Role of Gender, Age, Pubertal Status and Adiposity on Endothelium-Independent Dilation in Children and Adolescents

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Dedication

To my brilliant mother and diligent father, you both have sparked in me a love for learning and inspired me to always expect more out of life. And to my ever loving husband, thank you for your unfailing support, wisdom, and humor during this hectic time in our lives.

Abstract

In adults, gender, aging, and obesity have been identified as prominent risk factors for impaired endothelium-independent dilation (EID). Whether, these factors impact EID in youth has not been well documented. Therefore, we examined how gender, age, pubertal status, and obesity influence endothelium-independent dilation (EID) in youth. Three hundred twenty-two healthy youth (142 females), ages 9 to 18 years (mean ± SEM, age = 14.1 ± 0.14 years; body mass index percentile [BMI-percentile] = 70.0 ± 1.5 percentile) were included in this cross-sectional study. The change in brachial artery diameter following administration of 0.3 mg of sublingual nitroglycerin to induce EID was measured using a conventional ultrasound scanner (Acuson, Sequoia 512, Siemens Medical Solutions USA, Inc., Mountain View, CA, USA) with a 8-15 MHz linear array probe. Multiple linear regression models were created using gender, age, BMI-percentile, percent body fat and baseline diameter as predictor variables. Statistical analysis of pubertal status and BMI category was conducted using one-way ANOVA, adjusting for gender, age, BMI-percentile, race, and baseline diameter. Females had higher EID-area under the curve (EID-AUC) compared to males ($p=0.012$) but not EID% ($p=0.112$). Age was a significant predictor of EID% ($\beta=0.37$, $p=0.04$) and EID-AUC ($\beta=83.5$, $p=0.02$) in females but not in males (EID%, $p=0.83$; EID-AUC, $p=0.92$). No differences in EID% or EID-AUC were observed across Tanner stages. There were no differences in EID% or EID-AUC among normal-weight, overweight, and obese participants, and percent body fat was not a significant predictor of EID% or EID-AUC. These data suggest that gender differences in EID are apparent early in life, with aging-related changes observable in

females, but not males. Obesity does not appear to impact EID in children and adolescents. The mechanisms by which young males and females exhibit early differences in smooth muscle function require further investigation.

Key Words: Smooth muscle function, Gender Differences, Pediatric, Cardiovascular Disease Risk, BMI

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BMI: body mass index

CVD: cardiovascular disease

DXA: dual energy X-ray absorptiometry

EID: endothelium-independent dilation

EID%: endothelium-independent dilation percent

EID-AUC: endothelium-independent dilation area under the curve

VSM: vascular smooth muscle

CHAPTER 1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide (World Health Organization, 2013). The progression of CVD occurs over the course of the lifespan with its origins beginning in early life (Newman, Wattigney, & Berenson, 1991). Regulation of vascular tone via vascular smooth muscle (VSM) is vital for normal arterial function (Falk, 2006). Dysfunction of the VSM represents a key step in the early stages of the atherosclerotic process (Rudijanto, 2007).

A well-validated non-invasive measure of VSM function involves use of ultrasound imaging of the brachial artery following the administration of sublingual nitroglycerin (Corretti et al., 2002). Reduced amounts of this endothelium-independent dilation (EID) have been associated with many chronic diseases in adults including coronary artery disease (Heitzer, Schlinzig, Krohn, Meinertz, & Munzel, 2001), Type 1 (Johnstone et al., 1993) and Type II (McVeigh et al., 1992) diabetes, and inflammatory bowel disease (Kayahan et al., 2012). Although there are numerous studies examining EID in healthy and diseased adults, there is a lack of information available on EID in children.

In adults, males exhibit greater incidence of CVD (Lerner, Lerner, & Kannel, 1986) and reduced VSM function compared to females (Adams et al., 1998; McCue, Marlatt, Kelly, Steinberger, & Dengel, 2012; Thelen, Kelly, Williamson, & Dengel, 2008). Similar associations between gender and EID have not been verified in children, as minimal data on gender differences is available in this population (Jarvisalo et al., 2002). Aging is a primary contributor of VSM dysfunction which is caused by migration and proliferation

of smooth muscle cells (Herrera, Mingorance, Rodriguez-Rodriguez, & Alvarez de Sotomayor, 2010; Rudijanto, 2007). This phenomenon is mirrored in studies showing impairment of EID with increasing age in asymptomatic adults (Al-Shaer et al., 2006; Montero, Pierce, Stehouwer, Padilla, & Thijssen, 2014). While most studies in children utilize age as a covariate in analysis, no formal study has shown whether age is associated with differences in EID in children and adolescents (Jarvisalo et al., 2002) and only one study has examined the impact of pubertal status of EID response (Marlatt et al., 2013). Moreover, obesity in childhood is known to play a key role in the development of many CVD risk factors. However, only limited data exist on the role of obesity and EID in children and adolescents (Jarvisalo et al., 2002; Pena et al., 2006).

