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INFLUENCE OF AD36 SEROPOSITIVITY ON ADOLESCENT MUSCULOSKELETAL

DEVELOPMENT: A 5-YEAR PROSPECTIVE STUDY

by

STACI LYNNE BELCHER

(Under the Direction of RICHARD D. LEWIS)

ABSTRACT

This study examined the relationships between adenovirus (Ad)36 seropositivity and musculoskeletal development in children, 9-13 years of age through pubertal growth. Body composition was measured by dual-energy X-ray absorptiometry and cortical bone was assessed by peripheral quantitative computed tomography. Antibodies for Ad36 were detected from fasting blood samples using an indirect enzyme link immunosorbent assay (ELISA). Analysis of covariance quantified the difference between bone and body composition in seropositive [Ad36(+)] (n=36) and seronegative [Ad36(-)] (n=35) participants. There were no differences in changes in body composition or radial outcomes. Changes in tibia muscle cross sectional area (MCSA) (p=.016), periosteal circumference (p=.021), and total area (p=.035) were significantly lower in the Ad36(+) children; controlling for limb length or MCSA nullified these findings. Change in height and limb length was higher in the Ad36(-) participants. Our data indicates that infection does not alter body composition though it may impact longitudinal growth.

INDEX WORDS: Adenovirus 36, cortical bone, bone mineral accrual, body composition, obesity

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DEDICATION

This work is dedicated to my wonderful parents Steven and Jean Belcher for their unconditional

support and enthusiasm for my educational pursuits, and my grandparents: Moses and Opal

Belcher & Lucien and Irene Arel.

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CHAPTER 1

INTRODUCTION

Achieving optimal bone mass in childhood and adolescence is a key recommendation for the prevention of osteoporosis fractures in adulthood.[1] The rapid period of pediatric growth is important in establishing long-term bone health as bone density has been shown to track throughout childhood and into adulthood.[2] Considering that 90% of the skeleton is accrued by 18 years of age, optimizing bone mineral accrual during the adolescent growth period is essential.[3]

To achieve optimal bone growth, it is important that children engage in regular physical activity,[4] particularly high-impact, weight-bearing physical activity.[5,6] In addition, a balanced, nutrient-dense diet supplying sufficient vitamin D, calcium, protein, and minerals provides the body with the proper nutrients to physiologically support bone growth.[7] Unfortunately, only about one quarter of youth ages 12-15 years participate in moderate to vigorous physical activity for at least 60 minutes daily.[8] Additionally, many children's diets have shifted to diets high in solid fats and added sugars, and lower in fruits, vegetables, whole grains or dairy foods,[9] which are lifestyle factors that play a role in the development of childhood obesity.[10] This is of public health concern as over one third of children are defined as overweight or obese,[11] the projected costs of obesity-related comorbidities are a substantial economic burden,[12] and obesity is associated with greater childhood fractures.[13,14]

Why overweight children are at an increased risk for skeletal fractures[13–15] is unknown, but it is hypothesized that excess adipose tissue negatively affects bone strength. The findings,

however, regarding this hypothesis, are equivocal. For example, obesity has been shown to have both positive[16–18] and negative[19–21] influences on bone health depending on the definitions of obesity, age of participants, and whether or not skeletal muscle is controlled for. Further complicating the relationship, it has been shown that the impact of adiposity seems to be site specific. Increases in adiposity and muscle mass with weight gain generate forces on the lower limbs leading to greater bone mineral content and density than what is observed in the upper limbs.[22,23] This is particularly alarming because radial bones, a non-weight bearing site, are commonly fractured in children.[24] If a fall occurs on the forearm, the skeleton may not adapt sufficiently to compensate for the extra forces applied in a fall due to excess adiposity and body weight.[25] It appears that regardless of the discrepancy in findings regarding the fat-bone quality relationship, many agree with the conjecture that the bones of obese children may not adapt to the extra weight to help prevent a fracture during a fall.

Considering this intricate relationship with bone and adiposity, it is important to consider novel factors that can influence bone through adiposity. One such factor could be viral infections; most notable are adenovirus-5 (Ad5) and adenovirus-36 (Ad36), which have both been associated with adiposity.[26] Though Ad5 is adipogenic in rodents,[27] it has only more recently been associated with human obesity,[28] so the majority of the literature to date focuses on Ad36.[26] The contradictory effects of Ad36 infection, increasing adiposity, but improved clinical metabolic indices, has been the impetus for scientists to investigate this specific virus further. These effects have not been reported with Ad5 infection.

Ad36 is a common cold virus, which manifests as an upper respiratory tract infection or conjunctivitis.[29] Ad36 is adipogenic in animals,[30,31] is found with higher prevalence in overweight/obese adults[12,32,33] and children[34–38] and has been shown to induce differentiation

of preadipocytes and infiltration of adipocytes.[39,40] Though the research does not provide causation between Ad36 and obesity, such compelling cross-sectional and cellular evidence leads to the inquiry of the ramifications of an adipogenic virus on the fat deposition in children and adolescents. Considering preadipocytes and preosteoblasts share the same progenitor cell, the mesenchymal stem cell, an infection favoring adipogenesis could have a negative impact on bone formation.[39]

Preliminary data from our laboratory suggest a negative relationship between Ad36 infection and bone strength at the radius in obese late adolescent females[41] and obese female children.[42] These data show a link between suboptimal bone development and infection with Ad36, but there is a need to identify the long-term impact of the virus, especially throughout puberty when the rate of cortical bone growth is maximal. The adipogenic nature of Ad36 may be a novel factor that negatively impacts bone development during adolescent growth.

This purpose of this project was to investigate the long-term impact of Ad36 infection in 9-13-year-old children through 5-6 years of growth. Through the prospective analysis of bone and body composition measurements, we sought to extend the cross-sectional data and provide evidence of the impact of long-term infection on adolescent adiposity and bone development throughout growth. We hypothesized that Ad36 seropositive children would have smaller gains in bone strength and greater increases in fat mass through pubertal growth.

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CHAPTER TWO

REVIEW OF THE LITERATURE

In order to gain a better understanding of the background for this thesis project, this chapter reviews pediatric and adolescent skeletal growth and the lifestyle factors that influence this process. These include nutrition, physical activity, and body composition. Finally, biological factors including Adenovirus (Ad)36 infection will be discussed in the context of body composition and bone.

Bone development

Pubertal growth

Establishing healthy bones in childhood is thought to reduce the risk of fractures in adulthood. In the period of growth and development, many factors work synergistically to determine bone structure and composition. During the pubertal years, there is a 3- to 4-year window of rapid bone mineral accrual. This bone mineral accrual is influenced by environmental and genetic factors.[1] These factors start to take major effect beginning at the onset of puberty where bone mineral accretion occurs at a rapid rate.[2] The growth spurt has been shown to contribute a total of 51% of peak bone mass in girls.[3] In the two years surrounding peak bone mineral content (BMC) velocity, boys and girls have been shown to accrue ~26% of final adult bone.[4] The most substantial amounts of BMC have been shown to accrue in the five years surrounding peak height velocity (PHV).[2] Though specific skeletal growth patterns are dependent upon the site, the entire adolescent period is crucial for long-term bone accrual as 90%

of bone is accumulated by 18 years of age.[5] Longitudinal data suggest that bone area (BA) plateaus about five years post PHV and BMC accrual plateaus after seven years of PHV.[2]

