# ASSOCIATIONS OF ADENOVIRUS 36 INFECTION WITH INFLAMMATION, ADIPOSITY, AND BONE STRENGTH

by

PAIGE KIMBERLY BERGER

(Under the Direction of RICHARD D. LEWIS)

## ABSTRACT

Though animal and cell culture studies suggest that adenovirus 36 is linked to inflammation, obesity, and osteoporosis, human data are scant. The purpose of this research was to determine associations of Ad36 seropositivity [Ad36(+)], inflammatory-related markers, adiposity, and bone strength in children. The first study (Chapter 3) addressed relationships of Ad36(+), serum inflammatory-related markers, and fat mass, assessed by dual-energy X-ray absorptiometry, in 291 children ages 9-13 years. There was a higher prevalence of Ad36(+) in the highest tertiles of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) compared to their respective middle and lowest tertiles (both $P<0.03$). There was also a trend toward a higher prevalence of Ad36(+) in the highest tertile of vascular endothelial growth factor (VEGF) compared to tertiles 1 and 2 ($P=0.05$). Multinomial logistic regression, adjusting for age, race, sex, and fat-free soft tissue (FFST) mass, revealed that compared to participants with the lowest concentrations of TNF-α, IL-6, and VEGF (tertile 1), the adjusted odds ratios for Ad36(+) were 2.2 (95% CI: 1.2-4.0), 2.4 (95% CI: 1.4-4.0), and 1.8 (95% CI: 1.0-3.3), respectively, for those in the highest concentrations of TNF-α, IL-6, and VEGF (tertile 3). No association was observed between Ad36(+) and fat mass. These data suggest that Ad36(+) is associated with biomarkers implicated

in inflammation, but not with greater fat mass. The second study (Chapter 4) addressed relationships of Ad36(+), bone strength, assessed by peripheral quantitative computed tomography, and serum inflammatory-related markers in 78 obese females ages 9-12 years. After adjusting for age, race, fat mass, and FFST mass, Ad36(+) participants had lower radial strength-strain index (SSI) than Ad36-seronegative [Ad36(-)] participants (P=0.05). Ad36(+) participants had higher concentrations of TNF- $\alpha$ and VEGF versus Ad36(-) participants (both P<0.05). Radial SSI did not correlate with serum TNF- $\alpha$ and VEGF, suggesting that lower cortical bone strength is not attributed to higher concentrations of inflammatory-related markers observed in Ad36(+) obese females. This research supports associations of Ad36(+) with biomarkers implicated in inflammation and with cortical bone strength, but not with adiposity. Prospective studies are needed to determine the effects of Ad36(+) exposure in childhood on long-term health.

INDEX WORDS: ADENOVIRUS, INFLAMMATION, ADIPOSITY, OBESITY, BODY COMPOSITION, BONE STRENGTH, CHILDREN, PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

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by

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# DEDICATION

This thesis is dedicated to Andrea and Edward Berger for their unconditional love, support, and encouragement. It is also dedicated to Morris Berger and Robert Lieberman, whose hard work and commitment to our family made this possible.

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# CHAPTER 1

## INTRODUCTION

The topic of my dissertation research evolved from my contributions to a NIH-funded (R01) clinical trial, and a subsequent partnership formed through The University of Georgia Obesity Initiative. In 2010, I served as a member of the research team responsible for a multi-site (UGA, Purdue University, and Indiana University), randomized, controlled, vitamin D supplementation trial. In this study, 320 children between the ages of 9 to 13 years (50% female, 49% black/African American) participated. Data included measures of adiposity, bone strength, and biochemical markers, presenting the research team with the opportunity to investigate a host of health outcomes in a diverse group of children. During this time, Dr. Richard Lewis of the Department of Foods and Nutrition and Dr. Ralph Tripp of the Department of Infectious Diseases collaborated on a publication that identified a virus as a possible influence of obesity and osteoporosis in late adolescent females. Based on these findings, as well as access to serum samples from the vitamin D trial, I developed a project to determine the association of adenovirus 36 infection with inflammation, adiposity, and bone strength in young children, an area that had not been researched to date.

Childhood is a period of rapid growth, a time of both opportunity and vulnerability for optimizing bone and body composition development and diminishing risk for chronic disease. Indeed, childhood obesity, which affects one-third of 6 to 19 year-olds in the United States (U.S.), is a predisposing factor for adult obesity, as well as heart disease, type 2 diabetes, stroke, and some cancers (1, 2). While obesity poses a serious health burden to children and adults, it is

also a major financial burden. The lifetime cost of medical care for an obese child is approximately $19,000 more than that of a normal weight child (3), and the direct costs associated with overall obesity in the U.S. amounted to $147 billion in 2008 alone (4). Taken together, these findings show obesity as a major public health concern.

In addition to obesity, osteoporosis is also a serious national health problem that may have its origins in childhood. Osteoporosis is characterized by decreased bone mass, quality, and strength and increased susceptibility to skeletal fractures with age. Because 90% of adult bone mineral content (BMC) is achieved by the end of adolescence (5), the preceding years of rapid accretion are important for augmenting peak bone mass and presumably diminishing risk for osteoporotic fractures over the long-term. It is important to identify early life influences of suboptimal bone development and osteoporosis, a disease that affects 10 million men and women in the U.S. (6) and incurs an estimated $17 billion in medical expenses (7).

Though obesity and osteoporosis are two separate chronic health conditions, the parallels between fat and bone support the potential for overlapping etiologies (8, 9). One such influence is infection with adenovirus 36 (Ad36) (10-15), a common upper respiratory infection transmitted through person-to-person contact that elicits an antibody response ranging from days to years post-exposure (16). Ad36 has been implicated in obesity development through enhanced adipocyte differentiation in human-derived cells (17-19). It has been postulated that this may come at the expense of bone development given that adipocytes (fat-storing cells) and osteoblasts (bone-forming cells) share a mesenchymal progenitor (8, 9, 15). Though human data also support an association between Ad36 infection and obesity in children, findings are scant and equivocal (13, 20). Moreover, only one study to date has examined the relationship between Ad36 infection and bone strength in humans. Laing et al. (15) found that obese females who

tested positive for Ad36 antibodies exhibited lower cortical bone strength versus those who tested negative. While these findings support a potential link between Ad36 infection and adiposity and bone, more research is warranted. Additional studies have the potential to not only corroborate earlier findings, but may also help uncover underlying mechanisms.

It is possible that systemic inflammation, a common underpinning factor of viral infections, obesity, and osteoporosis, may provide insight into the associations of Ad36 with adiposity and bone (19, 21-25). Ad36 has been reported to induce cellular activation of an inflammatory pathway known as nuclear factor kappa B (NFkB). This signals the production of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1). These inflammatory-related factors have been shown to increase adipose tissue growth and bone degradation in vitro (19, 26-29), and were positively associated with body fatness and risk of bone fracture in vivo (22, 24, 25). While associations of Ad36 infection with inflammation, adiposity, and bone strength have been observed separately, no studies have examined these relationships concurrently in humans.

The purpose of this research was to determine associations of the human Ad36 infection, inflammation, adiposity, and bone strength in a cohort of otherwise healthy children. The first study presented in Chapter 3 addresses the relationships of Ad36 infection with total body fat mass, assessed by dual-energy X-ray absorptiometry, and serum inflammatory-related markers in 291 males and females ages 9 to 13 years. The second study presented in Chapter 4 addresses the relationships of Ad36 infection, bone strength, assessed by peripheral quantitative computed tomography at the radius and tibia, and serum inflammatory-related markers in a subsample of obese females ages 9 to 12 years.

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# CHAPTER 2

# REVIEW OF THE LITERATURE

## Bone

Bone is a dynamic tissue that must adapt to the stresses and strains of everyday living to support mechanical loading, metabolic demands, and mineral homeostasis. Bone is a composite of material properties (which determine bone mass) and structural properties (which dictate bone strength). Material properties of bone are made up of organic (20-25% by weight) and inorganic (70% by weight) components and water (5% by weight) (1). Organic components include collagen and non-collagenous proteins, which are produced by bone cells. Collagen is the major structural component of the bone matrix, and each molecule of collagen lies parallel to the next to form a collagen fibril. Small gaps between these molecules permit biological activities, such as mineralization (1).

Non-collagenous proteins, such as osteocalcin, osteonectin, integrins, and growth factors [i.e., transforming growth factor-beta (TGF-beta), insulin-like growth factor-I (IGF-I), and bone morphogenetic protein (BMP)] play an important role in bone's biological activities (1, 2). Osteocalcin is produced by osteoblasts (bone-forming cells), and is traditionally recognized as a late marker of osteoblast activity and bone formation. It is associated with recruitment of osteoclasts (bone-resorbing cells) to sites of bone resorption, and reflects matrix mineralization (3). Similarly, osteonectin is produced by osteoblasts, and is associated with adhesion of osteoclasts to bone surfaces (1). Integrins are a family of proteins that anchor bone cells and fibroblasts to the bone matrix, providing an interface between the cytoskeleton of cells and the

extracellular matrix (1). Finally, growth factors, bound to both bone mineral and bone matrix, are released during bone breakdown. Growth factors regulate cellular activation and differentiation of bone cells, and serve as coupling factors for bone formation and bone resorption. More specifically, TGF-beta and BMP may exhibit anti-resoprtive actions by stimulating osteoblast expression of the decoy receptor, osteoprotegerin (OPG), to inhibit osteoclast activation (1). IGF-I, which is produced primarily by the liver, is also a product of pre-osteoblasts and osteoblasts in response to growth hormone and parathyroid hormone (PTH) (4). It has been identified as a chemoattractant factor: free IGF-I activates the cellular pathway PI3K/PDK-1/Akt (5), which stimulates recruitment and maturation of osteoblasts, triggering the synthesis of bone collagen and matrix (6).

As briefly described above, many of the organic components of bone that help facilitate biological processes are the products of bone cells. Osteoblasts, osteocytes, and osteoclasts are the key regulators of bone metabolism (1). Osteoblasts are known as bone-forming cells, and they produce the osteoid (made of collagen) that forms the bone matrix. Osteoblast activity is influenced by endocrine (e.g., PTH, vitamin D and estrogen), paracrine, and autocrine (e.g., TGF-beta and IGF-I) factors (1, 7). Osteocytes are former osteoblasts that form a network of cells embedded in the bone matrix and facilitate communication in response to stimuli (1). Lastly, osteoclasts are known as bone-resorbing cells, which break down old bone tissue through production of lysosomal enzymes that degrade bone surfaces. Osteoclast activity is mediated by osteoblast-induced cytokines, receptor activator of nuclear factor kappa-B ligand (RANKL), and OPG (1, 8). When a pro-resorptive factor, such as PTH, binds to its receptor PTH1R (GPCR) on

osteoblasts, RANKL expression is induced. RANKL binds to RANK on osteoclast precursor cells, stimulating osteoclast differentiation and maturation [in the presence of macrophage colony-stimulating factor (M-CSF)], and results in acidification of hydroxyapatite on the osteoid matrix (9).

Hydroxyapatite [Ca10(PO4)6(OH)2] is the inorganic component of bone (mineral) (2). It is a combination of calcium and phosphorus crystals in and around collagen that are released into circulation following osteoclast-induced bone degradation. When osteoclast-induced bone resorption is immediately followed by osteoblast-induced bone formation (coupling), the process is known as bone remodeling (1). The function of bone remodeling is to replace damaged bone tissue with new tissue in the adaptive skeleton. This process is dominant in the non-growing skeleton (adulthood): bone resorption equals bone formation until the fourth or fifth decade of life when osteoclast activity exceeds osteoblast activity, resulting in net bone loss. When osteoclast-induced bone resorption is not coupled with osteoblast-induced bone formation, the process is known as bone modeling. This process is dominant in the growing skeleton (childhood and adolescence): osteoblast activity exceeds osteoclast activity, resulting in greater mass and diameter (1).

Bone is also characterized by its structural properties, such as size, shape, and architecture. Bone structure can be described in terms of its macro-architecture and micro-architecture. Macro-architecture refers to the axial and appendicular skeletons. The axial skeleton includes vertebrae, the skull, and flat bones, while the appendicular skeleton includes long bones, such as the radius and the tibia. Long bones consist of two wide extremities (epiphyses), a shaft between the epiphyses (diaphysis), and a zone between the epiphysis and the diaphysis (metaphysis). Each of these compartments is made up of bone tissue that forms the

micro-architecture: the epiphyses are predominantly trabecular bone while the diaphysis is predominantly cortical bone (1). Cortical bone provides structure: the inner surface facing bone marrow is the endosteum while the outer surface facing soft tissue is the periosteum. Trabecular bone is more metabolically active: it interacts with bone marrow, blood vessels, and connective tissue (1, 2, 10).

The properties of bone may be measured using non-invasive imaging technology employed in both clinical practice and research settings. Such technologies provide valuable information regarding the determinants of bone strength. Dual-energy X-ray absorptiometry (DXA) is a three-component imaging technique routinely used in the diagnosis of osteoporosis (11). It is able to perform site-specific bone scans to measure bone mineral content (BMC) and areal bone mineral density (aBMD). It is also provides measurements of body composition, such as fat mass and fat-free soft tissue (FFST) mass, which are determinants of bone material properties. Other advantages of DXA include low level of radiation exposure (equal to $1/10^{th}$ of a chest X-ray) (12), widespread availability, and a relatively short scanning time. However, with the advent of more advanced imaging technology, the use of DXA in research has come under scrutiny. DXA provides a two-dimensional estimate of bone's three-dimensional structure: aBMD captures bone length and breadth, but not depth (13). DXA measurements are also restricted to the material properties of bone: it does not provide information on architecture, and assumes that bone size is kept constant regardless of the individual being scanned (i.e., aBMD decreases as bone size increases) (13).

Because of the limitations of DXA, the use of peripheral quantitative computed tomography (pQCT) has been popularized in bone research. Peripheral QCT is a three-dimensional imaging technology that captures scans of the appendicular skeleton (i.e., radius and

tibia). Unlike DXA, pQCT is able to assess both material and structural properties of bone, and provides a true measurement of bone density [captures depth; volumetric bone mineral density (vBMD)]. In addition, pQCT differentiates between trabecular bone and cortical bone, and provides measurements that may be used to calculate muscle strength (14, 15). The pQCT scanner must be positioned at pre-measured distances from a reference point (i.e., endplate or growth plate) in order to capture compartments of the appendicular skeleton that are predominantly composed of trabecular bone (metaphyseal site, 4% or 8%) or cortical bone (diaphyseal site, 14%, 20%, 33%, or 66%), or to allow for determination of muscle density (diaphyseal site, 66%) (15-17). The most commonly used pQCT outcome measures are presented in **Table 2.1**, which has been adapted from Pollock (18) and Zemel et al. (15). These measures reflect BMC, BMD, and structural and geometrical indices that may be acquired at the metaphyseal and diaphyseal sites of both the radius and tibia.

As mentioned previously, bone responds to the mechanical and biological demands of everyday living through material and structural adaptations. Influences of bone health include physical activity as well as non-physical activity determinants, including age, race, sex, and soft tissue. To date, physical activity is the most widely studied influence of bone health. Frost's Mechanostat Theory, a refinement of Wolff's Law (19), postulates that bone adapts to the dynamic forces of muscle contraction by increasing mass and strength (20). High-impact activity not only imposes mechanical loads on the skeleton, but has also been shown to apply ground reaction forces of up to five and 12 times body weight in elementary school children and elite athletes (i.e., gymnasts), respectively (21, 22). This stimulates adaptations in bone modeling and remodeling to ensure that bone can withstand the physical demands it is placed under. Of the high-impact activities that have been examined with respect to bone, research in artistic

gymnasts suggests that the anabolic effects of high-impact activity largely occur in early childhood and accumulate. Nickols-Richardson et al. (23) found that pre-menarcheal artistic gymnasts exhibited significantly higher aBMD versus age-, height-, and weight-matched controls, and these differences were comparable to those observed in college gymnasts versus controls (24). In addition, studies of artistic gymnasts support the residual benefits of high-impact activity in youth on bone health in adulthood (25, 26). Pollock et al. (26) found that former college gymnasts (age 45 years), retired for 25 years, still exhibited significantly higher aBMD of the lumbar spine, femoral neck, proximal femur, and total body compared to controls. Together, these findings support the osteogenic potential of high-impact physical activity beginning in early childhood and maintained in late adulthood.

