The Effect of Aerobic Exercise on Endothelial Dysfunction in Overweight Children

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ABSTRACT

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Childhood obesity is an edpidemic in the United States, and is directly associated with risk factors for both cardiovascular disease (CVD) and type 2 diabetes. Endothelial dysfunction (EDF) is thought to be an initiating factor in both these diseases, and recent studies have established the presence of EDF in obese children. While the prevalence of EDF amongst obese children is unknown, EDF in the first two decades of life is predicted to increase both morbidity and mortality from CVD and type 2 diabetes. PURPOSES: 1) To determine the prevalence of EDF in obese children; 2) To determine whether aerobic exercise was effective in improving endothelial function (EF) in obese children; and 3) To determine whether tissue oxygenation could be used as a clinical marker for EF in obese children. METHODS: 49 (24 females) at-risk-for or currently overweight children were evaluated for flow-mediated dilation (FMD) of the brachial artery, tissue oxygentaion, lipids, insulin, glucose, waist and hip circumference and blood pressure. 35 children found to have EDF (17 females), were randomly assigned to an exercise (EX) group, who used Dance Dance Revolution™ (DDR) for a 12-week intervention period, or delayed treatment control (DTC) group. The children in both the EX and DTC groups were tested for all aforementioned measurements as well as for aerobic fitness, markers of nitric oxide production, and inflammatory markers at both baseline and post-intervention. RESULTS: Eighty percent of the 49 children presented with EDF. Children with normal EF (n=9) had lower BMI's (24.42 ± 3.73 to 29.44 ± 5.11, p=.008), lower diastolic blood pressures (63.78 ± 6.51 to 75.58 mmHg ± 8.77, p=.039) and lower systolic blood pressures (112.00 ± 8.77 to 119.48 ± 9.90 mmHg, p=.000) compared to those who presented with EDF (n=40). Change scores indicated that the EX had a significant improvement in FMD (5.56 ± 5.04 % compared to .263 ± 4.54 %, p=.008), mean arterial pressure (-5.62 ± 7.03 compared to -1.44 ± 2.16 mmHg, p=.05), weight (2.01± 3.38 compared to 5.35 ± 3.97 pounds, p=.017) and relative VO2 (2.38 ± 3.91 compared to -1.23 ± 3.18 mg/kg/min, p=.005) compared to the DTC group. At baseline, FMD was correlated with difference in oxygen saturation normalized by mean blood flow (r=-.342, p=.036) and difference in oxygen saturation normalized by peak blood flow (r=-.517, p=.001). No measurements of oxygen saturation at post-intervention correlated with FMD. CONCLUSIONS: While the prevalence of EDF in overweight children is high, 12 weeks of aerobic exercise using DDR not only significantly improved EF, but also aerobic fitness and mean arterial pressure without subsequent improvement in markers of NO production or inflammation. The comprehensive results of this study document the need to further assess the complex relationships between obesity, EF, inflammation and exercise in a larger population of overweight children.

Key Terms: Obesity, Children, Endothelial Function, Exercise

TABLE OF CONTENTS:

Dedication

This dissertation is dedicated to three people that not only gave me part of my genetic makeup, but also shared with me many life lessons that have shaped me into the person that I am today: Loretta Corbett, Michael Corbett, and Jack Spangler.

To my grandmother, Loretta Corbett: you are and will always be the original, strong-willed, opinionated and capable Corbett woman. You left us women in the family a lot to live up to. You taught me to stick up for myself, to not falter from what I believe in no matter what others may think, and that your nuclear family should be put ahead of everything else in life.

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To my Pop-pop, Jack Spangler: you taught me (although I do not practice it often) that you don't have to speak loudly to be heard. You taught me that situations change, but love is unfaltering.

To each of you, I miss you greatly and want you to know that even though you are not here in person, you are with me each day, and each of you have helped me persevere.

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The words that come to my mind when reflecting on the process of completing this journey are determination and perseverance. My journey has been somewhat longer than most, and at times I will admit I was ready to throw in the towel. Now that it is coming to an end, I look back over almost a decade of my life, and I am truly thankful for this experience. I am a firm believer in good things come to those who wait, and when I think of what I am truly thankful for, it is for all those who have helped me through this process, and have believed in me at times that I did not believe in myself.

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but to embrace. While I will never be able to call you Linda, I see you not only as a mentor and colleague, but also a dear friend.

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SPECIFIC AIMS:

Obesity has been increasing at an alarming rate over the last two decades in the United States (U.S.), and is now considered to be at epidemic proportions in both adults and children. From 1971 to 2000 obesity increased from 14.5 percent to 30.9 percent of the U.S. population, setting the stage for many other health problems, which cost more than 100 billion dollars annually[1]. Recently overweight and obese children have been diagnosed with impaired glucose tolerance, type 2 diabetes, insulin resistance syndrome, and cardiovascular risk factors at increasing rates[2]. Results of a recent cross-sectional study indicate that obese children have endothelial dysfunction[3], which is thought to be an initiating event in both cardiovascular disease (CVD) and type 2 diabetes mellitus. CVD is the number one cause of death in the U.S., and type 2 diabetes mellitus affects 16 million people[4]. Obesity is thought to be a major contributor to endothelial dysfunction because it is associated with a clustering of CVD risk factors (hypertension, high triglycerides, low HDL cholesterol, and abdominal adiposity) each of which are positively associated with endothelial dysfunction and collectively make up the metabolic syndrome, a precursor of type 2 diabetes mellitus. Although the prevalence of endothelial dysfunction in obese children is not known, any manifestation of endothelial dysfunction in the first two decades of life is predicted to significantly increase morbidity and mortality from CVD and type 2 diabetes and the consequent economic burden, while the cost in terms of human suffering is incalculable.

Although endothelial dysfunction is recognized as an initiating factor in both CVD and metabolic disease, it is not routinely evaluated because to do so requires expensive laboratory equipment and highly trained personnel; both of which are impractical in the clinical setting. A

clinical marker for endothelial dysfunction is needed to cue initiation of therapy and evaluate treatment. Tissue oxygenation is a potential marker since it is reasonable to assume that endothelial dysfunction affects oxygenation in the microcirculation and tissue. It could be used in the clinical setting because tissue oxygenation can be measured with near infrared spectroscopy (NIRS) which is relatively inexpensive, and easy to use.

Several pharmacologic therapies have been shown to improve endothelial function in certain disease states: for example, statins have been shown to be effective in improving endothelial function in individuals with hypercholesterolemia. Exercise has been shown to be effective in improving endothelial function in several adult populations, and several studies have shown exercise to be effective in improving endothelial dysfunction in obese children[5-7]. Exercise is a more appropriate treatment for endothelial dysfunction in children since it has no adverse side effects.

The long range goal of this research was to use exercise to decrease the incidence of metabolic and cardiovascular disease. The objectives of this project, which were the first steps toward achieving the long range goal, were to 1) determine the prevalence of endothelial dysfunction in obese children, 2) determine whether exercise was an effective therapy for improving endothelial function in obese children, and 3) determine whether tissue oxygenation can be used as a clinical marker for endothelial dysfunction in obese children. This was tested against the current "gold standard" for measurement of endothelial dysfunction, flow mediated dilation (FMD). The central hypotheses of the proposed research were: 1) obesity in children results in a high prevalence of endothelial dysfunction, 2) aerobic exercise would be an effective therapy for improving endothelial function in obese children, and 3) tissue oxygen saturation in response to hyperemic flow produced by five minutes of cuff occlusion would predict

endothelial function in overweight children. We tested our central hypotheses and achieved the objective of this application by:

1. Establishing the prevalence of endothelial dysfunction in children who were at risk for obesity or children who were obese.

Working hypothesis: Obesity will cause impaired endothelial function in children seven-12 years of age.

At risk for becoming overweight was defined as having a BMI ≥ 85th percentile for age and gender, while obesity was defined as a BMI ≥ to the 95th percentile for age and gender. Endothelial dysfunction was defined as less than an eight percent increase in the diameter of the brachial artery in response to hyperemic flow produced by five minutes of blood flow occlusion[3,8-9]. The average dilation in healthy individuals is an eight to ten percent increase in the diameter[10-11]. FMD was measured using ultrasound with two-dimensional imaging, color and spectral Doppler in 49 at-risk for overweight or overweight children aged seven to 12 years. In a sub-sample of subjects, blood samples were analyzed to determine whether biochemical markers for endothelial function (including nitric oxide oxidation products (NO2 + NO3 = NOx), asymmetric dimethylarginine, and arginine) and pro-inflammatory markers (including C-reactive protein (CRP), interleukin-6 (IL-6), tumor-necrosis factor alpha (TNF-α) and adiponectin were abnormal in these children[5,12-14].

2. Evaluating the effectiveness of an exercise intervention to improve endothelial function.

Working hypothesis: Twelve weeks of moderate intensity aerobic exercise will improve endothelial function in overweight children seven-12 years of age, and the improvement in endothelial function will be directly related to the improvement in aerobic capacity.

This hypothesis was tested by measuring endothelial function using FMD, and aerobic exercise capacity using a graded exercise test on a bicycle ergometer to volitional fatigue at baseline and after a 12 week exercise intervention. The aerobic exercise program included five, 30 minute aerobic exercise sessions per week for 12 weeks. The percent improvement in aerobic capacity would be directly related ($p < .05$) to the percent improvement in the diameter of the brachial artery in response to hyperemic flow due to five minutes of blood flow occlusion. Biochemical markers for endothelial function would be related to improvement in aerobic capacity ($p<.05$). Plasma levels of CRP, IL-6, TNF-α, and adiponectin were measured pre- and post-intervention to quantify the inflammatory state. The aerobic exercise intervention (n=23) group was compared to a delayed treatment control group that was matched for age, gender, ethnicity and weight (n=12).

3. Establish that flow mediated tissue oxygenation can be used as a clinical marker for endothelial function.

Working hypothesis: The increase in muscle tissue oxygenation in the forearm after five minutes of blood flow occlusion will be directly related to flow mediated dilation of the brachial artery.

This hypothesis was tested by measuring the increase in flow mediated tissue oxygenation in the forearm using near infrared spectroscopy and flow mediated dilation of the brachial artery using ultrasound simultaneously in 49 overweight children after five minutes of blood flow occlusion as described in specific aim 1, and at baseline and after the exercise intervention in the 30 children described in specific aim 2. This was done to determine if flow mediated increase in tissue oxygenation and brachial artery diameter were directly related ($p<.05$). Multiple regression using variables such as gender, blood pressure, time intervals involved in tissue oxygenation and percent change in tissue oxygenation were used to predict brachial artery diameter.