Considering females and males enter puberty at different chronological ages, utilizing a standard measure of maturation is critical in the interpretation of pubertal bone growth.[2] Many cross-sectional studies use the sexual maturation rating stages as described by Tanner (SMR) to classify maturational stage.[6] If longitudinal data are available, using the age at PHV as an indicator around which maturational age is constructed allows researchers to compare bone indices across sex[7] with high accuracy. The sexes differ in tempo and timing of bone mineral accrual as well as achievement of PHV. For example, females increase BMC and areal bone mineral density (aBMD) more rapidly in the earlier stages of puberty rather than the later stages of puberty. Conversely, males tend to experience increases in BMD in late puberty that are equal to or greater than the gains they experience in early puberty.[8] Figure 1 represents total body BMC gains in boys and girls in the context of PHV and shows that PHV is reached prior to peak BMC accrual in both sexes, albeit females reach this point first. On the other hand, males tend to increase BMC/BMD later in puberty (Wang et al. 1997) and ultimately reach a higher BMC than girls.[8] The difference in tempo and timing is evident in a prospective study that utilized the Saskatchewan Pediatric Bone Mineral Accrual Study cohort of children. In this study, growth indices at the proximal femur were followed in 165 boys and girls from late childhood to early adulthood. Females experienced their growth peaks for bone mass and geometry in the proximal femur one to two years before the boys. This difference was due to earlier maturation, defined by the PHV.[7]
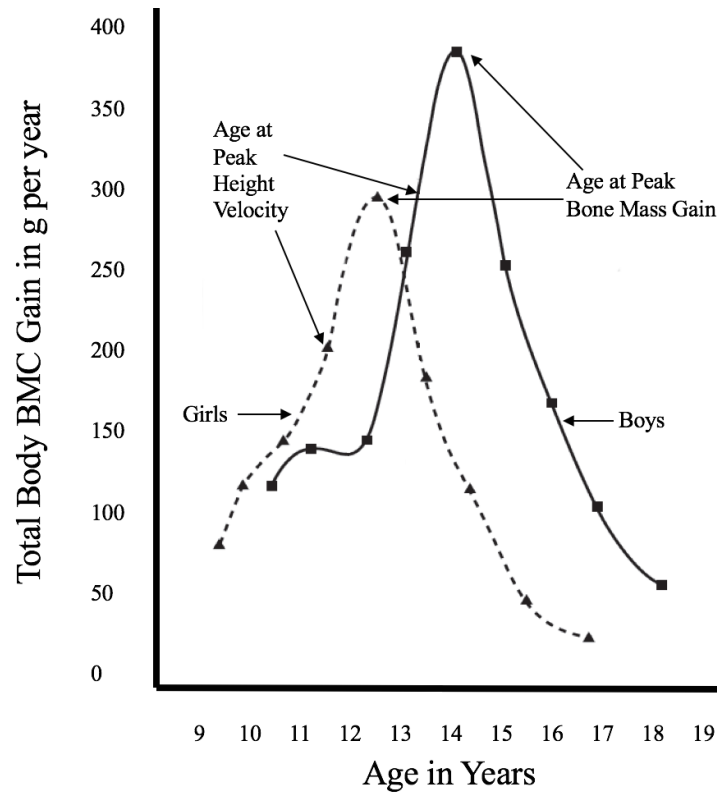
Figure 1: Peak bone mineral content (BMC) gains and peak height velocity (PHV) in boys and girls.[21]

Establishing strong bones during adolescent growth is an important step to promote bone health as an adult, as bone shows a high degree of tracking. Children with low bone density will likely have low bone density into young adulthood;[9] boys and girls with low bone mass grew into young men and women with BMC and aBMD below the average after six years of growth.[10]

Racial effects

African Americans have higher bone mass in adulthood than whites.[11] When evaluating prepubertal children, some studies see no differences in vertebral bone density between black and white children,[12] though this null relationship is not consistent. When examining the early phase of pubertal development, the lumbar spine and femoral neck BMC are greater in black

boys compared to white boys.[8] Warden et al. also observed differences in cortical volumetric bone mineral density (vBMD) and mass and size between black and whites in the early stages of puberty (stages 2 and 3) with blacks having greater values than whites.[13]

Other studies have revealed greater bone strength,[14] total body BMC,[15] and vBMD at the spine, hip and total body,[16] and femoral neck aBMD[17] in black vs. white children in varying stages of pubertal growth. These studies control for variables such as limb length, muscle cross sectional area (MCSA), height, weight and lean mass. In a study by Gilsanz et al., 80 white and black children were matched for chronological age, sex, SMR, height, and weight.[12] At SMR stage V, the density of vertebral bone was 9.8% higher in black girls and 11.7% higher in black boys compared to their matched white participants. Moreover, the cross-sectional area (CSA) of the femur was greater in black children no matter the sex or SMR stage analyzed. The driving force of the differences between black and white children appeared to be site specific. That is, the axial skeletal measures were greater due to increased trabecular density. In the appendicular skeleton, the bones were of greater size, which likely drove the observed differences.[12]

Growth trends in black and white children reveal that black participants do not accrue bone at a more rapid rate than white participants. Nor does it appear that black children spend more time in puberty. Hui et al. reported that black vs. white children spend approximately 0.2 more years in puberty.[18] However, maturation rates between the two races differ, as it has been reported that black children are younger than whites at the same SMR stage.[15,19,20]

Nutrition

Adequate nutrition is essential for bone development.[21] A recent systematic review of 18 studies reported that nutritional behaviors such as milk avoidance, high cheese intake, high

energy or sugar sweetened beverage intakes seem to be associated with increased fracture risk among 2-13-year-old children.[22] Vitamin D supplementation has been shown to increase total hip bone area and BMC[23,24] and geometrical properties of the femoral neck[25] in adolescent females. The benefit of vitamin D supplementation on bone has also been confirmed in a recent systematic review and the evidence was graded as 'B' or moderate.[21] Despite the health benefits of adequate vitamin D, many children are not reaching the recommended dietary intakes.[26] Supplementing to remedy a deficiency is preferable, as a meta-analysis suggested that supplementation has the most beneficial effect on total body BMC in children who are deficient in the vitamin.[24] Calcium supplementation has consistently been shown to enhance gains in skeletal mass and density.[21] In a three-year double-blind randomized placebo-controlled trial in identical twin boys and girls, calcium supplementation vs. placebo positively affected aBMD at several skeletal sites, especially in prepubertal children.[27]

Physical activity

The positive effects of physical activity on bone mass and density are well documented in a recent review.[21] Thus, for optimal bone health, it is widely recommended that children are physically active.[28] Establishing bone health in adolescents has been shown to have lasting effects into young adulthood. For example, one study showed that males who were active at a young age had greater total area and polar strength strain index (SSI_p) at the tibia diaphysis compared to their inactive peers in adulthood. Active females in this study also had lasting benefits at the tibia diaphysis in young adulthood, as they had greater cortical area and cortical content compared to inactive peers in adolescents.[29] A meta-analysis reviewing 17 studies showed a small influence of weight-bearing exercise on BMC and aBMD of the lumbar spine and BMC of the femoral neck.[30] High impact activities such as jumping[31] and gymnastics[32,33]

have proven to be highly osteogenic. Frequency of exercise is also important; more frequent physical activity (3 or more days per week) results in a greater positive effect for lumbar spine aBMD.[30] A six-year physical activity intervention demonstrated positive skeletal benefits for girls in BMC of the femoral neck as well as tibial cortical BMC, cortical area, and CSA at the end of the six years. Boys also benefitted from higher accrual of spine aBMD.[34]

Body composition and bone health

One of the most important determinants of bone development is lean muscle mass.[35–37] In healthy children, lean mass has been strongly related to bone mass, independent of muscular fitness. Children with greater lean mass had greater total body BMC, and upper and lower limb BMC/BMD.[38] In adolescents, higher levels of lean mass explain the greater bone mass which is found in those with higher adiposity.[39,40] A study of 9-11-year-old boys and girls observed similar trends in their participants. Over 16 months, the differences in tibial bone strength between overweight and normal weight participants was appropriate for their lean mass but not their total body weight.[41] Petit et al. also showed that though the bones of overweight children had higher bending strength, it was proportional to their levels of lean mass.[36] Higher adiposity can lead to increased lean mass, and this positive physiological adaptation to lean mass is likely site specific to the weight bearing bones. Ducher et al. analyzed bone variables at the tibia and radius in 7-10-year-old girls and boys,[42] and before controlling for MCSA, all bone variables were significantly greater in the overweight children except for cortical density at both diaphyseal sites and cortical thickness at both metaphyseal sites. After correcting for MCSA in this study, most of the skeletal differences disappeared. The exceptions were trabecular density at the radius and tibia and BMC, cortical area and cortical thickness at the tibia, which remained higher in the overweight children. Interestingly, at the radius, adjustment for MCSA led to

smaller values in overweight children for total area and cortical thickness. The authors concluded that the skeletal benefits in overweight children were only greater in the lower, weight bearing limbs as compared to the upper, non-weight-bearing limbs.[42]

To date, the research is quite conflicting, and considering the complex relationship between fat and bone, it is not surprising that some studies have reported a positive or neutral relationship between bone and fat mass specifically, or trends of positive BMC/BMD in overweight or obese populations. In a study of 51 obese male adolescents age-matched and bone-aged matched to controls, Vandewalle et al. reported that obese adolescents have stronger bones at the tibia and radius than normal weight peers. The researchers concluded that the increase in bone strength was related to estrogen levels, mechanical loading, and bone maturation.[43] It is important to note that the authors did not control for lean mass, which has nullified the positive relationship between obesity and bone strength in other studies.[41,42] Leonard et al. showed that tibia cortical section modulus was significantly higher in obese compared to non-obese adolescent girls and boys after controlling for sex, ancestry group, age and tibia length, and concluded that this difference was explained by advanced skeletal maturation, greater calf MCSA and greater muscle strength in the obese participants. Radial cortical section modulus was only slightly significantly greater in the obese participants, but the difference was nullified when maturation was accounted for in the statistical analyses.[44] However, El Hage et al. demonstrated that even after adjusting for body weight, lean mass and fat mass, overweight girls did not have lower BMC or aBMD at any bone site.[45] Additionally, in a cross-sectional 2-year analysis from the Avon Longitudinal Study of Parents and Children, in Bristol UK, fat mass of prepubertal children was a positive, independent predictor of bone mass and size, after controlling for height and lean mass.[46]