Age and sexual maturation are interrelated determinants of bone, particularly in early childhood. The onset of puberty generally occurs at age 12 years in males and age 11 years in females, at which time there is a considerable increase in bone area (attributed to length and width). There is a plateau in bone area followed by a plateau in bone mineralization 1 to 2 years later, and the lag in bone mineralization may account for the high incidence of fractures observed in growth (27). Peak bone mass is achieved as late adolescent males and females approach the second decade of life, and the tempo of bone accrual varies by skeletal site: peak bone mass of the lumbar spine is achieved by ages 19 and 17 years in males and females, respectively, while peak bone mass of the total body is achieved by ages 21 and 19 years (27). In adulthood, males exhibit greater total body BMC compared to females. There are also differences in cortical and trabecular bone between sexes. With respect to cortical bone, females lose more endosteal bone and gain less periosteal bone compared to males, resulting in greater bone porosity with age (1, 28, 29). With respect to trabecular bone, females exhibit a decline in both trabecular number and

trabecular thickness, while males only exhibit a decline in trabecular thickness (1, 30). Differences in cortical and trabecular bone between sexes, particularly with increasing age, may be attributed to sex hormones, namely estrogen. Estrogen maintains bone mass by limiting bone resorption. Estrogen binds to osteoblasts to induce expression of collagen and TGF-beta (material properties) (1, 31). In conjunction with weight-bearing exercise, estrogen sensitizes the bone adjacent to bone marrow, resulting in increased bone mineralization and bone area (1, 32). Because females experience reduced estrogen production with the onset of menopause or with amenorrhea, they experience greater bone loss than males, and trabecular bone is typically more affected than cortical bone. Cann et al. (33) found that females (ages 16 to 49 years) with amenorrhea exhibited significantly lower trabecular bone of the lumbar spine compared to eumennorheic controls.

Research suggests that bone also differs by race. In general, aBMD is highest in non-Hispanic blacks, followed by non-Hispanic whites and Asians (1, 34). It has been suggested that non-Hispanic blacks exhibit higher peak bone mass attainment (35), greater muscle mass (36), higher rates of bone turnover (37, 38), and longer periods of bone formation (39) during young adulthood compared to non-Hispanic whites, all of which are advantageous to bone strength (37). Warden et al. (40) conducted a cross-sectional study in children entering the early stages of puberty to compare measures of cortical bone in black and white participants. Black participants exhibited significantly greater pQCT-derived strength-strain index [measure of torsional strength (SSI)], cortical volumetric BMD, cortical BMC, cortical area, and total area of the tibia compared to white participants. Black participants also exhibited significantly higher markers of

bone formation (osteocalcin and bone alkaline phosphatase) as well as PTH compared to white participants. Therefore, differences in cortical bone between races may be explained by differences in circulating factors, such as osteocalcin, bone alkaline phosphatase, and PTH.

Bone is also affected by dietary intake and nutriture, with much of the research evidence focused on the effects of calcium and vitamin D and the potential to reduce bone loss in older adults (41-43) or augment bone mass in children (44, 45). Calcium, the most abundant mineral in the human body and an integral component of the skeleton, is essential for rigidity, strength, and elasticity of bone tissue (46). Though adequate calcium intake across the lifespan is essential, it is paramount during the growing years in order to support bone modeling (i.e., formation over resorption) and optimal peak bone mass attainment in children (46). Indeed, randomized controlled trials have shown that greater than 500 mg/d calcium supplementation increases bone mineral by approximately 1 to 3% in children entering the early stages of puberty (47-49). The long-term effects of calcium intake on bone, however, are equivocal, as studies suggest that calcium intake may be most influential in the two years of peak skeletal growth, while other determinants supersede later in life (1, 50).

Vitamin D, a lipid-soluble vitamin and steroid hormone acquired through dietary intake and cutaneous synthesis, is required for the regulation of serum calcium concentration and bone mineralization. Vitamin D maintains calcium homeostasis through three established mechanisms: intestinal absorption of calcium, mobilization of calcium from bone, and renal distal tubule reabsorption of calcium (46). Indeed, the importance of vitamin D for optimal bone accretion, proper bone maintenance, and reduced bone loss was recently reaffirmed by the 2010 Institute of Medicine Dietary Reference Intakes for Vitamin D (46), which concluded that there was sufficient research evidence to support a causal link between vitamin D status and bone health.

Research suggests that the effects of vitamin D supplementation on bone in children may be most pronounced in those with baseline vitamin D deficiency [i.e., serum 25-hydroxyvitamin D <30 nmol/L] (46). A 2010 Cochrane review found that children with low baseline concentrations of 25-hydroxyvitamin D exhibited a 2.6% and 1.7% greater increase in total body BMC and lumbar spine BMD, respectively, following vitamin D supplementation (51). In contrast, this systematic review and meta-analysis concluded that children with normal baseline concentrations of 25-hydroxyvitamin D [i.e., serum 25-hydroxyvitamin D >50 nmol/L] did not exhibit osteogenic benefits following vitamin D supplementation (51). Similarly, a recent randomized controlled trial in healthy children found that up to 4,000 IU/d vitamin D over 12 weeks increased serum concentrations of 25-hydroxyvitamin D in a dose-dependent manner, but had no effect on calcium absorption (52). These findings do not support a beneficial effect of vitamin D supplementation on bone via calcium absorption in children with normal vitamin D levels, though the potential osteogenic effects through an alternate mechanism may not be discounted (53, 54).

Other nutrients that have generated interest, but have been less heavily investigated with respect to bone health, include protein and zinc. While Chevalley et al. (55) found that total protein intake was associated with greater BMC of the forearm, hip, and spine in pre-pubertal males ($r = 0.18$-$0.27$; $P < 0.005$), Berger et al. (unpublished data) (56) found that zinc supplementation led to a significant increase in procollagen type 1 amino propeptide [bone formation marker (P1NP)] compared to placebo in pre-menarcheal females [mean change, 23.8 µg/L (95% CI, -14.9-62.5 µg/L) compared to -31.0 µg/L (95% CI, -66.4-4.2 µg/L); P=0.04]. It has been postulated that both protein and zinc augment bone in children via IGF-I, a mediator of linear growth and bone remodeling (57-61). However, it should be noted that the zinc-bone-IGF-

I association has only been observed in short stature children, and not those who are otherwise healthy (56, 58-62). Regardless of the exact mechanism, these findings indicate that both protein and zinc intakes may produce bone benefits in children entering the early stages of puberty.

Though research has shown the pronounced effects of individual nutrients on the skeleton, whole foods and dietary patterns have also generated interest with respect to bone health. Given the complex processes of bone modeling and remodeling required to build bone strength, it stands to reason that the singular components implicated in skeletal health may be more effective when packaged together and consumed contemporaneously (63). For example, Wosje et al. (64) found that diets rich in dark-green and deep-yellow vegetables were associated with higher bone mass in children ages 4 to 8 years. Similarly, Vatanparast et al. (65) found that higher intakes of fruits and vegetables were associated with higher total body BMC in males ages 8 to 20 years. These results support research examining the independent influences of magnesium, potassium, and vitamins C and K on bone (66, 67), as fruits and vegetables are important dietary sources of these individual micronutrients (63).

Bone is also influenced in part by soft tissue composition [i.e., fat-free soft tissue (FFST) mass and fat mass]. As discussed in the section on physical activity and bone, FFST mass exerts contractile forces on the skeleton that augment bone mass, and increasing muscle mass therefore creates the stimulus for increased bone mass and strength (68). Rauch et al. (68) conducted a multi-year longitudinal study to investigate the relationship between peak velocity in FFST mass accretion and peak velocity in BMC accretion during puberty. In both males and females, maximal rate of FFST mass accretion preceded maximal rate of BMC accretion, and the maximal rate of FFST mass accretion was positively associated with the maximal rate of BMC accretion at each site. More recently, Jackowski et al. (69) conducted a multi-year study in males

and females ages 8 to 18 years, and found that peak FFST mass accrual preceded peak cross-sectional area velocity and peak section modulus velocity at the proximal femur, and was a significant predictor of the magnitude of bone strength. While these findings do not establish a causal relationship, they do support a functional relationship between mechanical forces and bone development, also suggested by bone studies in athletes versus controls (68, 69).

**Adiposity and bone**

Like bone, adipose tissue is a dynamic tissue. It adapts to fluctuations in nutrient deprivation and excess through an ongoing process of remodeling (i.e., acute or chronic) (70). Adipose tissue is the major site of energy storage, and it exhibits a seemingly unlimited capacity to expand via adipocyte hypertrophy and hyperplasia in order to maintain total body energy homeostasis (70). Although adipose tissue accumulation has been traditionally defined in terms of mass via measures of total body weight and body mass index (BMI) (71), it is now recognized as a highly active endocrine organ with the potential to trigger metabolic dysfunction (70-72). The dual effects of adipose tissue may therefore have important implications for bone.

The intricate relationship between adipose tissue and bone is best evidenced by a shared lineage of adipocytes and osteoblasts from mesenchymal stem cells in bone marrow (73, 74). Commitment of mesenchymal stem cells to pre-adipocytes or pre-osteoblasts is influenced by systemic factors [e.g., proliferator-activated receptor gamma (PPAR-gamma), TGF-beta, leptin, and estrogen] (74). For example, PPAR-gamma is recognized as the master regulator of adipogenesis, which has been shown to occur at the expense of osteoblastogenesis (75). An environment that favors adipocyte differentiation may propagate adipose tissue growth and enlargement of adipocytes, reduced angiogenesis, and subsequent hypoxia (70). This course of events has been shown to trigger a heightened state of immune cell infiltration (a hallmark of

adipose tissue expansion) and subsequent inflammation and hormone imbalance (70, 76), with the potential to affect bone metabolism (77-79) (**Figure 2.1**). For the purposes of this review, the following section will address alternate effects of adipose tissue on bone separate from inflammation. A more detailed discussion related to inflammation will be provided in subsequent sections (please refer to **Inflammation and bone**).

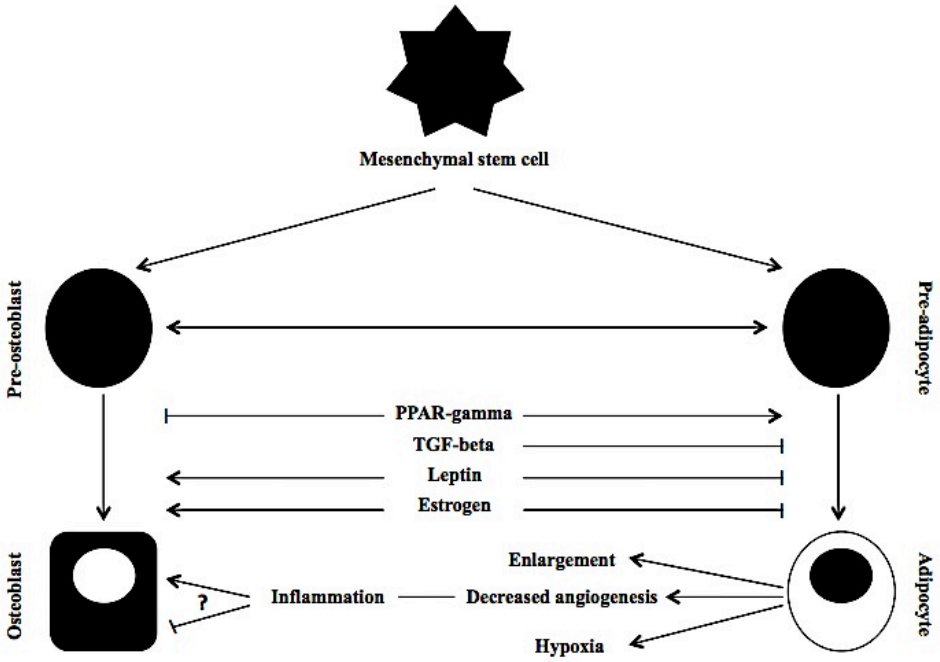


**Figure 2.1.** The differentiation of adipocytes and osteoblasts from a common mesenchymal stem cell progenitor [adapted from Pollock (18), Rosen and Bouxsein (74) and Sun et al. (70)].

Whether adiposity is advantageous to bone remains a topic of debate. Research findings that support a positive effect of adipose tissue on bone have been attributed to increased skeletal loading (80-82), increased protection against falls (83), and increased concentrations of hormones implicated in growth, such as insulin, estrogen, and IGF-I, all of which have been shown to stimulate bone formation (18, 84-86), though findings are equivocal (87, 88).

In support of the positive associations between fat and bone in children, Streeter et al. (82) found that increased body fat percent in 9 to 16 year-old females correlated with total body aBMD and bone area over seven years, which was attributed to extra mechanical loading from excess fat mass and early puberty (i.e., early attainment of peak height velocity). In accordance with these findings, Vandewalle et al. (89) observed higher pQCT-derived measures at the weight-bearing tibia in 10 to 19 year-old obese males versus bone-age matched controls, and trabecular vBMD and cortical area correlated with serum estrogen concentrations (i.e., estradiol and estrone). In addition to the potential osteogenic effects of sex hormones, Reinehr et al. (90) found that radiograph-derived bone age in obese children was positively associated with IGF-I. Because the interactions of mechanical loading, sex hormones, and IGF-I influence bone mass and strength during puberty (89, 91, 92), and these factors have been shown to be increased in obesity (89, 90), it is possible that obese children exhibit advanced bone maturation compared to non-obese children (93).

Conversely, findings to suggest deleterious effects of adipose tissue on bone have been attributed to the same mechanisms purported to optimize bone outcomes, such as increased mechanical loading and increased concentrations of hormones. For example, Pollock et al. (94) found that 18 to 19 year-old females classified as high-fat (i.e., ≥32% body fat) exhibited significantly lower cortical bone area, cortical bone BMC, and SSI at the tibia compared to those

classified as normal-fat (i.e., <32% body fat) after adjustment for muscle cross-sectional area. These findings suggest that the high-fat group did not benefit from increased mechanical loading at the weight-bearing tibia (94). Moreover, increased concentrations of insulin and risk of insulin insensitivity have been shown to be negatively associated with bone in children, and it has been hypothesized that these adverse effects may be related to increased calcium excretion, impaired regulation of the growth-hormone-IGF-I axis, and increased inflammation (95-98). Indeed, Afghani et al. (86) found that both fasting insulin and 2-hour insulin concentrations (i.e., measures of insulin resistance) were negatively associated with total body BMC in 8 to 13 year-old overweight Hispanic children. In support of these findings, Boucher-Berry et al. (99) observed negative relationships between BMI, body fat percent, and fat mass with serum osteocalcin (marker of bone formation) in a diverse sample of 11 to 14 year-old males and females. Indeed, prospective studies are needed to more definitively assess the relationships and underlying mechanisms governing the bone-fat association.

**Inflammation and bone**

The parallels between fat and bone are not limited to the shared lineage of adipocytes and osteoblasts from a common mesenchymal progenitor. Fat and bone are also individually influenced by systemic inflammation, an underpinning factor that has been implicated in both obesity and osteoporosis/osteopenia development. Though inflammation is traditionally recognized as a protective response of the innate immune system to tissue insult/injury by microbes and other antigens (100), inflammatory-related factors may be produced or bound by adipocytes, osteoblasts, and osteoclasts, in addition to immune cells (e.g., T lymphocytes), to

affect bone (71, 101, 102). While this highlights the considerable overlap that exists among the skeletal, metabolic, and immune systems, the following sections will be limited to a discussion of the association between inflammation and bone.

The inflammatory response has been characterized as the coordinated activation of various signaling pathways that regulate expression of cytokines, chemokines, and growth factors (103). A common signaling pathway inducible in all cell types is nuclear factor kappa B (NFkB), a family of related transcription factors that includes five genes and seven corresponding proteins that form homo- and heterodimers with differential effects on the production of inflammatory-related markers (104, 105).