SIGNIFICANCE:

More than half of adults in the United States are at risk for overweight and nearly one-quarter are overweight[14-16]. Between the completion of the second National Health Examination Survey (NHANES II) in 1980 and the NHANES III in 1994, the number of children and adolescents considered to be obese increased by 100 percent in the United States[16]. Currently, 15.5 percent of adolescents (ages 12 to 19) and 15.3 percent of children (ages six to 11) are obese (American Obesity Association), affecting more than 10 million children. Abdominal obesity is directly related to Metabolic Syndrome which currently has a prevalence of 23 percent (approximately 47 million Americans)[17]. Those with Metabolic Syndrome have twice the risk of developing CVD and four times the risk of developing type 2 diabetes compared with people who do not have this syndrome; the incidence of Metabolic Syndrome is expected to increase linearly with the increasing prevalence of obesity. Obesity in children will result in metabolic disorders much earlier in the life span with the potential for serious health consequences occurring as early as the third or fourth decade of life. This will translate into a significant increase in morbidity and mortality with an enormous economic burden as CVD and type 2 diabetes mellitus combined cost the nation $117 billion in 2000 alone, and are responsible for as many as 400,000 premature deaths each year[14]. Since endothelial dysfunction is thought to be an initiating factor in CVD and type 2 diabetes mellitus, finding an effective treatment for this condition and finding a clinical marker to cue initiation of therapy and determine the efficacy of treatment would have a clear benefit in reducing the incidence of CVD and metabolic disease and the consequent morbidity, mortality, and economic burden.

BACKGROUND:

ENDOTHELIAL FUNCTION

The endothelium is strategically located between circulating substances, cellular elements in the bloodstream, and vascular smooth muscle cells in the blood vessel wall. The endothelium was once viewed as an inert barrier separating the blood and tissues but it is now known that it acts as a biophysical sensor capable of responding to changes in both the physical and metabolic environment to maintain vascular homeostasis[18]. Its main purpose is to regulate the passage of substances from the bloodstream to the vascular wall. The healthy endothelium is an important regulatory organ in maintaining cardiovascular homeostasis[19] by controlling the balance between several opposing forces, including vasoconstriction and vasodilation, growth promotion and inhibition, pro-coagulation and anticoagulation, pro-inflammation and anti-inflammation, and oxidation and anti-oxidation[20]; all of which, if unbalanced, would contribute to atherogenesis. Pioneering experiments by Furchgott and Zawadzki[21] showed that the presence of an intact endothelium is essential for acetylcholine (ACh) to induce dilation of the arteries. Iganaro and Furchgott later revealed that ACh stimulated the release of nitric oxide (NO)[17].

NO is formed from the guanidine-nitrogen terminal of L-arginine by endothelial nitric oxide synthase (eNOS), which is activated by a receptor-dependent and receptor-independent agonist in a calcium dependent manner[22]. Shear stress causes NO production in a calcium independent manner[23]. The activation of eNOS requires several cofactors, including nicotinamide adenine dinucleotide (NADPH), 5,6,7,8-tetrahydrobiopterin (BH_4), and flavin adenine mono- and dinucleotides (FMN/FAD). eNOS binds calmodulin in a reversible, Ca2+-dependent manner and hence is activated by Ca2+ -elevating agonists. Since eNOS is a Ca2+/calmodulin-dependent enzyme, an elevation in the intracellular free Ca2+ is a prerequisite

for an acute increase in the synthesis of NO, regardless of the type of stimulus[23]. The bioavailability of NO is depicted in figure 1.

Like other nitrovasodilators, NO exerts its effect on the vascular smooth muscle by activating soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP), which is the intracellular second messenger of NO[24]. Shear stress and/or acetylcholine stimulate the release of NO from endothelial cells. Shear stress is thought to act by opening stress-sensitive cation channels, thereby raising intracellular Ca^{2+}. NO quickly diffuses into neighborhood smooth muscle cells, causing relaxation which results in vasodilatation[25]. Impaired release (or availability of NO) may be due to a down regulation of eNOS or decrease in the shear stress.

Recent work suggests that NO not only regulates vascular tone, it also has a key "antiatherogenic" role with the regulation of vascular permeability, the inhibition of platelet adhesion/aggregation, leukocyte/wall interaction, and smooth muscle proliferation[26]. These biologic actions of NO make it an important component in the endogenous defense against vascular injury, inflammation, and thrombosis, which are all key events in the progression of atherosclerosis[27].

ENDOTHELIAL DYSFUNCTION

While endothelial dysfunction is most often thought of as an impairment of endothelium-dependent vasodilation, it most likely also includes impairments in other endothelial dependent functions. The nature of endothelial dysfunction resulting in attenuation of NO-mediated responses is unclear, however several likely mechanisms of endothelial dysfunction include: alterations in signal transduction, reduced availability of L-arginine, modification of the expression of eNOS, altered availability of cofactors for eNOS, imbalance between the

production of endothelium-derived constricting and relaxing factors, increased destruction of NO by reactive oxygen species (ROS), intimal thickening as a diffusion barrier, and increased production of endothelium-dependent constricting factors (endothelin being the most prominent)[23].

Endothelial dysfunction has been shown to be present in a wide range of vascular disorders including, atherosclerosis, type 2 diabetes mellitus, and hypertension. It is thought that disruption of the functional integrity of the vascular endothelium plays an integral role in all stages of atherogenesis ranging from lesion initiation to plaque rupture. In a prospective study by Schachinger[28] and colleagues, 147 consecutive patients underwent coronary endothelial function testing in response to several stimuli (acetylcholine, cold pressor test, flow-mediated dilation) and subsequently were followed for 6.7 years. Patients who presented with endothelial dysfunction at the inception of the study had a significantly greater number of cardiovascular events. Obesity is associated with both endothelial dysfunction and increased risk of CVD in adults, therefore we hypothesize that a similar relationship will be found between these variables in children. While the mechanisms that lead to obesity-caused endothelial dysfunction remain unclear, it is thought that both insulin and a chronic pro-inflammatory state play a critical role.

OBESITY AND ENDOTHELIAL DYSFUNCTION

Obesity and its associated cardiovascular and metabolic complications are considered to be a major health problem in the US. The presence of endothelial dysfunction in small resistance vessels of obese adult subjects has been documented[29]. A study that evaluated the impact of obesity on coronary endothelial function in 397 patients with normal or mild coronary artery disease demonstrated that obesity was independently associated with coronary endothelial

dysfunction[26]. Arcaro et al.[9] studied a group of 18 obese, otherwise healthy premenopausal women, and found that body fat distribution, independent of body weight, was the greatest predictor of endothelial dysfunction in the femoral artery. In a similar study[31], waist hip ratio, independent of other cardiovascular risk factors, was shown to be a powerful predictor of vascular endothelial dysfunction in a sample of 32 healthy adults with a body mass index ≥27 kg/m^2.

There is substantial evidence that obesity in childhood predicts adult CVD. Tounian[2] et al. studied FMD in 48 obese children as compared to 27 normal-weight controls. Obese children had significantly lower arterial compliance, distensibility, and endothelium-independent and endothelium-dependent dilation than the controls. In addition, these authors also found that the endothelial dysfunction was related to indices of insulin resistance. These findings suggest that the current childhood obesity epidemic will translate into an increase in the incidence of metabolic disorders and CVD, unless interventions are found that decrease the complications of obesity, including endothelial dysfunction.

While the causes of endothelial dysfunction in obesity remain unclear and incompletely explored, insulin resistance and the presence of a proinflammatory state are two likely mechanisms linking obesity to endothelial dysfunction. There is a well-established relationship between reactive oxygen species (ROS) and NO. NO has a direct effect on oxidative stress by scavenging ROS, and NO inactivation is enhanced in the presence of excess ROS[32]. An overproduction of ROS may injure the endothelial cell membrane, inactivate NO, and cause oxidation of BH4. Hyperglycemia, which may be present in obese individuals, is associated with generation of ROS.

CRP is an acute-phase protein and a sensitive marker for systemic inflammation, and high levels have been shown to predict cardiovascular events[33]. Retrospective and cross-sectional studies have shown that overweight children have higher CRP levels than normal weight children[34-35]. The elevated CRP concentration associated with overweight might be explained by the expression of IL-6 in adipose tissue and its subsequent release into circulation[36]. Fichtlscherer et al. showed that elevated CRP levels indicative of a systemic inflammatory response reflected blunted systemic endothelial function, and normalization of CRP levels over time were associated with a significant improvement in endothelium-mediated blood flow responses[37].

IL-6 is a proinflammatory, endocrine cytokine that stimulates the production of acute-phase proteins, including CRP[34]. Within adipose tissue, both adipocytes and macrophages secrete IL-6, with roughly 30 percent of total production being initiated in the adipose tissue[38]. Production of IL-6 by adipose tissue increases with increasing adiposity, and circulating IL-6 concentrations are highly correlated with both percent body fat[39] and insulin resistance[40].

TNF-α, a proinflammatory cytokine has been shown to induce an impairment of endothelium-dependent vasodilation in a variety of vascular beds by increasing oxidative stress and decreasing the release of NO[41]. TNF- α may have a direct action on decreasing the amount of NO released and eNOS production. Further, it may directly activate NAD(P)H oxidase and increase ROS production in vascular smooth muscle[42]. TNF-α has a major role in adipose tissue and there is evidence of a three-fold increase in TNF-α mRNA protein and circulating levels in obese individuals[42]. Within adipose tissue, macrophages account for nearly all TNF-α production[38] and increased TNF- α expression has been linked to the development of insulin resistance. Within adipose tissue, TNF- α causes insulin resistance through serine

phosphorylation of both the insulin receptor and insulin receptor substrate, both of which result in a diminished activation of phosphoinositol-3-kinase, the essential second messenger signal that governs most of insulin's metabolic effects[43].

Adiponectin is a collagen-like plasma protein that is produced by the adipose tissue and that is abundant in the systemic circulation. In an obese state, plasma concentrations of adiponectin are decreased[44]. Adiponectin plays an important role in the regulation of insulin action[45-46], and has been shown to be negatively correlated with insulin resistance[47]. In addition to its effect on glucose metabolism, adiponectin appears to modulate endothelial function. Adiponectin has been shown to stimulate production of NO (and suppress adhesion molecule expression in vascular endothelial cells[48-50]. In three recent clinical studies in adults, hypoadiponectinemia has been found to be directly correlated with endothelial function of the peripheral arteries[48-50].