Other studies report a negative association between excess adiposity and bone. Ackerman et al. concluded that among adolescents matched for sex, height, and pubertal stage, those with greater fat mass have lower total body BMC.[37] In females between the ages of 10 and 19 years, body fat has been shown to have a negative impact on BMC and aBMD.[47] Goulding et al. showed that overweight and obese boys and girls had lower bone area and bone mass compared to those with healthy body weights.[48] Mosca et al. demonstrated an inverse relationship between body fat percentage and aBMD and BMC outcomes in overweight male adolescents. In females, the authors also observed differences at specific skeletal sites; there was a negative correlation between body fat percentage and aBMD at the spine and femur.[49] Janicka et al. reported that in males (ages 13-21 years), all dual-energy x-ray absorptiometry (DXA) measurements of lumbar and femoral cortical bone area were negatively related to fat mass. In females, there was a negative relationship between DXA leg aBMD and fat mass.[50] Farr et al. concluded that the effects of fat mass on bone were likely site-specific. For example, at the distal tibia, fat mass was weakly correlated with bone strength. Conversely, radial measures did not increase alongside the gains in adiposity during growth.[51] Findings from Pollock et al. suggest that excess body weight is not beneficial for bone strength in late adolescent females. The analyses revealed that body fat percentage was inversely related to radial cortical bone area, total bone CSA, cortical BMC, periosteal circumference and SSI. After controlling for MCSA and limb length, negative relations were observed between body fat percentage and tibia cortical bone outcomes. In this study, high fat ($\geq$32% body fat) vs. normal fat ($\leq$32% body fat) females had lower cortical bone area, cortical BMC, periosteal circumference and SSI at the tibia and lower bone cross-sectional area at the radius.[52]

The relationship between muscle, fat, and bone is complex. Though the literature presents conflicting information regarding the ultimate impact of excess adiposity on bone health, it is likely that the effects are site specific. Lean mass is an important predictor of bone density and BMC and may play an essential role in mediating the relationship between fat mass and bone. Weight bearing bones such as the tibia are more likely to benefit from the impact of compression forces, which may explain the more significant muscular adaptations in obese children compared to non-weight bearing sites such as the radius. Considering the observed relationship between lean mass and bone, the lean mass may be driving the conclusion that obese children have stronger bones as reported in some studies.[42,53]

Fracture risk and obesity

Many researchers analyze fracture risk and population prevalence of fracture to examine the etiology of fractures to aid in the assessment of bone health. Though some studies suggest overweight and obese children are not at an increased odds of extremity fracture,[54] many studies conclude that obese children are indeed at higher risk for fracture.[55–57] In one study, children who experienced a moderate or severe fracture were considerably heavier than the children suffering from a slight trauma fracture.[58] In fact, risk of forearm fracture has been shown to be 1.7x greater in obese compared to non-obese boys.[59] Additionally, overweight/obese children are more commonly fracturing their upper, rather than lower, weight-bearing limbs.[56,60] Overweight, moderately obese and extremely obese children have significantly higher odds of lower extremity injuries compared to normal weight children after controlling for sex, age, race/ethnicity and insurance status.[55] Similarly, in an analysis of over 910,000 children, Kessler et al. observed that overweight, moderately obese and extremely obese children all had increased

risk of fracture compared to normal-weight children after controlling for sex, race, age and socioeconomic status.[57]

Though the etiology of fractures is not known, it is suspected that the increased fracture risk is related to lower BMC or aBMD.[61] Goulding et al. (2005) showed that children with a history of multiple fractures of the distal forearm had lower BMC and aBMD and had a higher body weight and adiposity than their fracture-free peers. Others report an increased risk of fracture despite a higher total body less head bone area and BMC. Considering these projected risks with the complicated relationship between lean mass, adiposity and BMC/BMD, drawing conclusions on absolute risk of fracture is difficult. Despite some investigators suggesting that obese children have stronger bones,[46] researchers have raised the question of whether the observed higher BMC in overweight children is sufficient to compensate for the increased force if a fall occurs.[40,59,62]

Adenovirus-36 infection

Acquiring a viral infection is common among people at all stages of life. For example, the common cold infects adults, on average, 2-3 times per year; children are reported to have even more infections.[63] Viruses cause acute sicknesses like chicken pox; the latent form of the chicken pox virus, varicella, can cause shingles later in life. Similarly, latent infections like human immunodeficiency virus, varicella zoster, and human papilloma virus can increase long-term risk for health complications.[64–66] Upon exposure to a virus, three things must occur for infection to take hold in the human body. Firstly, sufficient viral load must be available to initiate infection. Then, cells at the site of infection must be accessible, susceptible and permissive to the virus. Finally, the host antiviral mechanism must be absent or their defenses blunted so the virus can attach to host tissues. Latent adenovirus infections may reside in lymphoid tissue, renal

parenchyma or other tissues for years, during which reactivation can occur in immunosuppressed patients.[67]

Considering the intricate relationship between obesity and bone, and the long-term impact of some viral infections, it is relevant to consider the potential impact of an adipogenic virus on bone health; most notable are adenovirus-5 (Ad5) and adenovirus-36 (Ad36), which have both been associated with adiposity. Though Ad5 is adipogenic in rodents,[68] it has only more recently been associated with human obesity,[69] and the majority of the literature to date focuses on Ad36. The contradictory effects of Ad36 infection, increasing adiposity, but improved clinical metabolic indices, has been the impetus for scientists to investigate this virus further. These effects have not been reported with Ad5 infection.[70] Though Ad36 has many biological effects, especially regarding metabolic health, the observed effect most pertinent to this project is its adipogenic nature.

Studies have shown an increased prevalence of Ad36 infection in obese compared to non-obese adults[71–73] and children.[74–79] On average, prevalence of infection ranges between 7.1% to as high as 73.9% in Korean, American, Hispanic, Swedish and Mexican children.[75,76,78,80,81] Additionally, the prevalence of Ad36 infection in Sweden has trended alongside the increasing trends in obesity; the prevalence increased from 7% in 1992-1998 to 15-20% in 2002-2009.[74] It also has been associated with increased body mass index (BMI) in adults[73,82,83] and increased BMI/BMI z-scores and waist circumference in children.[76,80] Gabbert et al. showed that obese Ad36 seropositive (Ad36(+)) children weighed, on average, 16.1 kg more than obese children who were Ad36 seronegative (Ad36(-)).[76] Identification of fat by bioelectrical impedance in Czech adolescents revealed Ad36(+) participants had greater amounts of total and trunk fat than those uninfected: 29.3% vs. 24.7% and 24.7% vs. 20.6%, respectively.[75]

Despite the association observed between infection and adiposity, it is important to note that some studies have shown no difference in BMI between infected and uninfected participants.[84,85] In a longitudinal study that followed participants for 29.3 years from childhood through adulthood, there was no significant difference in average BMI among seropositive or seronegative participants. A greater proportion of Ad36(+) participants among those who were normal weight as a child but became obese by adulthood when compared to those who maintained a normal weight throughout their lifetime. However, it is important to note that this difference was attributed to a decline in the percentage of seropositive individuals in the normal weight group.[86] A study that followed adults ten years past infection reported greater levels of adiposity in those who were Ad36(+) at baseline.[87] Conversely, a retrospective study in military personnel studied over an average of five years, did not find that infection predicted BMI gain. Despite this finding, Ad36 infection was shown to predict a new-onset clinical diagnosis of overweight/obesity in adult men.[88]

There has been one prospective study during adolescent growth that has assessed Ad36 infection. Over the course of one year, 79 Korean boys were followed to investigate changes in body composition and metabolic risk factors.[89] Participants were classified as Ad36(+) or Ad36(-) at baseline and cross-sectional analyses revealed that fat percentage and fasting insulin levels were significantly higher in the Ad36(+) group. In longitudinal analyses, the change in body weight, change in BMI, change in fat mass and change in body fat percentage seemed to be higher in the past Ad36(+) infection group; however, these changes did not reach statistical significance. Researchers hypothesized that this may be due to natural body changes that occur during adolescence and pubertal growth.[89]