Though there are two molecular mechanisms of NFkB activation, the canonical NFkB pathway may be triggered by pro-inflammatory factors (e.g., cytokines tumor necrosis factor-alpha and interleukin-1) and microbial products (e.g., viral infections) (103, 105). The canonical NFkB pathway is characterized by NFkB heterodimers, RelA and p50 (also known as p65 and NFkB1, respectively), which are retained in the cytoplasm of the target cell by inhibitor of NFkB (IkB), IkB-alpha. The Rel Homology Domain sequence of the p50 dimer serves as a binding site for IkB-alpha (104-106). Upon NFkB activation, the NFkB heterodimers and IkB-alpha are targeted by IkB kinases (IKK) that include IKK-alpha, IKK-beta, and IKK-gamma: the serine residues on IkB-alpha are phosphorylated by IKK-beta, and the protein undergoes ubiquitin-dependent degradation by the proteasome (105). This enables nuclear translocation of the NFkB heterodimers, and subsequent gene transcription of cytokines (e.g., tumor necrosis factor-alpha, interleukin-6), chemokines (e.g., monocyte chemoattractant protein-1), and other pro-inflammatory products (e.g., vascular endothelial growth factor) (104, 105) (**Figure 2.2**).

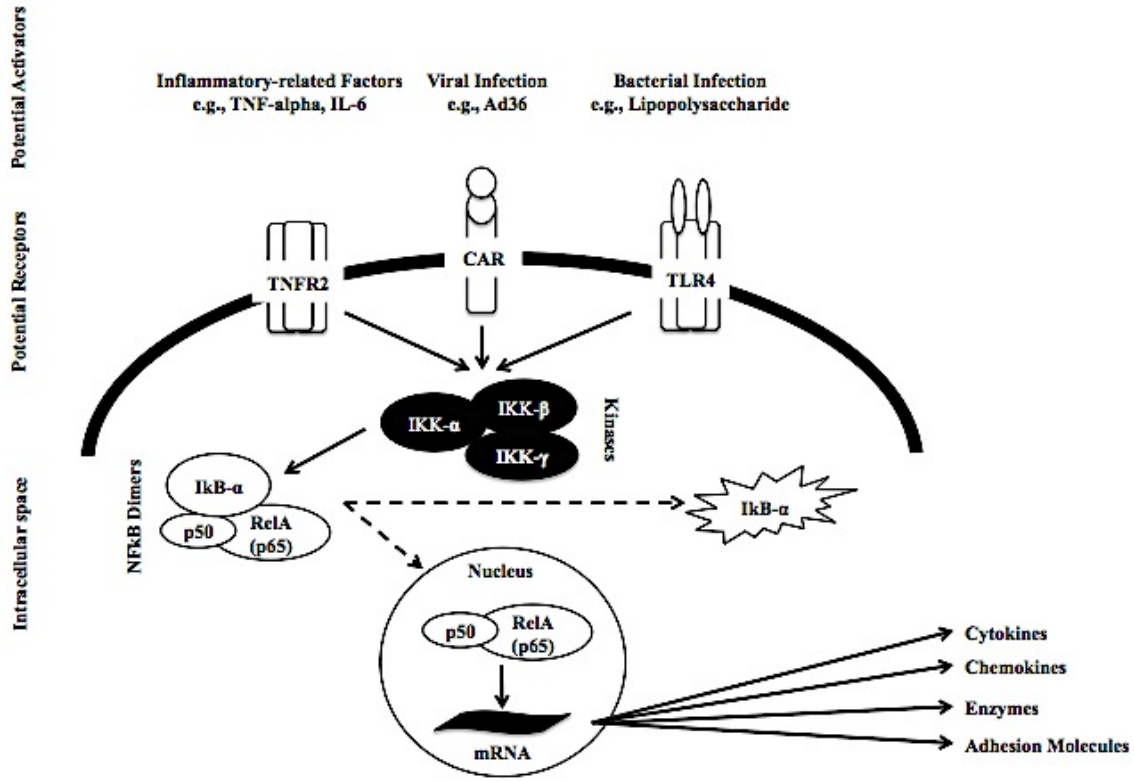


**Figure 2.2.** The canonical pathway of NFkB activation and subsequent production of inflammatory-related factors [adapted from Bonizzi and Karin (104) and Dolcet et al. (105)].

Cytokines, chemokines and growth factors are heterogeneous groups of secreted proteins produced through activation of various signaling pathways (e.g., NFkB) to regulate all aspects of immune response (100). Cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), are the principal mediators of communication between most cell types via pleiotropic, redundant, synergistic, and antagonistic properties (100). Chemokines, which include monocyte chemoattractant protein-1 (MCP-1), are low-molecular-weight cytokines that regulate leukocyte

movement and migration from blood to tissues (100). Growth factors, such as vascular endothelial growth factor (VEGF), are induced by cytokines and most often implicated in angiogenesis and vascular development. In addition to these cardinal functions, TNF-α, IL-6, MCP-1, and VEGF have also been recognized as potent bone-resorbing factors in inflammation (79, 107-109).

TNF-α has been described as the most potent osteoclastogenic cytokine produced in inflammation (79). Though a number of mechanisms have been proposed, TNF-α has been shown to enhance osteoclast differentiation and proliferation through increased expression of RANK mRNA in osteoclast precursors (78, 79, 110, 111). Indeed, Komine et al. (110) found that TNF-α markedly increased the formation of mononuclear osteoclast precursors, which were shown to develop into mature multinucleated osteoclasts with increased expression of RANK mRNA. In accordance with these findings, Lam et al. (78) observed that TNF-α acted directly on osteoclast precursors and stimulated RANKL-induced bone resportion (78, 79).

IL-6 is inducible by TNF-α and has also been implicated in bone biology. While the primary source of IL-6 in bone is osteoblasts rather than osteoclasts, it has been postulated that the main effect of IL-6 in bone is bone resorption (79, 112). O'Brien et al. found that IL-6 stimulated a common signal transducer (i.e., glycoprotein gp130 receptor) on stromal/osteoblastic cells, which resulted in increased expression of RANKL and increased formation of mature osteoclasts in a co-culture of osteoclast precursors (79, 113). Moreover, Devlin et al. (114) found that a neutralizing antibody to IL-6 inhibited formation of multinucleated osteoclasts in a culture of human-derived cells by decreasing the effects of TNF-α. Together, these data suggest that IL-6 may affect bone both independently and in combination with TNF-α (113, 114).

MCP-1 is a potent chemokine for monocytes and macrophages, which serve as osteoclast precursor cells (108, 115). It has been postulated that increased expression of MCP-1 stimulates recruitment of monocytes to sites of RANKL-activated cells, resulting in osteoclast differentiation (116). In vitro, Li et al. (108) found that MCP-1 derived from osteoblasts increased monocyte migration and RANKL-induced osteoclastogenesis via enhanced fusion, a hallmark of osteoclast formation. The effects of MCP-1 on bone resorption have also been shown in animal studies. Sul et al. (117) reported increased bone mass and decreased serum collagen type 1 cross-linked C-telopeptide (CTX; marker of bone resorption) in MCP-1 knock-out mice versus wild-type mice.

VEGF, the most critical growth factor for angiogenesis that is involved in the vascularization of developing bones, has also been shown to regulate osteoclast differentiation and activity (118). VEGF was first implicated in osteoclastogenesis once it was discovered that monocytes express VEGF receptor 1, suggesting that VEGF signaling affects osteoclast differentiation (119). Indeed, Niida et al. (109) found that VEGF injections induced recruitment and survival of osteoclasts in M-CSF knock-out mice, and it has been postulated that VEGF may therefore substitute for M-CSF as a co-stimulator (with RANKL) of osteoclast differentiation (109, 118). Moreover, Nakagawa et al. (120) found that VEGF treatment resulted in a dose- and time-dependent increase in bone resorption pits in a culture of rabbit-derived mature osteoclasts. Together, these data suggest that VEGF increases osteoclast differentiation and enhances osteoclast activity (109, 118, 120).

Studies that have examined the association of inflammatory-related markers and bone in humans have largely been conducted in adults with chronic diseases. For example, studies in untreated celiac disease patients found that serum IL-6 was negatively associated with BMD and

positively associated with carboxyterminal telopeptide region of type 1 collagen (ICTP, marker of bone resorption) (121, 122). Moreover, a recent systematic review found that rheumatoid arthritis patients exhibited increased BMD following treatment with a TNF-α antagonist (123).

While data have shown that adults diagnosed with inflammatory-based diseases are at risk for secondary osteoporosis, findings in otherwise healthy adults at risk for age-related bone loss may be more applicable to the general population. Indeed, 1 in 3 women and 1 in 5 men are likely to experience an osteoporotic fracture after the age of 50 years. A prospective study by Cauley et al. (124) found that higher serum concentrations of inflammatory-related markers, TNF-α and IL-6, predicted a higher incidence of fracture over 6 years in 70 to 79 year-old adults. In accordance with these findings, Barbour et al. (125) reported that 50 to 79 year-old females classified in the highest quartiles for 3 serum inflammatory-related markers [IL-6 soluble receptor (SR), TNF SR1, and TNF SR2)] had two times the risk of incident hip fracture versus those classified in the highest quartile for 1 or 0 serum inflammatory-related markers. These findings support systemic inflammation as a potential risk factor for bone fractures in well-functioning older adults.

Studies investigating the inflammation-bone relationship in children have generally targeted patients with chronic diseases, such as type-1 diabetes, juvenile idiopathic arthritis, and inflammatory bowel disease (126-128). However, it has been postulated that acute infections may affect linear growth as well. Indeed, a review by Stephenson (129) speculated that pneumonia and viral infections modulate long bone growth in otherwise healthy children via inflammatory-induced alterations in bone remodeling. However, no human studies to date have tested this hypothesis.

Of the inflammatory-related diseases that may alter bone, obesity affects 17% of US children and adolescents (130), and may therefore provide the greatest context and insight into the inflammation-bone relationship in otherwise healthy youth. Indeed, adipose tissue is a potent source of cytokines, chemokines, and growth factors, and while these products have been shown to influence bone outcomes, few studies in obese children have been conducted. Moreover, only one study to date has examined serum inflammatory-related markers, TNF-α and IL-6, with bone measures in obese and normal-weight children. Russell et al. (131) conducted a study in 12 to 18 year old females (50% obese) to determine whether associations of visceral and subcutaneous adipose tissues (VAT and SAT, respectively) with bone density measures were influenced by inflammatory-related cytokines. While the proportion of VAT to SAT in obese and normal-weight subjects was positively associated with adhesion molecule E-selectin, a negative predictor of BMD, there were no relationships with serum TNF-α and IL-6 (131). Other studies that have examined the inflammation-bone relationship in obese children have found that serum leptin is also a negative predictor of BMD and BMC (132-134). Together, these findings suggest that an adiposity-induced inflammatory state may have negative effects on bone in otherwise healthy children, though findings related to TNF-α, IL-6, MCP-1, and VEGF are limited.

**Adenovirus 36 infection, inflammation, adiposity, and bone**

The interaction among the skeletal, metabolic, and immune systems is not limited to the purported effects of inflammation on bone and adipose tissue remodeling. As discussed previously, inflammation is traditionally defined as a protective response of the innate immune system to tissue insult/injury by microbes that may be found on the surfaces of foreign pathogens, and a subgroup of pathogens that has garnered attention as it relates to bone and body composition is viruses. The hypothesis that viral infection may affect adipose tissue and bone has

been examined in studies of human immunodeficiency virus (HIV), which have reported increased abdominal fat mass and a prevalence of osteoporosis ranging from 55% to 89% in HIV patients (135-139). Moreover, these skeletal aberrations have been linked to HIV-induced inflammation (140-142), prompting research into other viral strains.

Adenoviruses are non-enveloped double-stranded DNA viruses that were first isolated from adenoidal cells to determine the etiology of the common cold (143). Of the known serotypes of human adenoviruses, adenovirus 36 (Ad36) has been proposed as a determinant of bone and adiposity (139, 144, 145), and its effects may be governed by an inflammatory-related response to infection (146, 147). Given that adenovirus infections are common, accounting for 5% to 15% of upper respiratory illnesses (143), and the short- and long-term health implications of osteoporosis and obesity, the effects of Ad36 related to inflammation, adiposity, and bone warrant further investigation.

Ad36 is an adipogenic human adenovirus derived from SMAM-1, an animal adenovirus first isolated from chickens that has been reported to impair immune function while augmenting body fatness (148-150). Ad36 is airborne and may be transmitted via respiratory droplets, as data have shown that natural infection occurs when animals inoculated with the virus are in contact with controls (150). Following initial exposure, Ad36 viral particles may invade upper respiratory epithelial cells and replicate in the nucleus without incorporation into the host cell DNA. This course of events induces epithelial cell death, releasing Ad36 viral antigens into circulation and spreading infection to peripheral tissues and organ systems (148). Though the live virus may be transient, Ad36 viral DNA has been detected in brain, lung, liver, muscle, and adipose tissues of the host for up to seven months following the initial infection (151), with the potential to induce biological changes over a prolonged period.

Ad36 has been shown to modify human cell metabolism, with the potential to ultimately decrease bone, increase adipose tissue, and augment an acute inflammatory-related response to a chronic inflammatory-related response. Indeed, infected pluripotent progenitor cells have a predisposition toward adipocyte differentiation, impeding osteoblast formation (144, 145). In differentiated cells, infected adipocytes have been shown to exhibit an enhanced rate of proliferation required for adipose tissue expansion (146). It is therefore plausible that Ad36 induces adipogenesis at the expense of osteoblastogenesis, though the long-term implications have not been examined.

Though the exact mechanism by which Ad36 may favor adipose tissue accumulation and compromise bone formation has not been established, it has been postulated that infection alters the expression of adiposity-related genes [e.g., PPAR-gamma, fatty acid synthase and phosphatidylinositide 3-kinase (PI3K)], many of which have been implicated in inflammation. On a cellular level, Ad36 enhances adipocytes that are potent sources of inflammatory-related factors that may affect bone. On a molecular level, Ad36 has the potential to increase expression of protein kinases such as PI3K, a regulatory enzyme that enables glucose uptake and metabolism via protein kinase B, which can in turn initiate NFkB-dependent gene expression (148, 152). The combined effects of infection therefore promote intracellular lipid accumulation, adipose tissue expansion, and systemic inflammation, with long-term consequences related to bone.

As discussed previously, factors that may initiate NFkB-dependent gene expression and subsequent systemic inflammation include viruses (e.g., Ad36) and also inflammatory-related products of the cascade itself (e.g., TNF-α), generating a positive feedback loop with potential effects on adipose tissue and bone. Indeed, animal and cell culture data support this causal link

between Ad36 and inflammation, though studies are limited to the effects on adiposity rather than bone. For example, infected wild-type mice have been shown to exhibit increased serum concentrations of TNF-α and MCP-1 with a concurrent increase in reproductive fat pad weight, whereas this relationship was not observed in infected MCP-1 knock-out mice (146).

In accordance with these findings, Na and Nam (146) found that Ad36 infected adipocytes increased TNF-α and MCP-1 production and decreased IkB-alpha production, which correlated with macrophage infiltration into adipose tissue, a hallmark of obesity development. Bouwman et al. (147) also reported that infected adipocytes increased IL-6 production, an additional inflammatory-related product of the NFkB signaling pathway (79). Together, these results suggest that Ad36 infection increases nuclear localization of NFkB in the activated state, triggering inflammatory-related marker production and subsequent adiposity development (146).

Findings from animal and cell culture studies prompted human investigations. Indeed, few human studies have been conducted to date, and initial findings were largely reported in adults with a primary focus on the association between Ad36 infection and adiposity-related outcomes (e.g., BMI and waist circumference). No studies in adults have investigated the relationship between Ad36 and bone, and only one human study to date has investigated the association between Ad36 and inflammatory-related factors (146). Therefore, a summary of studies in healthy adults related to Ad36 and adiposity is presented in **Table 2.2**.

