=.163) and NTX (r = -0.128, *P*=.033). After controlling for race, sex, stage of sexual maturation, and average total protein intake, these relationships were nullified except for OC (r=0.120, *P*=.049).

Discussion

The aim of this cross-sectional study was to determine whether whole egg intake was associated with cortical bone strength and biomarkers of bone turnover in healthy children. The secondary aim of this study was to determine whether FFST mediates the relationship between egg and bone. The primary finding was that egg intake was a positive predictor of mid-radius Ct.BMC and that the relationship between egg intake and

Ct.BMC was partially mediated through FFST. With the exception of dairy foods, including a recent study using yogurt, (9, 11, 34, 35) limited research exists regarding the relationships between whole food consumption and bone outcomes in children. The present study is the first to assess the relationships between whole egg intake and radius and tibia cortical bone in children. The only other study that examined egg intake and bone strength used a composite measure of eggs and dairy intake, which showed positive relationships between tibia cortical speed of sound in adult females (12). The degree to which eggs alone contributed to these positive relationships was not ascertained.

The positive relationship between whole egg intake and radius cortical bone observed in the current study could be related to the protein component of eggs, as cross-sectional (34-37) and prospective (4, 38-40) studies in children support the role of dietary protein on bone. For example, Bounds et al. (39) showed that protein intake in children is a positive predictor of total body BMC. Alexy et al. (4) reported that dietary protein intake over four years during growth is associated with increased diaphyseal bone strength, specifically increases in PC, Ct.Ar, Ct.BMC, and pSSI. Randomized-controlled trials have not been conducted and are needed to confirm the results of the cross-sectional and prospective protein studies.

Beyond protein, components of eggs that may benefit bone health include nutrients, such as vitamin D and zinc and bioactive components, including lutein and zeaxanthin. There is moderate evidence supporting a role of vitamin D for improving bone outcomes in children and adolescents (7) and zinc supplementation in children has been shown to increase bone formation (41). Lutein and zeaxanthin, bioactive components present in eggs, have known anti-inflammatory effects (42). These dietary

factors may mediate a potential benefit of egg consumption on bone geometry, as the pro-inflammatory cytokines tumor necrosis factor alpha, interleukin-6 and c-reactive protein have a negative effect on adult bone strength (43, 44). Assessments of biomarkers of inflammation in future studies can provide additional insight into the anti-inflammatory roles of whole eggs and bone outcomes.

In addition to a direct effect of whole eggs on bone, it is possible that eggs could improve bone strength through actions on lean body mass. In our path model, FFST mediated the relationship between egg intake and Ct.BMC. This novel finding is supported by previous studies showing that protein and leucine stimulate muscle protein synthesis (17, 45) and skeletal muscle mass is a strong determinant of pediatric bone mass (14, 15, 46). The significant positive relationships between 3-day egg intakes and FFST mass reported in the current study are supported by a short-term intervention study by Candow and colleges (45) who showed that independent of source, young adults who consumed a protein supplement during resistance training had increases in lean tissue mass and strength versus those who consumed an isocaloric placebo during resistance training ($P<.050$). Our data showing that FFST mass is a significant predictor of radius Ct.BMC is in agreement with Crabtree et al. (47) who demonstrated that among school children ages 5-18 years living in the United Kingdom, lean body mass was the strongest predictor of BMC at the total body and lumbar spine. In a recent review, the authors reaffirmed the importance of FFST mass as a strong positive predictor of cortical bone geometry (46).

Though our data showed a positive association between egg intake and radius Ct.BMC, we did not find significant relationships between egg consumption and mid-

tibia cortical bone outcomes and total body bone outcomes after adjusting for the covariates. Measures of areal bone mineral density may underestimate the strength of the bone given it does not provide information regarding trabecular and cortical bone properties. While there are no studies investigating egg intake on cortical bone outcomes, the site-specific differences reported in the present study are consistent with pediatric physical activity investigations. For example, Jackowski et al. (48) did not show a difference between recreational gymnastic, an activity known to exert positive influences on bone mineral accrual during growth, or control, on bone mineral content and bone area at the tibia in children ages 4-12 years. However, increased bone mineral content and area were seen at the distal radius in those children who participated in recreational gymnastics. Thus, it is plausible that mechanical loading associated with compression forces on the lower limbs with weight bearing masked any potential effects of gymnastic, or in the present study, egg intake, on tibia outcomes. It is also possible that the null findings of egg consumption on tibia bone outcomes were associated with weight bearing masking effects. Future intervention trials should account for site-specific effects of diet and load bearing, including physical activity, when assessing the relationship between egg intake and bone outcomes.

The positive relationship between egg consumption and the bone formation marker OC, following adjustments for key covariates, supports the mediation model and provides potentially, some insight into mechanisms for the link between eggs and bone. To our knowledge, there are no prior studies examining the relationships between egg consumption and markers of bone turnover in children. A number of clinical studies have investigated the effects of protein intake on bone biomarkers and the findings are

equivocal. For example, biomarkers of bone resorption and formation have been reported to decrease (49, 50) remain the same (51-53) or increase (54) following protein supplementation. It has been reported that milk protein supplementation in healthy women leads to a reduction in NTX and no change in BSAP or OC (55). Further, since OC is not the ideal marker for bone formation and most likely better reflects overall bone turnover (56), a more specific marker of pediatric bone formation like N-terminal propeptide of type 1 procollagen, should be assessed. Randomized controlled egg feeding trials will help to improve our understanding of the role of whole eggs on bone turnover.

The strength of this study is the utilization of path analysis statistical techniques to explore the mediation effects of FFST on the relationship of egg intake and Ct.BMC. However, when interpreting our results, certain aspects of the study should be considered. First, given its cross-sectional design, we cannot confirm causality when linking egg intake and bone. Second, participants self-reported their dietary intakes, which may have resulted in either over- or under-reporting of food consumption (57). Though the three-day dietary recall used in our study is a valid method in children (23-25), another limitation of the study is our single measure assessment of egg intakes. Given this study was a secondary analysis on baseline data obtained from a randomized controlled trial and not originally intended to assess egg intake, more complete egg intake data, including egg intakes from mixed dishes, was not collected and is needed in future trials. Despite these limitations, our results are hypothesis generating, and provide valuable insight into the relationship between egg consumption and musculoskeletal outcomes in children. Importantly, our results provide the basis to explore in clinical trials the viability of whole egg intake to increase bone strength and prevent childhood and adult fractures.

This cross-sectional study provides novel evidence of a positive link between whole egg consumption and cortical bone in healthy, black and white children in the early stages of puberty, and we showed for the first time that FFST mediated these relationships. Considering the liberalization of egg intake recommendations in the 2015 Dietary Guidelines for Americans report, coupled with the affordability of eggs in the marketplace, greater incorporation of eggs into children's diets is feasible, and could have a significant public health impact, including fracture risk reduction and osteoporosis prevention. Future randomized controlled trials are needed, however, to confirm the positive impact of egg intake on pediatric musculoskeletal development.

References

1. Dibba B, Prentice A, Ceesay M, Stirling DM, Cole TJ, Poskitt EM. Effect of calcium supplementation on bone mineral accretion in gambian children accustomed to a low-calcium diet. Am J Clin Nutr 2000;71(2):544-9.

2. Moyer-Mileur LJ, Xie B, Ball SD, Pratt T. Bone mass and density response to a 12-month trial of calcium and vitamin D supplement in preadolescent girls. J Musculoskelet Neuronal Interact 2003;3(1):63-70.

3. Ballard TL, Specker BL, Binkley TL, Vukovich MD. Effect of protein supplementation during a 6-month strength and conditioning program on areal and volumetric bone parameters. Bone 2006;38(6):898-904. doi: 10.1016/j.bone.2005.10.020.

4. Alexy U, Remer T, Manz F, Neu CM, Schoenau E. Long-term protein intake and dietary potential renal acid load are associated with bone modeling and remodeling at the proximal radius in healthy children. Am J Clin Nutr 2005;82(5):1107-14. doi: 10.1093/ajcn/82.5.1107.

5. El-Hajj Fuleihan G, Nabulsi M, Tamim H, Maalouf J, Salamoun M, Khalife H, Choucair M, Arabi A, Vieth R. Effect of vitamin D replacement on musculoskeletal parameters in school children: a randomized controlled trial. J Clin Endocrinol Metab 2006;91(2):405-12. doi: 10.1210/jc.2005-1436.

6. Viljakainen HT, Natri AM, Karkkainen M, Huttunen MM, Palssa A, Jakobsen J, Cashman KD, Molgaard C, Lamberg-Allardt C. A positive dose-response effect of vitamin D supplementation on site-specific bone mineral augmentation in adolescent girls: a double-blinded randomized placebo-controlled 1-year intervention. J Bone Miner Res 2006;21(6):836-44. doi: 10.1359/jbmr.060302.

7. Weaver CM, Gordon CM, Janz KF, Kalkwarf HJ, Lappe JM, Lewis R, O'Karma M, Wallace TC, Zemel BS. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. Osteoporos Int 2016;27(4):1281-386. doi: 10.1007/s00198-015-3440-3.

8. Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. Nutrition 2009;25(2):165-71. doi: 10.1016/j.nut.2008.07.019.

9. Okubo H, Sasaki S, Horiguchi H, Oguma E, Miyamoto K, Hosoi Y, Kim MK, Kayama F. Dietary patterns associated with bone mineral density in premenopausal Japanese farmwomen. Am J Clin Nutr 2006;83(5):1185-92. doi: 10.1093/ajcn/83.5.1185.

10. Moore LL, Bradlee ML, Gao D, Singer MR. Effects of average childhood dairy intake on adolescent bone health. The Journal of pediatrics 2008;153(5):667-73. doi: 10.1016/j.jpeds.2008.05.016.

11. Laird E, Molloy AM, McNulty H, Ward M, McCarroll K, Hoey L, Hughes CF, Cunningham C, Strain JJ, Casey MC. Greater yogurt consumption is associated with increased bone mineral density and physical function in older adults. Osteoporos Int 2017. doi: 10.1007/s00198-017-4049-5.

12. Inose T, Takano T, Nakamura K, Kizuki M, Seino K. Tibial cortical bone properties of preadolescents and their mothers in an urban area associated with lifestyle: a longitudinal study. Acta Paediatr 2006;95(3):276-82. doi: 10.1080/08035250500352169.

13. Zeng FF, Fan F, Xue WQ, Xie HL, Wu BH, Tu SL, Ouyang WF, Chen YM. The association of red meat, poultry, and egg consumption with risk of hip fractures in elderly Chinese: a case-control study. Bone 2013;56(2):242-8. doi: 10.1016/j.bone.2013.06.023.

14. Baptista F, Barrigas C, Vieira F, Santa-Clara H, Homens PM, Fragoso I, Teixeira PJ, Sardinha LB. The role of lean body mass and physical activity in bone health in children. J Bone Miner Metab 2012;30(1):100-8. doi: 10.1007/s00774-011-0294-4.

15. Hamrick MW. The skeletal muscle secretome: an emerging player in muscle-bone crosstalk. Bonekey Rep 2012;1:60. doi: 10.1038/bonekey.2012.60.

16. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. Am J Clin Nutr 2009;89(1):161-8. doi: 10.3945/ajcn.2008.26401.

17. Glynn EL, Fry CS, Drummond MJ, Timmerman KL, Dhanani S, Volpi E, Rasmussen BB. Excess leucine intake enhances muscle anabolic signaling but not net protein anabolism in young men and women. J Nutr 2010;140(11):1970-6. doi: 10.3945/jn.110.127647.

18. DeSalvo KB, Olson R, Casavale KO. Dietary Guidelines for Americans. JAMA 2016;315(5):457-8. doi: 10.1001/jama.2015.18396.

19. Shin JY, Xun P, Nakamura Y, He K. Egg consumption in relation to risk of cardiovascular disease and diabetes: a systematic review and meta-analysis. Am J Clin Nutr 2013;98(1):146-59. doi: 10.3945/ajcn.112.051318.

20. Griffin JD, Lichtenstein AH. Dietary Cholesterol and Plasma Lipoprotein Profiles: Randomized-Controlled Trials. Curr Nutr Rep 2013;2(4):274-82. doi: 10.1007/s13668-013-0064-0.

21. Lewis RD, Laing EM, Hill Gallant KM, Hall DB, McCabe GP, Hausman DB, Martin BR, Warden SJ, Peacock M, Weaver CM. A randomized trial of vitamin D(3) supplementation in children: dose-response effects on vitamin D metabolites and calcium absorption. J Clin Endocrinol Metab 2013;98(12):4816-25. doi: 10.1210/jc.2013-2728.

22. Tanner JM. The measurement of maturity. Trans Eur Orthod Soc 1975:45-60.

23. Crawford PB, Obarzanek E, Morrison J, Sabry ZI. Comparative advantage of 3-day food records over 24-hour recall and 5-day food frequency validated by observation of 9- and 10-year-old girls. J Am Diet Assoc 1994;94(6):626-30.

24. Bergman EA, Boyungs JC, Erickson ML. Comparison of a food frequency questionnaire and a 3-day diet record. J Am Diet Assoc 1990;90(10):1431-3.

25. Taylor RW, Goulding A. Validation of a short food frequency questionnaire to assess calcium intake in children aged 3 to 6 years. Eur J Clin Nutr 1998;52(6):464-5.

26. Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, Mei Z, Curtin LR, Roche AF, Johnson CL. CDC growth charts: United States. Adv Data 2000(314):1-27.

27. Warden SJ, Hill KM, Ferira AJ, Laing EM, Martin BR, Hausman DB, Weaver CM, Peacock M, Lewis RD. Racial differences in cortical bone and their relationship to biochemical variables in Black and White children in the early stages of puberty. Osteoporos Int 2013;24(6):1869-79. doi: 10.1007/s00198-012-2174-8.

28. Ferira AJ, Laing EM, Hausman DB, Hall DB, McCabe GP, Martin BR, Hill Gallant KM, Warden SJ, Weaver CM, Peacock M, et al. Vitamin D Supplementation Does Not Impact Insulin Resistance in Black and White Children. J Clin Endocrinol Metab 2016;101(4):1710-8. doi: 10.1210/jc.2015-3687.

29. Macdonald H, Kontulainen S, Petit M, Janssen P, McKay H. Bone strength and its determinants in pre- and early pubertal boys and girls. Bone 2006;39(3):598-608. doi: 10.1016/j.bone.2006.02.057.

30. Rauch F, Schoenau E. Peripheral quantitative computed tomography of the proximal radius in young subjects--new reference data and interpretation of results. J Musculoskelet Neuronal Interact 2008;8(3):217-26.

31. Pollock NK, Laing EM, Baile CA, Hamrick MW, Hall DB, Lewis RD. Is adiposity advantageous for bone strength? A peripheral quantitative computed tomography study in late adolescent females. Am J Clin Nutr 2007;86(5):1530-8.

32. Swinford RR, Warden SJ. Factors affecting short-term precision of musculoskeletal measures using peripheral quantitative computed tomography (pQCT). Osteoporos Int 2010;21(11):1863-70. doi: 10.1007/s00198-009-1151-3.

33. Preacher KJ, Hayes AF. Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. Behav Res Methods 2008;40(3):879-91.

34. Hoppe C, Molgaard C, Michaelsen KF. Bone size and bone mass in 10-year-old Danish children: effect of current diet. Osteoporos Int 2000;11(12):1024-30.

35. Iuliano-Burns S, Stone J, Hopper JL, Seeman E. Diet and exercise during growth have site-specific skeletal effects: a co-twin control study. Osteoporos Int 2005;16(10):1225-32. doi: 10.1007/s00198-004-1830-z.

36. Chevalley T, Bonjour JP, Ferrari S, Rizzoli R. High-protein intake enhances the positive impact of physical activity on BMC in prepubertal boys. J Bone Miner Res 2008;23(1):131-42. doi: 10.1359/jbmr.070907.

37. Ekbote VH, Khadilkar AV, Chiplonkar SA, Khadilkar VV. Determinants of bone mineral content and bone area in Indian preschool children. J Bone Miner Metab 2011;29(3):334-41. doi: 10.1007/s00774-010-0224-x.

38. Remer T, Krupp D, Shi L. Dietary protein's and dietary acid load's influence on bone health. Crit Rev Food Sci Nutr 2014;54(9):1140-50. doi: 10.1080/10408398.2011.627519.

39. Bounds W, Skinner J, Carruth BR, Ziegler P. The relationship of dietary and lifestyle factors to bone mineral indexes in children. J Am Diet Assoc 2005;105(5):735-41. doi: 10.1016/j.jada.2005.02.046.

40. Vatanparast H, Bailey DA, Baxter-Jones AD, Whiting SJ. The effects of dietary protein on bone mineral mass in young adults may be modulated by adolescent calcium intake. J Nutr 2007;137(12):2674-9. doi: 10.1093/jn/137.12.2674.

41. Berger PK, Pollock NK, Laing EM, Chertin V, Bernard PJ, Grider A, Shapses SA, Ding KH, Isales CM, Lewis RD. Zinc Supplementation Increases Procollagen Type 1 Amino-Terminal Propeptide in Premenarcheal Girls: A Randomized Controlled Trial. J Nutr 2015;145(12):2699-704. doi: 10.3945/jn.115.218792.

42. Andersen CJ. Bioactive Egg Components and Inflammation. Nutrients 2015;7(9):7889-913. doi: 10.3390/nu7095372.

43. Agrawal M, Arora S, Li J, Rahmani R, Sun L, Steinlauf AF, Mechanick JI, Zaidi M. Bone, inflammation, and inflammatory bowel disease. Curr Osteoporos Rep 2011;9(4):251-7. doi: 10.1007/s11914-011-0077-9.

44. Romas E, Gillespie MT. Inflammation-induced bone loss: can it be prevented? Rheum Dis Clin North Am 2006;32(4):759-73. doi: 10.1016/j.rdc.2006.07.004.

45. Candow DG, Chilibeck PD, Facci M, Abeysekara S, Zello GA. Protein supplementation before and after resistance training in older men. Eur J Appl Physiol 2006;97(5):548-56. doi: 10.1007/s00421-006-0223-8.

46. Kindler JM, Lewis RD, Hamrick MW. Skeletal muscle and pediatric bone development. Curr Opin Endocrinol Diabetes Obes 2015;22(6):467-74. doi: 10.1097/MED.0000000000000201.

47. Crabtree NJ, Kibirige MS, Fordham JN, Banks LM, Muntoni F, Chinn D, Boivin CM, Shaw NJ. The relationship between lean body mass and bone mineral content in paediatric health and disease. Bone 2004;35(4):965-72. doi: 10.1016/j.bone.2004.06.009.

48. Jackowski SA, Baxter-Jones AD, Gruodyte-Raciene R, Kontulainen SA, Erlandson MC. A longitudinal study of bone area, content, density, and strength development at the radius and tibia in children 4-12 years of age exposed to recreational gymnastics. Osteoporos Int 2015;26(6):1677-90. doi: 10.1007/s00198-015-3041-1.

49. Cassidy A. Dietary phytoestrogens and bone health. J Br Menopause Soc 2003;9(1):17-21.

50. Coon KA, Tucker KL. Television and children's consumption patterns. A review of the literature. Minerva Pediatr 2002;54(5):423-36.