Examining the relationships between gender, age, pubertal status and obesity with EID in children and adolescents may provide insight into early progression of atherosclerosis. However, limited data is available on how these variables influence EID in youth. Therefore, the purpose of this study was to examine the association between gender, age, pubertal status, and obesity with EID in children and adolescents.

The following chapters of this thesis include a review of literature, explanation of methods, summary of results, discussion and conclusion.

Chapter two provides a review of relevant literature related to the VSM function, influences of gender, age, pubertal status and adiposity on VSM function, and the use of EID as a validated measure of assessing vascular smooth muscle function non-invasively.

Chapter three includes information on the methods and procedures utilized in this study. Additionally, information on study population, techniques and equipment, data collected and statistical analyses used are included.

Chapter four summarizes the results of the study. Differences in EID between males and females, changes in EID across age, and comparison of EID between Tanner stages and BMI status are included.

Chapter five includes discussion of study results. Associations between gender, age, pubertal status, adiposity, and EID are reviewed. Findings are presented in light of previously published studies on, and related to, the current topic.

Chapter six provides final conclusions and clinical implications of the findings of this study. The significance of how certain clinical characteristics can influence EID in children is discussed along with recommendations for future studies.

Chapter seven provides a list of all cited works.

CHAPTER 2. LITERATURE REVIEW

The Endothelium and Vascular Smooth Muscle

The vascular system is a dynamic network of vessels that transport blood to tissues and clears waste (Cines et al., 1998). Blood vessels are composed of three layers: the innermost layer, the tunica intima; the tunica media; and the outermost layer, the tunica externa. (Marieb & Hoehn, 2007). The tunica media of the blood vessel is composed primarily of vascular smooth muscle (VSM) and is responsible for maintaining vascular tone by contracting and relaxing in response to endogenous and exogenous stimuli (Rudijanto, 2007). VSM cells display a high degree of plasticity and are able to make reversible transformations between several phenotypes in response to environmental cues (Frid, Dempsey, Durmowicz, & Stenmark, 1997). Each phenotype exhibits its own inherent set of physiological properties. Normal mature VSM cells are primarily of the contractile type, which contract and relax in response to stimuli (Louis & Zahradka, 2010). As a consequence of vascular injury and chronic inflammatory states, as seen in several diseased states including hypertension (Robinson, Dobbs, & Bayley, 1982), and diabetes (McVeigh et al., 1992; Williams, Cusco, Roddy, Johnstone, & Creager, 1996), VSM cells undergo transition from the healthy, contractile phenotype to the pathogenic, synthetic phenotype (Owens, Kumar, & Wamhoff, 2004). The synthetic phenotype is associated with vascular dysfunction, increased migration and proliferation of VSM cells, increased vessel constriction, and initiation of lesion formation (Schaper & Ito, 1996; Wolf et al., 1998). The migration and proliferation of VSM cells from the tunica media to the tunica intima results in reduced vascular function and is a fundamental process in the progression of atherosclerosis (Willis, Pierre-Paul, Sumpio, & Gahtan, 2004).

Although only a thin layer of cells, the endothelium functions as a paracrine organ that is involved in several physiological functions including participation in immune response, inhibition of platelet aggregation and adhesion, and regulation of vascular tone (Corretti et al., 2002; Luescher & Barton, 1997; S. Moncada, Radomski, & Palmer, 1988). Certain signals sent from the endothelium act directly on the smooth muscle and result in changes in vessel tone. Furchgott and Zawadzki (1980) were the first to observe that when prepared rabbit aortas were stripped of their endothelial cells, there was diminished VSM relaxation in response to the vasodilator acetylcholine. This finding indicates that endothelial cells play an imperative role in controlling vascular tone by releasing a substance, then named endothelium-derived relaxing factor, which acts on the VSM to cause vasodilation. Endothelium-derived relaxing factor was later recognized as the short-lived, humoral compound nitric oxide (NO) (Furchgott & Vanhoutte, 1989; Ignarro, Buga, Wood, Byrns, & Chaudhuri, 1987; Palmer, Ferrige, & Moncada, 1987). After being released from the endothelium in response to shear stress, NO exerts its effects on the VSM by increasing the levels of cyclic guanosine monophosphate (cGMP) (S. Moncada et al., 1988)

Molecular Mechanism of Vascular Smooth Muscle Relaxation

NO-mediated VSM relaxation was originally thought to be a cAMP pathway and it was not until the 1970's that it was determined that cGMP was the primary second messenger active in the pathway (Carvajal, Germain, Huidobro-Toro, & Weiner, 2000). This pathway is activated when NO binds to the iron-containing prosthetic heme group of

soluble guanylyl cyclase (s-GC) (Francis, Busch, Corbin, & Sibley, 2010; Lincoln & Cornwell, 1993; E. A. Moncada, 1991). This binding increases the level of cGMP in the cell by converting GTP to cGMP (Waldman & Murad, 1988). Increases in cGMP concentration activate cGMP-dependent protein kinases, which are a class of serine/threonine kinases that are found abundantly in the VSM. Activated c-GMP-dependent protein kinases alter intracellular calcium (Ca^{2+}) concentrations through several mechanisms including stimulating Ca^{2+}-K^{+} activated channels, inhibiting Ca^{2+}-voltage gated channels and activating Ca^{2+}/ATPase pump. Each of these mechanisms effectively reduces intracellular Ca^{2+} levels and results in VSM relaxation.