Insulin resistance classically refers to an impairment of the degree to which a given quantity of insulin lowers plasma glucose. Several factors including visceral adiposity, physical inactivity and genetic factors contribute to the development of insulin resistance. A morphological feature of an insulin resistant individual is a tendency towards android obesity. Data now shows that insulin resistance is associated with endothelial dysfunction[32] and insulin sensitivity is inversely proportional to the development of atherosclerosis. Petrie[51] et al. showed a close positive relationship between insulin sensitivity and basal endothelial NO production. Winkler[42] et al. showed that increased levels of TNF-α may be one of the linking factors in the insulin resistance and endothelial dysfunction relationship. A recent study by Arcaro et al.[9] revealed that modest hyperinsulinemia caused severe endothelial dysfunction in large conduit arteries, and that the endothelial dysfunction was prevented by vitamin C, which implies that

increased oxidant stress is a cause of the endothelial dysfunction. Figure 2 depicts some of the possible mechanisms that may link obesity and endothelial function.

EXERCISE AND ENDOTHELIAL FUNCTION

Increased physical activity and fitness are inversely related to the incidence of CVD[52]. The precise mechanisms of how physical activity decreases the incidence of CVD are not known, but the endothelium has become a focus for investigation in this relationship. Exercise causes an increase in shear stress (the force acting parallel to the blood vessels) and blood velocity. The increase in blood flow and shear stress that accompanies regular aerobic exercise elicits an adaptive response that alters the intrinsic responsiveness of the endothelium by increasing mRNA expression of eNOS[53]. This in turn increases the synthesis and release of NO which leads to augmented flow-mediated dilation and ultimately improves endothelial function. Figure 3 depicts the relationship between shear stress and endothelial function. Matching tissue oxygen and substrate supply to demand during exercise is controlled by both blood delivery and the capacity of cells to extract these substances. NO appears to play a role in both of these processes. Exhaled NO, urinary excretion of cGMP and the NO metabolite nitrate are elevated during exercise, indicating that NO plays a major role during exercise.

Results from animal studies suggest that chronic exercise results in improved endothelial function. Dogs exercised for as little as one week manifested an improvement in endothelium-dependent vasodilation in response to hyperemia and NO agonists[54]. The same study also found a two- to three-fold increase in eNOS after ten days of exercise. This suggests an upregulation of the eNOS gene in response to chronic increases in blood flow brought about by exercise.

Similar findings have been documented in humans. Endothelium-dependent responses of the brachial artery in young healthy men were improved after just ten-weeks of regular physical exercise training at a moderate intensity[55]. A 12-week exercise program improved endothelial function in patients who had polymetabolic syndrome, but who were otherwise asymptomatic for CVD[56]. Another recent study showed that endurance-trained men did not demonstrate an age-related decline in endothelium-dependent vasodilation; both middle aged and older men who regularly perform aerobic exercise exhibited greater acetylcholine-mediated vasodilation than sedentary peers; and that regular aerobic exercise restored the loss of endothelium-dependent vasodilation in previously sedentary middle aged and older men[3]. Patients with documented coronary artery disease showed an improvement in coronary endothelial function after just four weeks of vigorous exercise training[57]. Combined aerobic and resistance exercise training significantly improved both forearm blood flow and FMD, both indices of endothelial function, in patients with type 2 diabetes[58].

It is a continuing debate whether there is a critical proportion of the whole body muscle that has to be trained to achieve systemic effects on endothelial function. Kobayashi et al.[59] investigated whether lower-limb exercise on a bicycle ergometer in patients with congestive heart failure improved endothelial function in the trained lower limbs and also in the upper extremities which were essentially untrained. Results of this study indicated that flow-mediated dilation was improved in the trained lower limbs, but there was no improvement seen in the upper limbs. These results indicate that exercise training appears to correct endothelial function by a local effect in trained extremities only. Local training interventions like handgrip exercise[60-62] or bicycle ergometer training improve local flow-dependent, endothelium-mediated vasodilation[63] in the exercising muscles. These authors conclude that a systemic exercise

program should be used to enhance the efficacy of physical training on endothelial function. There are many studies which dispute these findings and have found a systemic effect of exercise on endothelial function as long as large muscle groups were recruited[56,64-65]. Clarkson et al.[55] recently showed that ten weeks of lower-limb endurance training in healthy subjects with normal endothelial function had systemic effects on agonist-mediated, endothelium-dependent vasodilation. Linke et al.[66] trained patients with congestive heart failure on a bicycle ergometer, and documented systemic effects on endothelial function. Studies showing systemic effects of exercises using large muscle groups indicate that increased vascular shear stress can alter nitric oxide production in non-exercising muscle beds.

A recent study by Goto[67] and colleagues examined the effects of different intensities of exercise on endothelium-dependent vasodilation in humans. Subjects (26 healthy males) were randomly assigned to one of three training groups: mild intensity (25 percent VO2 max), moderate intensity (50 percent VO2 max), or high intensity (75 percent of VO2 max). Results showed that twelve weeks of moderate-intensity exercise, but not mild- or high-intensity exercise, significantly increased acetylcholine-induced vasodilation of the brachial artery. Both 8-hydroxy-2'-deoxyguanosine and serum concentrations of mallondialdehyde-modified low-density lipoprotein were measured to evaluate the amount of oxidative stress present in each group. Both indices of oxidative stress were increased in the high-intensity group after exercise, whereas in the moderate-intensity group both were decreased after the exercise training. Those subjects that trained at a low-intensity had neither increased oxidative stress markers nor any improvements in endothelial function. These findings suggest that moderate-intensity aerobic exercise successfully augments endothelium-dependent vasodilation, while high-intensity exercise increases oxidative stress that may interfere with an increased production of NO.

Jodoin et al.[68] found conflicting results in relation to endothelial function improvements due to exercise in a study looking at the effects of a three month exercise training program in 22 middle-aged men and women. While peak oxygen uptake increased significantly in this population, flow-mediated dilation did not change significantly[68]. These authors suggested that longer and more aggressive programs may be needed to see significant changes.

A recent study of 45 children, ages five to ten years old, showed that habitual physical activity was a strong predictor of FMD in the brachial artery[7]. The population of children that participated in this study was classified as being moderately active and was normal-weight according to BMI measurements. A recent study conducted by Watts et al.[6] reported that FMD was impaired in obese children, and the exercise was successful in improving the impairment. In this study, 14 obese subjects (6 boys, 8 girls) with the mean age of 8.9 years, completed an 8-week exercise regimen consisting of three 1-hour sessions of whole-body exercises each week. Exercise was monitored during all exercise sessions by telemetry and heart rates were maintained between 140 and 180 beats per minute. FMD of the brachial artery significantly increased after exercise training from 6.00% ± 0.69% to 7.35% ± 0.99%. While improvements were seen after exercise, the obese children still did not achieve endothelial function similar to the normal weight controls (12.32% ± 3.14%).

EVALUATION OF ENDOTHELIAL FUNCTION

Assessment of endothelium-dependent, flow-mediated dilation of the brachial artery using high-resolution ultrasound provides a noninvasive technique to evaluate endothelial function of the peripheral vascular bed. The diameter of the brachial artery is measured two-dimensionally at rest, during reactive hyperemia, and after sublingual nitroglycerin, to assess

both endothelium-dependent and endothelium-independent vasodilation. Small changes in the vessel diameters can be reliably detected using a high-frequency transducer[69]. This technique for assessing FMD was first described by Celermajer et al. and is a widely accepted measurement of endothelial function. Reliability and validity of this technique have been established in both adults and children with and without endothelial dysfunction[69].

While FMD is a non-invasive technique, there is no clinical marker to measure endothelial function at this time. Near infrared spectroscopy (NIRS) is a non-invasive, optical technology used to measure local oxygen saturation of hemoglobin in the microcirculation. NIRS measures hemoglobin oxidation values in tissue based on spectrophotometric principles which relate light absorption to chemical concentration. By measuring changes in light absorption at different wavelengths (775, 805, 845 and 905 nm) changes in tissue oxygenation can be measured continuously. NIRS measurements in muscle have been used to investigate diseases associated with impaired tissue oxygenation, like heart failure and peripheral vascular disease[70]. Krageli et al.[70] used NIRS to explore the post-occlusive hyperemic response in healthy limbs, and a statistical analysis showed good reproducibility among repeated measures.

True capillaries are the most important functional unit of the microcirculation. The capillary wall consists of a single layer of endothelial cells and a basement membrane; the capillaries have no smooth muscle and are therefore incapable of active constriction. However, the endothelial cell protein does contain some contractile materials, small amounts of actin and myosin. Endothelial cells play a pivotal role in regulation of the microcirculation by virtue of a) their own contractile properties and b) their role as sensors of ischemia and flow variations. Because the capillaries are devoid of smooth muscle, blood flow regulation in the capillaries is

entirely endothelium-dependent. Measurement of muscle tissue O2 saturation by NIRS pre- and post-occlusion should therefore be indicative of endothelial function.

PILOT STUDIES:

Study #1:

A pilot study was done to test the reliability of near infrared spectroscopy (NIRS) for measuring endothelial function in adults. Fifteen young healthy adults (ages 18-30, eight females) served as subjects. Informed consent was obtained from each participant prior to beginning the study. Subjects reported to the Human Performance Laboratory at West Virginia University in Morgantown, West Virginia, after a 12-hour fast. Subjects were instructed to refrain from caffeine, medications and exercise for 24 hours prior to each visit. Upon arrival, subjects completed a medical history, a seven-day physical activity recall, and a previous day diet recall. The subject's height, weight and waist circumference were then measured. An electrocardiogram was monitored and a three-inch adhesive pad that holds the NIRS light probe was placed on the subject's right forearm. The subjects then rested quietly for fifteen minutes. Blood pressure was measured after the 15-minute rest period in the left arm and heart rate and tissue oxygenation were recorded. A blood pressure cuff was then placed loosely on the upper arm, and the subject rested for an additional ten-minute period. At the end of the ten minutes, the blood pressure cuff was inflated to 260 mmHg for five minutes. At the end of the five minutes of occlusion, the oxygen saturation value was recorded and the cuff was rapidly deflated. Once the oxygen saturation value returned to the resting value, the process was repeated on the arm.