51. Sadler LS, Pober BR, Grandinetti A, Scheiber D, Fekete G, Sharma AN, Urban Z. Differences by sex in cardiovascular disease in Williams syndrome. The Journal of pediatrics 2001;139(6):849-53. doi: 10.1067/mpd.2001.118889.

52. Alekel DL, Germain AS, Peterson CT, Hanson KB, Stewart JW, Toda T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. Am J Clin Nutr 2000;72(3):844-52.

53. Arjmandi BH, Khalil DA, Smith BJ, Lucas EA, Juma S, Payton ME, Wild RA. Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion. J Clin Endocrinol Metab 2003;88(3):1048-54. doi: 10.1210/jc.2002-020849.

54. Anderson JJ, Chen X, Boass A, Symons M, Kohlmeier M, Renner JB, Garner SC. Soy isoflavones: no effects on bone mineral content and bone mineral density in healthy, menstruating young adult women after one year. J Am Coll Nutr 2002;21(5):388-93.

55. Toba Y, Takada Y, Matsuoka Y, Morita Y, Motouri M, Hirai T, Suguri T, Aoe S, Kawakami H, Kumegawa M, et al. Milk basic protein promotes bone formation and suppresses bone resorption in healthy adult men. Biosci Biotechnol Biochem 2001;65(6):1353-7. doi: 10.1271/bbb.65.1353.

56. Kanbur NO, Derman O, Sen TA, Kinik E. Osteocalcin. A biochemical marker of bone turnover during puberty. Int J Adolesc Med Health 2002;14(3):235-44.

57. Subar AF, Freedman LS, Tooze JA, Kirkpatrick SI, Boushey C, Neuhouser ML, Thompson FE, Potischman N, Guenther PM, Tarasuk V, et al. Addressing Current Criticism Regarding the Value of Self-Report Dietary Data. J Nutr 2015;145(12):2639-45. doi: 10.3945/jn.115.219634.

Table 3.1. Participant characteristics

	Total Cohort N=294	Egg Consumers n=87	Non-Egg Consumers n=207	*P*[a]
Demographics				
Age, y	11.5 ± 1.2	11.7 ± 1.3	11.3 ± 1.2	.004
Sex (male), n	162 (55%)	34 (12%)	128 (44%)	.027
Race (black), n	165 (56%)	49 (17%)	116 (39%)	.014
Sexual maturation stage (2/3)	211(72%) / 83(28%)	50(17%) / 20 (7.0%)	161(55%) / 63 (21%)	.040
Anthropometrics				
Height, cm	150.7 ± 9.3	153.5 ± 9.6	149.9 ± 9.1	.002
Weight, kg	47.4 ± 12.2	53.1 ± 34.0	48.9 ± 12.5	.002
BMI percentile	68.0 ± 29.2	69.6 ± 27.0	66.3 ± 30.5	.352
BMI-for-age, Z-score	.67 ± 1.1	.75 ± 1.0	.57 ± 1.1	.196
Tibia length, cm	349.6 ± 35.2	360.3 ± 34.3	346.4 ± 35.6	<.001
Radius length, cm	245.8 ± 25.4	255.8 ± 25.3	242.5 ± 24.9	<.001
Body Composition				
Body fat, %	31.1 ± 9.4	29.6 ± 9.0	31.5 ± 9.5	.992
Fat mass, kg	14.7 ± 7.4	15.1 ± 7.7	14.5 ± 7.1	<.001
FFST mass, kg	30.4 ± 6.9	33.1 ± 7.4	29.4 ± 6.4	.352
Serum Biomarkers				
25(OH)D, ng/mL	28.0 ± 7.4	27.0 ± 7.4	28.4 ± 7.6	.182
Physical Activity				
Average 3-day EE, Mets/d	62.2 ± 10.0	63.2 ± 11.1	62.0 ± 9.5	.384
Nutrition				
Average kcal consumed, kcal/d	2004.3 ± 628.5	2097.0 ± 632.7	1975.4 ± 624.3	.132
Average 3-day PRO intake, g/d	77.1 ± 26.3	85.0 ± 29.5	74.4 ± 24.1	.004
Average 3-day PRO intake, gm/1000kcal	39.9 ± 11.4	42.2 ± 11.1	38.9 ± 11.4	.025
Average 3-day egg intake		0.7 ± 0.4		

Data presented as mean ± SD unless otherwise indicated

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

BMI, body mass index; FFST, fat-free soft tissue; EE, energy expenditure; Mets, metabolic equivalents; PRO, protein

Table 3.2. Bivariate and partial correlations between dietary egg intake and cortical bone outcomes

	Tibia				Radius			
	Unadjusted		Adjusted		Unadjusted		Adjusted	
	r	*P*	r	*P*	r	*P*	r	*P*
MCSA	0.188	.001			0.218	<.001		
Ct.vBMD	0.040	.506	0.032	.620	0.082	.184	0.016	.799
Ct.BMC	0.208	<.001	0.039	.539	0.224	<.001	0.138	.031
Tt.Ar	0.151	.011	-0.043	.504	0.150	.015	0.078	.223
Ct.Ar	0.202	.001	0.031	.628	0.181	.003	0.053	.406
Ct.Th	0.188	.002	0.073	.255	0.144	.020	0.025	.694
PC	0.146	.014	-0.047	.467	0.155	.012	0.086	.183
EC	0.070	.240	-0.071	.270	0.051	.408	0.045	.484
pSSI	0.186	.002	-0.001	.986	0.166	.007	0.054	.401

Statistically significant at $P<.05$
N=294
Adjusted for race, sex, stage of maturation, FFST, and protein intakes (g)
FFST, fat-free soft tissue; MCSA, muscle cross sectional area; Ct.vBMD, volumetric cortical bone mineral density; Ct.BMC, cortical bone mineral content; Tt.Ar, total area; Ct.Ar, cortical area; Ct.Th, cortical thickness; PC, periosteal circumference; EC, endosteal circumference; pSSI, polar strength strain index

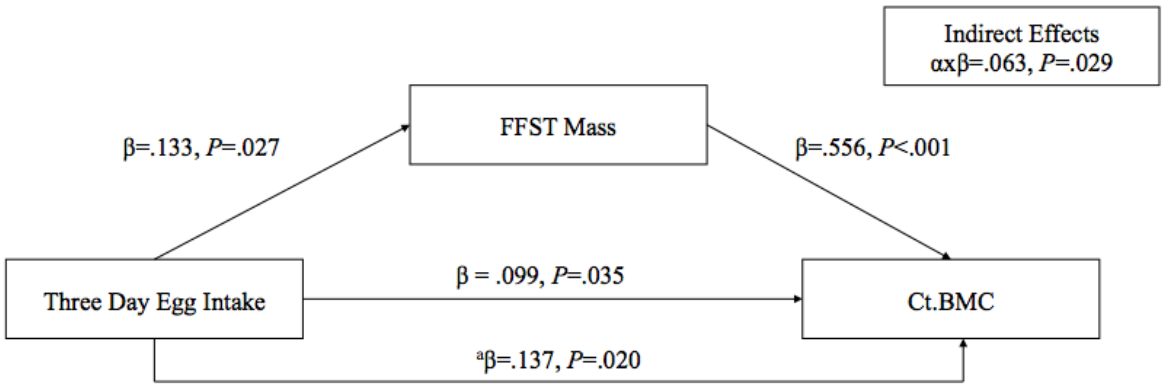


Figure 3.1. FFST mass is a partial mediator in the relationship between dietary egg intake and mid-radius Ct.BMC. Race, sex, and sexual maturation rating stage were included in this model.
[a]Indicates the relationship between 3-day egg consumption and mid-radius Ct.BMC after adjusting for race, sex, and sexual maturation rating stage.
FFST, fat-free soft tissue; Ct.BMC, cortical bone mineral content

CHAPTER 4[4]

DIETARY INFLAMMATORY INDEX® AND CORTICAL BONE OUTCOMES IN HEALTHY ADOLESCENT CHILDREN

[4] Coheley LM, Shivappa N, Hebert JR, Lewis RD. Accepted by Osteoporosis International. Reprinted here with permission of the publisher.

Abstract

Purpose: Examine the relationships between the dietary inflammatory index (DII)-scores and bone and biomarkers of inflammation in 290 adolescents, ages 9-13 years.

Methods: DII-scores were calculated from 3-day diet records and categorized into tertiles, low (<-1.34), medium (-1.34 to 1.41), and high (>1.41) inflammation. Radius and tibia bone were assessed via peripheral quantitative computed tomography (Stratec XCT 2000) at the 66%-site relative to the distal growth plate. Fasting serum was measured for tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1). The relationships between DII-score and bone and biomarkers of inflammation were assessed using bivariate and partial correlations adjusting for sexual maturation, sex, race, muscle cross-sectional area, and height. ANOVA/ANCOVA models were used to compare DII-tertiles with dependent variables.

Results: DII-score was negatively associated with tibia trabecular area (TtAr; r=-.141, *P*=.019), periosteal perimeter (PsPM; r=-.145; *P*=.016); endosteal perimeter (r=-.145, *P*=.016), strength strain index (SSI; r=-.129, *P*=.032), and radius TtAr (r=-.140, *P*=.020), PsPm (r=-.138, *P*=.027) and SSI (r=-.131, *P*=.036) but nullified when adjusting for covariates. Tibia PsPm was higher in the low DII group compared to the medium (*P*=.050) and high (*P*=.046) groups but nullified after controlling for covariates. DII-scores were not associated with TNF-α, VEGF, or IL-6, but were associated with MCP-1 only in the unadjusted model (r=.125, *P*=.042). In the adjusted model MCP-1 was inversely associated with tibia cortical thickness (r=-.150 *P*=.030).

Conclusion: The DII-scores were not related to biomarkers of inflammation or bone; however, the biomarker of inflammation, MCP-1 was negatively associated with tibia CtTh. Future prospective pediatric studies should be conducted to better understand this relationship and determine if there are long-term implications in adulthood.

Key Words: Diet, Inflammation, pQCT, DII, Adolescents

Mini Abstract

Diet is thought to modulate inflammation. This study shows no relationships between the DII and biomarkers of inflammation or bone after adjusting for covariates. Monocyte chemoattractant protein-1 was inversely associated with peripheral tibia cortical thickness and prospective childhood studies should be conducted to better understand these relationships and to determine if there are long-term consequences in adulthood.

Introduction

Systemic, low-grade inflammation is associated with several chronic diseases including cardiovascular disease (1, 2), type 2 diabetes mellitus (3), obesity (4), and osteoporosis (5, 6). Once considered adult diseases, children and adolescents are increasingly being diagnosed with these chronic conditions (7). Similar to adults, the relationships between inflammation and metabolic syndrome (8), obesity (8) and insulin resistance (9) are observed in youth. Low-grade, systemic inflammation in youth tracks into adulthood, putting these adolescents at increased risk for chronic diseases later in life (10).

Modifiable factors such as diet have been demonstrated to influence the inflammatory response process (11). The primary research approaches to test whether nutrition modifies inflammation is to target individual nutrients (12, 13). For example, consumption of omega-6 fatty acids and antioxidant vitamins (e.g., vitamin E, lutein and vitamin C) have been associated with lower inflammation (13), while consuming saturated fatty acids and excessive refined grains have been correlated with higher inflammation (12).

There are published reports that dietary patterns or whole foods may be more robust than individual nutrients in producing pro- or anti- inflammatory affects (14). The Dietary Inflammatory Index (DII) was developed by Cavicchia et al. (15) and updated by Shivappa et al. (16) to assess the overall quality of the diet on a continuum from maximally anti-inflammatory to maximally pro-inflammatory. Utilizing the first version of the DII, Cavicchia et al. demonstrated that the DII scores correlate with the inflammatory biomarker C-reactive protein (CRP) in a longitudinal study of apparently

healthy adults (15). These analyses were repeated for the next generation DII (16). Several cross-sectional studies have demonstrated significant relationships between the updated DII score and CRP in adults (17), as well as, IL-6, tumor necrosis factor alpha (TNF-α) and a combined inflammatory biomarker score in postmenopausal women (18). Among adolescents, pro-inflammatory diet (indicated by higher DII scores) was associated with increased levels of various inflammatory markers: TNF-α, interleukin-1, interleukin-2, interferon gamma, and vascular cell adhesion molecule (19).

The heightened interest in the link between chronic inflammation and pediatric bone health is derived from observations of low bone mass in children with a wide range of inflammatory disease states, including ulcerative colitis, cystic fibrosis, systemic lupus erythematous and rheumatoid arthritis (20-22). In a review addressing the link between the skeleton and immune system in children with clinical disorders, Cheung et al. noted that independent of pharmacological interventions, a high percentage of patients with chronic inflammatory conditions had low total body bone mineral density (BMD) and were at greater risk for fracture (23). For example, Roth et al. demonstrated low trabecular volumetric BMD and cortical bone strength in children with juvenile arthritis (24).

Given inflammation may negatively impact both cortical and trabecular bone density and volume, researchers have investigated the impact of the DII in relation to prospective changes in BMD and fracture. Orchard et al. found that a more anti-inflammatory diet, as measured by the DII, was associated with attenuated hip BMD loss in post-menopausal women, and consuming a higher inflammatory diet was associated with increased risk of hip fracture in younger women (25). A pro-inflammatory diet, as

indicated by a high DII score, also has been shown to be a risk factor for lower lumbar spine BMD in post-menopausal women (26). Similarly, body mass index (BMI)-adjusted mean BMD at the total femur, femoral neck, trochanter, and intertrochanter significantly decreased across increasing quartiles of DII score in US adults (27). These studies offer some evidence that chronic inflammation is related to adverse skeletal outcomes in adults. To our knowledge, there are no published studies examining the effects of a pro-inflammatory diet, as measured by the DII, on bone outcomes in healthy children. This could have important ramifications for peak bone mass attainment because bone mass tracks from youth into adulthood (28). Thus, the aim of this study is to assess the relationships between inflammatory diet patterns, as represented by DII scores, with cortical bone outcomes in otherwise healthy boys and girls.

Subjects and Methods

Study design and participant characteristics

This study was a secondary analysis of baseline data obtained from a randomized controlled clinical trial previously described (29). Healthy non-Hispanic Black and White participants were included if they were between the ages of 9-13 years, free from chronic diseases, and in the early stages of puberty with self-reported sexual maturation ratings of 2 or 3 for breast development for females and genital development for males as described by Tanner (N=323) (30). Participants were excluded from the parent study if they were menarcheal (for females), had growth disorders/chronic diseases (e.g., cerebral palsy), or used medications (e.g., corticosteroids) known to influence bone metabolism. Thirty-three participants were excluded from this ancillary analysis due to incomplete diet records. History of previous fracture and fracture site was assessed using a Health History

Questionnaire and was not considered in the exclusion criteria. Specific details surrounding the fracture incident (i.e., high vs. low impact fractures,) was not recorded. The Institutional Review Boards for Human Subjects at all study sites approved the study procedures. Participant informed assent and parental informed permission were obtained prior to all testing procedures.

Dietary assessment

Three-day diet records, a valid and reliable method for estimating energy and nutrient intakes in children (31, 32) were completed at home by participants with assistance from their parents or guardians for two weekdays and one weekend day. A registered dietitian nutritionist and one trained research assistant analyzed the diet records using Nutrition Data Systems for Research (NDSR®) software version 16 (Nutrition Coordinating Center [NCC], Minneapolis, MN). Test-retest coefficients of variation (CV) were determined for energy, protein, total fat, carbohydrates, vitamin D, and calcium (all≥0.85). Average measure (3-day) intraclass correlation coefficients (ICCs) were calculated in girls aged 6-10 years (N=10), whose 3-day diet records were completed twice during a 2-week period and calculated for vitamin D, calcium and energy (all≥0.86).

DII score calculation

The details of developing the DII is described by Shivappa et al. elsewhere (16). Briefly, high sensitivity CRP measurements were used to construct validity of the DII score in a longitudinal cohort using multiple (up to 15) 24-hour dietary recall interviews and up to five 7-day dietary recalls. The DII was subsequently validated in four studies among different populations with a variety of inflammatory biomarkers (i.e.,

interleukin, IL-6, high sensitivity-CRP, fibrinogen, homocysteine and TNF-α) (16, 18). In this updated version of the DII, 1,943 articles were reviewed and scored. Forty-five food parameters, including foods, nutrients, and other bioactive compounds, were identified based on their inflammatory effect on six specific inflammatory markers, including CRP, interleukin-1 beta, interleukin-4, IL-6, interleukin-10 and TNF-α. A regionally representative world database representing diet surveys from 11 countries was used as a comparative standard for each of the 45 parameters (i.e. foods, nutrients, and other bioactive food components). Intake values from this database were used to calculate the DII scores. This is explained in more detail in the DII Methods paper (16). Briefly, a standard mean for each parameter from the representative world database was subtracted from the actual individual exposure and divided by its standard deviation to generate Z-scores. These Z-scores were converted to proportions (thus minimizing effects of outliers/right-skewing). These values were then doubled, and one was subtracted to achieve symmetrical distribution with values centered on approximately zero. The resulting value was then multiplied by the corresponding inflammatory score for each food parameter and summed across all food parameters, to obtain the overall DII score. Using 3-day diet records, we calculated the DII based on 27 single food parameters of the 45 possible food parameters **(Table 1).**

Anthropometry

Height (to the nearest 0.1 cm) and body mass (to the nearest 0.1 kg) were measured using a wall-mounted stadiometer and electronic scale, respectively. BMI (kg/m^2) was calculated, and BMI age-percentile scores were derived using the 2000 CDC growth charts (33). BMI Z-scores were calculated using STATA® software (StataCorp.

2017. *Statistical Software: Release 15.* College Station, TX; StataCorp LLC). Single-measure ICCs and test-retest CVs were determined previously in our lab for standing height (0.99 and 0.4%) and body weight (0.99 and 1.4%) in females aged 6-10 years (N = 10) who were measured twice over a 2-week period by the same researcher.

Body composition and whole body bone measurements

Fat mass, percent fat, fat-free soft tissue, whole body BMD, and whole body bone mineral content (BMC) were assessed using dual energy X-ray absorptiometry (DXA; Delphi-A, Hologic Inc. [UGA]; Lunar iDXA, GE Medical Instruments [PU; Purdue University]; and Hologic Discovery-W [IU; Indiana University]) as previously described (29, 34, 35). The same technician at each site conducted scans and performed analyses using instrument-specific software and protocols. ICCs were calculated for body composition in females, aged 5-8 years (N=10), scanned twice over 7 days (all ≥ 0.98). Short- and long- term precision of DXA at IU was <2%. The UGA/PU sites were cross-calibrated by scanning 26 children on the Delphi-A and an iDXA, whereas the IU and PU sites were cross-calibrated by scanning 10 children on the Discovery-W and iDXA. Regression formulae between UGA/PU and IU/PU were derived and used to adjust data from UGA/IU to PU values.