Endothelium-Independent Dilation

Celermajer et. al. (1992) described a non-invasive technique to measure endothelium-independent dilation (EID) that involves the use of ultrasound to monitor changes in brachial artery diameter after administration of sublingual nitroglycerin. In vivo, the VSM dilates in response to NO released by the endothelium in response to shear stress. EID is used as a measure for VSM function (Corretti et al., 2002; Tomiyama & Yamashina, 2010) by bypassing the endothelium via direct stimulation of the VSM with exogenous nitrovasodilators such as sublingual nitroglycerin (Furchgott & Vanhoutte, 1989; E. A. Moncada, 1991), which utilizes the same molecular pathway as endogenous NO (Francis et al., 2010).

A multitude of studies over the years have verified this technique and found that impaired EID is associated with several disease states. In a study of 228 adults, Gokce et.al. (2001) reported impaired EID in those with hypertension compared with normotensive controls (14.9 ± 6.0% versus 18.5 ± 7.8%, p=0.003). In a long-term follow-up study (mean follow-up = 6.7 years) of 147 patients (mean age = 54 ± 9.9 years) undergoing vascular reactivity function testing, Schachinger, Briteen, & Zeiher (2000) found that those who experienced cardiovascular events had a blunted vasodilator response to nitroglycerin and the incidence of cardiovascular events was higher in those with lesser nitroglycerin response at baseline. Additionally, impaired EID has been observed in those at risk for atherosclerotic development but not yet experiencing symptoms. In a study by Adams et. al. (1998), 800 asymptomatic subjects (mean age = 38 ± 16 years) were assessed for EID. They found that EID negatively correlated with a number of CVD risk factors including cholesterol, smoking status, and diabetes. These findings suggest that impaired VSM function may be an early indicator of CVD.

Associations of Gender, age and obesity with EID

Gender

Males have been shown to have a higher risk of CVD (Lerner et al., 1986) and reduced VSM function (Orshal & Khalil, 2004) compared to females. Sex hormones may explain these differences as estrogen has been identified as having cardio-protective qualities, with premenopausal females demonstrating lower CVD risk than both males and post-menopausal females (Barrett Connor & Bush, 1991; Mendelsohn, 2002). Additionally,

testosterone deficiency in males has been associated with increased risk of CVD along with the CVD risk factors of increased plasma triglycerides, obesity, and insulin resistance (Jones, 2010; Schooling, 2014; Traish, Saad, Feeley, & Guay, 2009). A study using peripheral arterial tonometry, a technique which uses finger plethysmography to assess vascular function, assessed EID response in 86 healthy adults after administration of 0.4mg of sublingual nitroglycerin. They found that peak nitroglycerin mediated index, a calculated index score of EID, was found to be significantly reduced in males compared to females (2.50 ± 1.34 versus 3.11 ± 1.59, $p = 0.05$) (McCue et al., 2012). Additionally, a study by Adams et. al. (1998) used regression analysis to evaluate the correlation between traditional cardiovascular risk factors, including cholesterol and gender, and VSM function by measuring EID response to 0.4mg of sublingual nitroglycerin. In a sample of 800 adults, they found that gender was significantly correlated with VSM function ($r = -0.26$, $p < 0.001$), with males having significantly lower EID values compared to females.

In a review by Aggoun, Szezepanski, & Bonnet (2005) they noted that cardiovascular function in children and adolescents is impaired by traditional risk factors such as high cholesterol, hypertension, and diabetes, similar to reported observations in adults. However, there are limited data available on gender differences in EID in children and adolescents. Jarvisalo et. al. (2002) administered four doses of 50µg of sublingual nitroglycerin, five minutes apart, to assess the dose response curve and EID in 105 apparently healthy children. Using multiple regression analysis the researchers found that

gender was significantly correlated with EID (β = -1.856, p = 0.045) with males reporting lower EID response than females. This suggests that differences in VSM function between males and females may be distinguishable early in life.

Aging

Proliferation and migration of VSM cells is a hallmark trait of aging, and therefore leads to VSM dysfunction (Lacolley, Regnault, Nicoletti, Li, & Michel, 2012; Rudijanto, 2007). Some studies have observed reduced EID response in older adults compared with younger adults (Montero et al., 2014; Parker, Ridout, & Proctor, 2006). One study compared EID in healthy young adults (mean age = 22 ± 1 year, n = 15), healthy older adults (mean age = 62 ± 3 years, n = 17) and older adults with diagnosed atherosclerosis (mean age = 66 ± 2 years, n = 31) by measuring forearm blood flow via venous occlusion plethysmography in response to IV-infused nitroprusside. They found that both healthy older adults and atherosclerosis subjects had significantly less EID response than young healthy controls (Al-Shaer et al., 2006). However, other studies have reported no difference in EID response with age (Heiss et al., 2005; Pierce et al., 2011; K. S. Woo et al., 1997). Differences in the results of these studies may be due to the small sample sizes and differences in methods used and populations studied.