The reported prevalence of Ad36 infection is approximately 30 to 40% of adults (153). Though early studies have shown that the percentage of Ad36(+) adults is higher among obese versus non-obese groups, and that infection is associated with greater adiposity-related measures (153, 154), findings are not consistent (155-157). Indeed, a study of 502 white and black adults in the United States ages 30 to 46 years reported higher BMI in Ad36(+) versus Ad36(-) groups

(153), as did a study of 203 adults in Italy of approximately the same age (154). However, in a sample of 300 white, black, Hispanic, and Asian military personnel ages 17 to 30+ years, Broderick et al. (155) found no differences in BMI by Ad36 status. Similarly, in a more homogenous sample of 509 adults in Belgium, Goossens et al. (156) also reported no differences in BMI by Ad36 status. It is possible that these conflicting results may be attributed to differences in study design, as it is still unclear how age, race, geography, diet, and duration of infection may influence Ad36 status (148, 155).

It has been postulated that the prevalence of Ad36 among children may be even higher than that observed in adults, as the majority of adenovirus infections occur within the first 5 years of life with peak incidence reported at age 2 years (143, 158, 159). These findings, coupled with short- and long-term health complications of obesity, have prompted research related to Ad36 and adiposity in youth. In addition to the small number of studies examining the Ad36-adiposity link, one study to date has also investigated the association of Ad36 and bone in late adolescents. A summary of studies in healthy children related to Ad36, adiposity and bone is presented in **Table 2.3**.

Studies that have investigated the association of Ad36 and adiposity in children report disparate findings. Contrasting results may be related to differences in target populations and measures of body fatness, with findings in support of the adenovirus-adiposity link largely derived from obese cohorts and studies that rely on BMI-based outcomes. For example, in a cohort of 84 obese Korean children ages 8 to 13 years, Atkinson et al. (160) found that mean BMI z-score was higher in Ad36(+) versus Ad36(-) groups. However, in a cohort of 71 obese white children ages 10 to 17 years, Vander Wal et al. (161) observed no differences in baseline BMI, BMI-for-age percentile, or BMI z-score by Ad36 status.

In addition to these equivocal findings, it is also difficult to compare Ad36-adiposity studies that rely on indirect measures of body fatness because of differences in study criterion used to define obesity. In a sample of 157 Hispanic children ages 6 to 11 years, Parra-Rojas et al. (162) found no significant differences in mean BMI by Ad36 status, but did observe a higher prevalence of Ad36(+) in obese versus non-obese participants: obesity was defined using Centers for Disease Control and Prevention (CDC) criterion for BMI-for-age percentiles. Similarly, in a cohort of 318 Korean children ages 6 to 15 years, Na et al. (163) also observed no differences in mean BMI by Ad36 status, and found a higher prevalence of Ad36(+) in obese versus non-obese participants: however, obesity was defined using International Obesity Task Force (IOTF) criterion for BMI-for-age percentiles. Because these two sets of criteria differ, and CDC values for obesity have been shown to have a higher sensitivity and specificity versus IOTF values (164), it is difficult to generalize findings in support of an Ad36-adiposity association.

Only two studies to date have examined the association of Ad36 and adiposity using direct measures of body fatness (e.g., body fat percent) in children. In a recent study of 1,179 Czech adolescents ages 13 to 18 years, Aldhoon-Hainerová et al. (165) observed a positive correlation between total body fatness measured by bioelectrical impedance (BIA) and Ad36(+) status in the total sample of males and females. However, in the study of Korean children described previously, Na et al. (163) found no significant differences in BIA-derived total body fatness between Ad36(+) and Ad36(-) groups. While these disparate findings have been attributed to different assays used for the determination of Ad36 status (i.e., ELISA versus serum neutralization assay) (165, 166), additional research is warranted using more consistent methodology in order to draw conclusions on the Ad36-adiposity relationship.

One study to date has examined the association of Ad36 and bone in youth. In a sample of 115 predominantly white late adolescent females ages 18 to 19 years, Laing et al. (139) found that SSI of the radius was significantly lower in Ad36(+) versus Ad36(-) groups who were classified as high-fat (i.e., total body fat ≥32%), after adjustment for muscle cross-sectional area. There were no differences in SSI of the tibia by Ad36 status. Given these findings at the non-weight-bearing radius and the high incidence of forearm fractures that have been reported in children ages 8 to 14 years (167), the relationship between Ad36 and bone warrants further investigation in children entering the early stages of puberty.

**Summary**

- Bone is a dynamic tissue that must adapt to the stresses and strains of everyday living to support mechanical loading, metabolic demands, and mineral homeostasis.
- Bone modeling is the dominant process in childhood and adolescence by which osteoblast activity exceeds osteoclast activity, resulting in greater mass and diameter. Bone remodeling is the dominant process in adulthood by which osteoblast activity equals osteoclast activity. This persists until the fourth or fifth decade of life when osteoclast activity supersedes to drive net bone loss.
- Fat has a significant effect on bone metabolism, and the parallels between fat and bone go beyond a shared lineage of adipocytes and osteoblasts from a common mesenchymal progenitor. Both tissues are also affected by circulating proteins.
- Systemic inflammation, traditionally recognized as a protective response of the innate immune system to tissue insult/injury by microbes and other antigens, is not only an underpinning factor of obesity, but also of osteoporosis/osteopenia.

- In vitro, inflammatory-related markers have been shown to enhance osteoclast differentiation. In vivo, serum cytokines, chemokines, and growth factors have been shown to be negatively associated with bone outcomes, though findings in otherwise healthy adults and children are limited.
- It has been postulated that infection with human adenovirus 36 (Ad36) may enhance adipogenesis and compromise osteoblastogenesis via activation of a common inflammatory-related signaling pathway. Studies have shown that Ad36 is associated with increased adiposity and inflammatory-related markers and decreased bone strength in animals and humans.
- To date, no studies have examined the associations of Ad36 infection, adiposity, inflammatory-related markers, and bone in otherwise healthy children.

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**Table 2.1.** Peripheral QCT outcome measures and corresponding definitions [adapted from Pollock (18) and Zemel et al. (15)].

| Outcome measure | Definition (units) |
|---|---|
| Bone mineral content (BMC) | |
| Total BMC | Total bone mineral content (mg) |
| Trabecular BMC | Bone mineral content of trabecular bone (metaphyseal sites only) (mg) |
| Cortical BMC | Bone mineral content of cortical bone (mg) |
| Bone mineral density (vBMD) | |
| Total vBMD | Total volumetric bone mineral density ($mg/cm^3$) |
| Trabecular vBMD | Volumetric bone mineral density of trabecular bone (metaphyseal sites only, includes interspersed marrow fat) ($mg/cm^3$) |
| Cortical vBMD | Volumetric bone mineral density of cortical bone ($mg/cm^3$) |
| Structure and geometry | |
| Total area | Total cross sectional area of bone ($mm^2$) |
| Trabecular area | Cross sectional area of the trabecular portion of the total bone area ($mm^2$) |
| Cortical area | Cross sectional area of the cortical portion of the total bone area ($mm^2$) |
| Cortical thickness | Average thickness of the cortical shell (mm) |
| Periosteal circumference | Outer diameter of bone (mm) |
| Endosteal circumference | Inner diameter of bone (mm) |
| Cross-sectional moment of inertia | $\pi/4(R^4_o - R^4_i)$, where $R_o$ is the outer radius and $R_i$ is the inner radius; indication of |

| | |
|---|---|
| | bending strength ($mm^4$) |
| Polar moment of inertia | $\pi/2(R^4_o - R^4_i)$, where $R_o$ is the outer radius and $R_i$ is the inner radius; indication of strength in torsion ($mm^4$) |
| Section modulus | Polar moment of inertia/maximum distance to centroid; indication of shearing strength ($mm^3$) |
| Strength-strain index | Section modulus x (cortical vBMD/normal density), where normal density is equal to 1,200$mg/mm^3$; measure of strength of bone against density-weighted section modulus at cortical sites ($mm^3$) |
| Bone strength index | Total cross sectional area x total $vBMD^2$; measure of strength of bone against compression at trabecular sites ($mg^2/mm^4$) |

**Table 2.2.** Summary of studies in healthy adults related to Ad36 and adiposity.

| Author | Subjects | Materials and Methods | Results | Conclusions |
|---|---|---|---|---|
| Atkinson et al. 2005 (153) | White and black males and females (N=502), ages ~30-45 years | BMI; detection of Ad36 antibodies in sera via serum neutralization assay (SNA) | Ad36(+) adults exhibited higher BMI (44.9 vs. 35.8, P<0.01) vs. Ad36(-) in total sample; Ad36(+) obese had higher BMI vs. Ad36(-) obese, Ad36(+) non-obese had higher BMI vs. Ad36(-) non-obese (P<0.02) | Ad36 infection positively associated with obesity based on BMI in sample of adult males and females |
| Trovato et al. 2009 (154) | Italian males and females (N=203), mean age 46 years | BMI, BIA-derived body fat percent, fat mass and FFST mass, waist-hip ratio; detection of Ad36 antibodies in sera via SNA | Ad36(+) adults exhibited higher BMI (30.0 vs. 26.0, P<0.0001) and waist-hip ratio (0.90 vs. 0.87, P<0.001) vs. Ad36(-) in total sample; Ad36(+) non-obese had higher BMI and waist-hip ratio vs. Ad36(-) non-obese (P<0.01); no differences in obese by Ad36 status; no differences in body fat percent by Ad36 status | Ad36 status not associated with direct measures of adiposity (i.e., body fat percent); Ad36(+) positively associated with BMI in total sample and in non-obese adults |
| Broderick et al. 2010 (155) | Multi-racial/ethnic males and females (N=293), ages 17-30+ years | BMI; detection of Ad36 antibodies in sera via SNA | No differences in prevalence of Ad36(+) in obese vs. non-obese groups defined by BMI | Ad36 status not associated with obesity defined by BMI in adults |
| Goossens et al. 2011 (156) | Dutch and Belgian males and females (N=509), ages ~19-51+ years | BMI; detection of Ad36 antibodies in sera via SNA | No differences in BMI by Ad36 status; no differences in prevalence of Ad36(+) by BMI group | Ad36 status not associated with BMI in adults |
| Na et al. 2012 (157) | Korean males and females (N=540), ages 30-59 years | BMI, waist circumference; detection of Ad36 antibodies in sera via SNA | No differences in prevalence of Ad36(+) in normal-weight vs. overweight vs. obese groups based on BMI; overweight more likely to be Ad36(+) vs. normal-weight adults (adjusted OR, 2.03; 95% CI, 1.16–3.55), but not obese (adjusted OR, 1.56; 95% CI, 0.67–3.67) | Ad36 infection positively associated with overweight, but not obesity, based on BMI in adults |
| Lin et al. 2013 (168) | Hispanic males and females (N=1,400), mean age 40 years | Baseline BMI, BIA-derived body fat percent; detection of Ad36 antibodies in sera via SNA | No differences in baseline BMI by Ad36 status in total sample; Ad36(+) adults demonstrated higher baseline body fat percent | Ad36 infection positively associated with direct measures of adiposity (i.e., body fat percent) |

**Table 2.3.** Summary of studies in healthy children related to Ad36, adiposity, and bone strength.

| Author | Subjects | Materials and Methods | Results | Conclusions |
|---|---|---|---|---|
| Atkinson et al. 2010 (160) | Obese Korean children (N=84), ages 8-13 years | BMI z-score, waist circumference; detection of Ad36 antibodies in sera via SNA | Ad36(+) children exhibited higher BMI z-score (1.92 vs. 1.65, P<0.01) and waist circumference (96.3 vs. 90.7 cm, P=0.05) vs. Ad36(-) | Ad36 infection positively associated with measures of adiposity in a sample of obese children |
| Vander Wal et al. 2013 (161) | Predominantly obese white children (N=71), ages 10-17 years | BMI, BMI-for-age percentile, BMI z-score, waist circumference; detection of Ad36 antibodies in sera via SNA | No differences in baseline BMI, BMI-for-age percentile, BMI z-score, or waist circumference by Ad36 status | Ad36 infection was not associated with cross-sectional measures of adiposity in a sample of obese children |
| Almgren et al. 2012 (166) | Swedish children (N=424), ages 10-18 years | BMI z-score; detection of Ad36 antibodies in sera via ELISA | Obese children more likely to be Ad36(+) vs. normal-weight based on BMI z-score (OR, 1.6; 95% CI, 1.0-2.6) | Ad36 infection positively associated with obesity based on BMI z-score in sample of children and adolescents |
| Aldhoon-Hainerová et al. 2014 (165) | Czech adolescent males and females (N=1,179), ages 13-18 years | BMI, BMI z-score, BIA-derived fat mass; detection of Ad36 antibodies in sera via ELISA | Obese children more likely to be Ad36(+) vs. normal-weight (OR, 1.46; 95% CI, 1.07-1.99); Ad36(+) positively associated with BMI and fat mass in total sample (P<0.02) | Ad36 infection positively associated with overweight and obesity based on BMI-for-age percentile; Ad36(+) associated with higher fat mass in total sample of adolescent males and females |
| Gabbert et al. 2010 (169) | Predominantly Hispanic males and females (N=124), ages 8-18 years | BMI-for-age percentile, BMI z-score, waist circumference; detection of Ad36 antibodies in sera via SNA | Ad36(+) children exhibited higher BMI z-score (2.0 vs. 1.6, P=0.01) and waist circumference (106 vs. 89 cm, P<0.01) vs. Ad36(-); prevalence of Ad36(+) higher among obese vs. non-obese children defined by BMI-for-age percentile (22 vs. 7%, P=0.02) | Ad36 infection positively associated with obesity based on BMI-for-age percentile; Ad36(+) positively associated with measures of adiposity in total sample and in subsample of obese children |
| Parra-Rojas et al. 2013 (162) | Hispanic children (N=157), ages 6-11 years | BMI, waist circumference and skinfolds; detection of Ad36 antibodies in sera via ELISA | No differences in unadjusted mean BMI or waist circumference by Ad36 status in total sample; higher prevalence of Ad36(+) in obese vs. non-obese groups defined by BMI-for-age percentile (59 vs. 41%, P=0.01) | Ad36 infection associated with obesity based on BMI-for-age percentile |
| Na et al. 2010 (163) | Korean children (N=318), ages 6-15 years | BMI, BMI-for-age percentile, waist circumference, BIA-derived body fat percent; detection of Ad36 antibodies in sera via SNA | No differences in BMI, waist circumference or body fat percent by Ad36 status in total sample; prevalence of Ad36(+) higher among obese vs. non-obese children defined by BMI-for-age percentile (29 vs. 14%, P=0.02) | Ad36 status not associated with direct measures of adiposity (i.e., body fat percent); Ad36(+) associated with obesity based on BMI-for-age percentile |
| Laing et al. 2013 (139) | White late adolescent females (N=115), ages 18-19 years | DXA-derived fat mass, FFST mass and body fat percent; pQCT-derived cortical/trabecular bone; detection of Ad36 antibodies in sera via ELISA | No differences in body fat by Ad36 status; no differences in pQCT-derived bone by Ad36 status in total sample; lower radial SSI in Ad36(+), high-fat vs. Ad36(-), high-fat females | Ad36 status not associated with obesity based on percent body fat; Ad36(+) associated with lower cortical bone strength at the radius in high-fat females |

# CHAPTER 3

# ASSOCIATION OF ADENOVIRUS 36 INFECTION WITH ADIPOSITY AND INFLAMMATORY-RELATED MARKERS IN CHILDREN[1]

[1]Berger PK, Pollock NK, Laing EM, Warden SJ, Hill Gallant KM, Hausman DB, Tripp RA, McCabe LD, McCabe GP, Weaver CM, Peacock M, Lewis RD. 2014. *The Journal of Clinical Endocrinology and Metabolism*. 99:3240-6. Reprinted here with permission of the publisher.