Cortical bone measurements

Peripheral quantitative computed tomography (pQCT) was used to assess cortical bone outcomes using Stratec XCT 2000 (Stratec Medizintechnik GmbH, Pforzheim, Germany), as previously reported (29). To measure tibia length, subjects were asked to cross their non-dominant leg over their dominant leg. A pen mark was placed on the upper boarder of the medial condyle of the non-dominant tibia and at the tip of the medial

malleolus. Distance between the two points was measured in millimeters (mm) with a spreading caliper. To measure radius length, participants were asked to place their non-dominant forearm on a table forming a 90-degree angle. A pen mark was placed at the end the styloid process and the distance between the bottom of the forearm to the pen mark at the styloid process was measured in mm with a spreading caliper. All measurements were conducted by the same technician at all study sites. A single tomographic slice 66% relative to the distal growth plate was taken for the non-dominant tibia and radius. A cortical bone phantom specific to the pQCT with known properties was scanned a minimum of 20 times and the variation in phantom measures differed by < 1% (34).

Cort mode 1 (threshold, 710 mg/cm^3) was used to obtain cortical volumetric bone mineral density (Ct.vBMD, mg/cm^3), cortical bone mineral content (Ct.BMC, mg/mm), and cortical area (Ct.Ar, cm^2) and to define the outermost edge of the bone. Peel mode 2 (threshold, 400 mg/cm3) was used to separate the cancellous and cortical bone compartments. Total bone area (Tt.Ar, mm2), cortical thickness (Ct.Th, mm), periosteal perimeter (Ps.Pm, mm), and endosteal perimeter (Es.Pm, mm) were also measured. This same threshold was used to calculate polar-strength strain index (pSSI, mm^3), which represents the density-weighted section modulus and has been validated as a non-invasive measure of bone strength (36).The pSSI was determined in a separate analysis using cort mode 2 (threshold, 400 mg/cm^3), and was calculated as the section modulus multiplied by the ratio of CtBMD and normal physiologic density (i.e., 1200 mg/mm^3), as previous descripted (34). Section modulus (mm^3) was calculated as (a x d2)/dmax, where “a” is the cross-sectional area of a voxel (mm2), “d” is the distance of the voxel from the center of

gravity (mm), and "dmax" is the maximum distance (eccentricity) of one voxel to the center of gravity (mm).

$SSI_p = \Sigma [(a \times d^2)(\text{Ct.volumetric BMD / normal physiologic density BMD})] / \text{dmax}$

Tibia and radius muscle cross-sectional areas (MCSA) were assessed using a F03F05 filter (contour mode 3 [threshold of -100 mg/cm^3] and peel mode 2). All pQCT measures were performed and analyzed by one trained operator and the pQCT operator scanned the phantom daily to maintain quality assurance. Five healthy females (ages 18-24 years) were scanned twice over 7 days to determine test-retest reliability (37). One-way random effects model and single measure ICCs for all pQCT variables were $R \geq 0.97$. At the IU site, short-term precision for the pQCT scanning produce on 30 healthy individuals scanned six times with interim repositioning showed root mean square coefficients of variation of <1% for bone density, mass, structure, and estimated strength measures (38).

Biochemical analyses

Serum inflammation-related biomarkers, TNF-α, vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1) were quantified using the Luminex xMAP system, a high-throughput microsphere-based suspension array, with a MILLIPLEX MAP human cytokine/chemokine immunoassay (Millipore, St. Charles, MO). These biochemical measures were conducted in a single laboratory in batch analysis. The assay was analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX) using Luminex xPONENT 3.1 software. Additional analysis was performed using MILLIPLEX analyst (Millipore). The intra- and inter- assay coefficients of

variation were 2.6% and 13.0% for TNF-α, 3.7% and 10.4% for VEGF, and 1.5% and 7.9% for MCP-1, respectively (39). Serum IL-6 was quantified using a Meso Scale Discovery assay with a SECTOR Image plate reader. The intra- and inter-assay coefficients of variation were 4.4% and 12.3% for IL-6, respectively.

Statistical Analyses

Data were analyzed using SPSS® version 21 (SPSS, Inc.) for the Mac Os X. Histograms were visually inspected for outliers and normal distribution. Distributions were classified as skewed or kurtotic if > 2.0 standard deviations (SDs). Because serum IL-6 and TNF-α each had positive skewed distributions, they were log-transformed (i.e., IL-6) or square-root transformed (i.e., TNF-α) prior to analyses. Pearson's bivariate and partial correlations were conducted to determine the association between DII score and biomarkers of inflammation while adjusting for the covariates stage of sexual maturation, sex, and race. Additionally, Pearson's bivariate correlations were conducted to determine the associations between DII score and bone parameters and serum biomarkers of inflammation and bone outcomes. Because MCSA and height are considered key determinants of bone strength in children (40-42), these covariates were added to the Pearson's partial correlations models to determine the association between DII score and bone parameters and serum biomarkers of inflammation and bone outcomes. The DII score groups were created using tertile categories (low [<-1.34], medium [-1.34 to 1.41] and high [>1.41]) levels of inflammatory potential. Bone outcomes and biomarkers of inflammation were compared between groups using one-way analysis of variance and one-way analysis of covariance. *Post hoc* comparisons using the Bonferroni correction were utilized to compare DII ranking scores on serum inflammatory biomarkers and bone

parameters. The Bonferroni correction was used to correct *P*-values for multiple comparisons. A *P*-value of < 0.05 was considered statistically significant for all analyses.

Results

Participant characteristics

Descriptive participant characteristics are presented in **Table 2.** Participants were evenly distributed by sex and race (i.e., non-Hispanic White and Black). Average BMI-age percentile and BMI Z-score fell within the normal range. Participants had a mean ± SD DII score of 0.59 ± 1.36 (pro-inflammatory) and the means ± SDs are reported for the individual 27 nutrients and food components used to calculate DII score **(Table 2).** Participants met at least two-thirds the recommended dietary intakes for vitamin A, vitamin C, vitamin B6, selenium, iron, riboflavin, niacin, omega-3 fatty acids, omega-6 fatty acids, magnesium, and thiamine. Participants met 49% of the recommended dietary allowances (RDA) for fiber, 37% of the RDA for vitamin D, and 61% of the RDA for vitamin E. Approximately 23% (n=66) of participants had a history of a previous fracture (25 radius fractures, 8 tibia fractures, and 1 femur fracture).

Dietary inflammatory index and bone outcomes

DII score was negatively associated with tibia TbAr, PsPm, EsPm, and SSI and radius TtAr, PsPm and SSI **(Table 3)**. All relationships were non-significant when controlling for stage of sexual maturation, sex, race, MCSA (tibia and radius), and height. When comparing DII rank score categories, tibia PsPm was significantly higher in the low inflammation group compared to both medium (*P*=.050) and high (*P*=.046) inflammation groups, $F(2,277)=3.088$, *P*=.047. When controlling for the covariates, these group differences were non-significant.

Dietary inflammatory index and biomarkers of inflammation

The DII score was not significantly associated with VEGF, IL-6, and TNF-α; however, it was positively associated with MCP-1 in the unadjusted model (r=.125, *P*=.042) When adjusting for the covariates stage of sexual maturation, sex, and race, the relationship between DII and MCP-1 was nullified (r=.100; *P*=.166). There were no significant differences between DII rank score groups on MCP-1, VEGF, IL-6 and TNF-α (*P*=.120, *P*=.770, *P*=.250, and *P*=.637, respectively).

Biomarkers of inflammation and bone outcomes

In the unadjusted model, TNF-α was significantly negatively associated with tibia CtBMC, CtAr, and SSI; however, these relationships were nullified when adjusting for the covariates stage of sexual maturation, sex, race, tibia MCSA, and height (**Table 4**). In the unadjusted and adjusted models there were no significant relationships between IL-6 and VEGF and any tibia bone outcomes. MCP-1 was significantly negatively correlated with tibia CtBMD, EsPM, and SSI in the unadjusted model. When adjusting for the covariates there was a significant inverse relationship between MCP-1 and tibia CtTh.

Similar to the tibia, significant negative correlations were observed between TNF-α and radius CtBMC, CtAr, and SSI in the unadjusted model **(Table 5).** These relationships were nullified when controlling for the covariates, stage of sexual maturation, sex, race, radius MCSA, and height. In the unadjusted and adjusted models, there were no significant correlations between IL-6 and VEGF and radius bone outcomes. There were no significant correlations between MCP-1 and radius bone outcomes in the unadjusted model. When adjusting for the covariates the relationship between MCP-1 and radius CtBMD approached significance (*P*=.050).

Discussion

The aim of this cross-sectional study was to assess the relationships between dietary patterns reflective of varying degrees of inflammation, as represented by DII scores, and cortical bone outcomes in healthy children. A secondary aim of this study was to determine the relationships between baseline DII score and serum biomarkers of inflammation. The primary finding was that the DII score was not related to cortical bone strength or serum biomarkers of inflammation. Importantly, the pro-inflammatory biomarker of inflammation, MCP-1 was negatively associated with tibia CtTh.

The present study is the first to assess the relationships between the DII and bone outcomes as measured by pQCT in children and adolescents. The fact that no significant relationships existed between DII scores and cortical bone could be due to several factors. The studies that have reported positive findings were conducted in older adults. For example, Orchard et al. found that in comparison to a pro-inflammatory diet, postmenopausal women consuming a more anti-inflammatory diet had less hip BMD loss over a 6-year period, despite lower baseline hip BMD measurements (26). Additionally, a pro-inflammatory diet, as indicated by increasing DII scores, was associated with lower lumbar spine BMD in postmenopausal Iranian women (16). Consistent with our null findings, a study in young adults ages 18-25, found no associations between increased DII score and quantitative ultrasound of the right calcaneus (43). It is possible that the significant associations between low DII scores and better bone outcomes in older adults, but not in children, adolescents or young adults, could be due to more years of exposure to a pro-inflammatory diet in the older adults than shorter exposure intervals in children and adolescents, although this is unknown. One of the limitations of the current study and

others (44, 45) is the cross-sectional nature of the study designs and that dietary assessment targeted current intakes and not historical dietary information. Hence, based on the study design in the current study and the dietary methodology employed, the authors cannot address the question regarding long-term dietary exposures.

The DII was validated in adults using the pro-inflammatory biomarkers, CRP and IL-6 and an overall inflammatory biomarker score. In the current study, CRP was not assessed, but TNF-α, IL-6, VEGF, and MCP-1 were included as biomarkers of inflammation. We selected the biomarkers TNF- α, IL-6, VEGF and MCP-1 because of their potential detrimental effects on bone strength which was confirmed by the inverse relationships found between MCP-1 and tibia CtTh. To our knowledge it is not known whether CRP is higher in apparently healthy children due to lifestyle factors such as adhering to specific dietary patterns. Several factors including social (e.g., socioeconomic status) and physical (e.g., body fat) have been associated with higher levels of CRP in children (46). Because of the unavailability of additional serum samples, in the current study, CRP was not measured. It remains to be elucidated whether CRP is higher in apparently healthy children consuming pro-inflammatory diets; future studies of children should include assessment of CRP. The biomarkers IL-6, TNF-α, and VEGF were not related to DII scores in the present study. MCP-1 was the only biomarker found to be positively associated with DII scores, but once race was added as a covariate to the adjusted model, the significant relationships did not persist. In adults, Blacks have significantly lower MCP-I concentrations than Whites, but no data are published in children and adolescents. Our findings of lower MCP-I levels in Black children and

adolescents compared to Whites, is consistent with studies in several adult populations (47).

The most noteworthy finding in the current study was the significant inverse relationship observed between the pro-inflammatory biomarker of inflammation, MCP-1, and tibia cortical thickness. This inverse relationship makes sense given the biomarkers of inflammation investigated in this study are proresorptive and there is evidence for inflammation having a negative effect on bone outcomes (23). MCP-1 is a chemokine that regulates migration and infiltration of monocytes and macrophages into body tissues and has been implicated in multiple chronic inflammatory diseases. For example, it is well established that chronic inflammatory diseases such as rheumatoid arthritis and cystic fibrosis are associated with impairments in bone quality (20, 21). During growth, cortical bone expands and thickens via periosteal apposition and the cortical density and structural strength of the growing bone are determined by bone dimension and thickness. Thus, the negative association seen between MCP-1 and tibia cortical thickness is relevant and should be addressed in prospective trials to better ascertain if this negative association persists through growth and is linked with bone fragility and greater childhood fractures.

This is the first study to our knowledge that demonstrates a negative relationship between the serum biomarker of inflammation, MCP-1, and tibia cortical thickness in healthy children. This is important because it is during these years that children are developing maximum bone strength; thus, if inflammation attenuates bone strength in children this could have long-term fracture implications in adulthood. Future studies

should consider prospective study designs to confirm these cross-sectional findings, investigate potential mechanisms and explore potential strategies for intervention.

There were several strengths to this study including the large sample size, diverse population, and the use of advanced imaging technology (i.e., pQCT) for the assessment of bone indices. Despite these strengths this study is not without limitations. We calculated the DII score using 27 out of a possible 45 food parameters because these were the only components present in our database. Additionally, the DII score considers nutrients from foods but does not account for other bioactive components of foods such as zeaxanthin and lutein, which are thought to play a role in both inflammation and bone health (48, 49). A dietary measurement at one time point may not be a true reflection of a child's typical current dietary intake as many factors can influence a child's diet over time such as stage of development, seasons of the year, and family preferences (50). Another limitation when analyzing the biomarkers of inflammation is the high variability associated with the methodologies. It is possible that the high variability in the biomarkers could limit the ability to detect significant relationships between DII and the biomarkers. For quality control, to reduce assay variability, one individual conducted batch analyses in a single lab at the same time. Despite these limitations, our results provide valuable insight into the relationships between dietary patterns, biomarkers of inflammation and bone outcomes in a cohort of diverse healthy adolescents.

In conclusion, this was the first study to examine DII scores and bone outcomes in children and adolescents. In contrast to studies in older adults, the DII score was found not to be related to biomarkers of inflammation or bone strength. Because of the findings that the biomarker of inflammation, MCP-1, was negatively associated with tibia cortical

thickness, future prospective studies should be conducted to better understand the role of inflammation on bone quality in youth and to determine if there are long-term fracture implications in adulthood.

References

1. Libby P. Inflammation and cardiovascular disease mechanisms. Am J Clin Nutr 2006;83(2):456S-60S. doi: 10.1093/ajcn/83.2.456S.

2. Martinez BK, White CM. The Emerging Role of Inflammation in Cardiovascular Disease. Ann Pharmacother 2018:1060028018765939. doi: 10.1177/1060028018765939.

3. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 2011;11(2):98-107. doi: 10.1038/nri2925.

4. Skalicky J, Muzakova V, Kandar R, Meloun M, Rousar T, Palicka V. Evaluation of oxidative stress and inflammation in obese adults with metabolic syndrome. Clin Chem Lab Med 2008;46(4):499-505. doi: 10.1515/CCLM.2008.096.

5. Ginaldi L, Di Benedetto MC, De Martinis M. Osteoporosis, inflammation and ageing. Immun Ageing 2005;2:14. doi: 10.1186/1742-4933-2-14.

6. Ganesan K, Teklehaimanot S, Tran TH, Asuncion M, Norris K. Relationship of C-reactive protein and bone mineral density in community-dwelling elderly females. J Natl Med Assoc 2005;97(3):329-33.

7. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 2014;129(3):e28-e292. doi: 10.1161/01.cir.0000441139.02102.80.

8. Weiss R, Dziura J, Burgert TS, Tamborlane WV, Taksali SE, Yeckel CW, Allen K, Lopes M, Savoye M, Morrison J, et al. Obesity and the metabolic syndrome in children and adolescents. N Engl J Med 2004;350(23):2362-74. doi: 10.1056/NEJMoa031049.

9. Rubin DA, McMurray RG, Harrell JS, Hackney AC, Thorpe DE, Haqq AM. The association between insulin resistance and cytokines in adolescents: the role of weight status and exercise. Metabolism 2008;57(5):683-90. doi: 10.1016/j.metabol.2008.01.005.

10. Pirkola J, Vaarasmaki M, Ala-Korpela M, Bloigu A, Canoy D, Hartikainen AL, Leinonen M, Miettola S, Paldanius M, Tammelin TH, et al. Low-grade, systemic inflammation in adolescents: association with early-life factors, gender, and lifestyle. Am J Epidemiol 2010;171(1):72-82. doi: 10.1093/aje/kwp320.

11. Calder PC, Ahluwalia N, Albers R, Bosco N, Bourdet-Sicard R, Haller D, Holgate ST, Jonsson LS, Latulippe ME, Marcos A, et al. A consideration of biomarkers to

be used for evaluation of inflammation in human nutritional studies. Br J Nutr 2013;109 Suppl 1:S1-34. doi: 10.1017/S0007114512005119.

12. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, Teeling JL, Blaak EE, Fenech M, Vauzour D, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr 2015;114(7):999-1012. doi: 10.1017/S0007114515002093.

13. Sears B, Ricordi C. Anti-inflammatory nutrition as a pharmacological approach to treat obesity. J Obes 2011;2011. doi: 10.1155/2011/431985.

14. Nicklas TA, Baranowski T, Cullen KW, Berenson G. Eating patterns, dietary quality and obesity. J Am Coll Nutr 2001;20(6):599-608.

15. Cavicchia PP, Steck SE, Hurley TG, Hussey JR, Ma Y, Ockene IS, Hebert JR. A new dietary inflammatory index predicts interval changes in serum high-sensitivity C-reactive protein. J Nutr 2009;139(12):2365-72. doi: 10.3945/jn.109.114025.

16. Shivappa N, Steck SE, Hurley TG, Hussey JR, Hebert JR. Designing and developing a literature-derived, population-based dietary inflammatory index. Public Health Nutr 2014;17(8):1689-96. doi: 10.1017/S1368980013002115.

17. Shivappa N, Steck SE, Hurley TG, Hussey JR, Ma Y, Ockene IS, Tabung F, Hebert JR. A population-based dietary inflammatory index predicts levels of C-reactive protein in the Seasonal Variation of Blood Cholesterol Study (SEASONS). Public Health Nutr 2014;17(8):1825-33. doi: 10.1017/S1368980013002565.

18. Tabung FK, Steck SE, Zhang J, Ma Y, Liese AD, Agalliu I, Hingle M, Hou L, Hurley TG, Jiao L, et al. Construct validation of the dietary inflammatory index among postmenopausal women. Ann Epidemiol 2015;25(6):398-405. doi: 10.1016/j.annepidem.2015.03.009.

19. Shivappa N, Hebert JR, Marcos A, Diaz LE, Gomez S, Nova E, Michels N, Arouca A, Gonzalez-Gil E, Frederic G, et al. Association between dietary inflammatory index and inflammatory markers in the HELENA study. Mol Nutr Food Res 2017;61(6). doi: 10.1002/mnfr.201600707.

20. Cauley JA, Danielson ME, Boudreau RM, Forrest KY, Zmuda JM, Pahor M, Tylavsky FA, Cummings SR, Harris TB, Newman AB, et al. Inflammatory markers and incident fracture risk in older men and women: the Health Aging and Body Composition Study. J Bone Miner Res 2007;22(7):1088-95. doi: 10.1359/jbmr.070409.

21. Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol 2009;201(3):309-20. doi: 10.1677/JOE-08-0568.

22. Lien G, Selvaag AM, Flato B, Haugen M, Vinje O, Sorskaar D, Dale K, Egeland T, Forre O. A two-year prospective controlled study of bone mass and bone turnover in children with early juvenile idiopathic arthritis. Arthritis Rheum 2005;52(3):833-40. doi: 10.1002/art.20963.