As with gender differences, there is a dearth of information available on EID and aging in children and adolescents. In instances where studies involving youth have reported EID values in respect to age, the variable of age has only been utilized as a correcting factor as

opposed to a main outcome. One study used multiple linear regression to determine the impact of a variety of factors, including age, on EID in youth and reported that age was not a significant factor in predicting EID response in this population ($\beta = 0.140$, $p = 0.771$) (Jarvisalo et al., 2002). This suggests that aging during childhood and adolescents may not be profound enough to discern changes in EID.

Tanner staging is a technique used to estimate pubertal status in children and adolescents and is based on physical characteristics of sexual maturity, such as pubic hair and breast development (Tanner, 1975). Increased insulin resistance during puberty is a well-documented event that has the potential to influence vascular function (Amiel et al., 1986; Moran et al., 2002). Chronic insulin resistance, as seen in diabetes, has been reported to negatively impact VSM function (Trovati & Anfossi, 2002; Wang, Gurevich, & Draznin, 2003). It is possible that the state of insulin resistance during puberty may produce similar outcomes as those seen in diabetes and result in reduced EID response. Only one study has examined the impact of pubertal status on EID response. In a study by Marlatt et al. (2013), 344 children and adolescents were divided by Tanner Stage and examined for EID response after administration of 0.3 mg of sublingual nitroglycerin. No significant differences were reported in EID between Tanner stages, suggesting sexual maturity does not significantly impact VSM function

Obesity

Obesity is frequently accompanied by morbidities such as hypertension and diabetes and has been noted to be an independent risk factor for developing CVD (Hubert, Feinleib, McNamara, & Castelli, 1983; Zdrojewski et al., 2013). Studies examining EID in respect to obesity status in adults are inconclusive as to whether excess weight impacts VSM function. Some studies have reported no difference in EID response between obese and normal weight individuals (Perticone et al., 2001; Van Guilder, Stauffer, Greiner, & Desouza, 2008; Vigili de Kreutzenberg, Kiwanuka, Tiengo, & Avogaro, 2003) while others have noted decreased EID in obese subjects (Christou et al., 2012). A study by Ayer et. al. (2011) comparing 19 obese adults (mean age = 31.0 ± 1.2 years, mean BMI = 44.1 ± 2.1 kg/m^2) and 19 age-matched normal weight controls (mean BMI = 22.4 ± 0.4 kg/m^2) found those who were obese had lower EID% (13.4 ± 0.9% versus 18.3 ± 1.1%, p = 0.002) and EID-AUC (54,316 ± 362 %/s versus 55, 613 ± 375 %/s, p = 0.018) in response to incremental doses (total does 500µg) of the vasodilator glyceryl trinitrate. In contrast, Van Guilder et. al. (2008) determined that there was no difference in EID between obese (mean BMI = 23.4 ± 0.3 kg/m^2) and non-obese subjects (mean BMI = 30.3 ± 0.6 kg/m^2) in response to arterial infusion of sodium nitroprusside.

There is conflicting evidence on the impact of obesity on EID in children and adolescents, with some researchers noting differences in EID response between obese and non-obese youth (Pena et al., 2006; Tounian et al., 2001) and others finding no significant differences (Jarvisalo et al., 2002; K. Woo et al., 2004). One study examining obese

(mean BMI = 24.9 ± 4.7 kg/m^2) and lean (mean BMI = 15.5 ± 1.5 kg/m^2) children (mean age = 8.8 ± 1.5 years) found that obese children had significantly lower EID than their lean counterparts in response to 300µg of sublingual nitroglycerin (19.0 ± 9.0 versus 25.8 ± 6.1%, p = 0.0014) (Aggoun et al., 2008). This is in contrast to Woo et. al. (2004) who reported no difference in EID after sublingual nitroglycerin administration between overweight (mean age = 10.9 ± 0.9 years, mean BMI = 26.7 ± 3.0 kg/m^2) and age-and-gender matched controls (mean BMI = 17.1 ± 2.1 kg/m^2, 20.6 ± 5.9 versus 19.6 ± 2.8%). With no clear association between obesity and EID more research is needed to better understand the relationship between these two variables.

VSM dysfunction is characteristic of atherosclerosis, a progressive disease beginning in early life. As CVD is the leading the cause of death worldwide (World Health Organization, 2013), by examining EID in children and adolescents, who are typically free of the progressive effects of aging we are able to examine the effects of obesity and other CVD risk factors on vascular smooth muscle function. Therefore, the purpose of this study was to examine how gender, age, pubertal status and obesity affect EID response in a sample of children and adolescents.