**Abstract**

Though animal studies suggest that adenovirus 36 (Ad36) infection is linked to obesity and systemic inflammation, human data are scant and equivocal. Associations of Ad36 infection with total body adiposity and inflammatory-related markers were determined in 291 children ages 9-13 years (50% female, 49% black). Fasting blood samples were measured for presence of Ad36-specific antibodies and tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1). Fat mass and fat-free soft tissue (FFST) mass were measured by dual-energy X-ray absorptiometry. The overall prevalence of Ad36 seropositivity [Ad36(+)] was 42%. There were a higher percentage of Ad36(+) children in the highest tertiles of TNF-α and IL-6 compared to their respective middle and lowest tertiles (both $P<0.03$). There was also a trend toward a higher prevalence of Ad36(+) children in the highest tertile of VEGF compared to Tertiles 1 and 2 ($P=0.05$). Multinomial logistic regression, adjusting for age, race, sex, and FFST mass, revealed that compared to children with the lowest TNF-α, IL-6 and VEGF levels (Tertile 1), the adjusted odds ratios for Ad36(+) were 2.2 (95% CI: 1.2-4.0), 2.4 (95% CI: 1.4-4.0) and 1.8 (95% CI: 1.0-3.3), respectively, for those in the highest TNF-α, IL-6 and VEGF levels (Tertile 3). No association was observed between Ad36(+) and greater levels of fat mass or MCP-1 (all $P>0.05$). In children, our data suggest that Ad36(+) may be associated with biomarkers implicated in inflammation, but not with greater levels of fat mass.

KEY WORDS: Adenovirus, Adiposity, Obesity, Inflammation, Children

**Introduction**

As pediatric obesity continues to be an emergent concern, research efforts to disentangle its etiology have considered novel influences. One such influence that may trigger obesity development is infection with human adenovirus 36 (Ad36) (1-5), a common upper respiratory infection transmitted through person-to-person contact (6) that elicits an antibody response ranging from days to years post-exposure. In vitro, Ad36 has been shown to enhance adipocyte differentiation and lipid accumulation in human-derived cells (7-9), which prompted studies in children and adults (9-12). Indeed, children seropositive for Ad36 antibodies [Ad36(+)] have been shown to exhibit higher body mass index (BMI)-for-age percentile and waist circumference versus those who are seronegative [Ad36(-)] (10), although not all studies show consistent results (4, 12).

The discrepancies in the aforementioned studies can be attributed in part to differences in the populations examined and the study designs and instruments used. However, it is possible that systemic inflammation may explain the Ad36-adiposity link because it is an underlying characteristic of both viral infection and obesity (13-15). In vitro, Ad36 has been reported to induce cellular activation of an inflammatory pathway that signals the production of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1) (9, 16). In vivo, these inflammatory-related markers were recently shown to stimulate adipocyte and macrophage migration into adipose tissue of infected mice, a hallmark of obesity development (9, 17, 18). In addition, they have been implicated in the pathogenesis of cardiovascular diseases, diabetes mellitus, osteoporosis, and a host of adverse health outcomes (13).

To our knowledge, only one human study has investigated the association of Ad36 infection with adiposity and inflammatory-related markers. Na and Nam (9) found that Ad36(+) adults had higher serum MCP-1 versus those who were Ad36(-) (2.5-fold higher in the total sample and 3.5-fold higher in obese subjects). It is important to elucidate the Ad36-adiposity-inflammation link in youth, since measures may be introduced during childhood to prevent obesity and related disorders over the long-term. The primary objective of this study was to determine associations of the human Ad36 infection with total body adiposity and inflammatory-related markers in a cohort of otherwise healthy children.

**Subjects and methods**

The study included baseline data from 291 children who were part of a multi-site vitamin D supplementation trial conducted at the University of Georgia (UGA, Athens, Georgia), Purdue University (PU, West Lafayette, Indiana), and Indiana University (IU, Indianapolis, Indiana) (19). At each testing site, two cohorts of children were enrolled in the study, the first from October-December 2009 and the second from October-December 2010. Inclusion criteria were as follows: (1) age 9 to 12 years for females and age 10 to 13 years for males, (2) white or black/African American race, and (3) sexual maturation stages 2 and 3, estimated using self-administered questionnaires for genitalia or breast development (20). Children were excluded if they were taking medications or had any medical condition known to affect growth, maturation, nutritional status, metabolism, or inflammatory response. The Institutional Review Board for Human Subjects at each testing site approved the study procedures. All participants and their parent/guardian provided informed assent and consent, respectively.

**Anthropometry**

Standing height was measured using a wall-mounted stadiometer to the nearest 0.1 cm. Body weight was measured using an electronic scale to the nearest 0.1 kg (21). BMI z-scores and BMI-for-age percentiles were calculated (22), with obesity defined as having a BMI-for-age ≥95th percentile.

**Body composition**

Fat mass, fat-free soft tissue (FFST) mass, and body fat percent 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)]. The same technician at each site conducted scans and performed analyses using instrument-specific software and protocols. Cross-calibration was performed to facilitate comparability of data from each study site/scanner, as described in Warden et al. (23). Briefly, the UGA and PU study sites were cross-calibrated by scanning 26 individuals on the Hologic Delphi-A scanner and a Lunar iDXA scanner, whereas the IU and PU study sites were cross-calibrated by scanning ten individuals on the Hologic Discovery-W and Lunar iDXA scanners. Regression formulae between data obtained at UGA and PU, and IU and PU were derived for each variable and used to adjust data obtained at UGA and IU to PU values (23).

**Biochemical analyses**

Blood samples were collected from fasting participants for assessment of Ad36 seropositivity, and to determine the concentrations of TNF-α, IL-6, VEGF, and MCP-1 in sera. An indirect ELISA was used to determine Ad36 seropositivity [i.e., Ad36(+) or Ad36(-)] as described in Laing et al. (24). For the indirect ELISA assay, 10 μg Ad36 in 100 μL PBS was added to each well and incubated at 37ºC for 1 hour. Cells were washed and blotted, and then

primary antibody sera (1:10 dilution) or control (PBS) was added to the wells and incubated at 37ºC for 1 hour. Secondary antibody (goat anti-human immunoglobulin G [IgG] (HþL) whole molecule-alkaline phosphatase conjugated) diluted 1:500 was added to wells (100 mL/well) and incubated at 37ºC for 1 hour. Wells were washed and 100 mL para-nitrophenyl phosphate substrate/well was added and incubated at 37ºC for 10 to 15 minutes. Absorbance was measured at optical density (OD) 405/495 nm (24).

The Luminex xMAP system, a high-throughput microsphere-based suspension array, was used with a MILLIPLEX MAP human cytokine/chemokine immunoassay (Millipore, St. Charles, MO) for the quantification of serum inflammatory-related markers, TNF-α, IL-6, VEGF, and MCP-1, according to the manufacturer's guidelines. Biochemical measures were conducted in a single laboratory at UGA 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, 2.0% and 18.3% for IL-6, 3.7% and 10.4% for VEGF, and 1.5% and 7.9% for MCP-1, respectively.

**Statistical analyses**

Data were checked for outliers and normality with histograms and tests of skewness and kurtosis for normality. Because TNF-α, IL-6, VEGF, and MCP-1 had skewed distributions, they were log-transformed (i.e., TNF-α, VEGF, and MCP-1) or square root transformed (i.e., IL-6) for analyses, but back-transformed when we present the results in Table 3.1 for ease of interpretation. We used analysis of variance to compare age, sexual maturation stage, anthropometric, body composition, and the inflammatory-related variables between the Ad36(+) and Ad36(-) groups. Group differences in categorical variables were tested using chi-square

tests. Multinomial logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) for the presence of Ad36(+) according to tertiles of fat mass and the inflammatory-related variables (i.e., TNF-α, IL-6, VEGF, and MCP-1), after adjusting for age, sex, race, and FFST mass. Subsequent models for TNF-α, IL-6, VEGF, and MCP-1 were adjusted additionally for fat mass. All statistical analyses were conducted using SPSS software (version 21, IBM SPSS Statistics, Chicago, IL) and statistical significance was set at P-value less than 0.05.

**Results**

Participant characteristics are presented in **Table 3.1**. The sample was composed of 291 children, ages 9 to 13 years, with 50% female, 49% black, 21% obese, and 42% Ad36(+). There were no differences in the prevalence of Ad36(+) by testing site (P=0.17). No significant differences were observed between groups [Ad36(+) versus Ad36(-)] in age, sexual maturation stage, sex, FFST mass, fat mass, or body fat percent. In the Ad36(+) group, there was a greater proportion of whites (59%) versus blacks (41%), and obese (13%) versus non-obese (87%) than in the Ad36(-) group (both P<0.05). The Ad36(+) group had significantly lower BMI, BMI z-score, and BMI-for-age percentile, and significantly higher serum concentrations of TNF-α, IL-6, and VEGF than the Ad36(-) group (all P<0.05).

The prevalence and adjusted odds ratios for Ad36 seropositivity across tertiles of total body fat mass and serum concentrations of TNF-α, IL-6, VEGF, and MCP-1 are presented in **Table 3.2**. There were a higher percentage of children with Ad36 seropositivity in the highest tertiles of serum TNF-α and serum IL-6 compared to their respective middle and lowest tertiles (both P<0.03). There was also a trend for a higher percentage of children with Ad36 seropositivity in the highest tertile of serum VEGF compared to the middle and lowest tertiles (P=0.05). Multinomial logistic regression, adjusting for age, race, sex, and FFST mass, revealed

that compared to children with the lowest serum TNF-α, IL-6, and VEGF levels (Tertile 1), the adjusted odds ratios for Ad36(+) were 2.2 (95% CI: 1.2-4.0), 2.4 (95% CI: 1.4-4.0), and 1.8 (95% CI: 1.0-3.3), respectively, for those in the highest serum TNF-α, IL-6, and VEGF levels (Tertile 3). When fat mass was added as a covariate, these significant adjusted odd ratios for Ad36(+) persisted for TNF-α (OR: 2.2; 95% CI: 1.2-4.0), IL-6 (OR: 2.4; 95% CI: 1.4-4.1), and VEGF (OR: 1.9; 95% CI: 1.0-3.3)]. No association was observed between Ad36 seropositivity and greater levels of fat mass or increased serum MCP-1 concentrations (all $P>0.05$).

**Discussion**

To our knowledge, this is the first pediatric study to investigate associations of the human Ad36 infection with a robust measurement of adiposity and with serum markers implicated in inflammation. We did not observe a relationship between Ad36 infection and total body fat mass after controlling for age, sex, race, and FFST mass. On the other hand, Ad36 infection was associated with several biomarkers implicated in inflammation, including TNF-α, IL-6, and VEGF. These relationships were independent of potential confounding factors, such as age, sex, race, FFST mass, and fat mass.

The overall prevalence of Ad36(+) in our sample was 42%, which is similar to what has been reported in other pediatric studies (2, 10, 11). We found a higher prevalence of Ad36(+) in white versus black children, and though earlier adenovirus research has also shown differences by racial/ethnic group, findings are inconsistent. For example, while Gabbert et al. (10) reported that the prevalence of Ad36(+) was not different by race in 124 children, Broderick et al. (25) showed that Ad36(+) was significantly higher in black versus white adults. While determining the association of Ad36(+) with adiposity and inflammatory-related markers by race was not an objective of this study, future work in this area is warranted.

Several studies have investigated the association of Ad36(+) with adiposity in children, yet the findings have also been disparate. Contrasting results may be related to differences in target populations and measures of body fatness, with findings in support of the adenovirus-adiposity link largely derived from obese cohorts and studies that rely on BMI-based outcomes (1, 10, 26). For example, in a cohort of 84 obese children, Atkinson et al. (1) found that mean BMI z-score was higher in Ad36(+) versus Ad36(-) groups. In another sample of 157 children, Parra-Rojas et al. (26) found no significant differences in mean BMI by Ad36 status, but did observe a higher prevalence of Ad36(+) in obese versus non-obese participants (defined as BMI-for-age ≥95th percentile). Conversely, we observed a lower prevalence of Ad36(+) in obese versus non-obese children. While it is unclear why participants with a BMI-for-age ≥95th percentile were less likely to be classified as Ad36(+), it is important to note that there were no significant differences in fat mass or body fat percent by Ad36 status, and such robust measures of adiposity were not assessed in the previous studies. Our findings are in agreement with Na et al. (11), who also reported no significant differences in body fat percent between Ad36(+) and Ad36(-) groups in a cohort of 318 children who were obese and non-obese.

It has been postulated that systemic inflammation triggered in the early stages of Ad36 infection leads to the development of human obesity (9, 14). Given that there are no optimal ranges for serum concentrations of TNF-α, IL-6, VEGF, and MCP-1 for adults or children, we compared these inflammatory-related markers across tertile groups. In the present study, the prevalence of Ad36(+) was significantly associated with TNF-α, IL-6, and VEGF, but not MCP-1. These inflammatory-related markers represent a variety of cytokines, chemokines, and growth factors derived from a common cellular pathway that is activated by Ad36 infection (9, 27), and have been shown to act synergistically to induce obesity and related disorders (28, 29). Indeed,

Na and Nam (9) found that Ad36 infection increased expression of MCP-1 and TNF-α, which induced macrophage migration and infiltration, respectively, and culminated in increased adiposity in mice. Similarly, Bouwman et al. (16) reported that Ad36-infected human adipocytes had increased production of IL-6, which has been shown to stimulate VEGF and contribute to vascularization preceding adipose tissue growth (30, 31). While these inflammatory-related markers have also been implicated in the development of cardiovascular diseases (13) and diabetes mellitus (13, 32), our sample was relatively healthy with no history of metabolic disorders, and potential chronic effects were indeterminate at this time point.

While it is unclear why Ad36(+) was not associated with serum MCP-1 in children when Na and Nam (9) found a positive relationship in Ad36(+) adults, it is possible that age and the relative duration of Ad36 infection has differential effects on inflammatory response and long-term health. The sample of children in our study entering the early stages of puberty was younger than those of other studies that support the Ad36-adiposity association (mean age of 11 versus 16 years-old) (1, 3, 10). Though the chronic effects of Ad36 infection and inflammatory-related markers cannot be determined from the present study, it is possible that the Ad36-adiposity relationship emerges with age following years of exposure. This may be plausible given the proposed mechanism by which Ad36-induced inflammation triggers obesity development, and the underlying actions of the aforementioned inflammatory-related markers in the pathogenesis of progressive diseases (9, 16, 30). The hypothesis that viral infection may lead to chronic health outcomes must be explored through long-term prospective studies.

Strengths of the present study include the ethnically diverse sample of young children, and the use of both direct (fat mass and body fat percent via DXA) and indirect (BMI z-score and BMI-for-age percentile) measures of body fatness for comparisons. We acknowledge some

potential limitations. Though our study was conducted in a relatively large sample (1, 10), it is possible that our sample size was inadequate to detect differences in adiposity by Ad36 group given findings from larger-scale studies of children (2, 3). Our study also used cross-sectional data, and therefore we cannot determine how long Ad36(+) participants had been infected, nor can we be certain that Ad36(+) had a direct effect on serum inflammatory-related markers. We did not measure plasma C-reactive protein (CRP), a clinical marker of systemic inflammation, though it should be noted that CRP has been shown to highly correlate with TNF-α (33) and IL-6 (33, 34). Lastly, we assessed total body fatness rather than regional adiposity. Regional adiposity measures (e.g., subcutaneous and visceral fat deposition) differ following infection with the virus (4). Therefore, location of adipose tissue accumulation may provide greater insight into the long-term effects of Ad36(+) on production of inflammatory-related markers and chronic disease.

In conclusion, Ad36 seropositivity in young children was not associated with greater adiposity, assessed by total body fat mass, but was associated with several biomarkers implicated in inflammation. Research efforts in this area should focus on determining whether exposure to elevated serum inflammatory-related markers observed in Ad36(+) children has long-term effects on obesity and related outcomes. Moreover, future studies are warranted to examine the relationships between regional fat distribution, inflammation, and risk factors for chronic disease in children classified as Ad36(+) versus Ad36(-).