At the conclusion of the first visit, the subject was instructed to eat a similar diet for the 24 hours prior to the next visit. The second visit took place two to three days after the first visit. The procedure was repeated on the second visit, except subjects were not asked questions pertaining to their medical history or prior physical activity behavior.

Results:

Subject Number	Gender	Age	BMI	% Change in O_2 Trial 1/Day 1	% Change in O_2 Trial 2/Day 1	% Change in O_2 Trial 1/Day 2	% Change in O_2 Trial 2/Day 2
1	F	27	24.01	23.08	21.05	30.88	30.88
2	M	27	28.01	12.24	9.47	21.11	18.60
3	F	29	27.25	26.37	27.17	25.56	17.28
4	M	21	27.39	7.14	7.22	11.22	9.38
5	M	27	37.32	10.42	8.42	8.25	8.25
6	M	26	34.94	21.05	20.21	16.67	14.77
7	F	21	22.02	28.42	27.66	20.21	20.43
8	F	23	28.02	37.80	37.66	31.18	27.27
9	F	28	24.33	6.12	7.14	13.54	12.63
10	F	23	25.14	48.15	43.24	23.86	11.54
11	F	22	25.01	17.58	21.05	6.59	6.59
12	M	28	24.84	1.03	2.04	5.15	3.16
13	M	23	23.54	14.29	14.29	11.22	4.08
14	F	21	20.40	20.41	20.41	13.27	12.24

Analysis:

These data were analyzed using an ANOVA appropriate to the split-plot design. Mean values for O_2 percent change in the arm for each trial were as follows: trial 1/day 1, 19.58 ± 12.02; trial 2/day 2, 19.07 ± 12.03; trial 1/day2, 15.57 ± 8.37; trial 2/day 2, 14.08 ± 8.19. Mean values for O2 percent change in the arm were as follows: females, 22.42 ± 8.55; males, 10.82 ± 5.41 (p=.013). There was no gender by time interaction. Specifically, no significant differences were found between trial one, day one and trial one, day two values.

Study #2:

A second pilot study was conducted to test whether children ages seven-11 years were able to reach maximal oxygen consumption during a graded exercise test using a bicycle ergometer. Subjects (n=2) reported to the Human Performance Laboratory at West Virginia University in Morgantown, West Virginia. The subject's weight, height, and waist circumference were measured. Electrodes were then placed on the chest to monitor an electrocardiogram (ECG). Subjects sat quietly for five minutes. Resting heart rate and blood pressure were measured and recorded. Subjects were asked to exercise on a Monark bicycle ergometer to volitional fatigue. The bicycle test required the subject to maintain a set pedaling speed of 50 revolutions per minute (rpm). The test began at a workload of 25 watts, and was increased by an additional 25 watts every three minutes. The test was stopped when the subject could no longer maintain a pedaling speed of 50 rpm. Subjects wore a mask over their nose and mouth so that air could be continuously collected to determine aerobic capacity. Heart rate (by the ECG) was continually monitored throughout the test. Blood pressure was taken at the end of each minute. Subjects were asked to rate how hard they felt that they were working by indicating a number (between 1-10) on a children effort rating table (CERT) every other minute.

Results showed that the children did not reach their physiologic maximum as RER was less than .9. Peak VO2 was 49 ml x kg (-1) x min (-1), which is above average for children this age.

Results:

Sex/Age	BMI	HR Rest	BP Rest	RER Rest					
M/6	16.3	92	112/60	.72	Stage 1	HR 50 W	VO2 50W	RER 50W	
						133	24.4	.73	
					Stage 2	HR 75 W	VO2 75 W	RER 75W	
						145	38.0	.77	
					Stage 3	HR 100W	VO2 100W	RER 100W	
						150	45.1	.80	
					Stage 4	HR 125W	VO2 125W	RER 125W	Peak VO2
						182	46.8	.86	48.47
Sex/Age	BMI	HR Rest	BP Rest	RER Rest	Stage 1	HR 50W	VO2 50W	RER 50W	
F/7	20.2	72	110/70	.69		122	23.6	.70	
					Stage 2	HR 75W	VO2 75W	RER 75W	
						135	26.7	.75	
					Stage 3	HR 100W	VO2 100W	RER 100W	Peak VO2
						145	29.5	.81	50.34

Study #3:

A third study was conducted to test whether near infrared spectroscopy (NIRS) could be used to predict endothelial function. Flow-mediated dilatation measured by high-resolution ultrasound was used as the criterion measurement for this validation study. Sixteen subjects ranging in age from 18-30 (eight females) reported to the Human Performance Laboratory on the ground floor of the Health Sciences Center. Subjects were asked to fill out a seven-day physical

activity recall questionnaire, a previous day dietary recall questionnaire, and a medical history was recorded. Subjects rested for 15 minutes. At the end of the 15-minute rest period, electrodes were placed on the subject's chest to monitor heart rate by electrocardiogram and a resting blood pressure was taken. A three-inch adhesive pad that holds the NIRS light probe was placed on the subject's right forearm to monitor tissue oxygen saturation. Tissue oxygen saturation and ECG were monitored continuously during both rest and testing phases. A blood pressure cuff was placed loosely around the top portion of the subject's arm. A high-resolution ultrasound was taken of the subject's arm five-15 centimeters above the elbow. When a clear picture of the main artery of the arm was obtained, the arm was marked, so that the measurement could be repeated at the same location of the arm. Subjects were then asked to lie quietly for an additional ten minutes. A high-resolution ultrasound scan was taken during the last minute of the ten-minute rest period. At the end of the rest period, the blood pressure cuff was inflated to 50 mmHg above the resting systolic blood pressure value and remained at this pressure for five minutes (or until blood flow was occluded as seen on the ultrasound). A second scan was taken with the high resolution ultrasound beginning ten seconds before the blood pressure cuff was removed and was continued for 90 seconds after the cuff was removed.

Analysis:

In order for flow mediated tissue oxygen saturation to be used as a clinical marker for endothelial function the changes in tissue oxygen saturation must be able to predict (1) changes in the diameter of the brachial artery caused by hyperemic flow, and (2) blood flow (brachial artery diameter change is more meaningful if it is corrected by blood flow).

Predictive value was determined by multiple linear regression. Since there was a significant difference in percent change in flow mediated forearm oxygen saturation between genders, gender was entered into the regression model to predict the change in brachial artery diameter. The difference between baseline forearm oxygen saturation normalized by peak blood flow, and post occlusion oxygen saturation normalized by peak blood flow was also entered into the model to predict the change in brachial artery diameter pre and post occlusion normalized by peak blood flow. These two variables were strong predictors of the change in the brachial artery with $r=.946$, and an $R^2=.894$ ($p<.05$).

Multiple regression found that resting forearm oxygen tissue saturation, room humidity, the subject's BMI, resting heart rate, and gender predicted baseline peak blood flow with an $r=.858$, and $R^2=.735$ ($p<.05$) Gender, percent maximum forearm oxygen saturation at 21 seconds post occlusion, post occlusion heart rate, and room humidity predicted post occlusion peak systolic blood flow with an $r=.903$, $R^2=.815$ ($p<.05$).

Sex	Humidity	BMI	Resting heart rate	Diameter Rest	Diameter 1 min post	Post occlusion heart rate	Resting tissue O2 saturation	Maximum Tissue 02 saturation	Resting blood flow	Post occlusion peak blood flow	(Baseline O2 corrected by blood flow) – (post 02 corrected for blood flow)	Percent of maximum tissue O2 saturation at 21 seconds post occlusion
F	55	24.16	63	.33	.37	--	54	67	5.80	12.57	4.33	20.93
M	55	27.42	62	.44	.47	54	87	95	12.13	23.62	2.90	78.95
F	49	21.35	60	.40	.42	54	79	95	7.39	25.44	6.69	90.77
M	55	24.38	77	.43	.46	83	92	95	9.67	27.56	6.07	93.06
F	50	27.25	63	.41	.43	66	65	87	8.23	--	--	62.71
M	42	17.75	78	.33	.41	72	88	97	5.59	25.18	11.53	85.42
M	33	27.71	51	.47	.55	60	92	96	10.40	29.92	5.63	97.89
M	32	24.46	67	.48	.55	65	76	89	9.98	--	--	47.46
F	35	24.99	49	.39	.40	60	70	91	6.52	19.42	6.36	87.21
F	51	21.66	64	.41	.44	65	90	78	10.93	--	--	54.05
F	46	25.66	57	.37	.40	70	42	42	6.13	11.83	2.49	75.00
M	56	27.18	63	.49	.52	64	80	96	15.15	34.51	2.37	96.84
F	50	30.27	74	.36	.40	69	61	74	6.47	--	--	79.17
M	50	30.88	72	.45	.45	59	77	76	9.35	17.30	4.38	56.76
M	50	26.56	59	.55	.57	64	80	97	12.54	40.71	4.40	92.00

EXPERIMENTAL DESIGN AND METHODS:

The hypotheses of this study were: overweight children would have a higher prevalence of endothelial dysfunction than their normal-weight peers; that an aerobic exercise intervention for overweight children would result in an improvement in endothelial function; and that NIRS would be found to be an acceptable clinical marker to measure endothelial dysfunction.

Specific Aim #1: Establish the prevalence of endothelial dysfunction in overweight children.

Subjects

All subjects had to have West Virginia Public Employee Insurance Agency (WV PEIA) health insurance (Preferred Provider Participants) or Mountainstate Blue Cross Blue Shield health insurance. All subjects had to be between seven-12 years of age, and had to have a BMI above the 85th percentile for age and gender.

Human Subjects: Recruitment

Subjects were recruited from the Pediatric Clinics at the Physicians' Office Center in Morgantown, WV. Subjects were also recruited from advertisements sent to state employees, and a letter that was sent to local pediatricians to inform them of the study and to encourage them to refer patients.

Clinical Assessment

An initial phone interview was conducted to assess the volunteer's suitability and willingness to participate. Upon agreement to participate, an initial visit was scheduled and

subjects were instructed to report to the Human Performance Laboratory in the Health Sciences Building. Subjects were instructed to refrain from caffeine, medications, vitamins, and exercise for 24 hours prior to the first visit, and to fast for 12 hours.