Adenovirus36 and bone

Considering the strong correlation between Ad36(+) and obesity, the relationships between obesity and bone health and the fact that fat and bone cells are derived from the same bone marrow stem cells,[90] it is interesting to consider the potential impact of Ad36 infection on bone and body composition. This relationship was first explored by Laing et al.; in a sample of 115 late adolescent females, 53.9% were Ad36(+) and infection prevalence was greater in high-fat ($\geq$32% body fat) participants compared to those with normal levels of fat ($\leq$32% body fat) (66% and 46% seropositive, respectively).[91] In the high-fat group, Ad36 infection was positively associated with percent body fat (r=0.528, *p*=0.017) and negatively associated with SSI of the radius (r=-0.451, *p*=0.046). Having high levels of body fat in addition to Ad36(+) was associated with lower trabecular and cortical strength at the radius. These results were independent of MCSA, limb length and muscle density.[91] An additional study, currently under review, presented similar results in a younger cohort of children. Berger et al. showed that in obese 9-12 year old girls, the Ad36(+) group had significantly lower cortical area (P=0.030), total area (P=0.006), and SSI$_P$ (P=0.009) versus the Ad36(-) group.[92]

Assay methodology

The gold standard to detect Ad36 antibodies, and thus to identify a study participant as seropositive or seronegative, is a serum neutralization assay (SNA). However, this method is extremely time and labor intensive. Studies conducted up until 2012 relied exclusively on SNA for the evaluation of Ad36 antibodies. The development of the enzyme link immunosorbent assay (ELISA) to measure Ad36 antibodies in 2012 resulted in many studies utilizing the new assay method to conveniently assess seropositivity in their participants. To assess the validity of the ELISA assay, rabbit serum and human controls were tested via SNA and validated against

the newly designed ELISA assay. ELISA cutoffs to identify a sample as "positive" was based on analysis of human serum samples with known Ad36(-) SNA status. Human serum samples were used to compare the Ad36(-) ELISA scores to the Ad36(-) SNA scores. Researchers concluded the ELISA method was sensitive to and specific for Ad36.[74] Recently, accuracy of the ELISA assay has been questioned, as two studies reported widely different prevalence estimates that came from the same set of sera.[85,93] Identifying a method that is as time sensitive as the ELISA assay but as accurate as the SNA is a top priority in Ad36 research.[94]

Summary

Adolescent bone growth is a complex process integrating myriad physiological processes. Achieving peak bone mass during growth is the result of the symbiosis of these factors to promote optimal bone development. Adequate nutrition ensures the body has enough calcium and vitamin D to promote bone acquisition. Physical activity, especially high impact weight bearing physical activity, stimulates muscle growth and bone development. Children are at a high risk for skeletal fractures, so optimizing these preventative, bone-strengthening lifestyle factors can help to prevent fractures. High levels of adiposity have conflicting effects on bone across the literature; however, lean mass has consistently been shown to positively impact bone.

The common cold virus, Ad36 may have a negative effect on bone as observed in cross-sectional analyses. Long-term changes in bone and body composition in children infected with the virus are unknown. Thus, the aim of this project is to evaluate the influence of Ad36 on bone and body composition in children. These data will be insightful in the assessment of a novel risk factor for suboptimal bone development.

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CHAPTER THREE

INFLUENCE OF AD36 SEROPOSITIVITY ON ADOLESCENT MUSCULOSKELETAL

DEVELOPMENT: A 5-YEAR PROSPECTIVE STUDY

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Influence of Ad36 Seropositivity on Adolescent Musculoskeletal Development: A 5-Year Prospective Study

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ABSTRACT

Adenovirus 36 (Ad36) is a common cold virus that is more prevalent in obese versus normal weight children. Infection with the virus is also associated with compromised bone strength. Prospective data examining the relationships between Ad36 infection, obesity and bone health, are lacking. The aim of this approximately five-year prospective study was to examine the effects of Ad36 seropositivity on obesity and musculoskeletal development during pubertal growth. Participants included black and white boys and girls, 9-13 years of age at the baseline time point. Participants were divided into two groups: Ad36 seropositive {Ad36(+); n = 36} and negative {Ad36(-); n = 35}. At baseline and follow-up, fasting blood draws, dual-energy X-ray absorptiometry (total body), and peripheral quantitative computed tomography (tibia 66% site relative to the distal growth plate) were performed. Antibodies for Ad36 were detected from baseline fasting blood samples using an indirect ELISA immunoassay. To compare changes in body composition and bone outcomes between groups, ANCOVA was performed controlling for race, sex, and baseline sexual maturation rating (SMR) stage. Change in limb length was used as an additional covariate. There were no significant baseline group differences in age, height, weight, SMR, sex, race, dietary variables or any musculoskeletal endpoints. Overweight/obesity prevalence was 38.9% and 42.9% in the Ad36(+) and Ad36(-) groups, respectively (p=.734). Change in height (p=0.001), total body bone area (p=0.021), tibia MCSA (p=.016), periosteal circumference (p=.021), and total area (p=.035) were significantly lower in the Ad36(+) versus Ad36(-) children. After correcting for change in limb length, changes in tibia cortical bone endpoints did not differ between AD36(-) and AD36(+) groups. Our data indicate that, over five years of growth, infection with Ad36 does not significantly alter body composition. Cortical bone at the tibia was affected by seropositivity, though the observed significant changes were

explained by changes in limb length. In a cohort of adolescents within a normal range of body mass index-for-age percentiles, Ad36 infection does not appear to influence cortical bone strength. Ad36 infection may impact longitudinal growth, but more careful control of maturational status during this rapid growth would help ascertain that notion.

KEY WORDS: ADENOVIRUS; OBESITY; PROSPECTIVE; GROWTH; CHILDREN

INTRODUCTION

Achieving optimal bone mass during childhood and adolescence is thought to be protective against skeletal fractures in adulthood and is a key strategy recommended for the prevention of osteoporosis.[1] Multiple factors can modify bone accrual and strength during youth including lifestyle factors such as diet[1] and physical activity.[2] Moreover, overweight and obesity, which affect approximately one third of US youth,[3] have a negative relationship with total body bone area, bone mineral content (BMC), and areal bone mineral density (aBMD) at the spine and femur.[4–6] Obese children are at an increased risk for fracture,[7–9] yet it is unclear why excess fat mass contributes to the higher incidence of fractures. A biological factor linked with obesity and that may provide some insight into the bone and obesity relationship, is Adenovirus-36 (Ad36), a common cold virus. Human infection manifests as upper respiratory tract infections or conjunctivitis.[10] Ad36 is adipogenic in animals[11,12] and is found with higher prevalence in overweight/obese adults[13–15] and children.[16–20] In vitro, Ad36 has been shown to induce differentiation of human stem cells into preadipocytes, resulting in increased adipose accumulation.[21,22] Considering that preadipocytes and preosteoblasts share a common stem cell progenitor, diversion of mesenchymal stem cells to adipocytes over osteoblasts provides a logical mechanism for how Ad36 could indirectly reduce bone strength.

In the only published study to date that has linked Ad36, obesity and bone using peripheral quantitative computed tomography (pQCT), young adult obese females who were seropositive for Ad36 had lower total area at the 4% and 20% radial site, total BMC at the 4% radial site and lower measures of cortical BMC, cortical area and (SSI) of the 20% radial sites independent of muscle cross-sectional area (MCSA) and leg length.[23] This connection between Ad36 and lower radial bone outcomes was also observed in a cohort of younger obese adolescent

girls, where radial cortical area, total area and polar SSI (SSI_p) were lower in obese Ad36(+) girls when compared to obese Ad36(-) girls (unpublished observations).[24] The fact that the second study in younger children was in agreement with the earlier findings in young adults lends support to the hypothesis that Ad36 negatively impacts cortical bone in obese adolescents. However, more robust prospective data are needed to examine the impact of Ad36 infection on cortical bone strength over the long-term, particularly throughout rapid pubertal growth. The adipogenic nature of Ad36 may be a novel factor that negatively impacts bone development during adolescent growth; however, no prospective data have been collected to date to determine this. Therefore, the aim of this project was to investigate the long-term impact of Ad36 infection in 9-13-year-old children through approximately 5-6 years of growth. We hypothesize that Ad36 seropositive children will have smaller gains in bone structure and strength at the tibia and radius and greater increases in fat mass through pubertal growth.

MATERIALS AND METHODS

Subjects and Methods

This prospective study utilizes baseline data from 71 children who participated in a multisite randomized controlled vitamin D clinical trial conducted at the University of Georgia, Purdue University and Indiana University.[25] Recruitment of these participants took place between October and December of 2009 and 2010. The parent study inclusion criteria included non-Hispanic black and white females between the ages of 9 and 12 years and males between the ages of 10 to 13 years in sexual maturation rating stages 2 and 3. Sexual maturation was estimated using self-administered questionnaires for genitalia or breast development.[26] Children were excluded if they took medications or had medical diagnoses of conditions known to influence growth, maturation, nutritional status, metabolism or inflammatory response. Females

who had reached menarche were also unable to participate. The Institutional Review Board for Human Subjects at the University of Georgia approved all procedures for this follow-up study. All participants provided informed assent with permission provided by their parent/guardian.