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**Table 3.1.** Participant characteristics.

| | Total sample | Ad36(+) group | Ad36(-) group | *P* value[a] |
|---|---|---|---|---|
| *n* | 291 | 122 | 169 | |
| Age, y | 11.3 ± 1.2 | 11.4 ± 1.2 | 11.3 ± 1.3 | 0.23 |
| Sexual maturation stage | 2.4 ± 0.5 | 2.4 ± 0.5 | 2.4 ± 0.5 | 0.84 |
| Female, %[b] | 50 | 53 | 47 | 0.27 |
| Black, %[b] | 49 | 41 | 54 | 0.02 |
| Body mass index[c] | 20.7 ± 4.3 | 20.1 ± 4.0 | 21.3 ± 4.5 | 0.02 |
| *Z*-score | 0.7 ± 1.0 | 0.5 ± 1.0 | 0.8 ± 1.1 | 0.02 |
| Percentile | 69.1 ± 28.6 | 64.8 ± 27.4 | 72.1 ± 29.1 | 0.03 |
| Obesity, %[b] | 21 | 13 | 27 | 0.01 |
| FFST mass, kg | 30.7 ± 6.9 | 30.3 ± 7.2 | 30.9 ± 6.7 | 0.50 |
| Fat mass, kg | 14.7 ± 7.3 | 14.0 ± 7.2 | 15.2 ± 7.4 | 0.17 |
| Body fat, % | 30.9 ± 9.3 | 30.2 ± 9.2 | 31.4 ± 9.4 | 0.28 |
| TNF-α, pg/ml | 9.9 ± 1.7 | 10.9 ± 1.8 | 9.1 ± 1.6 | <0.01 |
| IL-6, pg/ml | 2.8 ± 2.0 | 3.5 ± 2.3 | 2.3 ± 1.8 | 0.03 |
| VEGF, pg/ml | 190.6 ± 2.6 | 239.8 ± 2.5 | 160.8 ± 2.6 | <0.01 |
| MCP-1, pg/ml | 428.4 ± 1.7 | 437.0 ± 1.7 | 419.9 ± 1.7 | 0.47 |

Values are means ± SD or %. FFST, fat-free soft tissue; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; MCP-1, monocyte chemoattractant protein-1.

[a]Tests of significance between groups were based on ANOVA.

[b]Tests of significance between groups were based on the chi-square test of goodness of fit.

[c]BMI is calculated as weight in kilograms divided by height in meters squared. Obesity indicates a BMI ≥95$^{th}$ percentile.

**Table 3.2**. Prevalence and adjusted odds ratios (95% CI) for Ad36 seropositivity across tertiles of total body fat mass and serum concentrations of TNF-α, IL-6, VEGF, and MCP-1 in children ages 9-13 years.

| | | | | |
|---|---|---|---|---|
| | **Total body fat mass**[a] | | | |
| | Tertile 1<br>7.8 kg (<10.4 kg) | Tertile 2<br>13.3 kg (10.4 - 16.1 kg) | Tertile 3<br>22.0 kg (>16.1 kg) | *P* |
| *n* | 98 | 97 | 96 | |
| Ad36 Seropositivity | | | | |
| Prevalence, % | 45 | 49 | 32 | 0.06 |
| Adjusted OR (95% CI)[b] | 1.0 | 1.2 (0.6, 2.1) | 0.6 (0.3, 1.2)[c] | 0.15 |
| | **Serum TNF-α**[a] | | | |
| | Tertile 1<br>6.2 pg/ml (<8.2 pg/ml) | Tertile 2<br>9.6 pg/ml (8.2-11.9 pg/ml) | Tertile 3<br>14.9 pg/ml (≥12.0 pg/ml) | *P* |
| *n* | 96 | 96 | 99 | |
| Ad36 Seropositivity | | | | |
| Prevalence, % | 31 | 44 | 51 | 0.02 |
| Adjusted OR (95% CI)[b] | 1.0 | 1.7 (0.9, 3.2) | 2.2 (1.2, 4.0)[c] | 0.01 |
| | **Serum IL-6**[a] | | | |
| | Tertile 1<br>0.9 pg/ml (<1.0 pg/ml) | Tertile 2<br>1.3 pg/ml (1.1-1.5 pg/ml) | Tertile 3<br>6.6 pg/ml (≥1.6 pg/ml) | *P* |
| *n* | 97 | 95 | 99 | |
| Ad36 Seropositivity | | | | |
| Prevalence, % | 35 | 50 | 55 | <0.01 |
| Adjusted OR (95% CI)[b] | 1.0 | 1.6 (0.2, 12.1) | 2.4 (1.4, 4.0)[c] | <0.01 |
| | **Serum VEGF**[a] | | | |
| | Tertile 1<br>82 pg/ml (<136 pg/ml) | Tertile 2<br>195 pg/ml (137-313 pg/ml) | Tertile 3<br>463 pg/ml (≥313 pg/ml) | *P* |
| *n* | 98 | 94 | 99 | |
| Ad36 Seropositivity | | | | |
| Prevalence, % | 33 | 44 | 49 | 0.05 |
| Adjusted OR (95% CI)[b] | 1.0 | 1.6 (0.9, 2.8) | 1.8 (1.0, 3.3)[c] | 0.04 |

| | Serum MCP-1[a] | | | |
|---|---|---|---|---|
| | Tertile 1<br>264 pg/ml (<332 pg/ml) | Tertile 2<br>419 pg/ml (334-536 pg/ml) | Tertile 3<br>694 pg/ml (≥536 pg/ml) | *P* |
| *n* | 95 | 97 | 99 | |
| Ad36 Seropositivity | | | | |
| Prevalence, % | 39 | 41 | 45 | 0.65 |
| Adjusted OR (95% CI)[b] | 1.0 | 0.8 (0.4, 1.5) | 0.8 (0.4, 1.6) | 0.53 |

Abbreviations: TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; MCP-1, monocyte chemoattractant protein-1.

[a]Values are median (range) in a given tertile.

[b]Data are adjusted for age, race, sex, and fat-free soft tissue mass.

[c]Significantly different from the reference group, tertile 1 ($P < 0.05$).

# CHAPTER 4

# ASSOCIATIONS OF ADENOVIRUS 36 INFECTION, BONE STRENGTH, AND INFLAMMATORY-RELATED MARKERS IN OBESE CHILDREN[2]

[2]Berger PK, Pollock NK, Laing EM, Warden SJ, Hill Gallant KM, Hausman DB, Bailey LB, Tripp RA, McCabe LD, McCabe GP, Weaver CM, Peacock M, Lewis RD. 2015. To be submitted to *The Journal of Bone Mineral Research*.

**Abstract**

We previously found that adenovirus 36 (Ad36) infection was associated with lower cortical bone strength in obese late adolescents, and with systemic inflammation in children. It is unclear whether these indices are correlated in Ad36 seropositive children. This study investigated associations of Ad36 infection, bone strength, and serum inflammatory-related markers in 78 obese females ages 9-12 years (≥32% body fat, 58% black). Fasting blood samples were measured for Ad36-specific antibodies and concentrations of inflammatory-related markers, tumor necrosis factor-alpha (TNF-$\alpha$), interleukin-6, vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1. Fat mass, fat-free soft tissue (FFST) mass, and body fat percent were measured by dual-energy X-ray absorptiometry. Cortical bone indices at the 66% non-dominant radius and tibia were measured using peripheral quantitative computed tomography, with strength-strain index (SSI) as the primary outcome measure. The overall prevalence of Ad36 seropositivity was 38%. After adjusting for age, race, fat mass, and FFST mass, the Ad36-positive [Ad36(+)] group had lower radial SSI, but not tibial SSI, than the Ad36-negative [Ad36(-)] group (P=0.05). The Ad36(+) group also had higher serum concentrations of TNF-$\alpha$ and VEGF versus the Ad36(-) group (both P<0.05). Radial SSI was not correlated with any of the inflammatory-related markers. In Ad36(+) obese children, our data show that lower cortical bone strength at the radius is not associated with higher biomarkers implicated in inflammation. Research efforts should determine whether exposure to Ad36 infection and inflammatory-related markers in childhood has long-term effects on osteoporosis in adulthood.

KEY WORDS: Adenovirus, Obesity, Bone strength, Inflammation, Children

**Introduction**

Osteoporosis is a major public health concern, with 1 in 3 women and 1 in 5 men age 50 years and older likely to experience an osteoporotic fracture in their lifetime (1-3). Peak bone strength, the result of rapid bone mineral accrual in childhood that is generally achieved by early adulthood (4-6), is an important determinant of long-term risk for osteoporosis (7). Therefore, a childhood disease or condition that impedes bone strength development may lead to suboptimal peak bone strength attainment and presumably a greater risk for fracture in later life. Given that the timing of peak bone strength varies by skeletal site (4, 6), and both children and older adults are at high risk for forearm fractures (8-10), research on suboptimal bone development at the radius may have important clinical implications.

A contributing factor to suboptimal bone development in children may be infection with human adenovirus 36 (Ad36), a common upper respiratory illness transmitted through person-to-person contact (11) that elicits an antibody response ranging from days to years post-exposure (12). Ad36 has been implicated in adiposity development through enhanced adipocyte differentiation in human-derived cells (13-15), and it has been postulated that this preferential fat differentiation comes at the expense of bone development given that adipocytes and osteoblasts share a mesenchymal progenitor (16-18). Indeed, a recent study from our laboratory showed that obese late adolescent females who tested positive for Ad36-specific antibodies exhibited lower cortical bone strength versus those who tested Ad36-negative (18).

Why obese late adolescent females exposed to Ad36 infection had lower cortical bone strength is unclear. It is possible that inflammation may play a role in the potential Ad36-bone association because it has been implicated in the progression of both viral infection and osteoporosis. In vitro, Ad36 has been reported to induce cellular activation of an inflammatory

pathway that signals the production of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1). These inflammatory-related markers have been shown to modulate bone formation and resorption (19-21). Moreover, our research team recently reported that infection with Ad36 was associated with inflammatory-related markers in otherwise healthy children (22). Given our findings in obese late adolescents regarding the virus and cortical bone indices (18), it may be that these inflammatory-related markers provide an important link between Ad36 infection and reduced bone strength. Therefore, the primary objective of this study was to determine associations of Ad36 infection, bone strength, and inflammatory-related markers in 78 obese females ages 9 to 12 years.

**Subjects and methods**

The study included data from a subsample of children who were part of a multi-site vitamin D supplementation trial conducted at the University of Georgia (UGA, Athens, Georgia), Purdue University (PU, West Lafayette, Indiana), and Indiana University (IU, Indianapolis, Indiana) (n=78) (23). These 78 subjects were chosen from the original baseline sample because they were female and classified as obese with a body fat ≥32% (18, 24, 25). Other inclusion criteria were as follows: (1) age 9 to 12 years, (2) white or black/African American race, and (3) sexual maturation stages 2 and 3, estimated using self-administered questionnaires for breast development (26). Children were excluded if they were taking medications or had any medical condition known to affect growth, maturation, nutritional status, metabolism, or inflammatory response. The Institutional Review Board for Human Subjects at each testing site approved the study procedures. All participants and their parent/guardian provided informed assent and consent, respectively.

**Anthropometry and body composition**

Standing height was measured using a wall-mounted stadiometer to the nearest 0.1 cm. Body weight was measured using an electronic scale to the nearest 0.1 kg (27).

Body composition was assessed using dual-energy X-ray absorptiometry [DXA, Delphi-A, Hologic Inc (UGA); Lunar iDXA, GE Medical Instruments (PU); and Hologic Discovery-W (IU)]. The same technician at each site conducted scans and performed analyses using instrument-specific software and protocols. Cross-calibration was performed to facilitate comparability of data from each study site/scanner, as previously described (28). Briefly, the UGA and PU study sites were cross-calibrated by scanning 26 individuals on the Hologic Delphi-A scanner and a Lunar iDXA scanner, whereas the IU and PU study sites were cross-calibrated by scanning ten individuals on the Hologic Discovery-W and Lunar iDXA scanners. Regression formulae between data obtained at UGA and PU, and IU and PU were derived for each variable and used to adjust data obtained at UGA and IU to PU values (28). Data obtained from the scans included total body areal bone mineral density (BMD), bone mineral content (BMC), bone area (BA), fat mass, fat-free soft tissue (FFST) mass, and body fat percent.

**Peripheral quantitative computed tomography**

Cortical bone measurements were performed using peripheral quantitative computed tomography [pQCT; Stratec XCT 2000, Stratec Medizintechnik GmbH (UGA, PU and IU)] at the 66% site of the non-dominant radius and tibia from the distal metaphysis. Tomographic slices

were analyzed for BMD, structure, and estimated strength, as described in Warden et al. (28). Cort mode 1 (threshold, 710 mg/cm$^3$) was used to obtain cortical BMD (CtBMD, mg/cm$^3$), BMC (CtBMC, mg/mm), and area (CtAr, cm$^2$). Total area (TtAr, mm$^2$), cortical thickness (CtTh, mm), and periosteal (PsPm, mm) and endosteal (EsPm, mm) perimeters were obtained by analyzing the slices using contour mode 1 (threshold, 710 mg/cm$^3$) to define the outer bone edge and peel mode 2 (threshold, 400 mg/cm$^3$) to separate the cortical and cancellous compartments.

Estimated strength was obtained by calculating the polar strength-strain index (SSI, mm$^3$), which served as the primary outcome measure. SSI represents the density-weighted section modulus and has been validated as a non-invasive measure of bone strength (29). It 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., 1,200 mg/mm$^3$), as previously described (28, 30, 31). Section modulus (mm$^3$) was calculated as (a × d2)/dmax, where "a" is the cross-sectional area of a voxel (mm$^2$), "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).

(1) $SSI = \Sigma [(a \times d2)(CtBMD / \text{normal physiologic density BMD})] / dmax$

Comparability of bone data from each study site/scanner was achieved by scanning a cortical bone phantom with known properties a minimum of 20 times on each scanner (28).

**Biochemical analyses**

Blood samples were collected from fasting participants for assessment of Ad36 seropositivity, and to determine the concentrations of TNF-α, IL-6, VEGF, and MCP-1 in sera. An indirect ELISA was used to determine Ad36 seropositivity [i.e., Ad36(+) or Ad36(-)] as described in Laing et al. (18). For the indirect ELISA assay, 10 μg Ad36 in 100 μL PBS was added to each well and incubated at 37ºC for 1 hour. Cells were washed and blotted, and then primary antibody sera (1:10 dilution) or control (PBS) was added to the wells and incubated at 37ºC for 1 hour. Secondary antibody (goat anti-human immunoglobulin G [IgG] (HþL) whole molecule-alkaline phosphatase conjugated) diluted 1:500 was added to wells (100 mL/well) and incubated at 37ºC for 1 hour. Wells were washed and 100 mL para-nitrophenyl phosphate substrate/well was added and incubated at 37ºC for 10 to 15 minutes. Absorbance was measured at optical density (OD) 405/495 nm (18).

Serum inflammatory-related markers, TNF-α, VEGF, and 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), according to the manufacturer's guidelines. These biochemical measures were conducted in a single laboratory at UGA 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. 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 checked for outliers and normality with histograms and tests of skewness and kurtosis for normality. Because TNF-α, IL-6, VEGF, and MCP-1 had skewed distributions, they were log-transformed for analyses, and back-transformed for ease of interpretation when presented in the results (Table 4.3). Analysis of variance was used to compare age, sexual maturation stage, anthropometric, and body composition variables between the Ad36(+) and Ad36(-) groups. ANOVA was also used to compare bone indices and inflammatory-related markers between groups, after adjusting for age, race, fat mass, and FFST mass. Group differences in categorical variables were tested using chi-square tests.

Partial Pearson's correlation coefficients were used to examine associations of cortical bone indices and inflammatory-related markers in the Ad36(+) group, after adjusting for age, race, fat mass, and FFST mass. All statistical analyses were conducted using SPSS software (version 21, IBM SPSS Statistics, Chicago, IL) and statistical significance was set at a P-value less than or equal to 0.05.

**Results**

Participant characteristics are presented in **Table 4.1**. The sample was composed of 78 obese females, ages 9 to 12 years, with 58% black and 38% Ad36(+). No significant differences were observed between groups [Ad36(+) versus Ad36(-)] in age, sexual maturation stage, weight, height, fat mass, FFST mass, or body fat percent. In the Ad36(+) group, there was a greater proportion of whites (57%) versus blacks (43%) than in the Ad36(-) group (P=0.04).

Bone indices at the total body, radius, and tibia are presented in **Table 4.2**. The Ad36(+) group had significantly lower TtAr, PsPm, and SSI at the radius versus the Ad36(-) group (P≤0.05). No differences were observed between groups in bone indices at the total body and

tibia. The Ad36(+) group had significantly higher serum concentrations of TNF-α and VEGF than the Ad36(-) group (both $P<0.05$). No differences were observed between groups in serum concentrations of IL-6 and MCP-1 (**Table 4.3**).