At the beginning of the first visit, informed consent was obtained from the parents and assent was obtained from the child; height and weight were measured to exclude those who were not above the 85th percentile for BMI. Subject's resting blood pressure was taken. A pediatric blood pressure cuff was placed on the subject's upper arm, and the cuff was inflated to 50 mmHg above resting systolic value for five minutes. This was done to determine whether the subject could tolerate the cuff occlusion. If the subject was unable to tolerate the occlusion, they were not eligible for the study. A medical and family history was completed to assess the presence of any disorders that affected study participation. Parents were asked to fill out a parental-report Tanner Staging questionnaire to assess sexual maturation.

Flow-mediated Dilation Testing

Testing was conducted in a quiet room with a constant temperature (22-23 °C). Upon arrival, subjects had electrodes placed on the chest for the electrocardiogram (ECG). A three-inch adhesive pad was placed on the subject's forearm that held a probe that monitored tissue oxygen saturation. A blood pressure cuff was placed on the upper portion of the arm. Subjects were then instructed to rest quietly in a supine position for 15 minutes. During this time, an investigator recorded what the subject had eaten the previous day. Subjects were asked to recall what they had done for physical activity during the last seven days. Subjects continued to rest for an additional ten minutes. During this ten minute rest period, subjects were asked to lie quietly, refraining from talking and moving as much as possible. At the end of the ten minutes,

the blood pressure cuff was inflated to 50 mmHg above resting systolic value, and a standard ultrasound with a 10 MHz linear array probe was used to obtain a B-mode image of the brachial artery. Tissue oxygen saturation was monitored continuously during both rest and testing phases.

Expected Outcomes

It is well established that obesity is associated with endothelial dysfunction in adults; therefore we expected to see endothelial dysfunction in overweight children defined as an increase in the brachial artery dilation after five minutes of blood flow occlusion of less than eight percent. We also expected to be able to predict the blood flow and brachial artery dilation due to hyperemia with tissue oxygenation measured by NIRS, gender, room humidity, heart rate, and BMI.

Potential Problems

Occluding blood flow to the arm is uncomfortable and some subjects may not tolerate this for five minutes. We familiarized subjects with the procedure at the time that consent and assent were received. Subjects were excluded if they found the procedure too uncomfortable.

Doppler blood flow must be measured at exactly ten seconds post occlusion to get maximal hyperemic flow. Vessels have a tendency to roll somewhat when the blood pressure cuff is inflated/deflated. If the center of the vessel at an angle of 90º could not be found exactly at ten seconds post occlusion, maximal flow could not be recorded. When looking at the increase in artery diameter post occlusion, it is important to be able to normalize that increase by flow. If the flow could not be measured accurately, a second trial was performed after a fifteen

minute rest period. If a second trial was needed during pre-testing, then a second trial was also performed at post-testing. It was estimated from the pilot study that a second trial would be necessary in no more than ten percent of all subjects. There is also a chance that the vessel would roll again on the second trial. In this case, flow would not be reported on those subjects. It was estimated from pilot data that this would not occur in more than five percent of the subjects.

Caffeine, certain medications, vitamins, exercise and certain foods in the diet can affect endothelial function. Therefore caffeine, medications, vitamins and exercise were restricted prior to testing. A 24- hour diet recall was taken during the initial visit. Subjects were instructed to eat the same diet the day before the post test for flow mediated dilation at the completion of the study.

***Specific Aim #2*: Evaluating the effectiveness of an exercise intervention to improve endothelial function.**

Subject Groups

Subjects that were classified as having endothelial dysfunction were randomly assigned to one of two groups. Group one (N=20) took part in a 12 week home-based aerobic exercise training program. Group two (N=15) served as delayed treatment controls, and was offered the exercise intervention after the study period. All subjects received baseline and follow-up measurements including flow-mediated dilation using a high-resolution ultrasound, flow mediated tissue oxygenation using NIRS, and a symptom limited maximal VO_2 test on a bicycle ergometer. Fasting (12 hours) blood samples were drawn on all subjects to measure total

cholesterol (TC), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL) triglycerides, blood glucose, insulin, and nitric oxide oxidation products (NO2 + NO3 = NOx), asymmetric dimethylarginine, arginine, and MDA. All subjects were asked to complete three-day dietary food logs the first and last week of the study period.

Symptom-limited Maximal Exercise Test

Subjects and their parents reported to the Human Performance Laboratory. Subjects were asked, prior to their visit, to refrain from caffeine for at least 12 hours, not to eat for at least two hours, and to wear comfortable clothing, such as a t-shirt and shorts, and athletic shoes. The bicycle seat was adjusted by an investigator to the proper height for each subject. Electrodes were placed on the chest to monitor an electrocardiogram (ECG). Subjects were asked to sit quietly for five minutes, and at the end of the five minutes heart rate and blood pressure were measured and recorded.

Subjects were instructed to ride the bicycle until they were too tired to continue. They wore a mask over their nose and mouth so that air could be continuously collected to determine aerobic capacity. Following the termination of the test, subjects were asked to cool down by cycling with no resistance for three to five minutes or until the heart rate had returned to 20 percent above the resting heart rate and blood pressure had returned to baseline.

Subjects then got off the bicycle and sat in a chair to continue to cool down. At this time, subjects were instructed on how to accurately record their daily food intake. Subjects were given food record sheets and were asked to write down everything that they ate and drank for three days (two week days and one weekend day) during the first week and the last week of the study period. Subjects were advised not to change dietary habits during the study period**.**

12 Week Aerobic Training Program

Subjects were enrolled in a home-based aerobic exercise intervention using Dance Dance Revolution (DDR) computer software as the mode of exercise. Subjects were required to attend a DDR training session prior to beginning the aerobic training period. DDR has two modes available to users, a workout mode and a game mode. The subjects' daily workout had to be completed using the workout mode. Subjects were asked to exercise using the DDR at least five days per week. Training progressed as follows:

Week 1: 10 min daily workout mode, unlimited game mode
Week 2: 15 min daily workout mode, unlimited game mode
Week3: 20 min daily workout mode, unlimited game mode
Week 4: 25 min daily workout mode, unlimited game mode
Week 5-12 30 min daily workout mode, unlimited game mode

Subjects recorded his/her exercise daily using a weekly exercise log. Subjects also recorded time spent per day watching TV, playing video games (other than DDR), and playing on the computer. Subjects were asked to wear a pedometer seven days per week, and to record accumulated steps daily. All records were mailed weekly in pre-addressed envelopes, provided by the investigator.

Expected Observations

We expected that the subjects may not reach maximal oxygen consumption during the graded exercise test. Other studies that have attempted to test children's aerobic capacity have not been successful in reaching maximum oxygen consumption in all subjects[71-72]. The bicycle ergometer may cause local muscle fatigue which limits the test instead of cardiorespiratory capacity[72]. Schober[71] conducted VO2 max tests on 20 ten year olds using a bicycle ergometer. Only half of the subjects were able to reach their physiological maximum. The range of VO2

max reported in the literature for a graded exercise protocol is 1.13 to 2.27 L/min (35.6 to 60.6 ml/kg/min) in nine to 14 year-olds. We expected to see an increase in VO2 and time to fatigue at the post-test in those subjects that completed the 12-week exercise program[6]. We did not expect to see any change in the delayed treatment control subjects.

Potential Problems

Recruitment of subjects could have been problematic. Typically overweight individuals are not likely to volunteer for exercise programs. Since 50 percent of West Virginia children of this age group are above the 85th percentile for BMI, the subject pool was very large. DDR is a very innovative and engaging mode of exercise. Overweight children are sometimes self conscious while exercising with a group of children. This exercise intervention was a home-based program where children felt comfortable to exercise with privacy. These two aspects of the exercise program were emphasized to encourage participation. The pedometer readings were compared to the exercise logs to evaluate whether there were any discrepancies between the two to ensure activity logs were filled out properly. Parents were asked to review and sign the exercise logs each week, which helped increase the accuracy of the logs.

The possibility that the children would not reach their VO2 max is a potential problem. During a VO2 max test, discomfort and fatigue become major factors. Children are less likely than adults to continue if they are uncomfortable. To help overcome this barrier, children were verbally encouraged throughout the test. VO2 and heart rate were recorded continuously and were used to quantify improvement in aerobic capacity at submaximal levels in cases that max VO2 was not achieved. Compliance with the exercise intervention was another potential problem. Patients were contacted weekly to encourage participation and subjects were called by

an investigator after any missed exercise session. Results of a statistical power analysis revealed the need for 14 subjects to complete the study in order to achieve 80 percent power for FMD. Attrition was addressed by recruiting more than 20 subjects for the intervention group.

Endothelial function could be affected by diet, therefore subjects were instructed not to change their diet. Three day dietary records were taken during the first and last week of the study to determine whether dietary habits changed during the study. Food portion size is difficult to estimate. Subjects were taught how to estimate food portions using food models. A weakness of the study design was that subjects may not accurately or honestly complete dietary records.

Specific Aim #3: Establish that flow mediated tissue oxygenation can be used as a clinical marker for endothelial function.

Measurement of tissue oxygenation using NIRS

FMD and tissue oxygenation were measured simultaneously. Tissue oxygenation was monitored throughout the testing session so that values at rest and post occlusion could be used to predict blood flow.

Expected Observations

We expected that we would be able to predict the increase in brachial artery diameter due to FMD using multiple regression and some combination of the following variables: tissue oxygenation changes, time intervals, heart rate, humidity, gender, and BMI.

Potential Problems

Measurement of the brachial artery is rater dependent. All measurements of the brachial artery were taken by the same trained researcher and recorded on a VHS tape. A second blinded researcher read all tapes. Intraobserver and interobserver variability were calculated and assessed for each condition [69].

GENERAL METHODS:

Flow-Mediated Dilation Testing

Subjects were instructed to lie quietly for a 15-minute rest period. Subjects were asked to recall what they ate the previous day at the pre-test and the post-test. At the post-test, subjects were given a sheet with what they ate the day before the pre-test, and were asked to eat exactly what they had eaten the day before the pre-test. An investigator took the subject's resting blood pressure and placed three electrodes on the subject to record an electrocardiogram (ECG). A pediatric-sized blood pressure cuff was placed loosely around the top portion of the subject's right arm. Subjects were then asked to rest for an additional ten minute period. They were instructed to refrain from moving and talking as much as possible. At the end of the rest period, the brachial artery was scanned longitudinally with a 10-MHz linear array probe two ten centimeters above the elbow. The brachial artery was chosen as the main conduit vessel of interest because previous data indicates that endothelial dysfunction of the brachial artery parallels that in coronary arteries[73]. Depth and gain settings were set to optimize images of the lumen/arterial wall. When a satisfactory transducer position was found, the position was marked on the skin and the arm remained in the same position throughout the procedure. All images were stored on a VHS tape for off-line analysis. A resting scan was then recorded. The arterial

diameter was measured at a fixed distance from an anatomic marker. Measurements were taken from the anterior to the posterior `m' line at end diastole, incident with the R wave on a continuously recorded ECG. The `m' line represents the edge of the intima-media interface in the ultrasound image of the arterial wall. A resting arterial flow velocity was measured by means of a pulse Doppler signal at a 90°angle to the vessel wall.