Follow-up recruitment began in May of 2015 and targeted only participants at the University of Georgia. Participants were contacted by phone and email and were enrolled if they had all baseline measurements completed, remained free of a conditions or medications known to influence bone metabolism and agreed to come in for testing (n=71). Two groups were created based on baseline analyses of Ad36, i.e., Ad36(+) (n=36) and Ad36(-) (n=35). At the follow-up study visit, a fasting blood draw was collected, and anthropometric and bone and body composition outcomes were measured. Follow-up testing concluded in March of 2016.

An online power analysis calculator (http://www.gpower.hhu.de/en.html) was used to estimate power. Alpha was set at $p=0.05$, power=80% with an ANCOVA design. Data from a study that assessed differences in Ad36 seropositivity and bone strength was used for estimations.[23] A sample size of $n=39$ per group (total sample of 78) would allow for detection of differences between groups for cortical SSI.

Anthropometry

Height and weight were measured with shoes removed and in light clothing. Height was measured using a stadiometer to the nearest 0.1 cm. Weight was recorded with an electronic scale to the nearest 0.1 kg. Height and weight were used to calculate body mass index (BMI) and BMI-for-age percentiles according to Centers for Disease Control and Prevention growth standards.[27] Validity was assessed using a one-way random-effects model, single-measure intraclass correlation coefficients (ICC) in 6-10-year-old females (n=10), measured twice over

two weeks by the same technician. The ICCs and test-retest coefficients of variation were 0.99%

and 0.4% for height and 0.99% and 1.4% for weight, respectively.[25]

Dietary Intake

With assistance from parents or guardians, 3-day diet records, a valid and reliable method

for estimating energy and nutrient intakes in children, were completed at home on 2 weekdays

and 1 weekend day.[28–30] Records were analyzed by one trained registered dietitian nutritionist

using Food Processor SQL version 9.7.3 (ESHA Research). Diet records were used to estimate

mean daily intakes for energy (kcals), protein (g), calcium (mg), and vitamin D (IU). Average

measure (3-day) intraclass correlation coefficients (ICCs) were calculated in girls ages 6-10

years (N=10), whose 3-day diet records were completed twice over 2 weeks and calculated for

vitamin D, calcium, and energy (≥ 0.86).

Biochemical Analyses

Blood samples were collected from participants following an overnight fast. Serum

samples were refrigerated immediately and stored at <-70°C until analysis. Ad36 antibody status

was measured using enzyme linked immunoabsorbent assay (ELISA) methodology. Whole Ad36

virus was coated on high binding plates overnight at 4°C at a concentration on 10ug/ml in 100ul.

The plate was then washed 3times with 1X KPL wash buffer (KPL 20X concentrate 50-63-00).

The plate was then blocked with 100ul of 5% dry milk in 1X KPL wash buffer for 1 hour at

room temperature. The plate was then washed 3times with 1X KPL wash buffer. Plasma samples

were added to 50µl/well in duplicate and incubated at 4°C overnight. The wells were washed

three times with 1X KPL wash buffer. Secondary antibody (goat anti-human immunoglobulin G)

(Thermo fisher A18805) was added to wells (diluted 1:800in blotto) and incubated for one hour

at room temperature. Wells were washed three times with 1X KPL. Wells were developed using

the 1-step Ultra TMB ELISA substrate solution (Thermo Fisher 34029). After the plate was developed about 10-30 minutes the Stop solution (Thermo Fisher N600) was added and the plate was read on a plate reader at 450 nm wavelength.

Body composition

Fat mass (in grams), percent body fat, fat-free soft tissue mass (FFST; in grams), and total body bone area, BMC and aBMD were quantified using dual-energy X-ray absorptiometry (DXA; Delphi-A Whole Body Analysis software, version 12.4; Hologic Inc. and Discovery-A APEX Software, version 3.3; Hologic Inc.) Whole body scans were performed in accordance with the manufacturers' standard protocols. The same technician analyzed all scans at baseline or follow-up, respectively. The two scanners were cross-calibrated by analyzing scans from 20 children ages 9-13 years using both software. Regression formulae were derived using the method of Carstenson[31] which were used to adjust data from the Delphi-A to Discovery-A values. For determination of measurement reliability, 12 females ages 18-24 years were scanned twice on the Hologic Discovery-A scanner over a two-week period. All one-way random effects model single measure ICCs were ≥ 0.96.

Peripheral quantitative computed tomography

Cortical bone measurements were performed using pQCT (Stratec XCT 2000, Stratec Medizintechnik GmbH) at the 66% site of the non-dominant radius and tibia from the distal metaphysis, as described previously.[32] In brief, a phantom was scanned daily to confirm proper calibration of the machine. Participants were positioned in the sitting position and their non-dominant leg was placed in the gantry. At 66% of the tibial length, proximal from the medial malleolus, a skin mark was placed and the tomographic slice (thickness, 2.3 mm; voxel size, 400 µm; and scan speed, 20 mm/s) was taken. MCSA, cortical volumetric BMD (Ct. vBMD,

mg/cm^3), BMC (Ct. BMC, mg/mm), and area (Ct.Ar, mm^2) were measured using cort mode 1 (threshold, 710 mg/cm^3). To obtain total area (Tt.Ar, mm^2), cortical thickness (Ct.Th, mm), and periosteal (Ps.Pm, mm) and endosteal (Es.Pm, mm) perimeters, contour mode 1 (threshold, 710 mg/cm^3) was used to define the outer bone edge and peel mode 2 (threshold, 400 mg/cm^3) was used to separate the cortical and cancellous compartments. Precision was assessed using 30 healthy individuals scanned six times with interim repositioning. Results revealed root mean square coefficients of variation (RMS-CVs) of <1% for bone density, mass, structure, and estimated strength measures and <1.5% for MCSA.[32]

Estimated strength was calculated by calculating the SSI$_P$ (in cubic millimeters). SSI has been validated as a noninvasive measure of bone strength. SSI is a representative measure of density-weighted section modulus and was obtained in analysis using cort mode 2 (threshold=400 mg/cm^3). The calculation multiplies the section modulus by the ratio of Ct.vBMD and physiologic density (1,200 mg/mm^3), as described previously.[32–34] Section modulus (mm^3) was calculated as $(a{\times}b^2)/d_{max}$, 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 d_{max} is the maximum distance (eccentricity) of one voxel to the center of gravity (mm).[32]

$$\text{Strength} - \text{strain index (SSI, mm}^3)$$

$$= [\ (a \times d^d)\ (\text{Ct.vBMD/ normal physiologic density BMD})\ ]\ /\ d_{max}$$

Statistical analyses

Data were checked for outliers and normality with histograms and tests of skewness and kurtosis for normality. Homogeneity of variance was assessed using Levene's tests. Categorical variables (race, gender, obesity prevalence) were tested for differences using chi-squared tests. Analysis of variance was used to compare continuous variables (age, sexual maturation stage,

anthropometric, dietary intake, and body composition measurements) between the Ad36(+) and Ad36(-) groups. Outliers were replaced with mean $\pm$ 2SD and all normality tests were repeated. Two separate models were utilized; covariates in Model 1 included sexual maturation stage at baseline, race and sex. In Model 2, limb length (tibia or radius) was added to the Model 1 covariates. All statistical analyses were conducted using SPSS software (version 21, IBM SPSS Statistics, Chicago, IL) and significance was set at a p-value <0.05.

RESULTS

Unadjusted baseline and follow-up participant characteristics are presented in Table 1, and are classified by Ad36 infection status. The total sample was 31% black, 51% female, and 51% Ad36(+). Overweight and obesity prevalence was 38.9% and 42.9% in the Ad36(+) and Ad36(-) groups, respectively (p=.734). No significant differences were observed between Ad36 groups at baseline or follow-up for age, race, sex, sexual maturation rating stage, weight, height, BMI-for-age percentile, fat-free soft tissue mass, fat mass, total body bone area, BMC or aBMD and calcium, vitamin D, protein, or calorie intakes (Table 1). Unadjusted tibial or radial bone outcomes were not different at baseline. At follow-up, unadjusted cortical vBMD at the tibia and radius was significantly higher in the Ad36(+) vs. the Ad36(-) participants (p=0.05 and p=0.04, respectively). Mean calcium intakes did not differ between the two groups at baseline, but were significantly lower in the Ad36- vs Ad36+ group at follow-up (p=0.021).

Table 2 summarizes the adjusted data indicative of change over time (Model 1). The Ad36(+) participants had significantly smaller gains in height (p=0.001) and total body bone area (p=0.021) than Ad36(-) participants. Changes in tibia MCSA (p=.016), periosteal circumference (p=.021), leg length (p=0.001), and total area (p=.035) were significantly lower in the Ad36(+) vs. Ad36(-) children. At the radius, arm length was significantly lower in the Ad36(+) vs. the

Ad36(-) participants (p=0.037). The addition of calcium intake at follow-up in Model 1 did not change the significance of Ad36 in gains in height (p=0.002), tibia MCSA (p=0.04), periosteal circumference (p=0.031), or leg length (p=0.005). Inclusion of follow-up calcium intake nullified total body bone area in the model (p=0.059), however calcium intake was not significantly associated with total bone area (p=0.986) (data not shown).