Associations of serum inflammatory-related markers and bone indices in the Ad36(+) group are presented in **Table 4.4**. TNF-α was negatively associated with tibial SSI ($r=-0.48$, $P=0.02$). IL-6 was positively associated with radial CtTh ($r=0.49$, $P=0.02$), and negatively associated with radial EsPm ($r=-0.54$, $P=0.01$). MCP-1 was positively associated with total body bone area ($r=0.42$, $P=0.03$), and negatively associated with radial TtAr ($r=-0.44$, $P=0.04$) and PsPm ($r=-0.44$, $P=0.04$). There were no correlations observed between VEGF and the cortical bone indices.

**Discussion**

To our knowledge, this is the first study in obese children to investigate associations of the human Ad36 infection, bone strength, and biomarkers implicated in inflammation. After adjusting for age, race, fat mass, and FFST mass, Ad36 infection was associated with lower cortical bone strength at the radius, but not at the tibia. Ad36 infection was also associated with higher serum concentrations of inflammatory-related markers, TNF-α and VEGF. Lower cortical bone strength at the radius, however, was not correlated with higher serum concentrations of inflammatory-related markers observed in Ad36(+) obese children.

Though several studies have investigated the association of Ad36(+) and adiposity in children (32-34), research related to Ad36(+) and bone is limited. Given the parallels in the biology of these tissues (16-18), it may be that the proposed effects of Ad36 on adiposity development diminish bone development (13). Yet to our knowledge, only one study has examined the relationship of Ad36(+) with bone strength in humans. Laing et al. (18) found that

Ad36(+) was associated with lower SSI at the radius, but not at the tibia, in obese females ages 18 to 19 years. There were no associations, however, observed in non-obese females. This prompted our study in a younger cohort to determine whether this relationship was evident in the years prior to peak bone strength attainment. We also found that Ad36(+) was associated with lower SSI at the radius, but not at the tibia, in obese females ages 9 to 12 years. It is possible that the combination of Ad36(+) plus obesity impedes bone strength development to a greater extent than obesity alone beginning in early childhood. This is important, as rapid bone mineral accretion occurs during childhood, and impediments to peak bone strength attainment in late adolescence are major determinants of long-term risk for osteoporosis (7). The hypothesis that Ad36 infection coupled with obesity may compromise long-term bone health, however, must be explored through prospective studies.

We postulated that lower cortical bone indices at the radius observed in Ad36(+) obese children were related to higher serum concentrations of inflammatory-related markers. This was plausible, as inflammation is an underpinning factor in viral infections and obesity (35), and data suggest that inflammatory-related markers are associated with bone loss in vitro and in vivo (19, 20, 36, 37). First, we found that serum concentrations of TNF-α and VEGF were higher in the Ad36(+) versus the Ad36(-) group. It is unclear why there were no differences in serum concentrations of IL-6 and MCP-1 given that all four inflammatory-related markers are derived from a common cellular pathway that is activated by Ad36 infection (15, 38). It is possible that these findings reflect variations in inflammatory-related marker production over the course of infection. In addition, while our results suggest that higher serum concentrations of TNF-α and VEGF are related to Ad36 infection, studies have shown that these inflammatory-related markers are also increased in human obesity. It is unclear how inflammatory-related markers may be

compounded by these two conditions to affect long-term health. This may explain why lower cortical bone strength at the radius has been observed in Ad36(+) obese subjects and not in Ad36(+) non-obese subjects (18).

Contrary to our hypothesis, however, lower TtAr, PsPm, and SSI at the radius were not associated with higher serum concentrations of TNF-α and VEGF observed in Ad36(+) obese children. It is unclear why there were no correlations, as cell culture studies have shown that several inflammatory-related markers induce bone resorption via enhanced osteoclast activity (19, 36, 37), and human studies have shown that cytokines are strong predictors of bone fracture (20, 39). It is possible that increased serum concentrations of TNF-α and VEGF with Ad36(+) reflect an acute, and not a chronic, inflammatory-related response. Short-term exposure to these inflammatory-related markers may therefore be inadequate to affect bone strength in children. Conclusions regarding temporal relationships, however, cannot be determined given the study design.

While lower cortical bone strength at the radius did not correlate with higher serum concentrations of TNF-α and VEGF, there were significant associations of alternate bone indices and inflammatory-related markers observed in Ad36(+) obese children. We found that serum TNF-α was negatively associated with SSI at the tibia, and serum MCP-1 was negatively associated with TtAr and PsPm at the radius. This supports the aforementioned findings in vitro and in vivo. Conversely, serum IL-6 was positively associated with CtTh and negatively associated with EsPm at the radius. This suggests that serum IL-6 is advantageous to bone. IL-6 has been recognized as a highly pleiotropic factor in bone remodeling. While IL-6 is most often

implicated in bone resorption (40), it has also been shown to activate bone formation (41, 42). While there are currently no standards for serum concentrations of IL-6 in children, our results may reflect physiologic concentrations with positive effects on bone versus elevated concentrations with potential negative effects on bone (40).

Strengths of the present study include the ethnically diverse sample of young children, and the use of advanced imaging technology (i.e., DXA and pQCT) for the assessment of body composition and bone indices. We acknowledge some potential limitations. Our study used cross-sectional data, and we cannot determine how long Ad36(+) obese participants had been infected. Moreover, we cannot be certain that Ad36(+) had a direct effect on serum inflammatory-related markers, or that serum inflammatory-related markers had a direct effect on bone indices. We did not measure plasma CRP as a clinical marker of systemic inflammation, though it should be noted that CRP has been shown to highly correlate with TNF-α (43) and IL-6 (43, 44).

In conclusion, Ad36 seropositivity in obese female children was negatively associated with cortical bone strength at the radius and positively associated with several biomarkers implicated in inflammation. The lower cortical bone strength at the radius, however, was not correlated with the higher serum inflammatory-related markers. Research efforts in this area should focus on determining whether Ad36(+) obese children exposed to higher serum concentrations of inflammatory-related markers exhibit long-term effects on bone health and related outcomes.

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**Table 4.1.** Participant characteristics.

| Variable | Total Sample | Ad36(+) Group | Ad36(-) Group | *P* value[a] |
|---|---|---|---|---|
| n | 78 | 30 | 48 | |
| Age, y | 10.3 ± 0.9 | 10.5 ± 0.9 | 10.2 ± 0.9 | 0.13 |
| Sexual maturation stage | 2.3 ± 0.5 | 2.3 ± 0.5 | 2.3 ± 0.4 | 0.63 |
| Black, %[b] | 58 | 43 | 67 | 0.04 |
| Weight | 49.7 ± 11.1 | 49.4 ± 13.1 | 49.9 ± 9.9 | 0.83 |
| Height | 146.3 ± 8.8 | 146.0 ± 9.4 | 146.5 ± 8.4 | 0.80 |
| Fat mass, kg | 19.7 ± 6.3 | 19.8 ± 7.9 | 19.6 ± 5.1 | 0.91 |
| FFST mass, kg | 27.9 ± 5.4 | 27.5 ± 5.5 | 28.2 ± 5.4 | 0.57 |
| Body fat, % | 40.0 ± 5.3 | 40.2 ± 6.3 | 39.9 ± 4.7 | 0.78 |

Values are means ± SD or percentage.

[a]Tests of significance between groups were based on ANOVA ($P \leq 0.05$).

[b]Tests of significance between groups were based on the $x^2$ test of goodness of fit.

**Table 4.2.** Bone indices at the total body, radius, and tibia between Ad36(+) and Ad36(-) groups.[a]

| Variable | Total Sample | Ad36(+) Group | Ad36(-) Group | *P* value[b] |
|---|---|---|---|---|
| Total body | | | | |
| BMD, g/cm$^2$ | 0.85 ± 0.2 | 0.84 ± 0.1 | 0.85 ± 0.2 | 0.87 |
| BMC, g | 1474.6 ± 324.7 | 1429.5 ± 254.3 | 1500.7 ± 359.2 | 0.34 |
| BA, cm$^2$ | 1749.1 ± 252.8 | 1723.5 ± 258.2 | 1763.8 ± 251.4 | 0.41 |
| Radius, 66% | | | | |
| CtBMD, mg/cm$^3$ | 1063.6 ± 39.1 | 1060.0 ± 47.1 | 1065.7 ± 34.2 | 0.71 |
| CtBMC, mg/mm | 58.9 ± 16.2 | 56.5 ± 11.8 | 60.2 ± 18.3 | 0.88 |
| CtAr, cm$^2$ | 56.8 ± 11.4 | 53.0 ± 9.7 | 58.9 ± 11.9 | 0.10 |
| TtAr, cm$^2$ | 88.0 ± 16.3 | 81.6 ± 10.2 | 91.7 ± 18.1 | 0.04 |
| CtTh, mm | 2.2 ± 0.4 | 2.1 ± 0.4 | 2.2 ± 0.3 | 0.81 |
| PsPm, mm | 33.1 ± 3.0 | 31.9 ± 2.0 | 33.8 ± 3.3 | 0.05 |
| EsPm, mm | 19.6 ± 3.0 | 18.7 ± 2.8 | 20.1 ± 3.1 | 0.18 |
| SSI, mm$^3$ | 160.2 ± 47.6 | 142.7 ± 32.7 | 170.2 ± 52.1 | 0.05 |
| Tibia, 66% | | | | |
| CtBMD, mg/cm$^3$ | 1066.4 ± 35.7 | 1059.1 ± 42.5 | 1070.6 ± 30.8 | 0.25 |
| CtBMC, mg/mm | 242.6 ± 46.7 | 231.5 ± 43.5 | 249.0 ± 47.7 | 0.12 |
| CtAr, cm$^2$ | 227.1 ± 40.5 | 218.1 ± 37.7 | 232.2 ± 41.6 | 0.19 |
| TtAr, cm$^2$ | 430.5 ± 85.5 | 411.6 ± 69.4 | 441.4 ± 92.5 | 0.55 |
| CtTh, mm | 3.7 ± 0.5 | 3.6 ± 0.6 | 3.7 ± 0.4 | 0.49 |
| PsPm, mm | 73.2 ± 7.2 | 71.7 ± 6.0 | 74.1 ± 7.8 | 0.66 |
| EsPm, mm | 50.1 ± 7.2 | 48.9 ± 6.9 | 50.8 ± 7.4 | 0.99 |
| SSI, mm$^3$ | 1599.3 ± 466.7 | 1479.4 ± 351.6 | 1668.5 ± 512.7 | 0.21 |

Values are means ± SD.

[a]Abbreviations: BA, bone area; BMC, bone mineral content; BMD, bone mineral density; CtAr, cortical area; CtBMC, cortical bone mineral content; CtBMD, cortical bone mineral density; CtTh, cortical thickness; EsPm, endosteal perimeter; PsPm, periosteal perimeter; SSI, strength strain index; TtAr, total area.

[b]Tests of significance between groups were based on ANOVA adjusting for age, race, FM, and FFST mass ($P \leq 0.05$).

**Table 4.3.** Serum concentrations of inflammatory-related markers between Ad36(+) and Ad36(-) groups.[a]

| Variable | Total Sample | Ad36(+) Group | Ad36(-) Group | *P* value[b] |
|---|---|---|---|---|
| TNF-α, pg/mL | 10.2 ± 1.9 | 13.2 ± 2.2 | 8.7 ± 1.6 | <0.01 |
| IL-6, pg/mL | 3.9 ± 1.6 | 2.8 ± 1.0 | 4.6 ± 1.9 | 0.21 |
| VEGF, pg/mL | 194.4 ± 2.4 | 267.7 ± 2.2 | 160.8 ± 2.4 | 0.01 |
| MCP-1, pg/mL | 376.2 ± 1.7 | 428.4 ± 1.7 | 347.2 ± 1.7 | 0.33 |

Values are means ± SD.

[a]Abbreviations: IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

[b]Tests of significance between groups were based on ANOVA adjusting for age, race, FM, and FFST mass ($P \leq 0.05$).

**Table 4.4.** Associations of serum inflammatory-related markers with bone indices at the total body, radius, and tibia in the Ad36(+) group.[a]

| | TNF-α [b] | | IL-6[b] | | VEGF[b] | | MCP-1[b] | |
|---|---|---|---|---|---|---|---|---|
| | r | *P* value | r | *P* value | r | *P* value | r | *P* value |
| Total body | | | | | | | | |
| BMD, g/cm$^2$ | -0.33 | 0.10 | 0.03 | 0.87 | -0.06 | 0.79 | -0.30 | 0.14 |
| BMC, g | -0.31 | 0.13 | 0 | 0.99 | 0.04 | 0.83 | -0.10 | 0.62 |
| BA, cm$^2$ | 0.22 | 0.27 | -0.08 | 0.70 | 0.08 | 0.70 | **0.42** | **0.03** |
| Radius, 66% | | | | | | | | |
| CtBMD, mg/cm$^3$ | -0.36 | 0.09 | 0.06 | 0.78 | 0.03 | 0.88 | 0.15 | 0.50 |
| CtBMC, mg/mm | -0.17 | 0.45 | 0.35 | 0.11 | 0 | 0.99 | -0.20 | 0.37 |
| CtAr, cm$^2$ | -0.09 | 0.69 | 0.38 | 0.08 | 0 | 1.0 | -0.27 | 0.22 |
| TtAr, cm$^2$ | 0.03 | 0.90 | -0.27 | 0.22 | 0.12 | 0.60 | **-0.44** | **0.04** |
| CtTh, mm | -0.11 | 0.61 | **0.49** | **0.02** | -0.06 | 0.79 | -0.05 | 0.83 |
| PsPm, mm | 0.03 | 0.89 | -0.29 | 0.20 | 0.12 | 0.59 | **-0.44** | **0.04** |
| EsPm, mm | 0.10 | 0.64 | **-0.54** | **0.01** | 0.11 | 0.61 | -0.21 | 0.34 |
| SSI, mm$^3$ | -0.03 | 0.88 | 0.01 | 0.96 | 0.15 | 0.50 | -0.31 | 0.15 |
| Tibia, 66% | | | | | | | | |
| CtBMD, mg/cm$^3$ | -0.34 | 0.10 | 0 | 1.0 | 0.10 | 0.65 | 0.18 | 0.39 |
| CtBMC, mg/mm | -0.21 | 0.32 | 0.35 | 0.10 | 0.18 | 0.41 | 0.16 | 0.46 |
| CtAr, cm$^2$ | -0.11 | 0.61 | 0.38 | 0.07 | 0.19 | 0.38 | 0.14 | 0.53 |
| TtAr, cm$^2$ | -0.25 | 0.25 | -0.07 | 0.74 | -0.29 | 0.17 | 0.18 | 0.41 |
| CtTh, mm | 0.03 | 0.87 | 0.31 | 0.15 | 0.29 | 0.18 | 0.02 | 0.93 |
| PsPm, mm | -0.24 | 0.26 | -0.07 | 0.77 | -0.30 | 0.15 | 0.15 | 0.47 |
| EsPm, mm | -0.16 | 0.47 | -0.20 | 0.36 | -0.32 | 0.13 | 0.08 | 0.71 |
| SSI, mm$^3$ | **-0.48** | **0.02** | 0.18 | 0.42 | -0.18 | 0.40 | 0.34 | 0.10 |

Values are presented as partial correlation coefficients (r) adjusted for age, race, FFST mass, and fat mass. Significant associations are in *bold*.

[a]Abbreviations: BA, bone area; BMC, bone mineral content; BMD, bone mineral density; CtAr, cortical area; CtBMC, cortical bone mineral content; CtBMD, cortical bone mineral density; CtTh, cortical thickness; EsPm, endosteal perimeter; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; PsPm, periosteal perimeter; SSI, strength strain index; TtAr, total area; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

[b]Log transformed for analyses.