CHAPTER 3. METHODS

Participants

Data from 322 children and adolescents (142 females), aged 9 to 18 years were used in this cross-sectional study (mean age = 14.3 ± 0.22 years; BMI-percentile = 70.0 ± 1.5). Data were obtained from two separate cross-sectional studies conducted at the University of Minnesota between 2006 and 2011. All studies used the same methods of data collection and all were approved by the Institutional Review Board at the University of Minnesota.

Anthropometric Measurements

Height was obtained by use of a standard stadiometer (Ayrton S100, Prior Lake, Minnesota) and weight was collected by use of an electric scale (ST Scale-Tronix, White Plains, New York). Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in meters-squared (m^2). BMI-percentile was calculated using CDC normality scales (National Center for Health Statistics (US), 2012) and stratified into three categories: normal (>5^{th} percentile - <85^{th} percentile), overweight (≥86^{th} percentile - <95^{th} percentile, and obese (≥95^{th} percentile). Two-hundred-sixty-four subjects (119 females) were assessed for percent body fat, lean mass, and fat mass as determined by dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy, General Electric Medical Systems) and analyzed using enCore™ software (platform version 13.6, GE Healthcare, Madison, WI, USA). Scans were performed using standard imaging and positioning protocol as previously described (Kaul et al., 2012). A sub-group of 266 subjects (121 females) were assessed by trained providers to determine Tanner stage using previously

described protocol (Tanner, 1975). Females were assessed for breast and pubic hair development and males were assessed for pubic hair development.

Measurement of Endothelium-Independent Dilation

Participants were fasted for at least 8 hours, abstained from caffeine and heavy exercise for at least 24 hours before their study visit. All data was collected in a quiet, temperature-controlled room (22-23^{o}C). Vascular images of the left brachial artery were obtained proximal to the antecubital fossa in the longitudinal plane using a conventional ultrasound scanner (Acuson, Sequoia 512, Siemens Medical Solutions USA, Inc., Mountain View, CA, USA) with a 8-15 MHz linear array probe held at a constant pressure on the skin and at a fixed point over the imaged artery by a stereotactic arm. All images were digitized and stored for later off-line analysis using electronic wall-tracking software (Vascular Research Tools 5, Coralville, IA). EID measurements were collected as previously described (Celermajer et al., 1992) following a 15-minute rest period subjects were given 0.3 mg sublingual nitroglycerin to assess EID. Brachial artery diameter was measured continuously for 5-minutes post-nitroglycerin administration. Peak dilation (EID%) was determined by taking the greatest percent change in artery diameter from baseline. Area under the curve (EID-AUC) was defined as the complete relaxation of the brachial artery after nitroglycerin administration.

Statistical Analysis

Results are presented as mean ± SEM unless otherwise stated. Data was analyzed using SAS Software Package (v. 9.4; SAS Inc., Cary, North Carolina). Males and females were compared by independent samples t-test for clinical characteristics and chi-square test (count, %) for demographic characteristics. The predictors of age, gender, percent body fat, BMI-percentile, and baseline diameter were modeled using multiple linear regression. Gender-specific regression models were also determined for the dependent variables of EID% and EID-AUC using the same predictors. A one-way analysis of variance (ANOVA) with Bonferroni post-hoc adjustment was used to assess differences in EID% and EID-AUC for gender, Tanner stage and BMI-percentile category with adjustment for age, gender, race, and baseline diameter. Significance level was set at $\alpha=0.05$.

CHAPTER 4. RESULTS

Participant's demographic and clinical characteristics, divided by gender, are presented in Table 1. Males were significantly taller ($p<0.001$) and heavier ($p=0.03$) than females and also had greater lean body mass ($p<0.001$) and lower percent body fat ($p<0.001$). There was a significant relationship between gender and Tanner stage ($\chi^2=14.67$, n=266, $p=0.005$), with male gender being associated with lower Tanner stage. Males had a significantly lower EID-AUC (3362±146%/s vs 3695±148%/s, $p=0.012$) but not EID% (22.7±0.8% vs 23.8±0.8%, $p=0.112$) compared to females, after adjustment for age, BMI-percentile, race, and baseline diameter, as shown in Table 2.