After baseline measurements had been taken, the pediatric-size pneumatic blood pressure cuff was inflated to 50 mmHg above the resting systolic pressure, and remained there for five minutes. A second ultrasound scan was taken continuously from 30 seconds before to 120 seconds after cuff deflation. Ten seconds post occlusion an arterial flow velocity was measured. Vessel diameters was measured and recorded at 60 seconds and 90 seconds post occlusion. Because there is often a shift in the brachial artery once the cuff is released, great care was taken to find and image the largest lumen diameter during this time.

NIRS

Near Infrared Spectroscopy (Inspectra, Hutchinson Technology, Hutchinson, MN) was used to measure tissue oxygen saturation in the brachioradialis muscle. This technique transmits light in wavelengths that pass through the skin and subcutaneous tissue but are either absorbed or reflected by hemoglobin. The absorption spectra of oxygenated and deoxygenated hemoglobin are well established and provide the means to calculate percent hemoglobin saturation. The NIRS measures tissue absorbance values at specific wavelengths between 650-900 nanometers (nm). A 25 mm probe was placed on forearm over the belly of the brachioradialis. The probe contains two bundles of optical fibers: one bundle emits light, and one receives reflected light. The depth of the penetration is about equal to the separation of the bundles. The instrument uses

a 25 watt tungsten halogen bulb for light and filtering system to remove wavelengths between 650-900 nm. The receiving filters transmit reflected light to the spectrometer where a computer processes the received signals and displays the calculated oxygen saturation value (oxygen saturation = oxyhemoglobin/deoxyhemoglobin).

Graded Exercise Test

Aerobic fitness was determined with a symptom limited graded exercise test to volitional fatigue performed on a bicycle ergometer. A MedGraphics oxygen analyzer was used to evaluate breath by breath oxygen consumption during the test. Electrodes were placed in the standard position for four-lead ECG. Heart rate was continuously monitored using the ECG. Subjects were then familiarized with the ergometer and taught how to estimate perceived exertion using the CERT scale. Blood pressure was recorded every minute of the test and for six minutes following the cessation of the exercise. Subjects were asked to give a rating of perceived exertion every two minutes using the ten-point CERT scale. The CERT scale is similar to the Rating of Perceived Exertion Scale (RPE) that is used with adults. It differs from the RPE scale in that it has fewer possible responses, a range of numbers one to ten, which is more familiar to children than six to 20[74]. The bicycle test required the subject to maintain a set pedaling speed of 50 revolutions per minute (rpm). The test began at a workload of 25 watts, and was increased by an additional 25 watts every three minutes. The test was stopped when the subject could no longer maintain a pedaling speed of 50 rpm, if the subject asked to stop, or if there were any abnormal ECG or blood pressure responses.

HUMAN SUBJECTS

Protection of Human Subjects

All subjects were informed of the study procedures and potential risk by one of the investigators and parents of the subjects signed an informed consent at the same time that the subjects signed the assent form. There was a possibility that the subjects may have felt discomfort due to the occlusion of the brachial artery. However, no subject became uncomfortable, and therefore the occlusion did not have to be stopped. Exercise soreness may result from exercise testing or training. The exercise test used was a symptom-limited maximal test, and the test could be stopped at any time upon subject's request. Exercise training was kept at a moderate intensity level to minimize discomfort. Subjects were instructed that they could stop any part of the study at any time. No subjects became injured or needed medical care during the study.

Minority Subjects

Every attempt was made to recruit subjects who are Hispanic/Latino, African American, Asian, Pacific Islander, and American Indian/Native Alaskan. It was difficult to recruit subjects from these ethnic or racial origins in the state of West Virginia because according to the U.S. Census Bureau data (1990-2000), the population of West Virginia is 95 percent white, 0.2 percent American Indian and Alaska Native, 0.5 percent Asian, 3.2 percent Black, and 0.0 percent Native Hawaiian or Pacific Islander.

Women

Prior to beginning the study, we anticipated that 50 percent or more of our subject volunteers would be females.

Children

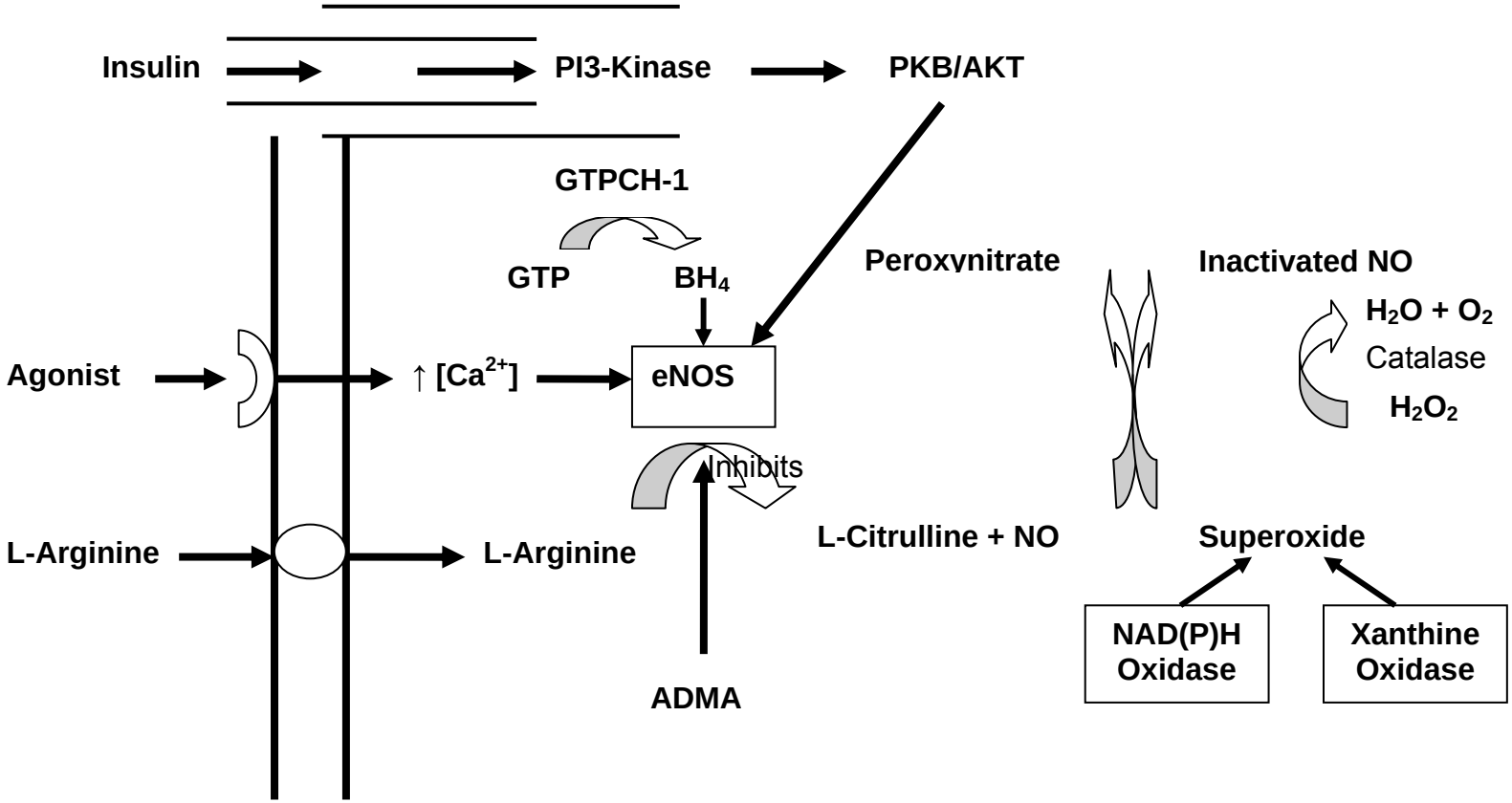
All subjects participating in this research project were between seven-12 years of age.

Occluding blood flow to the arm may be uncomfortable and some subjects may not tolerate this for five minutes. We familiarized all subjects with the procedure at the time that consent and assent was received. Subjects were excluded if they found the procedure too uncomfortable.