# CHAPTER 5

# SUMMARY AND CONCLUSIONS

This work was conducted to determine associations of the human Ad36 infection, inflammation, adiposity, and bone strength in a cohort of otherwise healthy children. Results from the study presented in Chapter 3 demonstrate that Ad36 infection is not associated with greater adiposity, but is associated with several biomarkers implicated in inflammation in children ages 9 to 13 years (N=291, 50% female, 49% black). In this study, there was a higher prevalence of Ad36 seropositivity in the highest tertiles of serum TNF-α and serum IL-6 compared to their respective middle and lowest tertiles (both $P<0.03$). There was also a trend for a higher prevalence of Ad36 seropositivity in the highest tertile of VEGF compared to the middle and lowest tertiles ($P=0.05$). Multinomial logistic regression, adjusting for age, race, sex, and FFST mass, revealed that compared to children with the lowest serum TNF-α, IL-6, and VEGF levels (tertile 1), the adjusted odds ratios for Ad36(+) were 2.2 (95% CI: 1.2-4.0), 2.4 (95% CI: 1.4-4.0), and 1.8 (95% CI: 1.0-3.3), respectively, for those in the highest serum TNF-α, IL-6, and VEGF levels (tertile 3). When fat mass was added as a covariate, the significant adjusted odds ratios for Ad36(+) persisted for TNF-α (OR: 2.2; 95% CI: 1.2-4.0), IL-6 (OR: 2.4; 95% CI: 1.4-4.1), and VEGF (OR: 1.9; 95% CI: 1.0-3.3)]. No association was observed between Ad36 seropositivity and greater levels of fat mass or increased serum concentrations of MCP-1 (all $P>0.05$).

The study presented in Chapter 4 was conducted to determine associations of the human Ad36 infection, bone strength, and inflammatory-related markers in obese females ages 9 to 12 years (N=78, ≥32% body fat, 58% black). This study was prompted by previous findings from our laboratory. After adjusting for age, race, fat mass, and FFST mass, we found that the Ad36(+) group had significantly lower TtAr, PsPm, and SSI at the radius versus the Ad36(-) group ($P \leq 0.05$). No differences were observed between groups in bone indices at the total body and tibia. The Ad36(+) group had significantly higher adjusted serum concentrations of TNF-α and VEGF than the Ad36(-) group (both $P<0.05$). No differences were observed between groups in serum concentrations of IL-6 and MCP-1. Overall, lower cortical bone indices at the radius did not correlate with higher serum concentrations of inflammatory-related markers observed in Ad36(+) obese children, after adjusting for age, race, fat mass, and FFST mass. These results suggest that lower cortical bone strength is not attributed to higher concentrations of biomarkers implicated in inflammation observed in Ad36 infection.

The results of the studies in chapters 3 and 4 provide insight into the interrelationships of Ad36 infection, inflammation, adiposity, and bone strength. However, many questions remain to be answered. Because these were cross-sectional studies, we cannot determine how long Ad36(+) children had been infected. Moreover, we cannot be certain that Ad36 infection had a direct effect on serum inflammatory-related markers, or that serum inflammatory-related markers had or would have a direct effect on bone indices. Given that these studies also used secondary data, it is possible that the sample sizes were too small to detect differences in total body fat mass and tibial bone strength between Ad36(+) and Ad36(-) groups, and to observe correlations

between radial bone strength and serum inflammatory-related markers. Given these limitations, prospective studies are needed to determine whether Ad36(+) children exposed to higher serum concentrations of inflammatory-related markers exhibit changes in adiposity, bone health, and related outcomes.

Investigations such as these are important for identifying novel influences of obesity and osteoporosis beginning in early childhood. Overall, the findings provide evidence that Ad36 infection is associated with higher serum concentrations of inflammatory-related markers, which may reflect an acute response to infection in children entering the early stages of puberty. In addition, Ad36(+) plus obesity is associated with lower cortical bone strength at the non-weight-bearing radius. There is no correlation, however, between higher serum concentrations of inflammatory-related markers and lower cortical bone strength observed in Ad36(+) children, suggesting that an alternate mechanism is at work. Continued research is necessary to determine the long-term health implications of Ad36 infection in children.

# APPENDIX I

## SUPPLEMENTAL VITAMIN D IN EARLY ADOLESCENCE

CONSENT FORMS

ANTHROPOMETRICS/DXA/PQCT DATA RECORDING SHEET

HEALTH HISTORY QUESTIONNAIRE

SEXUAL MATURATION QUESTIONNAIRES

# APPENDIX I-A

CONSENT FORMS

**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, I and/or my child 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 3 years.

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 the payment for my child's participation, the researcher(s) need to collect my child's name, mailing address and social security number 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, social security number 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 transport 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
Telephone: 542-4901
Email: rlewis@fcs.uga.edu

_____ _____

Signature Date

| _____ | _____ | _____ |
|---|---|---|
| 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**.**

**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**.**

Consent Form for the Use of the Hologic Delphi A
X-Ray Bone Densitometry and the XCT 2000 Peripheral Quantitative Computed Tomography (pQCT) Machines

**Are you pregnant or do you think you might be pregnant? YES NO**
*If yes, please do not participate in this study using the Delphi A bone densitometer and the XCT 2000 pQCT.

I, _____, am hereby giving my consent to be used for research conducted by Dr. Richard D. Lewis, University of Georgia, Foods and Nutrition Department, 279 Dawson Hall.

I understand that by giving my consent I am agreeing to be scanned on the Hologic Delphi A X-Ray Bone Densitometer and on the XCT 2000 peripheral Quantitative Computer Tomography machine. Both of these instruments use a low dose X-ray to determine bone mineral density and body composition.

I understand that the Hologic Delphi A X-Ray Bone Densitometer uses a very low level of X-ray and that under most operating conditions, the entrance dose to the patient is 0.5mRem-10mRem. This equals about 3% to 30% of the exposure of a standard chest X-ray and is of no danger to me.

I understand that the XCT 2000 pQCT uses a very low level of X-ray and that under most operating conditions, the maximum entrance dose to the patient is less than 1 mRem.

I understand that The University of Georgia is responsible for my safety during my participation in this study. However, any illness or injury not related to this study is not the responsibility of the investigator or the University of Georgia.

I understand that my participation is entirely voluntary. I can withdraw my consent at any time without penalty and have the results of my participation returned to me, removed from records or destroyed.

_____ _____
Signature of Investigator Date Signature of Participant Date

# APPENDIX I-B

ANTHROPOMETRICS/DXA/PQCT DATA RECORDING SHEET

***Supplemental Vitamin D in Early Adolescence***

# Participant Information Sheet
## Anthropometrics/DXA/pQCT

Subject ID: _____ Visit Date:_____

Race/Ethnicity: _____ Sex: _____

DOB: Month _____ Day _____ Year _____

Weight (kg): _____ _____ _____

Measure 1 Measure 2 Average of 1 and 2

Height (cm): _____ _____ _____

Measure 1 Measure 2 Average of 1 and 2

Sitting Ht (cm): _____ _____ _____

Measure 1 Measure 2 Average of 1 and 2

BMI (g/cm$^2$): _____

Maturity Offset (years): _____ (Eligible to participate if -1.5 to 0 years)

Hand Grip: _____ _____ _____ _____ _____

Grip 1 Grip 2 Grip 3 Average SD

| DXA operator use | PQCT operator use |
|---|---|
| ❑ Total Body<br><br>Scan date: _____<br>Completed by: _____<br>initials of operator | **Non-Dominant Limb: R L** circle one<br>❑ Arm Length _____ and MCSA _____<br>❑ Leg Length _____ and MCSA _____<br><br>Scan date: _____<br>Completed by: _____<br>initials of operator |

# APPENDIX I-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? _____

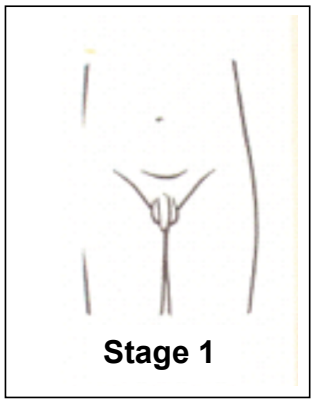
11. How many hours, on average, do you spend watching TV, or on the computer? _____

# APPENDIX I-D

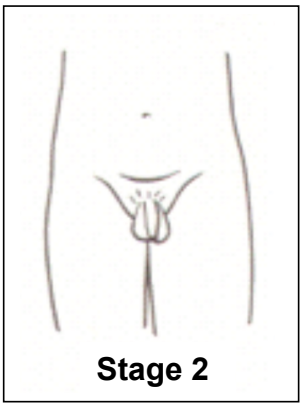
SEXUAL MATURATION QUESTIONNAIRES

## Sexual Maturation Questionnaire

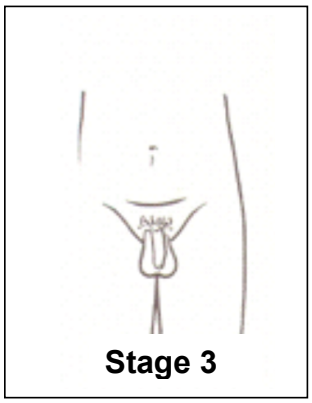
We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



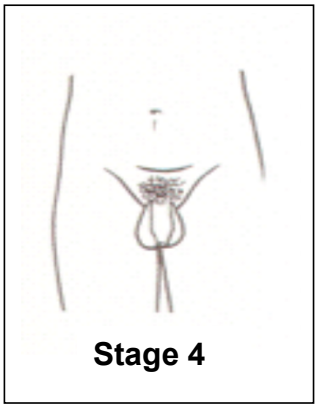

**STAGE 1:** The penis, scrotum, and testes are of the same size and proportion as in early childhood.



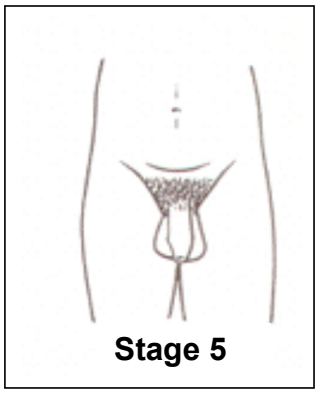

**STAGE 2:** The scrotum and testes have enlarged. The size of each testis can be judged by looking at the scrotum and also by feeling each testis through the skin of the scrotum. The skin of the scrotum becomes thinner, wrinkled and slightly red but this is difficult to see in a photograph. There is 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.



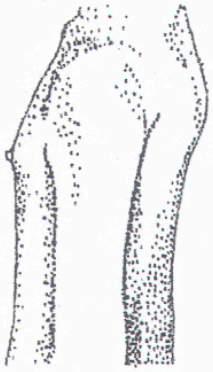

**STAGE 5:** The penis, scrotum, and testes are adult in size and shape.

Lewis, Richard D.

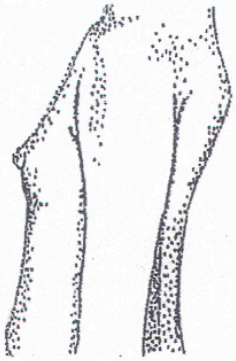
**SEXUAL MATURATION QUESTIONNAIRE (GIRLS)**

**Subject ID#: _____**
**Date: _____**

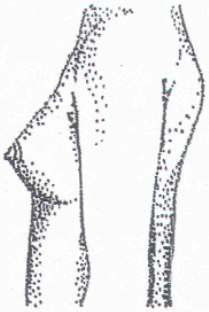
**We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.**



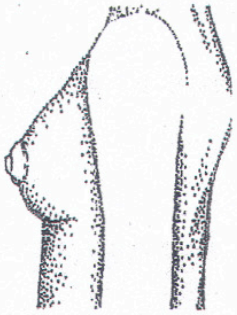
**Stage 1: Elevation of papilla only.**



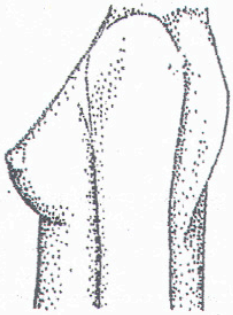
**Stage 2: Elevation of breast and papilla as small mound, areola diameter enlarged.**



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



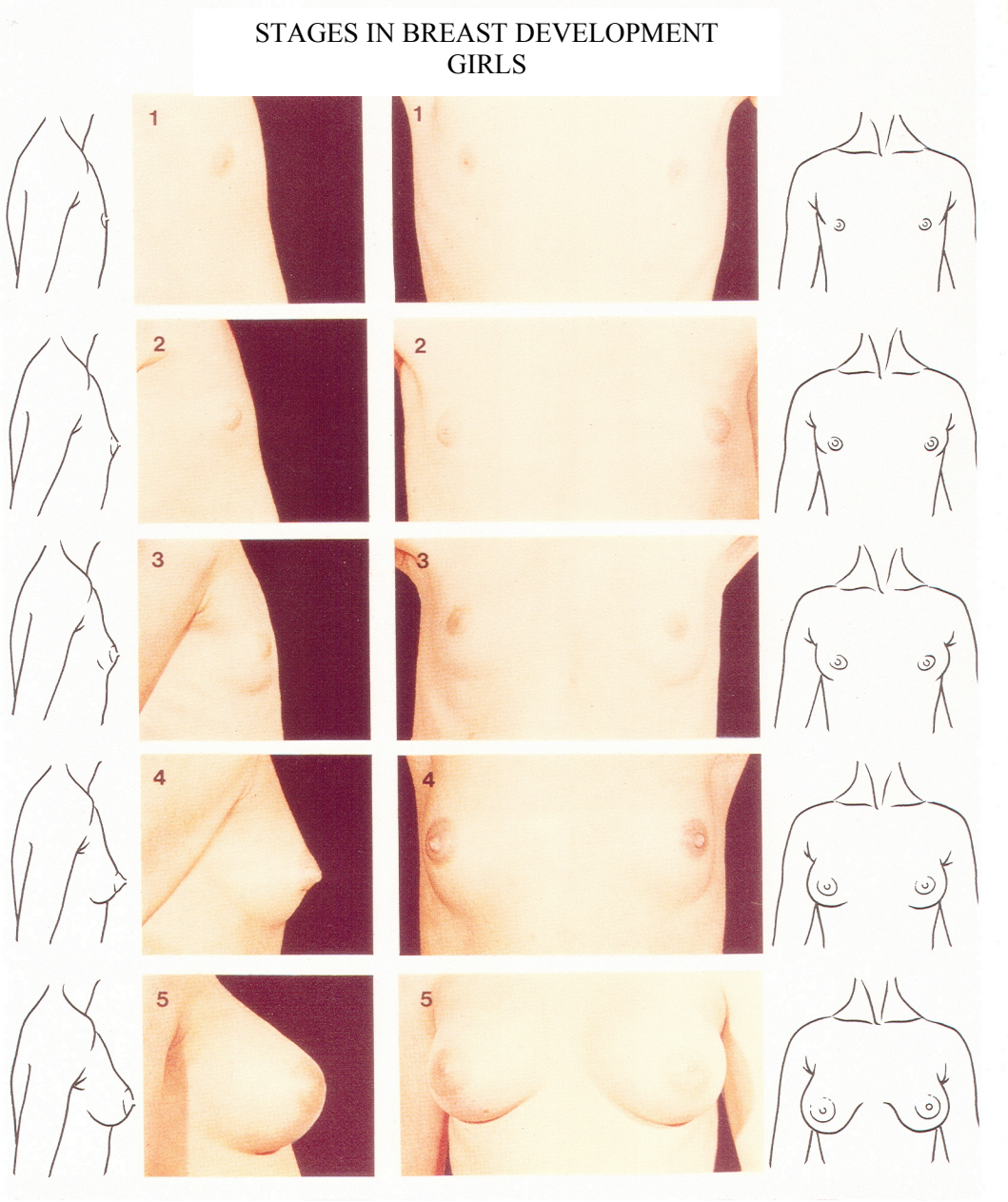
**Stage 4: Secondary mound of areola and papilla above the breast.**



**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.




**STAGE 1:** Only the nipple is higher than the general level of the chest wall in this area, as in a child.

**STAGE 2:** There is an elevation of the areola, which is the colored area around the nipple. The areola is larger than in childhood and fairly hard tissue can be felt deep to it.

**STAGE 3:** There is further enlargement of the breast and the areola, without any separation of their contours. The breast is now clearly feminine in appearance.

**STAGE 4:** The nipple and the areola project from the general level of the breast to form a mound.

**STAGE 5:** The breast is now adult. The nipple projects from the general contour but the areola does not. The areola is markedly colored.