Table 3 includes limb length as a covariate in the model (Model 2). Height gains were significantly less in Ad36+ vs Ad36-, but no significant differences between groups for tibia total area, MCSA, periosteal circumference, cortical BMC, cortical vBMD, cortical area, cortical thickness, SSI, or endosteal circumference. No significant differences between groups were found for any bone outcome at the radius in either model (all p>0.05).

DISCUSSION

To our knowledge, the current investigation is the only prospective study to examine the effects of Ad36 infection on changes in bone and body composition over approximately five years, through the period of rapid pubertal growth. The primary findings of this study revealed that tibial bone area and periosteal circumference during pubertal growth were less pronounced in children infected with Ad36 vs. Ad36(-) children. However, after controlling for changes in limb length (Model 2), these differences were nullified. Changes in total body bone area and height were significantly greater in Ad36(-) vs. Ad36(+) children.

To date, only one published study has examined the effects of Ad36 infection on obesity and bone strength, and this was a cross-sectional study. After adjusting for MCSA and limb length, Laing et al. showed that late adolescent females classified as high-fat (having $\geq$ 32% body fat) and infected with Ad36 had lower total area at the 4% and 20% radial sites, lower total BMC at the 4% radial site, and lower measures of cortical BMC, cortical area and SSI of the

20% radius when compared to the high-fat participants not infected with the virus.[23] In a younger cohort of girls, the unpublished work of Berger et al. found lower Ct. Ar (p=0.03), Tt. Ar (p=0.006) and SSI_p (p=0.009) in obese Ad36(+) vs. obese Ad36(-) participants, supporting the findings of Laing et al.[24] In contrast to results from these studies, after controlling for limb length, we did not observe changes in cortical bone. Several factors could help explain the differences in study outcomes. First, in the Laing et al. and Berger et al. studies, the authors were able to account for adiposity and grouped participants by obesity status in the statistical analyses. There were no effects of Ad36 infection on bone in non-obese participants. If the effects of Ad36 infection are augmented by the state of obesity, possibly related to an inflammatory condition, we were unable to detect that in this study. Because of the relatively small number of overweight/obese participants per group (n=14, n=14, in Ad36(+) and Ad36(-), respectively), we were unable to classify subgroups by adiposity status in our statistical analyses. In the current study, there were no statistical differences in mean BMI-for-age percentiles, fat mass or percent fat at baseline or follow-up between the Ad36 groups. Moreover, though Ad36 infection is considered an obesogenic virus, the prevalence of overweight/obesity across groups was very similar, 38% and 40% in the Ad36(+) and Ad36(-) group, respectively (p=.924).

It is not entirely surprising that inclusion of limb length as a covariate nullified the effects of the virus on cortical bone. While we used limb length in Model 2, statistically controlling for changes in MCSA or height produced similar results. This period of rapid growth, combined with the profound effects of increasing muscle mass on bone, may have dwarfed any effects of the virus on bone. In addition, geometric forces that positively impact bone are greater at the tibia compared to the radius and the increased compression and torsional forces at this site[35] could explain the nullification of our results at the tibia upon inclusion of limb length in Model 2.

Controlling for MCSA/limb length in the Laing et al. study did not eliminate significant differences in radius cortical bone between Ad36 groups; however, it is important to note that participants were older and had completed pubertal maturation.[23]

Despite no observed differences in leg length or height at baseline, Ad36(+) children gained less leg length and height than Ad36(-) children throughout growth. Though no height differences were observed cross-sectionally at follow-up between groups in the present study, other cross-sectional studies have revealed differences in standing height with Ad36(-) females being taller than Ad36(+) females of the same age.[17] In a study of 8-18 year-old males and females, Ad36(+) children were significantly taller, albeit older.[18] However, several other studies report no difference in height[23,24,36] or leg length[23,24] between Ad36(+) and Ad36(-) subjects. Further, a one-year prospective study in boys observed no difference in height between groups after one year of growth.[37] Our data suggest that there is a more complex relationship between growth, adiposity, and Ad36 status. Many factors work synergistically during growth; however, some may have had a profound effect on the results of this study, such as age, pubertal maturation, and dietary intakes of bone-related nutrients. Indeed, maturational status,[38] chronological timing of bone growth,[39] and prevalence of Ad36 infection[17] differ between boys and girls. From a growth standpoint, it is important to note that females enter puberty at an earlier chronological age[38] and gain lateral growth earlier than their male counterparts.[1] Though these lateral growth trends could reflect effects of infection, it is important to note that our Ad36(+) group was 61% female, as opposed to the Ad36(-) group, which was 40% female (p=0.08). This difference, though not statistically significant, could have impacted changes in maturational status of the groups, given that the Ad36(+) children were plausibly more mature at baseline. Despite no age difference or SMR stage difference between the groups in the present

study, the larger proportions of girls in the Ad36(+) group may have entered the study at a more advanced stage of pubertal growth, and therefore may have led to the observed differences in rate of longitudinal growth. Dietary intake analyses revealed that calcium intake was significantly lower in the Ad36(-) group at follow-up. Though this difference did not appear to impact our results in Model 1, the low intakes of both calcium and vitamin D are noteworthy, as the majority of the children in this study did not meet Recommended Dietary Allowances (RDA) for calcium and vitamin D. At baseline, only 38% of children met $\geq$ 80% of the RDA for calcium; only one child met $\geq$ 80% of the RDA for vitamin D. At follow up, a mere 16% of children met $\geq$80% of the RDA for calcium; vitamin D was also very poor, with only one child meeting $\geq$80% of the RDA.

We hypothesized that body composition measurements would differ between Ad36(+) and Ad36(-) participants after five years of growth. Our data contrast with studies that showed higher BMI z-scores among Ad36(+) participants[16,18,40] as well as studies suggesting differences in body weight between Ad36(+) and Ad36(-) participants.[18,41] Though we did not find absolute differences in these measures between groups, we did observe some hypothesis-generating trends in the data. For example, Ad36(+) adolescents appeared to have a higher amount of FFST (p=0.07) at baseline. The difference was negligible at follow-up (p=0.37) and the change data support this trend, as Ad36(-) adolescents appeared to gain more FFST mass over time (p=0.066). We expected to observe differences in body fat percentage or fat mass between groups, but neither was significantly different cross-sectionally, and there were no significant differences in the body fat changes over time. Park et al. followed Korean boys through one year of adolescent growth, and in their cross-sectional analyses of baseline and follow-up data, the authors reported greater fat percentage at follow-up in Ad36(+) participants (p=0.02), but no

difference in body weight, BMI or fat mass at baseline or follow-up.[37] The researchers observed trends toward differences in body composition over time, specifically toward a greater change in weight, BMI, fat mass and fat percentage in the Ad36(+) group. However, in agreement with our data, the Park et al. analyses revealed no statistical differences in changes between Ad36(+) and Ad36(-) children over one year of growth.[37] Though some longitudinal studies report greater levels of adiposity in Ad36(+) vs Ad36(-) adults ten years post collection of baseline data,[42] our study across approximately five years of adolescent growth agrees with studies showing no association between higher BMI and Ad36 infection[43] and no difference between BMI in Ad36(+) or Ad36(-) adults over time.[44] While there are uncertainties surrounding the reason for these discrepancies, one possibility might be due to our inability to determine how long participants have been infected.

Strengths of this study include the availability of prospective data in an ethnically diverse sample of boys and girls over five years of growth. Furthermore, the utilization of sophisticated technologies (i.e., DXA and pQCT) allowed for 2- and 3-dimensional quantification of bone and body composition measurements. However, this study is not without limitations. Though we can confirm participants were Ad36 seropositive at baseline, it is unknown when their exact infection occurred. Moreover, we did not have Ad36 measures at follow-up and whether or not they maintained their seropositive status at follow-up is unknown. Additionally, this more normal weight, homogenous sample, prevented us from classifying children by adiposity status as we have been able to accomplish in previous studies.[23,24] Physical activity data were available and inclusion of an objective measure of physical activity could have strengthened the findings. Finally, because of the high degree of variability in maturation during these pubertal years, the

use of chronological age or sexual maturation stages assessed by self-assessment are limiting in aligning participants on similar maturational trajectories.

In conclusion, our data indicate that, over five years of growth, infection with Ad36 does not significantly alter body composition. Cortical bone at the tibia was affected by seropositivity, though the observed significant changes were explained by changes in limb length. Finally, our results show that Ad36 infection was associated with smaller height gains through puberty. If infection with a common cold virus negatively affects linear growth during puberty, early detection in childhood would be critical. In future work, assessment of biological age could provide a landmark to help control for the highly variable pubertal growth and enhance our ability to detect and better understand the impact of Ad36 infection on long-term bone health.