23. Cheung WW, Zhan JY, Paik KH, Mak RH. The impact of inflammation on bone mass in children. Pediatr Nephrol 2011;26(11):1937-46. doi: 10.1007/s00467-010-1733-5.

24. Roth J, Palm C, Scheunemann I, Ranke MB, Schweizer R, Dannecker GE. Musculoskeletal abnormalities of the forearm in patients with juvenile idiopathic arthritis relate mainly to bone geometry. Arthritis Rheum 2004;50(4):1277-85. doi: 10.1002/art.20128.

25. Haring B, Crandall CJ, Wu C, LeBlanc ES, Shikany JM, Carbone L, Orchard T, Thomas F, Wactawaski-Wende J, Li W, et al. Dietary Patterns and Fractures in Postmenopausal Women: Results From the Women's Health Initiative. JAMA Intern Med 2016;176(5):645-52. doi: 10.1001/jamainternmed.2016.0482.

26. Shivappa N, Hebert JR, Karamati M, Shariati-Bafghi SE, Rashidkhani B. Increased inflammatory potential of diet is associated with bone mineral density among postmenopausal women in Iran. Eur J Nutr 2016;55(2):561-8. doi: 10.1007/s00394-015-0875-4.

27. Mazidi M, Shivappa N, Wirth MD, Hebert JR, Vatanparast H, Kengne AP. The association between dietary inflammatory properties and bone mineral density and risk of fracture in US adults. Eur J Clin Nutr 2017;71(11):1273-7. doi: 10.1038/ejcn.2017.133.

28. Gordon CM, Zemel BS, Wren TA, Leonard MB, Bachrach LK, Rauch F, Gilsanz V, Rosen CJ, Winer KK. The Determinants of Peak Bone Mass. The Journal of pediatrics 2017;180:261-9. doi: 10.1016/j.jpeds.2016.09.056.

29. Lewis RD, Laing EM, Hill Gallant KM, Hall DB, McCabe GP, Hausman DB, Martin BR, Warden SJ, Peacock M, Weaver CM. A randomized trial of vitamin D(3) supplementation in children: dose-response effects on vitamin D metabolites and calcium absorption. J Clin Endocrinol Metab 2013;98(12):4816-25. doi: 10.1210/jc.2013-2728.

30. Tanner JM. The measurement of maturity. Trans Eur Orthod Soc 1975:45-60.

31. Crawford PB, Obarzanek E, Morrison J, Sabry ZI. Comparative advantage of 3-day food records over 24-hour recall and 5-day food frequency validated by observation of 9- and 10-year-old girls. J Am Diet Assoc 1994;94(6):626-30.

32. Bergman EA, Boyungs JC, Erickson ML. Comparison of a food frequency questionnaire and a 3-day diet record. J Am Diet Assoc 1990;90(10):1431-3.

33. Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, Mei Z, Curtin LR, Roche AF, Johnson CL. CDC growth charts: United States. Adv Data 2000(314):1-27.

34. Warden SJ, Hill KM, Ferira AJ, Laing EM, Martin BR, Hausman DB, Weaver CM, Peacock M, Lewis RD. Racial differences in cortical bone and their relationship to biochemical variables in Black and White children in the early stages of puberty. Osteoporos Int 2013;24(6):1869-79. doi: 10.1007/s00198-012-2174-8.

35. Ferira AJ, Laing EM, Hausman DB, Hall DB, McCabe GP, Martin BR, Hill Gallant KM, Warden SJ, Weaver CM, Peacock M, et al. Vitamin D Supplementation Does Not Impact Insulin Resistance in Black and White Children. J Clin Endocrinol Metab 2016;101(4):1710-8. doi: 10.1210/jc.2015-3687.

36. Kontulainen SA, Johnston JD, Liu D, Leung C, Oxland TR, McKay HA. Strength indices from pQCT imaging predict up to 85% of variance in bone failure properties at tibial epiphysis and diaphysis. J Musculoskelet Neuronal Interact 2008;8(4):401-9.

37. Pollock NK, Laing EM, Baile CA, Hamrick MW, Hall DB, Lewis RD. Is adiposity advantageous for bone strength? A peripheral quantitative computed tomography study in late adolescent females. Am J Clin Nutr 2007;86(5):1530-8.

38. Swinford RR, Warden SJ. Factors affecting short-term precision of musculoskeletal measures using peripheral quantitative computed tomography (pQCT). Osteoporos Int 2010;21(11):1863-70. doi: 10.1007/s00198-009-1151-3.

39. Berger PK, Pollock NK, Laing EM, Warden SJ, Hill Gallant KM, Hausman DB, Tripp RA, McCabe LD, McCabe GP, Weaver CM, et al. Association of adenovirus 36 infection with adiposity and inflammatory-related markers in children. J Clin Endocrinol Metab 2014;99(9):3240-6. doi: 10.1210/jc.2014-1780.

40. Binkovitz LA, Henwood MJ. Pediatric DXA: technique and interpretation. Pediatr Radiol 2007;37(1):21-31. doi: 10.1007/s00247-006-0153-y.

41. Wetzsteon RJ, Zemel BS, Shults J, Howard KM, Kibe LW, Leonard MB. Mechanical loads and cortical bone geometry in healthy children and young adults. Bone 2011;48(5):1103-8. doi: 10.1016/j.bone.2011.01.005.

42. Cole ZA, Harvey NC, Kim M, Ntani G, Robinson SM, Inskip HM, Godfrey KM, Cooper C, Dennison EM, Southampton Women's Survey Study G. Increased fat mass is associated with increased bone size but reduced volumetric density in pre pubertal children. Bone 2012;50(2):562-7. doi: 10.1016/j.bone.2011.05.005.

43. Correa-Rodriguez M, Rueda-Medina B, Gonzalez-Jimenez E, Correa-Bautista JE, Ramirez-Velez R, Schmidt-RioValle J. Dietary inflammatory index, bone health and body composition in a population of young adults: a cross-sectional study. Int J Food Sci Nutr 2018:1-7. doi: 10.1080/09637486.2018.1446915.

44. Berteus Forslund H, Torgerson JS, Sjostrom L, Lindroos AK. Snacking frequency in relation to energy intake and food choices in obese men and women compared to a reference population. Int J Obes (Lond) 2005;29(6):711-9. doi: 10.1038/sj.ijo.0802950.

45. Sjoberg A, Hallberg L, Hoglund D, Hulthen L. Meal pattern, food choice, nutrient intake and lifestyle factors in The Goteborg Adolescence Study. Eur J Clin Nutr 2003;57(12):1569-78. doi: 10.1038/sj.ejcn.1601726.

46. Dowd JB, Zajacova A, Aiello AE. Predictors of inflammation in U.S. children aged 3-16 years. Am J Prev Med 2010;39(4):314-20. doi: 10.1016/j.amepre.2010.05.014.

47. Deo R, Khera A, McGuire DK, Murphy SA, Meo Neto Jde P, Morrow DA, de Lemos JA. Association among plasma levels of monocyte chemoattractant protein-1, traditional cardiovascular risk factors, and subclinical atherosclerosis. J Am Coll Cardiol 2004;44(9):1812-8. doi: 10.1016/j.jacc.2004.07.047.

48. Palozza P, Parrone N, Catalano A, Simone R. Tomato Lycopene and Inflammatory Cascade: Basic Interactions and Clinical Implications. Curr Med Chem 2010;17(23):2547-63.

49. Chung RWS, Leanderson P, Lundberg AK, Jonasson L. Lutein exerts anti-inflammatory effects in patients with coronary artery disease. Atherosclerosis 2017;262:87-93. doi: 10.1016/j.atherosclerosis.2017.05.008.

50. Birch L, Savage JS, Ventura A. Influences on the Development of Children's Eating Behaviours: From Infancy to Adolescence. Can J Diet Pract Res 2007;68(1):s1-s56.

Table 4.1. Mean, standard deviation (SD), RDAs and typical intakes of nutrients and food components included in the calculation of DII score

DII Food Parameters	RDA	Mean	SD	Typical Intakes*
Energy, kcal/day		1804	497	1813-2247
Carbohydrate, g/day		227	71	233-282
Protein, g/day	34-52	69	20	63-85
Total fat, g/day		70.8	21.8	72.1-88.5
Alcohol, g/day		0.26	3.80	
B-Carotene, μg/day		2019	2513	1086-1409
Vitamin A, μg /day	600	504	430	506-653
Caffeine, g/day		9.98	3.63	11.5-50.0
Cholesterol, mg/day		224.4	110.4	214-276
MUFA, g/day		24.1	7.91	24.3-30.1
n-3 Fatty acids, g/day		1.62	0.69	
n-6 Fatty acids, g/day		15.0	5.56	
PUFA, g/day		16.9	6.24	15.6-19.1
Saturated fat, g/day		24.2	8.20	24.8-31.0
Trans fat, g/day		2.37	1.52	
Fiber, g/day	25-30	14.0	5.11	13.9-16.0
Iron, mg/day		14.1	5.18	13.2-16.7
Magnesium, mg/day		211.5	70.2	223-276
Niacin, mg/day	12	21.4	6.61	19.9-27.8
Riboflavin, mg/day	0.9	1.95	0.66	1.70-2.26
Selenium, mg/day	40	102.7	31.5	91.9-122.8
Thiamin, mg/day	0.9	1.79	0.53	1.46-1.83
Vitamin B6, mg/day	1.0	1.64	0.60	1.56-2.11
Vitamin C, mg/day	45	64.2	50.2	63.5-67.9
Vitamin D, μg/day	15	5.52	3.06	4.2-5.7
Vitamin E, mg/day	11	6.72	3.07	7.1-8.5

*From *What We Eat in America, National Health and Nutrition Examination Survey 2015-2016, range for boys and girls ages 6-19*
RDA, Recommended Dietary Allowances; DII, dietary inflammatory index; kcal, calories; MUFA, monosaturated fatty acids; PUFA, polyunsaturated fatty acids; RE, retinol equivalents; SD, standard deviation
N=290

Table 4.2. Participant characteristics

	Mean	SD
Age, years	11.38	1.23
SMR stage, 2	188 (65%)	
Race (Black), n	137 (47%)	
Height, cm	150.74	9.23
Weight, kg	47.7	12.44
BMI	20.8	4.52
BMI-age-percentile	68.1	29.3
BMI Z-score	0.68	1.08
Fat Mass. kg	15.15	7.3
FFST mass, kg	30.30	6.90
DII score	0.59	1.36
TNF-α, pg/mL	10.69	5.16
IL-6, pg/mL	0.73	0.89
MCP-1, pg/mL	481.41	228.82
VEGF, pg/mL	269.35	220.74

N = 290

DII, dietary inflammatory index; SMS, sexual maturation rating; BMI, body mass index; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; VEGF, vascular endothelial growth factor

Table 4.3. Bivariate and partial correlations between DII score and cortical bone outcomes

	Tibia				Radius			
	Unadjusted		Adjusted		Unadjusted		Adjusted	
	R	P	R	P	R	P	R	P
CtBMD	-0.07	.27	-0.31	.61	-0.01	.99	-0.03	.64
CtBMC	-0.11	.06	-0.06	.30	-0.07	.30	-0.02	.77
TtAr	-0.14	**.02**	-0.03	.97	-0.14	**.02**	-0.09	.15
CtAr	-0.10	.10	-0.08	.19	-0.09	.14	-0.01	.85
CtTh	-0.02	.74	-0.09	.14	-0.02	.76	-0.04	.54
PsPM	-0.15	**.02**	-0.01	.94	-0.14	**.03**	-0.09	.18
EsPM	-0.15	**.02**	-0.05	.44	-0.11	.07	-0.08	.21
SSI	-0.13	**.03**	-0.05	.40	-0.13	**.04**	-0.07	.25

Statistically significant at $P < 0.05$; N = 290; adjusted stage of sexual maturation, sex, muscle cross-sectional area and height; CtBMD, cortical bone mineral density; CtBMC, cortical bone mineral content; TtAr, trabecular area; CtAr, cortical area; CtTh, cortical thickness; PsPM, periosteal perimeter; EsPM, endosteal perimeter; SSI, strength strain index

Table 4.4. Bivariate and partial correlations between biomarkers of inflammation and tibia bone outcomes

	TNF-α				IL-6				MCP-1				VEGF			
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P
CtBMD	-0.12	.06	-0.09	.16	-0.06	.36	-0.06	.41	-0.20	**<.01**	-0.02	.82	-0.04	.95	-0.05	.44
CtBMC	-0.14	**.03**	-0.09	.19	0.05	.46	-0.02	.72	-0.07	.24	-0.10	.14	0.02	.75	-0.09	.18
TtAr	-0.10	.11	-0.02	.75	0.06	.35	-0.04	.50	-0.11	.09	-0.02	.82	-0.02	.78	-0.01	.89
CtAr	-0.12	**.05**	-0.07	.32	0.06	.36	-0.04	.54	-0.04	.58	-0.11	.09	0.03	.69	-0.08	.21
CtTh	-0.11	.09	-0.06	.36	0.04	.54	-0.02	.81	-0.06	.34	-0.15	**.03**	0.05	.40	0.08	.23
PsPM	-0.09	.14	-0.01	.89	0.07	.31	-0.05	.42	-0.10	.11	-0.01	.89	-0.02	.77	-0.01	.92
EsPM	-0.05	.42	-0.02	.73	0.05	.43	-0.03	.61	-0.14	**.03**	-0.08	.24	-0.04	.49	-0.03	.62
SSI	-0.12	**.05**	-0.05	.41	0.05	.49	-0.02	.80	-0.13	**.05**	-0.02	.82	0.04	.96	-0.06	.33

Statistically significant at $P < 0.05$; $N = 290$; adjusted for stage of sexual maturation, sex, race, tibia muscle cross-sectional area, and height
CtBMD, cortical bone mineral density; CtBMC, cortical bone mineral content; TtAr, trabecular area; CtAr, cortical area;
CtTh, cortical thickness; PsPM, periosteal perimeter; EsPM, endosteal perimeter; SSI, strength strain index

Table 4.5. Bivariate and partial correlations between biomarkers of inflammation and radius bone outcomes

	TNF-α				IL-6				MCP-1				VEGF			
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P
CtBMD	-0.06	.35	-0.03	.62	0.09	.17	-0.12	.07	-0.02	.73	-0.13	.05	0.051	.43	0.08	.23
CtBMC	-0.14	**.04**	-0.05	.47	-<0.01	.95	-0.03	.62	-0.03	.62	-0.04	.55	-0.03	.64	-0.06	.36
TtAr	-0.12	.06	-0.03	.64	-0.02	.79	-0.09	.21	-0.10	.13	-0.07	.31	-0.06	.39	-0.03	.71
CtAr	-0.14	**.03**	-0.05	.48	0.01	.83	-0.02	.82	-0.10	.11	-0.01	.94	-0.06	.37	-0.06	.41
CtTh	-0.10	.12	-0.02	.76	0.03	.71	-0.04	.57	-0.06	.33	-0.08	.27	-0.05	.41	-0.02	.79
PsPM	-0.12	.07	-0.03	.68	-0.02	.80	-0.08	.23	-0.10	.14	-0.60	.35	-0.05	.47	-0.04	.60
EsPM	-0.05	.49	-0.01	.91	-0.03	.63	-0.08	.24	-0.05	.46	-0.09	.20	-0.01	.89	-0.02	.82
SSI	-0.14	**.03**	-0.05	.46	<0.01	.99	-0.06	.35	-0.11	.09	-0.04	.57	-0.07	.31	-0.04	.54

Statistically significant at $P < 0.05$; $N = 290$; adjusted for stage of sexual maturation, sex, race, muscle cross-sectional area, and height
CtBMD, cortical bone mineral density; CtBMC, cortical bone mineral content; TtAr, trabecular area; CtAr, cortical area; CtTh, cortical thickness; PsPM, periosteal perimeter; EsPM, endosteal perimeter; SSI, strength strain index

CHAPTER 5

Dietary Protein and Bone Health in Apparently Healthy Children and Adolescents: A Systematic Review5[5]

[5] Coheley LM, Duckam RD, Lewis RD. To be submitted to the Journal for Bone and Mineral Research

Abstract

Modfiable lifestyle factors such as the influence bone development in children and adolescents. Optimizing these factors in youth can be important strategies aimed at reducing the risk osteoporosis or low bone mass later in life. Recently, considerable attention has focused on the role of dietary protein on bone health in children and adolescents. Currently there are no systematic reviews assessing the role of dietary protein on pediatric bone. Therefore the aim was to systematically review cross-sectional and prospective studies and clinical trials in healthy children and adolescents and to determine whether dietary protein intake impacts bone health outcomes. Searches across 3 databases were conducted from January 2000 to December 2018. Two investigators independently conducted abstract and full-text screenings and data extraction. Strength of evidence was rated by group consensus. Seven cross-sectional studies, 9 prospective studies, and 1 randomized controlled trial were included in this systematic review. Overall, this systematic review demonstrated limited evidence (Grade C) that protein intake has a positive role on bone health outcomes as determined by dual energy x-ray absorptiometry and peripheral quantative computed tomography in healthy children. It is likely that physical activty as well as calcium intakes, in addition to protein status may be important factors that mediate the effects of protein on bone. This trial was registered at www.crd.york.ac.uk as CRD4201811655.

Introduction

Osteoporosis is a major public health concern, with 1 in 3 women and 1 in 5 men age 50 years or older experiencing an osteoporotic fracture during their lifetime (1-3). The main determinant of osteoporotic fractures is low bone mass and this low bone mass can be caused by failure to obtain optimal peak bone mass during childhood and accelerated age-related bone loss (4). While it is esimated that 60-80% of the variability in bone mass and osteoporosis risk is attributed to heritable factors (e.g., genetic and hormonal influences) (5, 6), modfiable lifestyle factors such as diet and physical activity (PA) impact peak bone mass; thus, optimizing these factors in youth can be important strategies aimed at reducing the risk of osteoporosis or low bone mass later in life.

Previous investigations examining dietary factors that influence bone strength in children have focused primarily on the micronutrients calcium and vitamin D. Other nutrients, specifically the macronutrient protein (7, 8), have been examined with respect to bone mass in adults and children. The rationale for examing the role of dietary protein in the developing skeleton is related to the fact that protein comprises ~50% of bone volume and approximately ~33% of its mass (9). The organic bone matrix is comprised primarily of collagen and non-collagenous proteins which provides the bone structural integrety. Moreover, dietary protein intake stimulates the activity of anabolic hormones such as growth hormone (GH) and growth factors including insulin-like growth factor-1 (IGF-1) (10). During pubertal growth, bone mineral accrual and linear bone growth are markedly influenced by GH and IGF-1 (11). GH and IGF-1 stimulate osteoblast proliferation and activity, promoting bone formation (12). Low protein intakes can contribute to low GH concentrations which results in a reduced rate of bone remodeling

and a gradual loss of bone mineral density (BMD) (12). Similarly, low dietary protein intakes are associated with low IGF-1 concentrations (13, 14), which lowers bone mineral accrual and linear growth (13, 14). Low protein intakes may also impact bone through intestinal calcium absorption. For example, low protein intakes (< 0.80 grams per kilogram of body weight per day [g/kgBW/day]), reduces intestinal calcium absorption, causing elevated parathroid hormone concentrations, and greater release of calcium from the bone (15). Thus, adequate dietary protein intake appears to be essential for optimal bone growth during childhood.