VERTEBRAE ANIMALS

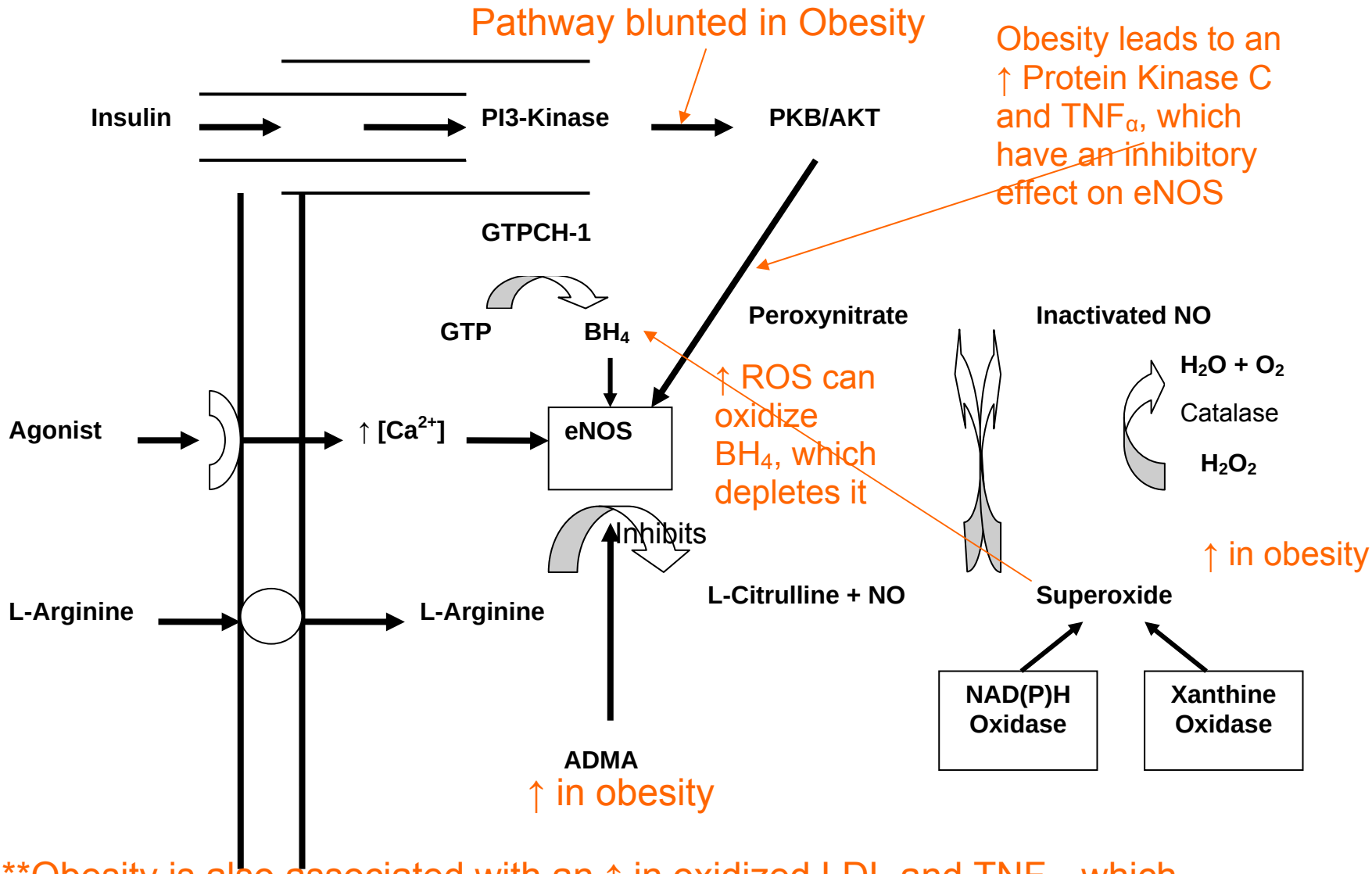
Not applicable.

FIGURE 1:



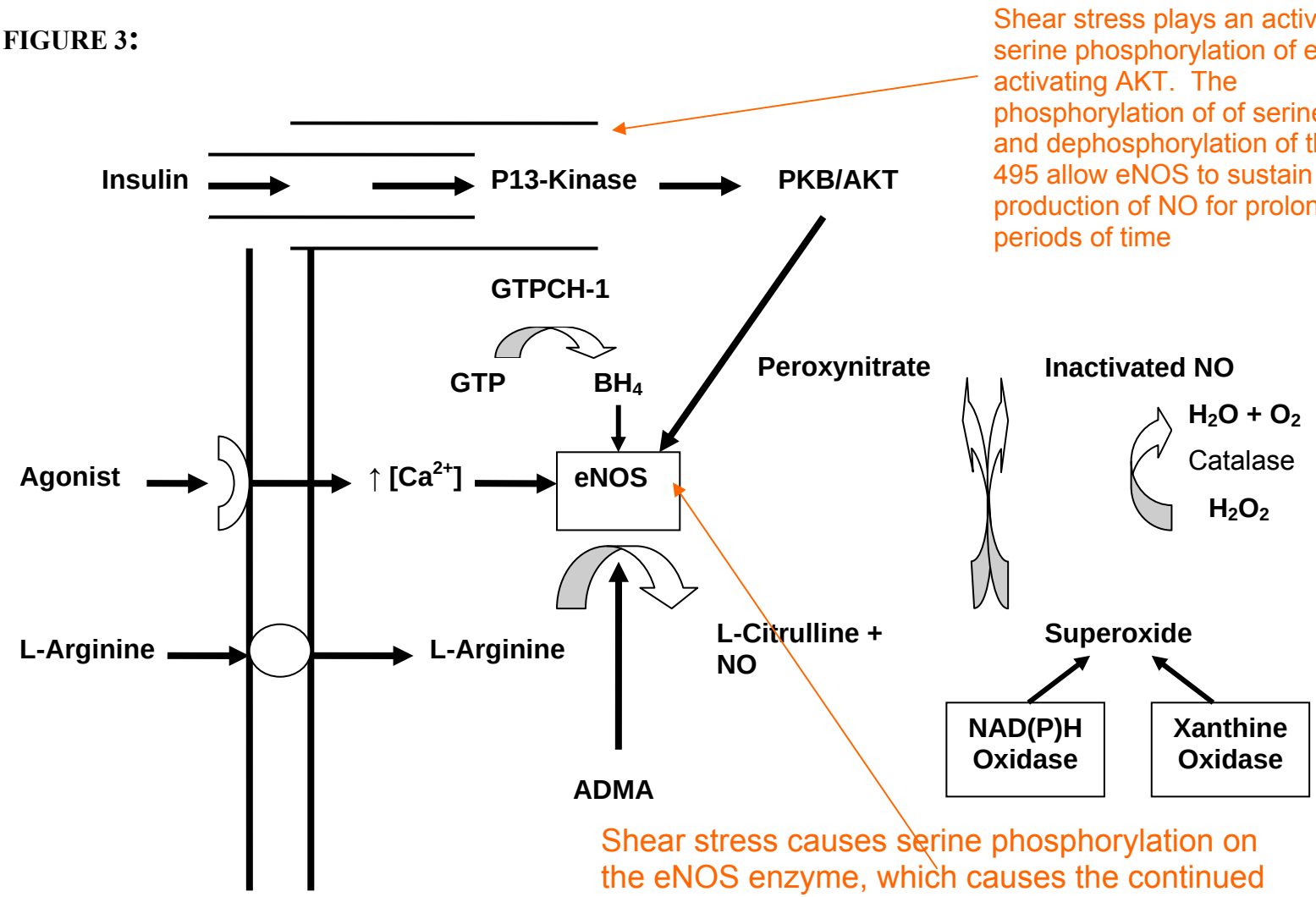
NO Bioavailability: NO is synthesized by eNOS from L-arginine. NO production is dependent on L-arginine availability and endogenous synthesis by the kidneys and vascular endothelium, via specific cell membrane amino-acid transporters, and the availability of cofactors (mainly BH_4), which is synthesized from GTP. ADMA competitively inhibits L-arginine from binding to eNOS. eNOS activity may be stimulated by a Ca^{2+}-dependent pathway, or in the case of insulin and shear stress by a Ca^{2+}-independent pathway (this pathway is mediated by pI3-kinase and Akt. Reactive oxygen species may interact with NO to inactivate it, and potentially produce the peroxynitrate radical.

FIGURE 2:



**Obesity is also associated with an ↑ in oxidized LDL and TNFα, which leads to a downregulation of eNOS through a reduction in mRNA ½ life

FIGURE 3:


Shear stress plays an active role of serine phosphorylation of eNOS by activating AKT. The phosphorylation of of serine 1177 and dephosphorylation of threonine 495 allow eNOS to sustain production of NO for prolonged periods of time
Insulin
P13-Kinase
PKB/AKT
GTPCH-1
GTP
BH4
Peroxynitrate
Inactivated NO
H2O + O2
Catalase
H2O2
Agonist
↑ [Ca2+]
eNOS
L-Arginine
L-Arginine
L-Citrulline + NO
Superoxide
NAD(P)H Oxidase
Xanthine Oxidase
ADMA
Shear stress causes serine phosphorylation on the eNOS enzyme, which causes the continued activation of eNOS and ultimately the production of NO

BUDGET

Item	Cost Each	Total	Funding Source
Adhesive pads for NIRS	$15	$600	Human Performance Lab
FMD		$12,000	Human Performance Lab
Masks for Med Graphics	$4	$280	Human Performance Lab
Lipid Profiles including Fasting Insulin	$22	$1540	CARDIAC
Phlebotomy supplies (Tubes, Butterfly Needles, alcohol pads, 2x2 pads, gloves)	Butterfly Needle ($59.99 for 50) Tubes ($25.99 per box 100)	$250	Human Performance Lab
Biochemical testing	To be determined		Dr Baylis's Lab
Shipping	$25	$50	Human Performance Lab
Pedometers	$12	$360	PEIA
DDR Software	$30	$1050	PEIA
Red Octane Pad	$56	$1960	PEIA
X-Box or Playstation II	$150	$5250	PEIA
Printing supplies		$500	PEIA
Student Helpers		$1500	PEIA
Total (excluding biochemical testing)		$25,340	

Justification:
Adhesive pads for NIRS- number needed based on the fact that one needed per subject. Labeled each adhesive pad with child's name and used the same one for both pre and post testing.

Masks for Medgraphics- Needed one mask per child for both the pre-test and post-test.

Pedometers- Walk4life 2505 –My Life Stepper Series allowed the quantification of both step counts and exercise time. The retail price for these monitors was $25/unit. Because they were used for research purposes were given a price of $12/unit.

DDR software- Subjects were given DDR software and Red Octane pad to keep if the entire study was completed. Subjects were given either a Playstation II or X-Box if they did not already own one.

Lipid profiles- Lipid profiles were run through Lab Corp for $7 dollars per child. Lipid profiles were performed both pre and post. Fasting Insulin was run by Lab Corp for $15.

Phlebotomy supplies- Blood draws were performed at both the pre-test and post-test. Three tubes of blood were be drawn per child (one green top for lipid profile and two purple tops for NO and oxidative markers)

Biochemical testing- Was done at both pre-test and post-test.

Shipping- Pre-test and post-test plasma samples from all subjects were shipped to Dr Baylis's Lab as a batch.

Printing- Consent and Assent forms were printed for each subject. Health histories, Tanner staging questionnaires, parent questionnaires, seven-day physical activity questionnaires, and three-day food/beverage records were printed for each subject. Subjects were asked to keep daily exercise logs.

Student workers- Student workers were hired to assist in testing subjects, data entry and to interact with subjects throughout the duration of the study.

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ARTICLE 1:
Presence of Endothelial Dysfunction, Metabolic Syndrome, and Other Cardiovascular Risk Factors in Overweight Children

ABSTRACT

Objectives To explore the prevalence of endothelial dysfunction in overweight children and to explore the relationship between cardiovascular risk factors, insulin resistance, subclinical inflammation, endothelial dysfunction, and the metabolic syndrome in overweight children.