Table 1: Participant characteristics[a]

	Baseline				Follow-up			
	Total sample	Ad36(+)	Ad36(-)	p [b]	Total sample	Ad36(+)	Ad36(-)	p [b]
	N=71	n=36	n=35		N=71	n=36	n=35	
Female (%)[c]	51%	61% (22)	40% (14)	0.08	-	-	-	-
Black (%)[c]	31%	33.3% (12)	28.6% (10)	0.66	-	-	-	-
Tanner Stage	2.4 ± 0.6	2.5 ± 0.6	2.3 ± 0.5	0.23	-	-	-	-
Age (y)	11.4 ± 1.3	11.6 ± 1.3	11.2 ± 1.2	0.25	17.0 ± 1.4	17.2 ± 1.5	16.8 ± 1.3	0.25
Weight (kg)	46.5 ± 10.5	48.3 ± 10.8	44.6 ± 10.0	0.14	73.0 ± 17.3	71.4 ± 17.3	74.7 ± 17.4	0.43
Height (cm)	151.2 ± 9.4	152.5 ± 1.7	149.9 ± 8.4	0.24	171.0 ± 9.4	169.0 ± 9.5	173.0 ± 8.9	0.07
BMI (kg/m^2)	20.2 ± 3.6	20.7 ± 3.5	19.8 ± 3.6	0.30	25.0 ± 5.4	25.0 ± 5.4	25.0 ± 5.4	0.98
BMI-for-age (%)	66.7 ± 29.8	68.6 ± 30.6	64.7 ± 29.3	0.58	67.5 ± 28.6	67.6 ± 27.7	66.7 ± 30.1	0.90
Total Fat Mass (g)	12858.5 ± 5949.4	13249.9 ± 5860.6	12455.9 ± 6098.0	0.58	17821.6 ± 9054.2	17514.4 ± 8466.4	18137.6 ± 9735.9	0.77
Percent body fat (%)	26.8 ± 8.4	26.8 ± 8.6	26.8 ± 8.4	0.98	23.5 ± 8.4	23.9 ± 8.0	23.2 ± 9.0	0.72
FFST mass (g)	32099.5 ± 6887.7	33553.2 ± 7659.0	30604.3 ± 5722.5	0.07	52935.6 ± 10661.2	51809.7 ± 11531.8	54093.7 ± 9715.7	0.37
TB BA (g^2)	1568.0 ± 159.7	1589.7 ± 167.2	1545.7 ± 150.7	0.25	2079.1 ± 203.3	2044.5 ± 208.3	2114.7 ± 194.6	0.15
TB BMD (g/cm^2)	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.21	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.94
TB BMC (g^2)	1357.2 ± 263.8	1402.3 ± 298.2	1310.7 ± 217.6	0.14	2457.6 ± 431.7	2423.7 ± 480.6	2492.5 ± 378.8	0.51
Calcium (mg)[d]	960.7 ± 400.5	953.0 ± 473.9	968.5 ± 324.6	0.87	659.9 ± 392.4	777.8 ± 367.4	541.9 ± 387.5	0.021*
Vitamin D (IU)[e]	164.5 ± 110.8	171.2 ± 126.4	158.2 ± 96.1	0.63	105.0 ± 109.4	114.9 ± 124.8	94.4 ± 93.0	0.50
Protein (g)[d]	72.1 ± 21.9	72.1 ± 23.6	72.3 ± 20.7	0.98	75.5 ± 25.8	75.6 ± 26.7	76.2 ± 25.5	0.94
Total Calories[d]	1976.7 ± 538.9	1956.5 ± 570.9	1991.2 ± 519.9	0.79	1937.8 ± 724.7	1873.6 ± 627.2	2000.6 ± 816.8	0.51
Tibia (66%)								
MCSA (mm^2)	4880.7 ± 851.2	5030.2 ± 950.9	4722.4 ± 711.1	0.13	6935.7 ± 1175.2	6744.0 ± 1154.6	7133.0 ± 1180.4	0.17
Leg length (cm)	34.4 ± 2.6	34.8 ± 2.8	34.0 ± 2.4	0.21	39.4 ± 3.1	38.7 ± 3.3	40.1 ± 2.8	0.06
Ct.vBMD (mg/m^3)	1056.9 ± 34.2	1058.7 ± 38.3	1054.9 ± 29.8	0.65	1129.2 ± 23.2	1134.6 ± 22.6	1123.6 ± 22.9	0.05*
Ct.BMC (mg/mm)	240.2 ± 42.5	245.3 ± 47.2	234.8 ± 36.8	0.31	356.6 ± 56.5	352.1 ± 62.9	361.3 ± 49.5	0.50
Tt.Ar (mm^2)	431.0 ± 85.4	438.0 ± 88.4	423.6 ± 82.8	0.49	570.8 ± 103.4	553.5 ± 96.3	588.7 ± 108.9	0.16
Ct.Ar (mm^2)	227.2 ± 39.6	231.5 ± 44.0	222.6 ± 34.4	0.36	315.9 ± 50.6	310.4 ± 55.9	321.6 ± 44.7	0.36
Ct.Th (mm)	3.7 ± 0.5	3.8 ± 0.6	3.7 ± 0.4	0.48	4.5 ± 0.6	4.5 ± 0.7	4.6 ± 0.6	0.85
Ps.Pm (mm)	73.2 ± 7.2	73.8 ± 7.4	72.6 ± 7.2	0.49	84.4 ± 7.6	83.1 ± 7.0	85.6 ± 8.0	0.17
Es.Pm (mm)	49.9 ± 6.9	50.3 ± 6.7	49.6 ± 7.2	0.69	55.9 ± 7.8	54.7 ± 6.8	57.0 ± 8.6	0.21
SSI (mm^3)	1533.3 ± 387.3	1575.3 ± 407.7	1488.8 ± 365.5	0.36	2562.1 ± 609.4	2472.4 ± 601.3	2654.5 ± 612.6	0.22
Radius (66%)								
Arm Length (cm)	24.2 ± 1.7	24.5 ± 1.8	23.8 ± 1.6	0.09	27.0 ± 2.1	26.7 ± 2.3	27.3 ± 1.9	0.25
Ct.vBMD (mg/cm^3)	1060.6 ± 36.8	1062.3 ± 40.1	1059.0 ± 33.9	0.71	1144.9 ± 29.1	1152.0 ± 26.3	1137.6 ± 30.4	0.036*
Ct.BMC (mg/mm)	59.2 ± 1.3	60.8 ± 2.1	57.6 ± 1.6	0.24	95.4 ± 15.5	95.1 ± 16.7	95.8 ± 14.4	0.84
Tt.Ar (mm^2)	91.5 ± 16.2	91.7 ± 17.2	91.3 ± 15.4	0.92	119.7 ± 23.4	117.6 ± 24.8	121.9 ± 22.1	0.45
Ct.Ar (mm^2)	55.7 ± 9.7	57.2 ± 11.2	54.3 ± 8.0	0.23	83.8 ± 14.8	83.1 ± 16.6	84.4 ± 12.9	0.72
Ct.Th (mm)	2.0 ± 0.3	2.1 ± 0.3	2.0 ± 0.3	0.33	2.8 ± 0.4	2.8 ± 0.4	2.8 ± 0.4	0.96
Ps.Pm (mm)	33.8 ± 3.0	33.8 ± 3.1	33.8 ± 2.9	0.94	38.7 ± 4.0	38.4 ± 4.4	39.0 ± 3.5	0.54
Es.Pm (mm)	20.9 ± 3.5	20.7 ± 3.4	21.1 ± 3.7	0.64	21.0 ± 4.2	20.8 ± 4.2	21.3 ± 4.1	0.58
SSI (mm^3)	-	-	-	-	296.2 ± 89.2	295.1 ± 106.8	297.4 ± 68.1	0.92

Values are unadjusted means $\pm$ SD or percentages.