A number of national and international organizations have set dietary reference values or recommendations for protein. According to the World Health Organization/ Food and Agriculture Organization, for boys ages 3-18 years and girls ages 3-15 years the reference value for protein intake is 0.9 g/kgBW/day (16). The recommended reference value decreases sightly for girls ages 15-18 years to 0.8 g/kgBW/day. The recommended dietary allowance (RDA) for protein ranges from 13-56 g/day for children ages 3-18 years (17). Most children worldwide are meeting the protein recommendations. According to the 2015-2020 Dietary Guidelines for Americans, both boys and girls are either meeting or exceeding the RDA for protein in all age categories (18). Similalry in European countires, average protein intakes varies in infants and young children from about 29-63 g/day (19). Protein intakes increase with age to about 61-116 g/day in adolescents (19). Only a few European countries present data as g/kgBW/day. The estimated mean intakes vary from ≥3 g/kgBW/day in the youngest group to approximately 1.2-2.0 gm/kgBW/day in children and adolescents aged 10-18 years (19).

Studies examining the associations between protein intake and bone health outcomes are limited and focus primarily on adult populations. A meta-analysis published in 2009 found null effects of protein consumption on fracture risk in healthy adults (20), while two more recent meta-analyses demonstrated a slight reduction in hip fractures with increased protein intake (21) and protective effects on lumbar spine bone mineral density (BMD) with increased protein intakes in adults (22). Simiarly, in a recent systematic review and meta-analysis, protein intake accounted for 0-4% of bone mineral content (BMC) and areal bone mineral density (aBMD) variance in adults. Additionally, there were no associations between total protein, vegetable protein, or animal protein intake and relative risk of osteoporotic fractures for lumbar spine BMD and femoral neck BMD. Together these limited studies suggest there is slight beneficial effects of increasing protein intake for bone health in adults and no detrimental effects of protein intake on bone health outcomes.

To our knowledge, there is only one systematic review and meta-analysis investigating the role of protein intake in children and adolescents, but the study focused primarily on adults and assessed evidence published over the last 40 years. They did find that protein intake accounted for 0-14% of areal BMC variance in children and adolescents. Given establishing healthy bones during childhood serves as a blueprint for bone health in adulthood, it is of interest to determine whether nonpharmalogic dietary approaches, such as nutrition, can increase bone mineral accrual and peak bone mass during childhood. The primary aim of this paper is to review cross-sectional and prospective studies and clinical trials in healthy children and adolescents and to determine whether dietary protein intake impacts bone health outcomes.

Methods

Data sources and searches

The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement, consisting of a 27-item checklist and four-phase flow diagram, was followed when reporting this systematic review (23). A specific systematic review protocol was developed and registered on PROSPERO (ID=CRD4201811655) (24).

We conducted a comprehensive search of the literature in three databases: PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Web-of-Science (webofknowledge.com) and Scopus (www.scopus.com) for articles published from January 2000 through December 2018. For PubMed the following search strategies were implemented (((((search term [Title/Abstract]) AND bone [Title/Abstract]) AND child* [Title/Abstract]) OR adolescent [Title/Abstract]) NOT review [Publication Type]). Due to differences in the search engine search bar, for Web-of-Science and Scopus the following search strategies were implemented, TITLE: (dietary protein) AND TITLE: (bone) AND TITLE: (child) Timespan: 2000-2018 and TITLE: (dietary protein intake) AND TITLE: (bone) AND TITLE: (adolescent*) Timespan: 2000-2018. For Web of Science the following indexes were also included: SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC. All searches were limited to the English language. Specific filters were applied to the list of search results to eliminate articles published outside of the years of 2000-2018 and any articles reporting animal studies. MeSH terms were utilized rather than “Title/Abstract” in some instances to yield more viable results.

Study eligibility criteria

Cross-sectional, prospective, and intervention trials were considered for inclusion in this systematic review. Dietary protein interventions included studies in which participants were asked to either a.) Increase consumption of dietary protein or protein containing food products (e.g., dairy products) or b.) Consume protein supplement in their regular diet. Interventions that examined protein and calcium or protein and exercise interactions were also included. Studies examining dietary patterns (e.g., Mediterranean, Western, etc.) rather than protein foods were excluded. We also excluded studies in children and adolescents with diseases known to affect bone metabolism (e.g., cerebral palsy). A complete list of bone outcomes included in this systematic review are depicted in **Table 1.** Reference lists of included studies were manually searched to identify additional articles. One reviewer independently assessed each article, generated a list of articles recommended for inclusion and this recommendation was reviewed and confirmed by the other co-authors. Any discrepancies were discussed among the research team and resolved via group consensus. The studies included in this systematic review were rated based on the extent of scientific evidence. This grading system has been previously utilized by prominent organizations such as the American Society for Nutrition (25) and is recommended by other experts (26). Recommendations are assigned ratings of A (strong), B (moderate), C (limited), or D (inadequate) depending on the quality of evidence **(Table 2).**

Data extraction

We extracted the following: descriptive information regarding the study reference (authors and year of publication), study design/characteristics, participant characteristics (e.g., sex, sample size, etc.), background dietary data, dietary assessment methods,

dietary intervention (in randomized controlled trials and intervention studies) method of assessing bone outcomes, anatomical sites assessed, bone variables reported, confounders and effect modifiers used in statistical analyses and results. A summary table was organized by study type (i.e., cross-sectional and prospective designs and intervention trials). Results were quantitatively and qualitatively summarized by study type and outcome of interest.

Results

Our search yielded 231 citations since January 2000. After removal of duplicate articles, twenty-three articles were identified for abstract screening. Of those, six articles were deemed irrelevant based on the abstract or title; thus, seventeen articles were identified for full-text screening and data extraction (7 cross-sectional studies, 9 prospective cohorts, and 1 randomized-controlled trial). These studies taken together accounts for 5,620 children and adolescents (1,164 males and 1,500 females). Two studies did not differentiate results based on males and females (N=2,956). The details are summarized in **Table 3.** Overall the evidence was limited (grade C) for the effect of protein intake on pediatric bone.

Characteristics of included studies

The age of the participants varied between 2 months and 29 years. The total sample size was 5,620 participants with a mean sample size of 331 boys and girls. Thirteen studies measured bone outcomes with dual energy x-ray absorptiometry (DXA). Three studies measured the non-dominant forearm with peripheral quantitative computed tomography (pQCT) 2000, 1 study measured the distal tibia with pQCT 3000, and 1

study measured the distal tibia with high-resolution-pQCT (HR-pQCT). Two of the studies using pQCT to assess bone outcomes also used DXA for aBMD measures.

Dietary intake was not measured objectively in any of the studies. Four studies measured dietary intake via 7-day food records. Three studies measured dietary intake via 3-day diet records. Three-day weighed food diaries were used to measure typical intakes in three studies while four studies used food-frequency questionnaires. A Registered Dietitian administered interview to determine typical intakes in one study and one study used multiple 24-hour dietary recalls.

The other characteristics and the main results (described below) are presented in **Table 2.**

Cross-sectional associations between protein intake and bone outcomes

Four of the seven cross-sectional studies examined the relationships between total protein intake and total body BMD outcomes (27-30). In the unadjusted model, Hoppe et al, determined that total protein intakes among Danish children ages 6-8 years were positively associated with total body bone area (BA) and total body BMC; however, when adjusting for the covariates, height, weight, and sex, only the relationship between total protein intake and total body BA persisted (27). In a group of healthy and malnourished children ages 2-3 years, total protein intakes were associated with total body BA and BMC (29). Budek et al., investigated the relationships between total protein intakes but also, milk, dairy, and meat protein intakes on total body BMC (27). After adjusting for BA, weight, height, and sex, only total protein and milk protein intakes were associated with total body BMC (27). Additionally, when calcium, energy intake, and PA, as measured by a 24-hour recall were added to the model, only the relationship

between milk protein intakes and total body BMC persisted (28). Lastly, in a study of 56 twin pairs, differences in total body BMC were not explained by protein intakes (30).

Four of the seven cross-sectional studies examined the relationship between protein intake and lumbar spine as measured by DXA (28, 30-32). One of the studies demonstrated a positive effect of milk protein, but not total protein on lumbar spine BMC after adjusting for BA, weight, height, sex, calcium, energy intake, and PA (determined by 24-hour recall questionnaire) (28). Similarly, Esterle et al. reports in postmenarcheal girls, lumbar spine BMC and BMD are positively associated with milk consumption and girls with milk intakes <55 mL/day have significantly lower lumbar spine BMC and BMD in comparison to those consuming over 260 mL/day (32). In pre-pubertal boys, total protein intakes were associated with lumbar spine BMC in the unadjusted model and when adjusting for PA and calcium intakes (31). Similar to the total body, in the 56 twin pairs, differences in lumbar spine BMC were not explained by differences in protein intakes (30).

Other bone outcomes measured in the 56 twin pairs included differences in arm and leg BMC (30). Differences in protein intake was not associated with differences in BMC at the legs; however, differences in arm BMC between twins was partially explained by protein intakes, such that a 1-g increase in protein intake resulted in a 0.4% difference in arm BMC (30). In addition to demonstrating that protein intake was associated with lumbar spine BMC in prepubertal boys, total protein intake was associated with radial metaphysis, total radius, femoral neck, and femoral diaphysis when adjusting for PA and calcium intake (31).

Only one cross-sectional study measured mid-radius (65% site) cortical bone outcomes with pQCT. In healthy children who participated in the Dortmund Nutritional and Anthropometric Longitudinally Designed Study (DONALD) study, after adjusting for muscle area, body mass index standard deviation scores, body fat percentage, age, sex, androstenediol, calcium, vitamin D, and potential renal acid load (PRAL [measures the dietary component of net endogenous acid production and is highly correlated with analyzed urinary net acid excretion]), protein intake showed a trend for an association with mid-radius cortical BMC (β=0.11, *P*=.073) and cortical area (β=0.11, *P*=.056) (33).

Prospective associations between protein intake and bone outcomes

Four out of the nine prospective studies examined the associations between protein intake and total body bone outcomes. One study demonstrated that protein intakes in boys and girls between the ages of 2 months and 8 years were positively associated with total body BMC at age 8 (34). Another study demonstrated that over a period of 5 years, protein consumption (average 57.3 g/day) in children with low calcium intakes were negatively associated with total body BMC (35). In young adults who participated in the Saskatchewan Pediatric Bone Mineral Accrual Study, protein intake predicted total body BMC net gains. Additionally, in females at peri-adolescents or early adulthood with calcium intakes >1000 mg/day, protein was a positive predictor of total body BMC, total body BMC net gains, and total body BMD (36). Dairy intake, but not total protein intake, was shown to predict bone mineral accrual in children and adolescents (36). Children who consumed ≥ 2 servings of dairy per day during the ages of 3-5 years had increases in trunk, ribs, and pelvis BMC and trunk and ribs bone area at ages 15-17 years when compared to children consuming <2 servings of dairy per day (37).

In a cohort of healthy boys, areal BMC at the femoral neck and total hip tracked from prepuberty to mid-adolescents. Additionally, healthy adolescent boys aged 6.5 to 8.5 years, who consumed more protein (approximately 80% above the RDA) and had higher PA levels during childhood, had greater distal tibia volumetric BMD after 8 years compared to adolescent boys who had lower protein intakes and high PA levels (38). Interestingly, approximately 75% of the protein intake came from meats and 70% of the PA came from weight bearing activities. In a follow-up study of this cohort, Chevalley and colleagues determined that in boys with high PA levels, higher vs. lower protein intake was associated with 9.8% greater femoral neck BMC at 7.4 years and this difference was maintained at 15.2 and 22.6 years with 12.7% and 11.3% greater femoral neck BMC, respectively. Moreover, HR-pQCT assessment of the distal tibia at years 15.2 and 22.6 indicated that high protein intake and high PA was associated with greater cross-sectional area, stiffness, and failure load (39).

Alexy et al., demonstrated in a cohort of German children that protein intakes over 4 years were positively associated with, and were predictors of, forearm periosteal circumference, cortical area, BMC, and stress-strain index (SSI). Additionally, the investigators showed that long-term dietary PRAL, was negatively associated with forearm BMC and cortical area (40). In the same cohort, Remer et al. demonstrated that urinary nitrogen, an accepted recovery biomarker of protein intake (41) was positively associated with forearm periosteal circumference, cortical area, BMC, SSI, and urinary PRAL was negatively associated with forearm BMC and cortical area (42). Similarly, a secondary study from the Generation R Study demonstrated that dietary PRAL measured at 1-2 years was not consistently associated with childhood bone at age 6 (43). In children

with high protein intake (> 42 g/d), a 1-unit increase in dietary PRAL was inversely associated with total body BMC differences (-0.32 grams). No associations were observed in the group of children with low protein intakes (43).

Dietary protein intervention on bone health outcomes

In the one RCT, there was no effect of protein supplementation (2 supplements per day with 42 grams of protein each) in conjunction with a 6 month strength and conditioning training program (5 sessions per week) on trabecular or cortical bone at the 4% and 20% sites of the distal tibia in young adolescents males and females ages 18-25 years (44). Interestingly, there were no differences in energy or macronutrient intake between supplemented and non-supplemented groups a baseline.

Discussion

In a recent systematic review of dietary protein and bone in adults the authors concluded that although there were positive trends for protein intake improving DXA derived aBMD at most bone sites, only the lumbar spine showed moderate evidence to support the benefits of higher protein intakes (22). The authors also concluded that there are no adverse effects of higher protein intakes on aBMD at various bone sites (22). In children and adolescents, there are fewer published reports addressing the role of protein on bone. Therefore, the aim of this systematic review was to systematically review the evidence on dietary protein intake and bone health outcomes in healthy children and adolescents. To the best of our knowledge, this is the first systematic review examining the relationships between dietary protein and pediatric bone health outcomes as measured by both DXA and pQCT. Overall, the level of evidence was limited (grade C) for the benefit of protein on bone.

The search yielded 17 articles for inclusion, 7 cross-sectional studies, 9 prospective cohorts, and 1 randomized-controlled trial, studying 5,620 children and adolescents. For many of the cross-sectional and prospective studies, protein intake positively influenced pediatric bone health outcomes for nearly all bone sites; however, the findings were inconsistent across studies. Intervention studies examining the independent role protein intake alone on bone outcomes in children do not exist. The one published RCT in adolescents (44) used an intervention combining protein supplementation with an exercise intervention. There were no beneficial effects on trabecular or cortical bone outcomes by adding protein to a strength and conditioning program. Based on the bone remodeling cycles in growing children, it is possible that the study duration of 6 months was not long enough to detect improvements in bone outcomes. Additionally, due to the absence of a non-exercise group, it is not clear if protein intake or exercise intake alone would have improved bone outcomes. In college-aged individuals, protein supplementation during 6 months of exercise did not result in any additional benefit on bone outcomes compared to a carbohydrate supplement of equal caloric value (45). It remains unknown whether protein independently improves bone health outcomes in healthy adolescent children.

A majority of the prospective studies demonstrated positive effects of dietary protein on pediatric bone health outcomes. For example, protein intake over a period of 8 years was associated with total body BMC net gains in healthy boys and girls (34). Similarly, Alexy et al. demonstrated that protein intakes over 4 years were positively associated with forearm pQCT derived outcomes, periosteal circumference, cortical area, BMC, and SSI (40). Like the one RCT, two prospective studies examined the

combination of protein intake and weight-bearing PA on bone (38, 39). In contract to the RCT, these studies support the synergistic role of protein intake and PA on bone size, longitudinal growth, and cross-sectional dimensions of the bone. Participants in the RCT were males and females aged 18-25 years while participants in the prospective studies were only males and aged 7.4 years at baseline testing. Participants in the RCT had already attained their peak bone mass which may be why they were not responsive to the dietary and exercise intervention. It is unclear if there are sex differences related to the response of dietary protein interventions and exercise on bone mass. Future studies should consider targeting children during the period of peak bone mass accrual and should assess whether there are sex differences related to the response of dietary protein on bone.

It is possible that the effects of protein intakes on bone depend on dietary calcium intakes or protein status. One prospective study demonstrated a negative relationship between dietary protein intake and bone outcomes (35). The children in this study had low calcium intakes (432.7 mg/day), which may have attenuated the impact of protein intake on bone health outcomes. In children and adolescents ages 9-13 the recommended dietary allowance (RDA) for calcium is 1,300 mg/day; thus, children in this study were consuming calcium at approximately 67% below the RDA (35). Independently, calcium is known to influence pediatric bone in children. For example, several intervention studies have demonstrated that calcium supplementation positively effects aBMD and/or BMC accrual as measured by DXA (46-49). Additionally, protein and calcium intake from dairy foods is known to positively influence pediatric bone. For example, 1000 mg of supplemented dairy foods resulted in 1.5% greater gain in spine BMD compared to no

supplementation (50). Whether protein and calcium intakes from non-dairy sources work synergistically to influence pediatric bone health outcomes remains to be elucidated. In adults, Shani et al. demonstrated that a greater risk of hip fractures was associated with higher protein intakes, but only when calcium intakes were low (<800 mg/day). Conversely, there was a lower risk of hip fracture with high protein and high calcium intakes (≥800 mg/day) (51). It is possible that higher protein intakes accompanied by low calcium intakes may lead to increased urinary calcium excretion and therefore, lower bone mass. However, critics of this hypothesis indicate that higher protein diets lead to increased intestinal calcium absorption and therefore there is little concern that high-protein diets would adversely influence skeletal health (15). RCTs are needed to further investigate the association between a lower calcium/protein ratio and bone mass outcomes in children (52).

Four of the studies used dietary PRAL as a surrogate measure of protein intake (33, 40, 42, 43). The dietary PRAL measures the dietary component of net endogenous acid production and is highly correlated with analyzed urinary net acid excretion. The effect of dietary PRAL on bone outcomes in children was mixed with some studies finding a detrimental effect of high dietary PRAL (40, 42) on bone while others found no effect (33, 43). Differences in sample size, smaller variations in PRAL scores, and different dietary techniques to determine PRAL (i.e., 3-day diet records vs. FFQ) may have accounted for the conflicting findings.

In all of the cross-sectional studies, protein intake was positively associated with bone outcomes; however, the magnitude of the effects varied based on the covariates used in the model and the skeletal sites measured. For example, all studies investigating

the role of protein intake on total body bone outcomes showed positive associations but controlled for different covariates making it difficult to compare studies. Only one cross-sectional study investigated the role of protein on pQCT derived bone outcomes and found a positive trend with mid-radius cortical BMC and bone area. Collectively, these cross-sectional studies lend support for a positive effect of protein on bone in children, but RCTs are needed to prove this assumption.

For children ages 1-8, the dietary reference intakes (DRIs) for protein range from 13-19 g/day (53). In adolescents and young adults ages 9-18 the DRI for protein ranges from 34-52 g/day (53). In only one of the studies was there a deficient intakes of protein (29) and these children were malnourished. Despite malnutrition status, intakes of protein were positively associated with total body BMC and BA after adjusting for energy intakes (29), further supporting the importance of dietary protein intakes for optimal bone outcomes in children.