In regression models for both EID% and EID-AUC (Table 3) age was not a significant predictor of EID%:($p=0.10$) or EID-AUC ($p=0.09$). Similarly, BMI-percentile was not a significant predictor of EID% ($p=0.98$) or EID-AUC ($p=0.35$). Baseline diameter was significant in both models (EID%: $\beta=-7.40$, $p<0.001$; EID-AUC: $\beta=-1197.1$, $p<0.001$). Gender-specific regression models are represented in Table 4. Age was a significant and positive predictor of EID% ($\beta=0.37$, $p=0.04$) and EID-AUC ($\beta=83.5$, $p=0.02$) in females, but not in males (EID%: $p=0.83$; EID-AUC: $p=0.92$). Greater baseline diameter in both females (EID%: $\beta=-8.58$, $p<0.001$; EID-AUC: $\beta=-1384.8$, $p<0.001$) and males (EID%: $\beta=-6.57$, $p<0.001$; EID-AUC: $\beta=-1039.8$, $p<0.001$) was associated with a lower EID response. One-way ANOVA showed no significant differences between normal-weight, overweight, and/or obese participants (Table 5). Additionally, a regression analysis of 264 subjects (119 females, Table 6) showed that the percent body fat was not a significant predictor of EID% ($p=0.83$) or EID-AUC ($p=0.11$). A sub-analysis of Tanner

stage (266 subjects, 121 females) showed no significant differences in EID% or EID-AUC between Tanner stages (Table 7).

CHAPTER 5. DISCUSSION

The main findings from the current study reveal that gender may be associated with smooth muscle function in children and adolescents, with males having lower EID-AUC, but not EID%, compared to females. Gender also appears to impact EID response differently across age, with age being a significant and positive predictor of EID in females but not in males. Additionally, pubertal status did not have significantly impact EID response in our sample. Another main finding of this study is that obesity status, as determined by both BMI and body composition based criteria, is not associated with smooth muscle dysfunction in children and adolescents. Taken together these data suggest that gender may play a role in smooth muscle function in children and adolescents independent of body weight and adiposity.

In adults, gender differences in smooth muscle function have been reported with males exhibiting lower EID than females (Adams et al., 1998; McCue et al., 2012). The difference in EID response between males and females might be explained by the hormonal influence of testosterone and estrogen. The influence of testosterone on the vascular system is controversial and information from *in vivo* studies is limited to adult samples. *In vitro* studies have demonstrated conflicting findings, with some reporting increases in VSM proliferation and migration in response to testosterone (Campelo, Cutini, & Massheimer, 2012; Fujimoto et al., 1994) and others demonstrating vasodilation of VSM in response to testosterone (Deenadayalu, White, Stallone, Gao, & Garcia, 2001). It may be that testosterone has both positive and negative effects on the vascular system, complicating our understanding of how it may contribute to gender

differences in EID. In contrast, the influence of estrogen on vascular function has been well-studied and has been shown to have cardio-protective properties that directly affect vascular smooth muscle function and limit cell migration and proliferation (Akishita et al., 1997; White et al., 2002). Studies have shown that in pre-menopausal females, where estrogen levels are high, there is a lower incidence of CVD compared to males and post-menopausal women (Barrett Connor & Bush, 1991; Boukhris et al., 2014). Greater estrogen levels in females may explain why females in our study reported significantly greater values for EID-AUC than males but not EID%. Estrogen receptors are located on the VSM (Mendelsohn & Karas, 1994) and cause K^+-channels to open (Mugge, Riedel, Barton, Kuhn, & Lichtlen, 1993; White et al., 2002) while also reducing amounts of NADPH oxidase, a free radical that degrades NO (Chambliss & Shaul, 2002). These influences of estrogen cause VSM relaxation and could extend relaxation time by keeping intracellular Ca^{2+} levels low and increasing bioavailability of NO.

The impact of aging on atherosclerotic development is well documented in adults (Falk, 2006; Herrera et al., 2010; Rudijanto, 2007). Studies examining EID response in adults have determined that, with age, vascular smooth muscle function deteriorates (Al-Shaer et al., 2006; Montero et al., 2014). However, the current body of literature examining age and EID response in youth is extremely restricted, with most studies using age only as an adjustment factor and not as a variable of interest. We observed differences in EID response across age between genders, with age being a significant and positive predictor of EID in females but not in males. This difference in regression models may be

explained by hormonal interactions in cardiovascular function, which are complex and differ between genders (Bruck et al., 1997; Traish & Kypreos, 2011). It may be that the cardio-protective mechanisms of estrogen and testosterone vary between genders and possibly result in gender differing EID response across time.

Another possibility for the difference in multiple linear regression models between genders could involve baseline diameter. Males have been noted to have greater baseline diameter measurements (Herrington et al., 2001) than females and baseline diameter has been previously reported to be inversely related with EID response (Brown, Bolson, Petersen, Pierce, & Dodge, 1981; Herrington et al., 2001). It may be that the effect of greater baseline diameter could possible overshadow the impact of age on EID in males. Our findings support the observation that baseline diameter greatly impacts EID and should therefore be adjusted for during statistical analysis. Additionally, we found no differences in EID response between Tanner stages. This is in agreement with a study by Marlatt et. al.(2013) which reported no difference in EID response between three pubertal stage groups: Tanner I, Tanner II-IV, and Tanner V. This suggests that pubertal status may not significantly impact EID response and therefore may not be necessary to determine during vascular testing.