*Significance

[a]Abbreviations: FFST, fat-free soft tissue; TB, total body; BA, bone area; BMD, bone mineral density; BMC, bone mineral content; MCSA, muscle cross sectional area; Ct.vBMD, cortical bone mineral density; Ct.BMC, cortical bone mineral content; Tt.Ar, total area; Ct.Ar, cortical area; Ct.Th, cortical thickness; Peri Circ, periosteal circumference; Endo Circ, endosteal circumference; SSI, strength strain index

[b]Tests of significance between groups were based on ANOVA (P < 0.05)

[c]Tests of significance between groups were based on x^2 test

[d]Follow-up data includes N=58 (n=29; n=29, for Ad36(+) and Ad36(-), respectively)

[e]Follow-up data includes N=54 (n=28, n=26, for Ad(+) and Ad36(-), respectively)

Table 2. Changes in total body composition and cortical bone indices at the tibia and radius

(Model 1)

	Ad36(+)			Ad36(-)			p
Body Composition		$n=36$			$n=35$		
Height (cm)	17.4	$\pm$	0.9	22.1	$\pm$	1.0	0.001*
Weight (kg)	24.0	$\pm$	1.9	29.2	$\pm$	1.9	0.066
Total Fat Mass (g)	3967.4	$\pm$	912.5	5958.5	$\pm$	925.9	0.137
FFST (g)	19089.5	$\pm$	1148.5	22201.3	$\pm$	1165.3	0.066
BF Percentage (%)	-3.3	$\pm$	0.9	-3.1	$\pm$	0.9	0.878
TB BMC (g^2)	1035.4	$\pm$	46.9	1138.4	$\pm$	47.6	0.134
TB BMD (g/cm^2)	0.3	$\pm$	0.0	0.3	$\pm$	0.0	0.378
TB BA (cm^2)	472.5	$\pm$	22.8	550.8	$\pm$	23.1	0.021*
Leg length (cm)	4.2	$\pm$	0.3	5.9	$\pm$	0.3	0.001*
Arm length (cm)	2.4	$\pm$	0.3	3.2	$\pm$	0.3	0.037*
Tibia (66% site)							
MCSA (mm^2)	1840.5	$\pm$	142.1	2347.5	$\pm$	144.3	0.016*
Ct.vBMD (mg/cm^3)	73.8	$\pm$	4.6	68.3	$\pm$	4.7	0.414
Ct.BMC (mg/mm)	109.1	$\pm$	6.1	119.9	$\pm$	6.2	0.227
Tt.Ar (mm^2)	128.0	$\pm$	9.1	156.3	$\pm$	9.2	0.035*
Ct.Ar (cm^2)	80.6	$\pm$	5.5	94.1	$\pm$	5.6	0.095
Ct.Th (mm)	0.8	$\pm$	0.1	0.8	$\pm$	0.1	0.439
Peri.Circ. (mm)	10.1	$\pm$	0.7	12.4	$\pm$	0.7	0.021*
End.Circ (mm)	5.5	$\pm$	0.7	7.1	$\pm$	0.7	0.092
SSI (mm^3)	958.6	$\pm$	57.7	1081.0	$\pm$	58.5	0.148
Radius (66% site)							
Ct.vBMD (mg/cm^3)	89.9	$\pm$	5.3	80.2	$\pm$	5.3	0.204
Ct.BMC (mg/mm)	35.8	$\pm$	1.8	35.7	$\pm$	1.8	0.977
Tt.Ar (mm^2)	25.6	$\pm$	2.0	28.8	$\pm$	2.0	0.277
Ct.Ar (cm^2)	26.8	$\pm$	1.6	27.8	$\pm$	1.6	0.671
Ct.Th (mm)	0.7	$\pm$	0.0	0.8	$\pm$	0.0	0.764
Peri.Circ. (mm)	4.4	$\pm$	0.3	5.0	$\pm$	0.3	0.184
End.Circ (mm)	-0.3	$\pm$	0.3	0.0	$\pm$	0.3	0.435

Values are means $\pm$ SD or percentages.

*Significance

Model 1 controlled for sex, race and sexual maturation rating (SMR) stage at baseline

[a]Abbreviations: FFST, fat-free soft tissue; TB, total body; BA, bone area; aBMD, areal bone mineral density; BMC, bone mineral content; MCSA, muscle cross sectional area; Ct.vBMD, cortical bone mineral density; Ct.BMC, cortical bone mineral content; Tt.Ar, total area; Ct.Ar, cortical area; Ct.Th, cortical thickness; Peri Circ, periosteal circumference; Endo Circ, endosteal circumference; SSI, strength strain index

Table 3: Changes in cortical bone indices at the tibia and radius (Model 2)

Tibia (66% site)	Ad36(+) n=36			Ad36(-) n=35			p
	Mean	±	SD	Mean	±	SD	
Muscle cross sectional area (mm^2)	1942.9	±	139.8	2242.1	±	142.1	0.16
Cortical vBMD (mg/cm^3)	71.8	±	4.8	70.5	±	4.8	0.85
Cortical BMC (mg/mm)	113.0	±	6.2	116.0	±	6.3	0.75
Total area (mm^2)	139.2	±	7.8	144.8	±	7.9	0.63
Cortical area (cm^2)	84.8	±	5.5	89.7	±	5.6	0.52
Cortical Thickness (mm)	0.8	±	0.1	0.8	±	0.1	0.66
Periosteal Circumference (mm)	10.9	±	0.6	11.6	±	0.6	0.40
Endosteal Circumference (mm)	6.3	±	0.6	6.3	±	0.6	0.93
SSI (mm^3)	1015.6	±	54.3	1022.3	±	55.2	0.93
Radius							
Cortical vBMD (mg/cm^3)	88.3	±	5.3	81.7	±	5.2	0.40
Cortical BMC (mg/mm)	37.0	±	1.7	34.6	±	1.7	0.35
Cortical vBMD (mg/cm^3)	88.3	±	5.3	81.7	±	5.2	0.40
Cortical area (mm^2)	28.0	±	1.4	26.7	±	1.4	0.52
Cortical Thickness (mm)	0.8	±	0.0	0.8	±	0.0	0.90
Periosteal Circumference (mm)	4.6	±	0.3	4.7	±	0.3	0.82
Endosteal Circumference (mm)	-0.2	±	0.3	-0.1	±	0.3	0.98

Values are means ± SD or percentages.

*Significance

Model 2 controlled for sex, race, sexual maturation rating (SMR) at baseline and limb length

[a]Abbreviations: Tt.Ar, total area; Ct.vBMD, cortical bone mineral density; Ct.BMC, cortical bone mineral content; Ct.Ar, cortical area; Ct.Th, cortical thickness; Peri Circ, periosteal circumference; Endo Circ, endosteal circumference; SSI, strength strain index; MCSA, muscle cross sectional area

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CHAPTER 4

SUMMARY AND CONCLUSIONS

This project was conducted to determine the prospective effects of Ad36 infection on body composition measures such as muscle and fat mass as well as indices of cortical bone development in a cohort of apparently healthy children through five years of adolescent growth. In our cohort of children, prevalence of infection was not higher in overweight participants, nor did infected participants have a higher percent body fat or increased fat mass at either time point. Results do not suggest that Ad36 significantly alters body composition in children throughout pubertal growth, as no differences were observed between the two groups for any body composition measure when key covariates, including dietary intake, were incorporated into the model. A prospective study in adolescent boys similarly observed non-significant trends in body composition over one year.[1] Interestingly, in our study, Ad36(-) children did gain more height and leg length over time compared to Ad36(+) participants. They also trended towards being taller than the Ad36(+) participants at follow-up, though this was not statistically significant (p=0.07). This difference in magnitude of growth may be explained by differences in baseline maturational status of the children that we were unable to capture by our SMR staging questionnaire. Though non-significant, there was a higher proportion of girls in the Ad36(+) group, and girls have been shown to enter puberty at an earlier chronological age than boys. If a high proportion of participants in the Ad36(+) were more mature at baseline, it is plausible that they would gain less height over time. Previous cross-sectional studies have shown differences in

height between groups, but longitudinal data have been conducted primarily in adults, whose height doesn't change between visits or has been omitted from results.[2,3]

This project added an additional layer of complexity through body composition analyses of cortical bone at the radius and tibia. Surprisingly, in contrast to two previous projects that showed effects of Ad36 at the radius, there were no differences in radial bone outcomes over time between groups prospectively.[4,5] Considering the aforementioned work was conducted in obese females, it is suggestive of a more complicated relationship between obesity, Ad36 infection and bone health. This cohort of children included a wide range of BMI-for-age percentiles and the distribution may not have been extreme enough to detect differences.

In the future, it would be important to utilize the most sensitive and specific method to assess Ad36 seropositivity such as the serum neutralization assay method. Recently, concern has been raised for the enzyme linked immunoassay (ELISA) methodology,[6] especially related to cross-reactivity with other viruses. Considering the small sample size in this study, a small variation in identification of true infection could have influenced results and conclusions. Further, it would be extremely insightful to test participants at baseline and follow-up for infection, as a major gap in the literature relates to the duration participant's blood remains seropositive for the virus. Considering that maturational timing is so crucial in this study design, utilization of a more specific measure of maturation such as hand wrist radiograph for determination of biological age, would strengthen the project.

Overall, the findings from this thesis provide evidence that Ad36 is likely influential in adolescent growth. Though no direct measures of adiposity differed, a difference in lateral growth was observed in height and limb length. If infection with a common cold virus negatively affects linear growth during puberty, early detection in childhood would be critical. These data

are important to help elucidate the long-term impact of Ad36 infection on development of adiposity and suboptimal bone development.

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