A strength of this systematic review is the fact that we included studies from randomized controlled trials, prospective, and cross-sectional studies. Additionally, this study included both DXA and pQCT bone measures. There was only one RCT and that study combined protein and resistance training into the intervention. Additionally, the study duration of six months may not have been long enough to see an effect on bone. The lack of protein RCTs highlights a priority research area. Despite seeing positive effects of protein intake on bone outcomes, several of the cross-sectional and prospective studies controlled for different covariates making it difficult to compare the findings of these studies. Finally, estimates of dietary protein intake from the cross-sectional and prospective studies relied on 24-hour dietary recalls and/or food-frequency

questionnaires, which are subjective measures of dietary intake and not a true estimate of intake. Future research needs to include larger-scale intervention studies to better understand the role of dietary protein intake on pediatric bone.

Overall, this systematic review demonstrated limited (grade C) evidence that dietary protein intake during childhood is beneficial for bone growth and development. Prospective studies demonstrate that calcium intakes and physical activity, in addition to protein status may be important factors that influence pediatric bone health. Childhood and adolescence is a period of rapid bone growth, areal bone expansion and bone mineral accrual and serves as blueprint for bone health later in life; thus, future long-term intervention trials are needed to further elucidate the role of dietary protein on pediatric bone growth and development.

References

1. Melton LJ, 3rd, Atkinson EJ, O'Connor MK, O'Fallon WM, Riggs BL. Bone density and fracture risk in men. J Bone Miner Res 1998;13(12):1915-23. doi: 10.1359/jbmr.1998.13.12.1915.

2. Melton LJ, 3rd, Chrischilles EA, Cooper C, Lane AW, Riggs BL. Perspective. How many women have osteoporosis? J Bone Miner Res 1992;7(9):1005-10. doi: 10.1002/jbmr.5650070902.

3. Kanis JA, Johnell O, Oden A, Sembo I, Redlund-Johnell I, Dawson A, De Laet C, Jonsson B. Long-term risk of osteoporotic fracture in Malmo. Osteoporos Int 2000;11(8):669-74.

4. Ferrari S, Rizzoli R, Slosman D, Bonjour JP. Familial resemblance for bone mineral mass is expressed before puberty. J Clin Endocrinol Metab 1998;83(2):358-61. doi: 10.1210/jcem.83.2.4583.

5. Seeman E, Hopper JL, Bach LA, Cooper ME, Parkinson E, McKay J, Jerums G. Reduced bone mass in daughters of women with osteoporosis. N Engl J Med 1989;320(9):554-8. doi: 10.1056/NEJM198903023200903.

6. Soroko SB, Barrett-Connor E, Edelstein SL, Kritz-Silverstein D. Family history of osteoporosis and bone mineral density at the axial skeleton: the Rancho Bernardo Study. J Bone Miner Res 1994;9(6):761-9. doi: 10.1002/jbmr.5650090602.

7. Tucker KL, Hannan MT, Chen H, Cupples LA, Wilson PW, Kiel DP. Potassium, magnesium, and fruit and vegetable intakes are associated with greater bone mineral density in elderly men and women. Am J Clin Nutr 1999;69(4):727-36. doi: 10.1093/ajcn/69.4.727.

8. New SA, MacDonald HM, Campbell MK, Martin JC, Garton MJ, Robins SP, Reid DM. Lower estimates of net endogenous non-carbonic acid production are positively associated with indexes of bone health in premenopausal and perimenopausal women. Am J Clin Nutr 2004;79(1):131-8. doi: 10.1093/ajcn/79.1.131.

9. Heaney RP. Protein intake and the calcium economy. J Am Diet Assoc 1993;93(11):1259-60.

10. Ekbote VH, Khadilkar VV, Khadilkar AV, Mughal Z, Chiplonkar SA, Palande SA, Phanse-Gupte SS, Patwardhan VG, Shilvant DS. Relationship of insulin-like growth factor 1 and bone parameters in 7-15 years old apparently, healthy Indian children. Indian J Endocrinol Metab 2015;19(6):770-4. doi: 10.4103/2230-8210.167549.

11. Breen ME, Laing EM, Hall DB, Hausman DB, Taylor RG, Isales CM, Ding KH, Pollock NK, Hamrick MW, Baile CA, et al. 25-hydroxyvitamin D, insulin-like growth factor-I, and bone mineral accrual during growth. J Clin Endocrinol Metab 2011;96(1):E89-98. doi: 10.1210/jc.2010-0595.

12. Olney RC. Regulation of bone mass by growth hormone. Med Pediatr Oncol 2003;41(3):228-34. doi: 10.1002/mpo.10342.

13. Hoppe C, Udam TR, Lauritzen L, Molgaard C, Juul A, Michaelsen KF. Animal protein intake, serum insulin-like growth factor I, and growth in healthy 2.5-y-old Danish children. Am J Clin Nutr 2004;80(2):447-52. doi: 10.1093/ajcn/80.2.447.

14. Tessema M, Gunaratna NS, Brouwer ID, Donato K, Cohen JL, McConnell M, Belachew T, Belayneh D, De Groote H. Associations among High-Quality Protein and Energy Intake, Serum Transthyretin, Serum Amino Acids and Linear Growth of Children in Ethiopia. Nutrients 2018;10(11). doi: 10.3390/nu10111776.

15. Kerstetter JE, O'Brien KO, Insogna KL. Dietary protein, calcium metabolism, and skeletal homeostasis revisited. Am J Clin Nutr 2003;78(3 Suppl):584S-92S. doi: 10.1093/ajcn/78.3.584S.

16. Who. Protein and Amino Acid Requirements in Human Nutrition: Report of a Joint WHO/FAO/UNU Expert Consultation. Who Tech Rep Ser 2007;935:1-270.

17. Gassmann B. Dietary reference intakes (DRI), report 6, part 2: Protein and amino acids. Ernahrungs-Umschau 2003;50(5):178-+.

18. Casavale KO, Stoody EE, Rihane C, Olson R. Recommendations of the 2015-2020 Dietary Guidelines for Americans. Faseb J 2016;30.

19. Agostoni C, Bresson JL, Fairweather-Tait S, Flynn A, Golly I, Korhonen H, Lagiou P, Lovik M, Marchelli R, Martin A, et al. Scientific Opinion on Dietary Reference Values for protein EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Efsa J 2012;10(2). doi: UNSP 2557 10.2903/j.efsa.2012.2557.

20. Darling AL, Millward DJ, Torgerson DJ, Hewitt CE, Lanham-New SA. Dietary protein and bone health: a systematic review and meta-analysis. Am J Clin Nutr 2009;90(6):1674-92. doi: 10.3945/ajcn.2009.27799.

21. Wu AM, Sun XL, Lv QB, Zhou Y, Xia DD, Xu HZ, Huang QS, Chi YL. The relationship between dietary protein consumption and risk of fracture: a subgroup and dose-response meta-analysis of prospective cohort studies. Sci Rep 2015;5:9151. doi: 10.1038/srep09151.

22. Shams-White MM, Chung M, Du M, Fu Z, Insogna KL, Karlsen MC, LeBoff MS, Shapses SA, Sackey J, Wallace TC, et al. Dietary protein and bone health: a systematic review and meta-analysis from the National Osteoporosis Foundation. Am J Clin Nutr 2017;105(6):1528-43. doi: 10.3945/ajcn.116.145110.

23. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. J Clin Epidemiol 2009;62(10):1006-12. doi: 10.1016/j.jclinepi.2009.06.005.

24. Sideri S, Papageorgiou SN, Eliades T. Registration in the international prospective register of systematic reviews (PROSPERO) of systematic review protocols was associated with increased review quality. J Clin Epidemiol 2018;100:103-10. doi: 10.1016/j.jclinepi.2018.01.003.

25. Cho SS, Qi L, Fahey GC, Jr., Klurfeld DM. Consumption of cereal fiber, mixtures of whole grains and bran, and whole grains and risk reduction in type 2 diabetes, obesity, and cardiovascular disease. Am J Clin Nutr 2013;98(2):594-619. doi: 10.3945/ajcn.113.067629.

26. Woolf SH. Weighing the evidence to formulate dietary guidelines. Journal of the American College of Nutrition 2006;25(3):277s-84s. doi: Doi 10.1080/07315724.2006.10719578.

27. Hoppe C, Molgaard C, Michaelsen KF. Bone size and bone mass in 10-year-old Danish children: effect of current diet. Osteoporos Int 2000;11(12):1024-30.

28. Budek AZ, Hoppe C, Ingstrup H, Michaelsen KF, Bugel S, Molgaard C. Dietary protein intake and bone mineral content in adolescents-The Copenhagen Cohort Study. Osteoporos Int 2007;18(12):1661-7. doi: 10.1007/s00198-007-0422-0.

29. Ekbote VH, Khadilkar AV, Chiplonkar SA, Khadilkar VV. Determinants of bone mineral content and bone area in Indian preschool children. J Bone Miner Metab 2011;29(3):334-41. doi: 10.1007/s00774-010-0224-x.

30. Iuliano-Burns S, Stone J, Hopper JL, Seeman E. Diet and exercise during growth have site-specific skeletal effects: a co-twin control study. Osteoporos Int 2005;16(10):1225-32. doi: 10.1007/s00198-004-1830-z.

31. Chevalley T, Bonjour JP, Ferrari S, Rizzoli R. High-protein intake enhances the positive impact of physical activity on BMC in prepubertal boys. J Bone Miner Res 2008;23(1):131-42. doi: 10.1359/jbmr.070907.

32. Esterle L, Sabatier JP, Guillon-Metz F, Walrant-Debray O, Guaydier-Souquieres G, Jehan F, Garabedian M. Milk, rather than other foods, is associated with

vertebral bone mass and circulating IGF-1 in female adolescents. Osteoporos Int 2009;20(4):567-75. doi: 10.1007/s00198-008-0708-x.

33. Libuda L, Wudy SA, Schoenau E, Remer T. Comparison of the effects of dietary protein, androstenediol and forearm muscle area on radial bone variables in healthy prepubertal children. Br J Nutr 2011;105(3):428-35. doi: 10.1017/S0007114510003508.

34. Bounds W, Skinner J, Carruth BR, Ziegler P. The relationship of dietary and lifestyle factors to bone mineral indexes in children. J Am Diet Assoc 2005;105(5):735-41. doi: 10.1016/j.jada.2005.02.046.

35. Zhang Q, Ma G, Greenfield H, Zhu K, Du X, Foo LH, Hu X, Fraser DR. The association between dietary protein intake and bone mass accretion in pubertal girls with low calcium intakes. Br J Nutr 2010;103(5):714-23. doi: 10.1017/S0007114509992303.

36. Vatanparast H, Bailey DA, Baxter-Jones AD, Whiting SJ. The effects of dietary protein on bone mineral mass in young adults may be modulated by adolescent calcium intake. J Nutr 2007;137(12):2674-9. doi: 10.1093/jn/137.12.2674.

37. Moore LL, Bradlee ML, Gao D, Singer MR. Effects of average childhood dairy intake on adolescent bone health. J Pediatr 2008;153(5):667-73. doi: 10.1016/j.jpeds.2008.05.016.

38. Chevalley T, Bonjour JP, van Rietbergen B, Ferrari S, Rizzoli R. Tracking of environmental determinants of bone structure and strength development in healthy boys: an eight-year follow up study on the positive interaction between physical activity and protein intake from prepuberty to mid-late adolescence. J Bone Miner Res 2014;29(10):2182-92. doi: 10.1002/jbmr.2247.

39. Chevalley T, Bonjour JP, Audet MC, Merminod F, van Rietbergen B, Rizzoli R, Ferrari S. Prepubertal Impact of Protein Intake and Physical Activity on Weight-Bearing Peak Bone Mass and Strength in Males. J Clin Endocrinol Metab 2017;102(1):157-66. doi: 10.1210/jc.2016-2449.

40. Alexy U, Remer T, Manz F, Neu CM, Schoenau E. Long-term protein intake and dietary potential renal acid load are associated with bone modeling and remodeling at the proximal radius in healthy children. Am J Clin Nutr 2005;82(5):1107-14. doi: 10.1093/ajcn/82.5.1107.

41. Adams GR. Exercise Effects on Muscle Insulin Signaling and Action - Invited review: Autocrine/paracrine IGF-I and skeletal muscle adaptation. J Appl Physiol 2002;93(3):1159-67. doi: 10.1152/japplphysiol.01264.2001.

42. Remer T, Manz F, Alexy U, Schoenau E, Wudy SA, Shi L. Long-term high urinary potential renal acid load and low nitrogen excretion predict reduced diaphyseal bone mass and bone size in children. J Clin Endocrinol Metab 2011;96(9):2861-8. doi: 10.1210/jc.2011-1005.

43. Garcia AH, Franco OH, Voortman T, de Jonge EA, Gordillo NG, Jaddoe VW, Rivadeneira F, van den Hooven EH. Dietary acid load in early life and bone health in childhood: the Generation R Study. Am J Clin Nutr 2015;102(6):1595-603. doi: 10.3945/ajcn.115.112821.

44. Ballard TL, Specker BL, Binkley TL, Vukovich MD. Effect of protein supplementation during a 6-month strength and conditioning program on areal and volumetric bone parameters. Bone 2006;38(6):898-904. doi: 10.1016/j.bone.2005.10.020.

45. Vincent KR, Braith RW, Feldman RA, Magyari PM, Cutler RB, Persin SA, Lennon SL, Gabr AH, Lowenthal DT. Resistance exercise and physical performance in adults aged 60 to 83. J Am Geriatr Soc 2002;50(6):1100-7. doi: 10.1046/j.1532-5415.2002.50267.x.

46. Iuliano-Burns S, Saxon L, Naughton G, Gibbons K, Bass SL. Regional specificity of exercise and calcium during skeletal growth in girls: a randomized controlled trial. J Bone Miner Res 2003;18(1):156-62. doi: 10.1359/jbmr.2003.18.1.156.

47. Stear SJ, Prentice A, Jones SC, Cole TJ. Effect of a calcium and exercise intervention on the bone mineral status of 16-18-y-old adolescent girls. Am J Clin Nutr 2003;77(4):985-92. doi: 10.1093/ajcn/77.4.985.

48. Bass SL, Naughton G, Saxon L, Iuliano-Burns S, Daly R, Briganti EM, Hume C, Nowson C. Exercise and calcium combined results in a greater osteogenic effect than either factor alone: a blinded randomized placebo-controlled trial in boys. J Bone Miner Res 2007;22(3):458-64. doi: 10.1359/jbmr.061201.

49. Courteix D, Jaffre C, Lespessailles E, Benhamou L. Cumulative effects of calcium supplementation and physical activity on bone accretion in premenarchal children: a double-blind randomised placebo-controlled trial. Int J Sports Med 2005;26(5):332-8. doi: 10.1055/s-2004-821040.

50. Merrilees MJ, Smart EJ, Gilchrist NL, Frampton C, Turner JG, Hooke E, March RL, Maguire P. Effects of diary food supplements on bone mineral density in teenage girls. Eur J Nutr 2000;39(6):256-62.

51. Sahni S, Cupples LA, McLean RR, Tucker KL, Broe KE, Kiel DP, Hannan MT. Protective effect of high protein and calcium intake on the risk of hip fracture in the Framingham offspring cohort. J Bone Miner Res 2010;25(12):2770-6. doi: 10.1002/jbmr.194.

52. Weaver CM, Proulx WR, Heaney R. Choices for achieving adequate dietary calcium with a vegetarian diet. Am J Clin Nutr 1999;70(3 Suppl):543S-8S. doi: 10.1093/ajcn/70.3.543s.

53. Yon BA, Johnson RK. US and Canadian Dietary Reference Intakes (DRIs) for the macronutrients, energy and physical activity. Nutr Bull 2005;30(2):176-81. doi: 10.1111/j.1467-3010.2005.00498.x.

Table 5.1. Included bone outcomes of interest

Measurement Technique	Sites	Outcome Measure
DXA	Total Body	aBMD BA
	Hip Lumbar Spine (L1-L4 or L2-L4) Radius Pelvis Leg and arm BMC	
pQCT	Radius Tibia	BA Trabecular Density Trabecular area BSI SSI vBMD vBMC TtA CtCSA EC PC
HR-pQCT	Radius Tibia	Trabecular Number Trabecular Separation Trabecular BMD Connective Density Cortical Porosity Trabecular Thickness

DXA, dual-energy x-ray absorptiometry; pQCT, peripheral quantitative computed tomography; HR-pQCT, high resolution peripheral quantitative computed tomography; aBMD, areal bone mineral density; BA, bone area; BSI, bone strength index; SSI, strength strain index; vBMD, volumetric bone mineral density; vBMC, volumetric bone mineral content; TtA, total area; CtCSA, cortical cross-sectional area; EC, endosteal circumference; PC, periosteal circumference

Table 5.2. Evidence grading system

Level of evidence	Description
A (Strong)	Clear evidence from at least one large, well-conducted, generalizable RCT that is adequately powered with a large effect size and is free of bias or other concerns OR Clear evidence from multiple RCTs or many controlled trials that may have few limitations related to bias, measurement imprecision, inconsistent results, or other concerns
B (Moderate)	Evidence obtained from multiple, well-designed, conducted, and controlled prospective cohort studies that have used adequate and relevant measurements and that gave similar results from different populations OR Evidence obtained from a well-conducted meta-analysis of prospective cohort studies from different populations
C (Limited)	Evidence obtained from multiple prospective cohort studies from diverse populations that have limitations related to bias, measurement imprecision, or inconsistent results or have other concerns OR Evidence from only one well-designed prospective study with few limitations OR Evidence from multiple well-designed and conducted cross-sectional or case-controlled studies that have very few limitations that could invalidate the results from diverse populations OR Evidence from a meta-analysis that has designed limitations
D (Inadequate)	Evidence from studies that have one or more major methodological flaws or many minor methodological flaws that result in low confidence in the effect estimate OR Insufficient data to support a hypothesis OR Evidence derived from clinical experience, historical studies (before and after), or uncontrolled descriptive studies or case reports

RCT, randomized controlled trial

[a] Refers to the body of evidence

Table 5.3. Study characteristics

Cross-sectional

Reference	Study Description	Population Description	*N*	Outcome Measures	Results
Hoppe et al., 2000 (27)	The objective of this study was to identify associations between dietary factors and whole body bone measures determined by DXA in a random sample of healthy Danish children. Dietary intakes were assessed by 7-day food records.	Sex: male and female Age: 6-8 y Race: Caucasian Location: Dortmund, Germany Year(s): 1997-1998; sub-cohort of the DONALD study	105	Data are shown for the overall group (*N*=105)	Protein (g/day) Pearson's r; *P* Total Body Bone area (cm^3): 0.31; <0.01 BMC (g): 0.33; <0.01 • These data are unadjusted • In the multiple linear regression models with height, weight, sex and intakes of energy, fat, carbohydrate, protein, and other bone-related nutrients, there were no significant relationships between protein and bone outcomes • After backward elimination where height, weight, and sex were forced to stay in the model, intake of protein was positively associated with bone area (*P*=.003) • Including stage of sexual maturation in the analyses did not alter the outcomes
Budek et al., 2007 (28)	The aim of this study was to test the hypotheses that total protein intake is positively associated with bone mass as measured by DXA, and that milk and meat protein intake is differently associated with bone mass in adolescents. The dietary intakes of total, milk, dairy, and meat protein, and the dietary intake of selected micronutrients, were calculated for each subject from 7-day food records.	Sex: male and female Age: 17 y Race: Caucasian Location: Danish, otherwise unspecified Year(s): 2004-2005; sub-cohort of the Copenhagen Cohort Study	109	Data are shown for the overall group (*N*=109)	Protein (g/d) Total protein: ß, *P*; Milk protein: ß, *P*; Dairy protein: ß, *P*; Meat protein: ß, *P*; Mode [a] T-BMC: 0.04, 0.047, 0.02, 0.003, 0.02, 0.11, 0.01, 0.55, 1 LS-BMC: 0.02, 0.68, 0.03, 0.01, 0.001, 0.97, -0.02, 0.48, 1 T-BMC: -0.02, 0.78, 0.04, 0.007, -0.01, 0.72, 0.01, 0.62, 2 LS-BMC: -0.08, 0.46, 0.06, 0.01, -0.06, 0.42, -0.01, 0.78, 2 • [a]After adjustment for bone area, weight, height and sex (model 1), and additional adjustment for calcium and energy intake, and physical activity (model 2)