Information available on the influence of BMI status on EID response is conflicting. There are several studies in adults reporting no difference in EID between normal weight and obese individuals (Perticone et al., 2001; Van Guilder et al., 2008; Vigili de

Kreutzenberg et al., 2003) while other studies contend that there is a difference in EID between the groups (Ayer et al., 2011; Christou et al., 2012). This disagreement also exists in the pediatric population with some studies reporting differences in EID between normal and obese subjects (Aggoun et al., 2008; Pena et al., 2006; Tounian et al., 2001) and others reporting no association between EID and BMI (Jarvisalo et al., 2002; K. Woo et al., 2004). In the present study, there were no significant differences in EID response between normal, overweight and obese children. Additionally, percent body fat was not a predictive variable of EID response. This is in agreement with a study by Woo et al. (2004), which found no difference in EID response between obese participants and age-and-gender matched controls. However, a separate study involving a total of 75 participants reported significantly lower EID response in severely obese subjects compared with normal weight controls (Tounian et al., 2001). The mean BMI values for obese subjects in this study was greater than the values of our sample. It may be that impaired EID response is only detectable in youth who are severely obese, leaving those who are overweight/mildly obese unaffected (Fernhall & Agiovlasitis, 2008). Furthermore, accounting for baseline diameter, as we did in our analysis, may explain differences in results as baseline has been shown to be inversely related to EID% (Schroeder et al., 2000). Of the studies examining the relationship between obesity and EID in youth, only one reported adjustment for baseline. Using univariate regression, Pena et. al. (2006), found that BMI Z-score was significantly correlated with EID. However, when baseline diameter was entered into the model, the relationship was no longer significant.

Strengths of our study include a large sample, with a range of adiposity and age in children and adolescents. The inclusion of DXA and Tanner stage data provided accurate information on body composition and pubertal development as compared to using age and BMI-percentile alone. However, we are limited by the cross-sectional nature of our study. While we observed difference in the effect of age on EID between genders in our sample, future studies which collect data in a longitudinal manner would be more appropriate to examine the trajectory of change in smooth muscle function during youth.

CHAPTER 6. CONCLUSION

In conclusion, we have demonstrated that gender may be associated with smooth muscle function in children and adolescents and that differences in EID-AUC between the two genders are apparent at an early age. Additionally, resting baseline diameter significantly impacts EID response, making it an important variable to adjust for during statistical analysis of EID, particularly when comparing males vs. females or normal weight vs. obese populations.

Table 1. Cohort demographics and anthropometric characteristics.

	Female (*n*=142)	**Male (*n*=180)**	***p*-value**
Age (years)	14.3 ± 0.22	13.9 ± 0.19	0.23
Height (cm)	159.8 ± 0.84	164.6 ± 1.06	<0.001*
Weight (kg)	58.9 ± 1.43	63.5 ± 1.70	0.03*
BMI (kg/m^2)	22.8 ± 0.43	22.9 ± 0.42	0.83
BMI-percentile	69.1 ± 2.08	70.8 ± 2.05	0.58
BMI Z-Score	0.65 ± 0.07	0.76 ± 0.08	0.28
Percent Body Fat^	29.2 ± 0.92	22.6 ± 1.11	<0.001*
Lean Mass (kg)^	37.6 ± .8	44.7 ± 1.3	<0.001*
Fat Mass (kg)^	16.6 ± 1.0	14.2 ± 1.0	0.08
Race	*n* (%)	*n* (%)	0.73
Caucasian	97 (30.2)	123 (38.3)	
Black	32 (10.0)	38 (11.8)	
American Indian	3 (0.9)	5 (1.6)	
Asian/Pacific Islander	3 (0.9)	3 (0.9)	
Other/Mixed	4 (1.3)	2 (0.6)	
Unknown	3 (0.9)	8 (2.5)	
Total	142	179	
Tanner Stage	*n* (%)	*n* (%)	0.005†
I	12 (4.5)	30 (11.3)	
II	7 (2.6)	21 (7.9)	
III	15 (5.6)	20 (7.5)	
IV	35 (13.2)	27 (10.2)	
V	52 (19.6)	47 (17.7)	
Total	121	145	

Abbreviations: BMI, body mass index; BMI Z-score, body mass index Z-score
Age, height, mass, BMI, BMI-percentile, BMI Z-score, percent body fat, lean mass and fat mass are presented as mean ± SEM.
Race and Tanner Stage are presented as count (% of total)
^*n* = 264 (119 females, 145 males)
*indicates significance as determined by independent t-test
†indicates significance as determined by Chi-squared test

Table 2. EID in males and females.