Ekbote et al., 2011 (29)	The aim of this study was to examine lifestyle factors as determinants of total body BMC and bone area as measured by DXA in Indian preschool children. Dietary intakes were assessed via 3-day food diaries.	Sex: male and female Age: 2–3 y Race: Indian Location: Pune, India Year(s): 2009	71	Data are shown for the overall group (*N*=71)	Protein (g/d)					
					Normal Children		Malnourished Children		All	
					r	*P*	*r*	*P*	*r*	*P*
				Total body						
				Bone area	0.65	<0.01	0.57	<0.01	0.58	<0.05
				BMC	0.62	<0.01	0.44	<0.05	0.55	<0.01
				• Data presented above are from Pearson's correlation coefficients correlating protein intake and bone among normal children, malnourished children, and all (normal and malnourished children combined)						
Iuliano-Burns et al., 2005 (30)	This study was conducted to test the following hypotheses: 1) Associations between bone mass and dimensions and exercise are greater than between bone mass and dimensions and protein and calcium intakes; 2) Exercise or nutrient intake are associated with appendicular bone mass before puberty and axial bone mass during/after puberty. Dietary intakes were assessed using 3-day weighed food diaries. Total body and posteroanterior lumbar spine BMC and mid-femoral shaft dimensions were measured by DXA.	Sex: male Age: 7–20 y Race: not specified Location: Melbourne, Australia Year(s): 1997–2001	112 (56 twin pairs)	Data are shown for the overall group (*N*=112)	Differences in protein					
					Univariate		Size adjusted		All lifestyle/ size adjusted	
					ß	*P*	ß	*P*	ß	*P*
				Differences in BMC (g)						
				Total body	3.5	NS	1.3	NS	1.3	NS
				Arms	0.8	<0.05	0.7	<0.05	0.8	<0.05
				Legs	1.6	NS	0.3	NS	0.3	NS
				Lumbar spine	0.0	NS	0.0	NS	0.0	NS
				Differences in BMC (%)						
				Total body	0.3	<0.05	0.2	<0.05	0.1	NS
				Arms	0.4	<0.05	0.4	<0.01	0.4	<0.05
				Legs	0.3	NS	0.1	NS	0.1	NS
				Lumbar spine	0.1	NS	0.1	NS	0.2	NS
				Differences in bone dimensions (mm)						
				Cortical thickness	0.0	NS	0.0	NS	0.0	NS
				Periosteal diameter	0.0	NS	0.0	NS	0.0	NS
				Endosteal diameter	0.0	NS	0.0	NS	–0.0	NS
				Differences in bone dimensions (%)						
				Cortical thickness	0.2	NS	0.1	NS	0.4	NS
				Periosteal diameter	0.1	NS	0.0	NS	0.0	NS
				Endosteal diameter	–0.0	NS	–0.1	NS	–0.3	NS
				• ß coefficients for within-pair differences in protein versus [a]within-pair differences in BMC and bone dimensions; [b]within-pair differences in size-adjusted BMC and bone dimensions; [c]all within-pair differences in protein, calcium, exercise duration, and size are included in the regression equation • A 1-g difference in protein intake was associated with a 0.8-g (0.4%) difference in arm BMC ($P < 0.05$). These relationships were only present in peripubertal and postpubertal pairs						
Chevalley et al., 2008 (31)	The aim of this study was to assess the interaction between physical activity and protein (assessed via FFQ) compared to	Sex: male Age: 6.5–8.5 y (M=7.44 ± 0.30) Race: white	232	Data are shown for the overall group (*N* = 232)	Correlation with protein intake (g/d)					
					r	*P*	ß Adjusted	*P*		
				BMC (g)						

	calcium (assessed via FFQ) intakes on bone mineral mass at several skeletal sites measured by DXA in healthy prepubertal boys	Location: Geneva, Switzerland Year(s): 1999-2000		Radial metaphysis	0.26	<0.01	0.20	0.01
				Radial diaphysis	0.21	<0.01	0.12	NS
				Total radius	0.27	<0.01	0.20	0.01
				Femoral neck	0.20	<0.01	0.19	0.03
				Total hip	0.18	<0.01	0.12	NS
				Femoral diaphysis	0.23	<0.01	0.19	0.03
				Lumbar spine	0.24	<0.01	0.22	<0.01
				Data presented above are from univariate (r) and multivariate ($ß_{Adjusted}$) analyses; the latter takes into account the respective contribution of physical activity, protein, and calcium intakes				

Libuda et al., 2011 (33)	The aim of this study was to identify the strongest long-term dietary predictors of prepubertal diaphyseal bone status in healthy children who participated in the DONALD study. 3-day weighed dietary records were used to assess dietary intakes. pQCT was used to measure bone outcomes at the non-dominant forearm at the 65% site.	Sex: male and female Median age: 8.1 y Race: white Location: Dortmund, Germany Year(s): 1998–1999 subcohort of the DONALD study	107	Data are shown for the overall group (N = 107)	Protein (g/MJ)			
					ß	$ß_{stand}$	R^2	P
				Forearm				
				Polar SSI (mm^3)	—	—	—	NS
				Periosteal circumference (mm)	—	—	—	NS
				BMC (mg/mm)	1.49	0.11	0.01	NS
				Cortical area (mm^2)	1.37	0.11	0.01	NS
				• Data presented above are from stepwise linear regression analyses, considering muscle area, BMI standard deviation scores, body fat %, age, sex, and androestenediol, as well as intakes of protein, calcium, vitamin D, and PRAL • Of all nutrients considered, only protein showed a trend for an association with BMC (P = 0.073) and cortical area (P = 0.056) in stepwise linear regression models • None of the other dietary variables were associated with bone parameters • The protein effect did not differ between sexes				

Esterle et al. 2009 (32)	The aim of this study was to identify dietary foods and nutrients associated with lumbar bone mineral content and bone mineral density in adolescent girls with a special emphasis on milk, dairy products, and nutrients likely to have an impact on bone mass. Dietary intake was assessed via 7-day food recalls. DXA was used to assess lumbar spine outcomes.	Sex: female Age: 12–22 y Race: Caucasian Location: France, otherwise unspecified Year(s): not specified	192	Data are shown for the postmenarchal group only (n = 142)	Protein from milk			Protein from other foods		
					Adj R^2	$ß_{stand}$	P	Adj R^2	$ß_{stand}$	P
				Lumbar spine						
				BMC (g)						
				Absolute	0.61	0.14	<0.01	0.61	<0.01	NS
				Adjusted	0.60	0.13	0.02	0.60	<0.01	NS
				BMD (g/cm^2)						
				Absolute	0.21	0.20	<0.01	0.21	0.01	NS
				Adjusted	0.21	0.18	0.02	0.21	0.01	NS

- Data presented above are from multivariate linear analyses
- Absolute protein intake is expressed in g/d
- Adjusted protein intake for weight, years after menarche, and vertebral area is expressed in g/kg/d
- Girls with milk intakes <55 mL/d had significantly lower BMC compared to girls consuming >260 mL/d

Prospective

Reference	Study Description	Population Description	*N*	Outcome Measures	Results

Alexy et al., 2005 (40)

The aim of this study was to examine the association of long-term protein intake and dietary potential renal acid load with diaphyseal radius bone. In a prospective study design, long-term dietary intakes were calculated from 3-day weighted diet records that were collected yearly over a 4-year period before a one-time pQCT bone scan.

Sex: male and female
Age: 6–18 y
Race: white
Location: Dortmund, Germany
Year(s): 1998–1999 sub-cohort of the DONALD study

N = 229

Data are shown for the overall group (*N* = 229)

	Protein (g/d)			
	ß	$ß_{stand}$	r^2	*P*
Forearm				
Periosteal circumference (mm^2)	0.07	0.17	0.03	<0.01
Cortical area (mm^2)	0.42	0.27	0.04	<0.01
BMC (mg/mm)	0.46	0.26	0.03	<0.01
Polar SSI (mm^3)	1.83	0.29	0.06	<0.01

- Data presented above are results from stepwise multiple regression and after adjustment for age, sex, and energy intake and controlling for forearm muscularity, BMI, growth velocity, and pubertal development
- $ß_{stand}$ is the standardized parameter estimate
- Children with a higher dietary PRAL had significantly less cortical area ($P < 0.05$) and BMC ($P < 0.01$)
- Long-term calcium intake had no significant effect on any bone variable

Bounds et al., 2005 (34)

The aim of this study was to identify factors related to children's bone mineral indexes at age 8 years, and to assess bone mineral indexes in the same children at ages 6 and 8 years. Children's dietary intake were assessed via RD interview from the ages 2-8 years. BMC was assessed at 6 y and 8 y using DXA.

Sex: male and female
Age: 6 y (baseline) and 8 y (follow-up)
Race: white
Location: Knoxville, TN
Year(s): not specified

52

Data are shown for the overall group (*N* = 52)

	Protein intake (g) over ages 2–8 y		
	(M±SD)	*r*	*P*
Protein intake (g)	55 ± 12		
Total body			
BMC at age 8 y		0.37	≤0.05
BMD at age 8 y		0.33	≤0.05
	ß	Partial R^2	*P*
BMC model 1	(+) 2.40	0.08	<0.01
BMD model 1	(+) .001	0.07	0.04

- Data presented above show the Pearson's correlation coefficient (*r*) relating longitudinal protein intake over ages 2– 8 y, representing 27 d of dietary data
- BMC Model 1: significant predictors of BMC included longitudinal protein intake, height, weight and age
- BMD Model 2: the only significant positive predictor of total BMD was longitudinal protein intake
- The only significant negative predictor for BMC and BMD was female sex

Sex: male

176

Correlation with protein intake (g/d)

Chevalley et al., 2014 (38)	The aim of this study was to examine whether consuming protein during childhood led to increased bone outcomes as measured by DXA in healthy prepubertal to mid-late adolescent boys. Protein and calcium intakes were assessed by a frequency questionnaire. Physical activity was assessed by a questionnaire and expressed as physical activity energy expenditure.	Age: 7-15 y Race: white Location: Geneva, Switzerland Year(s): recruitment period September 1999– September 2000, otherwise unspecified		Data are shown for the overall group (N = 179)	*PA<Med; PRO<Med (n=52)*	*PA>Med; PRO<Med (n=38)*	PA<Med; PRO>Med (n=36)	*PA>Med; PRO>Med (n=49)*	*P*
				aBMC (g)					
				FN	4411±795	4405±858	4645±788	5075±894	<0.001
				Total hip	34381±7863	35303±7863	36389±7995	40913±8451	<0.001
				vBMD					
				DT					
				Total	259±44	263±53	276±39	274±41	0.234
				Cort	723±52	733±59	736±53	739±53	0.486
				Trab	196±26	733±59	736±53	739±53	0.070
				• Values are means ± SD • When comparing based on level of PA, participants in the high PA and high PRO group had significantly greater femoral neck and hip BMC than those in the low PA and low PRO group • When PA level was high but PRO intake was low, there was significantly less femoral neck and hip BMC gains than when PA intake was low but protein intake was high • FN, femoral neck; DT, distal tibia; Cort, cortical; trab, trabecular					
Chevalley et al., 2017 (39)	The aim of this study was to further report the tracking over 15 years of the positive impact of protein intake on physical activity from prepuberty to young adulthood on both bone strength and structure in healthy males (DXA and HR-pQCT measures). Protein intakes were assessed by food frequency questionnaires and physical activity was assessed via PA questionnaire.	Sex: male Age: 7-22.5 Location: Geneva, Switzerland Year(s): recruitment period September 1999-September 2000, overwise unspecified	124	Data are shown for the overall group	Correlation with protein intake (g/d)				
					PA < Med; PRO < Med (n=40)	*PA > Med; PRO < Med (n=26)*	PA < Med; PRO > Med (n=24)	*PA > Med; PRO> Med (n=34)*	*P*
				15.2 years					
				Tb.N, mm-1	2.04 ± 0.23	2.00 ± 0.23	2.10 ± 0.25	2.21 ± 0.35	0.015
				Tb.Sp, um	415 ± 0.50	424 ± 57	402 ± 61	385 ± 65	0.044
				CSA, mm2	833 ± 126	854 ± 120	847 ± 111	966 ± 172	0.001
				22.6 years					
				Tb.N, mm-1	2.06 ± 0.26	2.06 ± 0.21	2.18 ± 0.27	2.19 ± 0.40	0.128
				Tb.Sp, um	403 ± 56	403 ± 51	379 ± 64	383 ± 79	0.304
				CSA, mm2	834 ± 131	845 ± 110	821 ± 104	907 ± 166	0.055
				• $P < 0.05$ • Values are means ± SD • When comparing based on level of PA, participants in the high PA and high PRO group had significantly greater femoral neck BMC, femoral neck area, and femoral neck width at age 7.4 years compared to those in the high PA, low protein group • When comparing based on level of PA, participants in the high PA and high PRO group participants had significantly greater femoral neck BMC and femoral neck aBMD at age 15.2 years compared to those in the high PA, low protein group.					

				• When comparing based on level of PA, participants in the high PA and high PRO group participants had significantly greater femoral neck BMC than those in the high PA, low protein group.				
Garcia et al., 2015 (43)	The aim of this study was to examine the associations between dietary acid load at the age of 1 and 2 years and bone health outcomes, as measured by DXA at age 6 years. Protein intakes were assessed by a food frequency questionnaire	Sex: males and females Age: 1-6 Location: Rotterdam, Netherlands Year(s): April 2002-January 2006, otherwise unspecified	2850	Data are shown for the overall group Protein intake below the mean <. 42 g/d (*n*=1505)	BMD	BMC	aBMC	Bone Area
				dPRAL	0.08 (-0.22, 0.38)	0.10 (-0.29, 0.49)	0.11 (-0.18, 0.40)	-0.01 (-0.46, 0.43)
				Tertile 1	Reference	Reference	Reference	Reference
				Tertile 2	1.11 (-3.02, 5.23)	-0.62 (-5.93, 4.70)	1.69 (-2.29, 5.67)	-2.98 (-9.05, 3.09)
				Tertile 3	3.75 (-1.33, 8.84)	5.37 (-1.19, 11.92)	3.02 (-1.89, 7.92)	3.04 (-4.44, 10.51)
				P-trend	0.16	0.19	0.20	0.68
				Protein intake above the mean > 42 g/d (*n*=1345)				
				dPRAL	-0.04 (-0.33, 0.24)	-0.07 (-0.47, 0.33)	-0.04 (-0.28, 0.27)	-0.09 (-0.52, 0.35)
				Tertile 1	Reference	Reference	Reference	Reference
				Tertile 2	-3.79 (-9.31, 1.75)	-5.47 (-13.20, 2.26)	-2.06 (-7.42, 3.30)	-4.40 (-6.67, 2.14)
				Tertile 3	-2.86 (-7.93, 2.21)	-6.58 (-13.66, 0.50)	-0.89 (-5.80, 4.03)	-7.36 (-15.01, 0.30)
				P-trend	0.38	0.09	0.86	0.06
				• Significant at $P < 0.05$ • Confidence intervals are in parentheses • Adjusted for child sex, age at DXA< height at DXA, and ethnicity				
Moore et al., 2008 (37)	The aim of this study was to evaluate the effects of average dairy intake throughout childhood on adolescent bone mineral content, area, and density. Dietary intake was assessed by means of multiple sets of three-day food diaries. Bone outcomes were measured via DXA.	Sex: male and female Age: 3-5 y at baseline; 15-17 y at bone measurement Race: Caucasian Location: Framingham, Massachusetts	106	Data are shown for the overall group (*N* = 106)				

	n	Arms	Legs	Trunk	Ribs	Pelvis	Spine
		mean ± SD					
		Bone Mineral Content (gm)					
Dairy ≥ 2 servings	49	334.5 ±6.8	1089.9 ±13.6	1069.3 ±16.7	403.1 ±8.4	399.1 ±6.8	267.1 ±5.1

		Year(s): 1987-1999		Dairy < 2 servings	35	309.7 ±8.4	1042.0 ±16.9	982.4 ± 20.7	361.5 ±10.4	368.3 ±8.4	252.6 ±6.3
				p-value		*P*=.05	*P*=0.05	*P*<.01	*P*<.01	*P*=.01	*P*=.11
								Bone Area (cm2)			
				Dairy ≥ 2 servings	49	365.2 ±4.8	842.4 ±5.5	1037.7 ± 9.9	501.8 ±6.6	315.3 ±3.8	220.4 ±3.3
				Dairy < 2 servings	35	354.9 ±5.9	836.1 ±6.9	990.8 ±12.3	469.2 ±8.2	304.7 ±4.7	216.9 ±4.0
				p-value		*P*=.22	*P*=.52	*P*<.01	*P*<.01	*P*=.11	*P*=.55
				• All models are adjusted for gender, physical activity, age, height, BMI and percent body fat at the time of the bone scan (15-17 y) • Higher intakes of meats/other proteins (≥ 4 servings per/day) were also associated with higher mean BMC and BA values. • Children with higher intakes of both dairy and meats/other proteins had the highest adjusted BMC, and children consuming less of each had the lowest BMC.						
Remer et al., 2011 (42)	The aim of this study was to examine the association of long-term protein intake and dietary potential renal acid load with diaphyseal radial bone in a sample of healthy children and adolescents. Data were collected in 197 healthy children during the 4 y preceding proximal forearm bone analyses by pQCT	Sex: male and female Age: 6–18 y Race: white Location: Dortmund, Germany Year(s): 1998–1999 subcohort of the DONALD study	197	Data are shown for the overall group (*N* = 197)		Urinary uN		Urinary PRAL			
						ß	*P*	ß	*P*		
				Diaphyseal Bone Variables							
				BMC (mg/mm) [log 10]		0.03	<0.01	–0.02	0.03		
				Cortical area (mm^2) [log 10]		0.02	<0.01	–0.02	0.03		
				Polar SSI (mm^3) [log 10]		0.02	<0.01	–0.01	NS		
				Periosteal circumference (mm)		0.50	0.03	0.02	NS		
				BMD (mg/cm^3)		5.40	NS	–8.70	NS		
				• Data presented above are from multivariate regression models showing independent associations of both long-term protein intake (as uN) and PRAL as explanatory variables with forearm bone variables. Urinary uN and PRAL were adjusted for age, sex, pubertal stage, forearm muscle area, forearm length, and urinary calcium							
Vatanparast et al., 2007 (36)	The aim of this mixed-longitudinal study was to investigate the influence of protein intake on bone mass measures in young adults, considering the influence of calcium intake through adolescence. Dietary intake was assessed via 24-h recalls carried out at least once yearly. DXA was used to assess total body BMC and BMD.	Sex: male and female Age: 8–21 y during phase I of the study; 17–29 y for phase II Race: majority Caucasian Location: Saskatoon, Saskatchewan, Canada Year(s): 1991–1997 (phase I); 2003–2006 (phase II); participating in the Saskatchewan Pediatric Bone	133	Data are shown for the overall group (*N* = 133) and a subgroup (*n* = 44)		Protein intake (g)					
						Regression coefficient		Partial R^2		*P*	
				Total body (*N* = 133)							
				BMC		NS		—		NS	
				BMC net gain		0.11		0.21		0.02	
				Total body (*n* = 44)							
				BMC		0.21		0.33		0.04	
				BMC net gain		0.21		0.37		0.02	
				The net gain of TB-BMC and the net gains in height and weight from age of peak height velocity to early adulthood were entered into the model. Sex, current height, weight, physical activity level, protein intake, vegetable and fruit							