Gender (*n*)		Female (142)	Male (180)	p-value
EID%				
Mean (%)	Unadjusted	26.6 (0.5)	22.5 (0.5)	<0.001*
	Adjusted†	23.8 (0.8)	22.7 (0.8)	0.112
95% CI	Unadjusted	25.7, 27.6	21.4, 23.5	
	Adjusted†	22.2, 25.4	21.1, 24.2	
EID-AUC				
Mean (%/sec)	Unadjusted	4182 (92)	3341 (90)	<0.001*
	Adjusted†	3695 (148)	3362 (146)	0.012*
95% CI	Unadjusted	4001, 4363	3163, 3519	
	Adjusted†	3404, 3985	3075, 3650	

Reported as mean (SE)
*Indicates significance as determined by ANCOVA analysis with post-hoc Bonferroni adjustment
† Adjusted for race, age, BMI-percentile and baseline diameter

Table 3. Multiple linear regression examining the associations of gender, age, baseline diameter and BMI-percentile with EID.

Variable	β	Standard Error	P-value
EID%			
Baseline Diameter	-7.40	0.77	<0.001*
Gender	-1.08	0.71	0.13
Age	0.23	0.14	0.10
BMI-percentile	0.0003	0.01	0.98
EID-AUC			
Baseline Diameter	-1197.1	142.5	<0.001*
Gender	-340.0	132.1	0.01*
Age	44.1	25.9	0.09
BMI-percentile	2.2	2.3	0.35

*Denotes significance as determined by multiple linear regression

Table 4. Multiple linear regression examining the associations of age, baseline diameter and BMI-percentile with EID by gender.

	Females			*Males*		
Variable	**β**	**Standard Error**	**P-value**	**β**	**Standard Error**	**P-value**
EID%						
Baseline Diameter	-8.58	1.30	<.001	-6.57	1.05	<0.001
Age	0.37	0.17	0.038	0.05	0.22	0.83
BMI-percentile	0.003	0.018	0.887	-0.005	0.02	0.79
EID-AUC						
Baseline Diameter	-1384.8	250.3	<.001	-1039.8	187.9	<0.001
Age	83.5	33.7	0.015	-3.7	39.6	0.92
BMI-percentile	-0.45	3.4	0.894	3.2	3.1	0.30

*Denotes significance as determined by multiple linear regression

Table 5. EID according to obesity status.

BMI Category (*n*)		Normal (181)	Overweight (78)	Obese (59)
EID%				
Mean (%)	Unadjusted	24.9 (0.5)	24.2 (0.7)	22.7 (0.9)
	Adjusted†	24.3 (0.4)	24.7 (0.6)	24.4 (0.8)
95% CI	Unadjusted	23.9, 25.9	22.8, 25.6	20.8, (24.5)
	Adjusted†	23.4, 25.1	23.4, 25.9	22.9, 25.9
EID-AUC				
Mean (%/sec)	Unadjusted	3765 (91)	3438 (172)	3859 (130)
	Adjusted†	3652 (78)	3940 (119)	3766 (138)
95% CI	Unadjusted	3585, 3945	3600, 4119	3094, 3782
	Adjusted†	3499, 3805	3707, 4174	3495, 4036

Reported as mean (SE)
† Adjusted for gender, race, age, and baseline diameter
No significant differences found between groups prior to and after adjustment (p-value > 0.05)

Table 6. Multiple linear regression examining the associations of gender, age, baseline diameter and percent body fat with EID.

Variable	β	Standard Error	P-value
EID%			
Baseline diameter	-8.61	1.15	<0.001*
Gender	-0.58	1.04	0.52
Age	0.23	0.23	0.38
Percent body fat	-0.02	0.04	0.63
EID-AUC			
Baseline diameter	-1455.2	195.0	<0.001*
Gender	-181.4	177.6	0.31
Age	66.4	38.8	0.09
Percent body fat	8.5	8.3	0.31

*Indicates significance as determined by multiple linear regression

Table 7. EID measures by Tanner Stage.

Tanner Stage (*n*)		**Stage I (42)**	**Stage II (28)**	**Stage III (35)**	**Stage IV (62)**	**Stage V (99)**
EID%						
Mean (%)	Unadjusted	26.4 (1.3)	25.0 (1.1)	23.7 (1.0)	24.4 (0.7)	24.1 (0.7)
	Adjusted†	23.1 (1.5)	22.7 (1.4)	23.2 (1.0)	24.7 (0.7)	26.3 (1.0)
95% CI	Unadjusted	23.7, 29.1	22.7, 27.3	21.6, 25.8	22.9, 25.9	22.7, 25.56
	Adjusted†	20.1, 26.1	19.9, 25.5	21.2, 25.2	23.2, 26.1	24.4, 28.2
EID-AUC						
Mean (%/sec)	Unadjusted	4031 (208)	3814 (209)	3506 (181)	3833 (147)	1305 (126)
	Adjusted†	3423 (274)	3419 (261)	3407 (185)	3842 (133)	4135 (174)
95% CI	Unadjusted	3611, 4450	3406, 4292	3139, 3874	3503, 4099	3474, 4006
	Adjusted†	2882, 3964	2903, 3934	3043, 3771	3580, 4103	3793, 4478

Reported as mean (SE)
† Adjusted for gender, race, age, BMI-percentile and baseline diameter
No significant differences found between groups prior to and after adjustment (p-value > 0.05)

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