		Mineral Accrual Study		intake, periadolescence intakes of vegetables and fruits, protein, and physical activity were entered into the multiple regression model (stepwise). Protein intake predicted total body BMC net gain in all subjects. In females at periadolescence or early adulthood with adequate calcium intake (>1000 mg/d) (n = 44), protein intake positively predicted total body BMC and BMC net gain
Zhang et al., 2008 (35)	The aim of this study was to assess the association between protein intakes and bone mass accrual in girls who participated in a 5-y study including 2 y of milk supplementation (intervention groups only) and 3 y of follow-up study. Dietary intakes were assessed by 7-day food records and bone outcomes were assessed by DXA.	Sex: female Mean age: 10.1 y Race: Chinese Location: Beijing Year(s): 1999–2004	757	Data are shown for the overall group (N = 757) — Average protein intake ß — P value Total body Bone area — —; NS BMC — –1.92; 0.02 Proximal forearm Bone area — –9.11; <0.01 BMC — –10.2; <0.01 Distal forearm Bone area — —; NS BMC — –4.82; <0.01 • Data presented above (ß) represent the percentage change in the dependent variable associated with intake of protein after controlling for baseline bone mass and pubertal development, age and physical activity, survey time, group, and clustering by schools. Protein, among other nutrients, was included in an initial model and flowed by backward elimination with $P < 0.01$ as the standard for retention, excluded by the regression model • When protein intake was considered according to animal or plant food sources, protein from animal foods, particularly meat, had significant negative effects on BMC accrual at the proximal and distal forearm ($P < 0.05$)

Randomized Controlled Trials

Reference	Study Description	Population Description	*N*	Outcome Measures	Results	
Ballard et al., 2006 (44)	The aim of this study was to determine if 6 months of protein supplementation in conjunction with a strength and conditioning training program improves areal and volumetric bone mineral density. pQCT was used to assess the 4% and 20% sites of the distal tibia. 3-day food records were used to determine typical dietary intakes.	Sex: male and female Age: 18–25 y Race: not specified Location: South Dakota, USA Year(s): not specified	68	Data are shown for the protein supplemented group (n = 36)	Mean change, protein group (n = 36)	P
				4% site		
				Total vBMD (mg/cm^3)	0.20	NS
				Trabecular vBMD (mg/cm^3)	–0.50	NS
				Total area (cm^2)	5.0	NS
				20% site		
				Cortical vBMD (mg/cm^3)	2.4	NS
				Cortical area (cm^2)	1.7	NS
				Cortical thickness (mm)	0.05	NS
				Periosteal circumference (mm)	–0.20	NS
				Endosteal circumference (mm)	–0.50	NS
				Polar SSI (mm^3)	57	NS
				Total body		
				BMC (g)	–3.5	NS

Bone area (cm^2)	–3.9	NS
Leg		
BMC (g)	1.3	NS
Arm		
BMC (g)	5.7	NS

Data presented above are least-squares means determined by ANCOVA while controlling for initial height and weight and baseline bone value

Chapter 6

SUMMARY AND CONCLUSIONS

This work was conducted to determine associations egg intake, dietary and serum inflammation, and dietary protein in otherwise healthy boys and girls. The work generated from this dissertation provided preliminary data for the funding of the SCENE (Skeletal and Cognitive Effects of Nutrition from Eggs) Study that is currently being conducted in the Bone and Body Composition Lab at the University of Georgia.

The study presented in Chapter 3 was conducted with the primary objective to determine the relationship between whole egg intake and cortical bone parameters, total body bone outcomes, and biomarkers of bone turnover in children entering the early stages of puberty. The secondary aim was to determine if FFST has a mediating role on the relationship between whole egg intake and bone. Egg intake was positively correlated with radius and tibia Ct.BMC, total bone area, cortical area, cortical thickness, periosteal circumference, and polar strength strain index in the unadjusted models (r=0.14-0.22, all $P<.05$). After adjusting for race, sex, stage of sexual maturation, FFST, and protein intakes, tibia relationships were nullified; however, egg intake remained positively correlated with radius Ct.BMC (r=.14, $P=0.03$). After adjusting for the covariates, egg intake was a positive predictor of radius FFST ($\beta = 0.11$, $P<0.05$) and FFST was a positive predictor of Ct.BMC ($\beta=0.56$, $P<0.05$) in path analyses. There was a direct influence of egg on radius Ct.BMC ($\beta=0.10$, $P=0.04$), even after adjusting for the mediator, FFST ($\beta=0.14$, $P=0.02$). Egg intake was positively correlated with osteocalcin

in both the unadjusted (P=<0.05) and adjusted (P=<0.05) models. With the expectation of dairy foods (1-4) limited research exists regarding the relationship between whole food consumption and bone outcomes in children. The study presented in Chapter 3 is the first study to assess the relationship between whole egg intake and cortical bone outcomes in children and provides novel evidence of a positive link between whole egg consumption and cortical bone in children.

The study presented in Chapter 4 aimed to assess the relationships between inflammatory dietary patterns, as represented by DII scores, and serum inflammation on cortical bone outcomes in healthy boys and girls. DII-scores were negatively associated with tibia trabecular area (TtAr; r= −.14, P=0.02), periosteal perimeter (PsPM; r= −.15, P=0.02), endosteal perimeter (r= −.15, P=0.02), strength strain index (SSI; r= −.13, P=0.03), and radius TtAr (r= −.14, P=0.02), PsPM (r= −.138, P=0.03) and SSI (r= −.13, P=0.04) but nullified when adjusting for covariates. Tibia PsPM was higher in the low DII group compared to the medium (P=0.05) and high (P<0.05) groups but nullified after controlling for covariates. DII-scores were not associated with TNF-α, VEGF, or IL-6, but were associated with MCP-1 only in the unadjusted model (r=.13, P=0.04). In the adjusted model, MCP-1 was inversely associated with tibia cortical thickness (r= −.15 P=0.03). These results suggest that based on the food parameters we were able to generate with our dietary software it would be premature to use the DII to determine the inflammatory potential of the diet in healthy children. Future studies should consider using additional dietary data programs to get a more inclusive analysis of the food parameters included in the DII calculation. Because the findings that the biomarker of inflammation, MCP-1, was negatively associated with tibia CtTh, future prospective

studies should be conducted to better understand the role of serum inflammation on bone quality in youth and to determine if there are long-term fracture implications in adulthood.

In children and adolescents, there are few published reports addressing the role of protein on bone. Chapter 5 was a systematic review examining the relationship between dietary protein intake and pediatric bone health. Overall, the systematic review demonstrated limited (grade C) evidence that dietary protein intake during childhood is beneficial for bone mass and strength. A grade C was given due to there being only one randomized controlled trial of protein intake on pediatric bone and this intervention study was in conjunction with an exercise intervention (6). To date, there are no published intervention studies investigating the role of protein independently on bone in healthy children and adolescents. A systematic review investigating the role of dietary protein over the past 40 years indicates that future large-scale intervention studies, particularly in understudied population subgroups such as children and adolescents is a high research priority (7).

The findings from this dissertation provided preliminary data to support the funding of the SCENE study, a 9-month randomized controlled trial in normal weight and obese children. The primary aim of the SCENE study is to determine if consumption of a formulated whole egg snack replacement over 9 months augments cortical bone strength more so than milk powder or placebo during pubertal growth. The secondary aim of the SCENE study is to determine if consumption of a formulated whole egg snack replacement over 9 months augments memory and executive functioning during pubertal growth compared to milk powder or placebo. The final aim of the SCENE study is to test

the intermediary role (i.e., mediation) of inflammation on the effect of egg product consumption on changes in cortical bone strength, as well as memory and executive function. This innovative project will advance our knowledge about egg nutrition, bone and cognitive health, and inflammation in a pediatric population, and determine the efficacy of a novel nutrition strategy to improve childhood bone health, reduce the risk of osteoporosis and enhance cognitive function. If successful, this intervention could be readily disseminated to children in the home, via school nutrition breakfast and lunch programs, or via other avenues within communities throughout Georgia, the nation and worldwide. Additionally, results from this study could also significantly benefit egg farms and agriculture in Georgia and the United States. We have completed study enrollment (N=182) and are currently conducting 4.5-month and 9-month testing sessions. **Table 6.1** shows our participant characteristics and our study consort diagram can be seen in **Figure 6.1.**

In summary, the results of the studies in chapters 3, 4, and 5 provide insight into the interrelationships of egg intake, dietary and serum inflammation, and protein consumption on pediatric bone strength. These are the first data to investigate the role of egg and protein intake and dietary inflammation on bone health outcomes in healthy boys and girls. Given the cross-sectional design in chapters 3 and 4, we cannot confirm causality when linking egg intake and bone and the dietary inflammatory index and biomarkers of inflammation on bone. The systematic review in chapter 5 demonstrated limited (grade C) evidence that dietary protein intake during childhood is beneficial for bone growth and development. A limited grade was given due to the lack of dietary protein interventions in children and adolescents.

References

1. Okubo H, Sasaki S, Horiguchi H, Oguma E, Miyamoto K, Hosoi Y, Kim MK, Kayama F. Dietary patterns associated with bone mineral density in premenopausal Japanese farmwomen. Am J Clin Nutr 2006;83(5):1185-92. doi: 10.1093/ajcn/83.5.1185.

2. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. Am J Clin Nutr 2009;89(1):161-8. doi: 10.3945/ajcn.2008.26401.

3. Hoppe C, Udam TR, Lauritzen L, Molgaard C, Juul A, Michaelsen KF. Animal protein intake, serum insulin-like growth factor I, and growth in healthy 2.5-y-old Danish children. Am J Clin Nutr 2004;80(2):447-52. doi: 10.1093/ajcn/80.2.447.

4. Iuliano-Burns S, Saxon L, Naughton G, Gibbons K, Bass SL. Regional specificity of exercise and calcium during skeletal growth in girls: a randomized controlled trial. J Bone Miner Res 2003;18(1):156-62. doi: 10.1359/jbmr.2003.18.1.156.

5. Shams-White MM, Chung M, Du M, Fu Z, Insogna KL, Karlsen MC, LeBoff MS, Shapses SA, Sackey J, Wallace TC, et al. Dietary protein and bone health: a systematic review and meta-analysis from the National Osteoporosis Foundation. Am J Clin Nutr 2017;105(6):1528-43. doi: 10.3945/ajcn.116.145110.

6. Ballard TL, Specker BL, Binkley TL, Vukovich MD. Effect of protein supplementation during a 6-month strength and conditioning program on areal and volumetric bone parameters. Bone 2006;38(6):898-904. doi: 10.1016/j.bone.2005.10.020.

7. Darling AL, Manders RJF, Sahni S, Zhu K, Hewitt CE, Prince RL, Millward DJ, Lanham-New SA. Dietary protein and bone health across the life-course: an updated systematic review and meta-analysis over 40 years. Osteoporos Int 2019;30(4):741-61. doi: 10.1007/s00198-019-04933-8.

Table 6.1. Enrollment by sex and race

Ethnicity	Male	Female
White	67	45
Black	7	14
White/black	4	4
Hispanic	21	10
Asian/other	7	3
Total	106	76

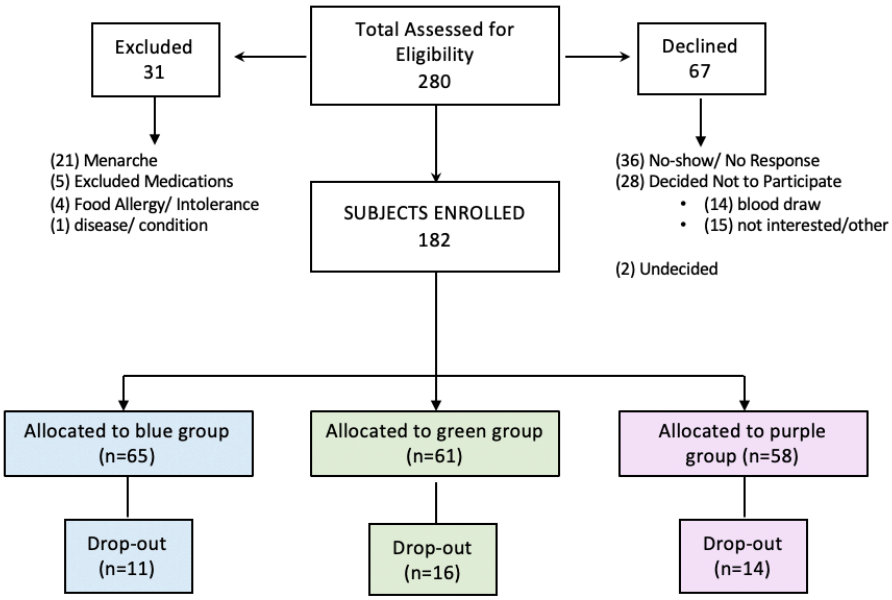


Figure 6.1. SCENE study consort diagram

APPENDICES I

Supplemental vitamin D in early adolescence

ASSENT FORM (CHILD)

PARENTAL PERMISSION FORM

HEALTH HISOTRY QUESTIONNAIRE

SEXUAL MATURATION QUESTIONNAIRES

APPENDIX A

ASSENT FORM (CHILD)

Assent Form (Child)

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

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

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

Before entering the study:

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

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

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

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

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

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

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

Sincerely,

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

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

_____ _____
Signature Date

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

_____ _____
Signature Date

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

_____
Signature of the Participant/Date

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

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

APPENDIX B

PARENTAL PERMISSION

PARENTAL PERMISSION FORM

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

_____ _____
Signature Date

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

_____ _____
Signature Date

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

Richard Lewis/Emma Laing _____ _____

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

_____ _____

Name of Parent or Guardian Signature Date

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

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

APPENDIX C

HEALTH HISTORY QUESTIONNAIRE

Supplemental Vitamin D in Early Adolescence

Health History Questionnaire

Subject ID#_____
Interviewer _____
Date _____

Surgery/Medication/Fracture History

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

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

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

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

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

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

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

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

Other History

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

   b. How long have you been using them? _____

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

8. Are you on any nutritional supplements? _____

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

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

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

APPENDIX D

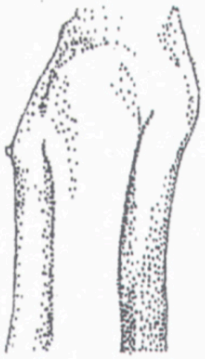
SEXUAL MATURATION QUESTIONNAIRES

Lewis, Richard D.

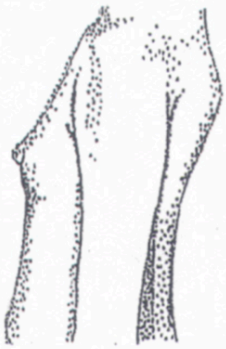
SEXUAL MATURATION QUESTIONNAIRE (GIRLS)

Subject ID#: _____
Date: _____

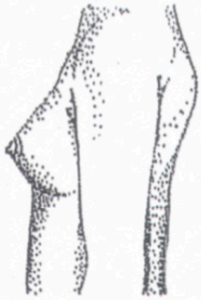
We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



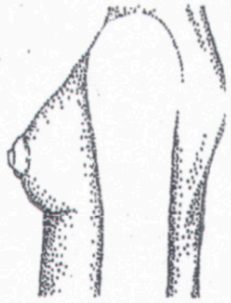
Stage 1: Elevation of papilla only.



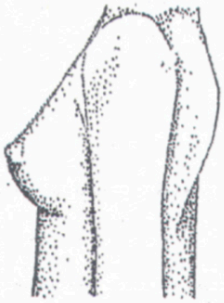
Stage 2: Elevation of breast and papilla as small mound, areola diameter enlarged.



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



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



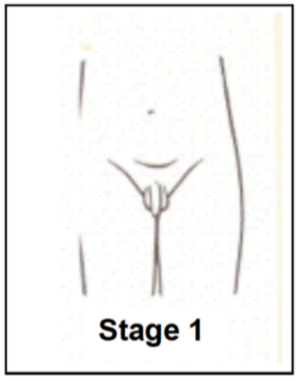
Stage 5: Recession of areola to contour of breast.

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

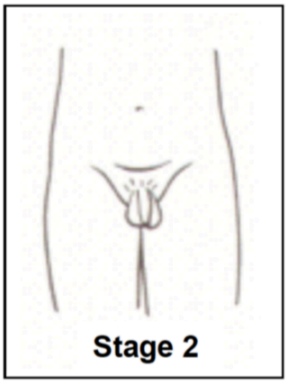
J. Tanner (1962) Growth and Adolescence, 2nd ed. Blackwell Scientific Publications, Oxford.

Sexual Maturation Questionnaire

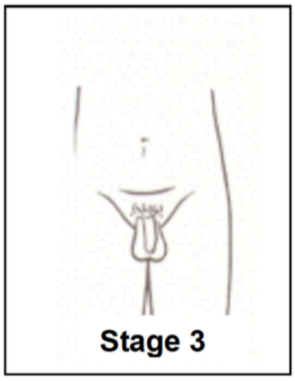
We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



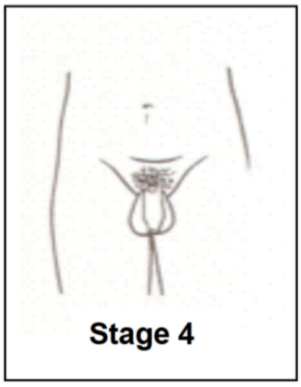
STAGE 1: The penis, scrotum, and testes are of the same size and proportion as in early childhood.



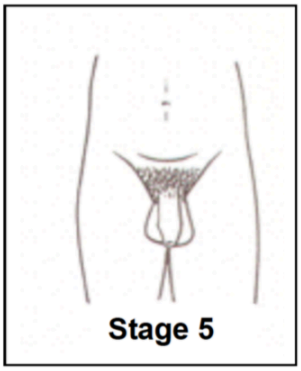
STAGE 2: The scrotum and testes have enlarged. The size of each testis can be judged by looking at the scrotum and also by feeling each testis through the skin of the scrotum. The skin of the scrotum becomes thinner, wrinkled and slightly red but this is difficult to see in a photograph. There is little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



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



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



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