Study design Forty-nine overweight children (body mass index (BMI) $\geq 85^{th}$ percentile, 24 females) were evaluated for flow-mediated dilation of the brachial artery (FMD), lipids, insulin, insulin sensitivity was calcaulated using the homeostasis model assessement(HOMA), glucose, waist (WC) and hip circumference (HC), blood pressure, and the metabolic syndrome. In a subsample of subjects, C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), adiponectin, $NO_2 + NO_3$ (NOx, the stable inert products of NO production), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), l-arginine, and aerobic fitness were also measured.

Results Eighty two percent of these children presented with impaired endothelial function. Pearson Product Correlations revealed that BMI (-.524, p<.01), WC (r=-.479, p<.01), HC (r= -.430, p<.01), SBP (r= -.538, p<.01) and DBP (r= -.608, p<.01) were all significantly correlated with FMD. Children who presented with normal function (n=9) had lower BMI's (24.42 ± 3.73 to 29.44 ± 5.11, p=.008), lower diastolic blood pressures (63.78 ± 6.51 to 75.58 mmHg ± 8.77, p=.039) and lower systolic blood pressures (112.00 ± 8.77 to 119.48 ± 9.90 mmHg, p=.000) compared to those who presented with abnormal function (n=40). In those subjects with abnormal function, all markers of inflammation measured were found to be elevated. Thirty one of the 49 subjects presented with the metabolic syndrome. Those subjects with the metabolic syndrome had significantly higher BMI, SBP, DBP, resting heart rates, TRIG, and HOMA, and

they had lower HDL cholesterol, aerobic capacity, FMD, and adiponectin values compared to those subjects without the metabolic syndrome.

Conclusions The coexistence of both endothelial dysfunction and the metabolic syndrome in most overweight children emphasizes the need for effective preventive and intervention strategies to improve the health profiles of children in the United States.

INTRODUCTION

Obesity has reached epidemic proportions nationwide, with the prevalence of childhood overweight tripling between 1980 and 2000[1]. According to data from the Coronary Artery Risk Detection in Appalachian Communities Project, the prevalence of childhood obesity in West Virginia is nearly double the national average[2]. Childhood overweight is associated with an elevated risk of premature death[3], and the American Heart Association has reclassified obesity as a 'major, modifiable risk factor' for coronary artery disease[4]. The metabolic and cardiovascular consequences of childhood obesity are well documented and have a major impact on lifetime cardiovascular risk and the subsequent development of both atherosclerosis and type 2 diabetes[5].

Endothelial cell dysfunction is a critical early event in the pathogenesis of both atherosclerosis and type 2 diabetes[6], and it is now well established that obesity is associated with systemic[7] and coronary[8] endothelial dysfunction in adults. More recently, endothelial dysfunction has been documented in obese children[9-11], but whether all obese children present with endothelial dysfunction is not known.

It is generally assumed that traditional risk factors for cardiovascular disease (CVD), a low-grade pro-inflammatory state, and insulin resistance play a role in endothelial dysfunction associated with obesity, and that a clustering of these, defined as the metabolic syndrome,

creates a state of constant and progressive damage to the vascular wall[12-14]. However, there has been relatively little research investigating the exact interactions that result in endothelial dysfunction.

Interleukin-6 (IL-6), an endocrine cytokine, is secreted within the adipose tissue by both adipocytes and macrophages. Both endothelial cells and vascular smooth muscle cells are targets of IL-6 action, resulting in increased expression of adhesion molecules and activation of the local renin-angiotensin pathways, both modifications that favor inflammation and endothelial dysfunction[16]. In addition, IL-6 enhances the production of C-reactive protein (CRP) by the liver. CRP is an acute-phase reactant, and has been used for decades in the diagnosis and monitoring of chronic inflammatory diseases. In adults, plasma CRP levels are directly related to body fat as well as specific components of the metabolic syndrome[17-18]. In children, data are limited, but associations between CRP and cardiovascular risk have been observed and have been similar to those found in adults[19]. Tumor necrosis factor alpha (TNF-α) is an important mediator of many cardiovascular diseases, and has been shown to induce endothelial dysfunction in a variety of vascular beds by increasing oxidative stress and decreasing the release of nitric oxide (NO)[20]. Adiponectin is also emerging as an important mediator of risk for both CVD and diabetes. Adiponectin has been found in several studies to be inversely related to markers of endothelial dysfunction and systemic inflammation, such as TNF-α and CRP[21]. Insulin is known to have a direct vasodilatory effect that is mediated through the stimulation of NO production in the endothelial cells[22]. With insulin resistance, the ability of insulin to stimulate NO is diminished[23].

While the mechanisms linking childhood obesity and endothelial dysfunction remain unclear, some of the proposed mechanisms include altered plasma lipids, insulin resistance,

hypertension, elevated pro-inflammatory markers, and the clustering of these factors associated with increased adiposity. Therefore, the aims of the present study were 1) to explore the incidence of endothelial dysfunction in overweight children and 2) to determine the relationship between cardiovascular risk factors, insulin resistance, pro-inflammatory markers, and endothelial dysfunction in overweight children with and without the metabolic syndrome.

METHODS

Subjects and screening measures

Forty-nine overweight subjects (body mass index [BMI] $\geq 85^{th}$ percentile for age and gender) between the ages of seven-12 years (24 females) were recruited for the study. All subjects were covered by West Virginia Public Employee Insurance Agency or Mountain State Blue Cross/Blue Shield Insurance Agency. Subjects were recruited through website and newsletter advertisements of the participating insurance companies. Children were screened for the study by telephone interview, which included parent-report of height and weight, so that an estimate of BMI could be calculated.

All participants and a parent/guardian provided informed assent/consent prior to study participation. Participation in the study was voluntary and subjects were free to withdraw at any time. The study protocol was reviewed and approved by the Institutional Review Board for the Protection of Human Subjects at West Virginia University.

Anthropometric Measurements and Questionnaires

Health history was obtained via interview with both the subject and consenting parent. Family history of cardiovascular disease, diabetes, obesity and dyslipidemia was recorded.

Tanner growth staging for secondary sexual development was estimated by parental completion of the Pubertal Development Scale[24].

Body weight was measured in pounds and height was measured in inches. BMI was calculated using the following equation (BMI= [weight in pounds/ (height in inches)^2*703][25]. Waist circumference was measured in inches at the smallest area of the waist, which is typically right above the navel. Hip circumference was measured in inches at the largest area of the hips and buttocks. Waist-hip ratio was then calculated. These circumference measurements were included to assess central adiposity as an important predictor of health status.

Vascular Testing

Flow-mediated dilation, induced by reactive hyperemia to a 5-minute ischemic stimulus, is a well-established measure[26] of endothelium-dependent vasodilator function. Vascular function was assessed after a 12 hour fast and abstinence from caffeine and exercise for 24 hours prior to testing. Brachial artery diameter was assessed using a high-resolution vascular ultrasound (ATL UM-9 HDI Ultrasound System) with a 10-MHz linear-array probe, after subjects rested in a supine position for ten minutes. A pediatric-size blood pressure cuff was placed loosely around the top portion of the subject's right arm. Resting artery diameter was measured five to ten centimeters proximal to the elbow. The arm was marked, so that subsequent measurements could be repeated at the same location. The blood pressure cuff was inflated to 50 mmHg above resting systolic blood pressure and remained at this pressure for five minutes. A scan was started ten seconds before the blood pressure cuff was deflated and continued for 90 seconds after deflation. All images were stored on a VHS tape for off-line analysis. The arterial diameter was measured at a fixed distance from a specific anatomic marker. Measurements were taken from the anterior to the posterior `m' line at end diastole, incident with the R wave on a

continuously recorded ECG. Subjects were classified into two groups based on their FMD response: those with a FMD response of less than eight percent were said to have impaired endothelial function and those that presented with a FMD response of greater than eight percent were said to have normal endothelial function.

All ultrasound scans were performed by a single experienced vascular sonographer. A second trained, blinded observer re-measured vessel diameters from the VHS tapes. The inter-observer coefficient of variation for brachial artery diameter was 2.17 ± 1.97%. The correlation coefficient between paired measurements was .973 ($p<.01$) for diameter.

Assessment of Aerobic Exercise Capacity

Those subjects presenting with endothelial dysfunction (n=40), completed a test of aerobic fitness, quantified as peak oxygen uptake, assessed with a symptom-limited, ramped intensity protocol on a bicycle ergometer, starting at 0 Watts and increasing 15 Watts every minute until exhaustion. Subjects were required to maintain a set pedaling speed of 50 revolutions per minute (rpm) throughout the test. Expired oxygen and carbon dioxide concentrations and volumes were collected and analyzed using a MedGraphics metabolic cart (MedGraphics, St. Paul, Minn).

Subjects were familiarized with the ergometer and taught how to estimate perceived exertion prior to beginning the test. Subjects were asked to give a rating of perceived exertion every two minutes using the ten-point Child's Effort Rating Table (CERT). CERT is similar to the Rating of Perceived Exertion Scale (RPE) that is used with adults. Electrodes were placed in the standard position for a four-lead ECG, and a resting blood pressure was measured prior to

beginning the exercise test. Heart rate was monitored using the ECG. Blood pressure was measure and recorded every minute of the test and for five minutes after the test was completed.

Assessment of Blood Chemistry

Blood samples were collected after a 12 hour fast from a vein in the antecubital fossa to determine concentrations of cholesterol (total cholesterol, high-density lipoprotein cholesterol [HDL], and triglycerides), insulin, and glucose on all subjects. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation[27]. Plasma glucose and lipid levels were measured with the use of an Autoanalyzer (SYNCHRON LX Systems, Beckman Coulter, Inc., Fullerton, CA). Plasma insulin levels were measured by immunoassay system by Warde Medical Laboratory (Ann Arbor, Michigan). Insulin sensitivity was calculated using the homeostasis model assessment (HOMA)[28], a mathematical estimate of insulin sensitivity based on fasting glucose and insulin concentrations. HOMA yields an equation where insulin resistance = [fasting insulin (μIU/ml)*fasting glucose (mmol/l)]/22.5.

An additional tube of blood was drawn on those subjects who presented with endothelial dysfunction. The samples were separated into aliquots after centrifugation (2000 rpm, 15 minutes), and immediately placed at -80º C until analysis. Samples were thawed only once immediately prior to analysis. The following markers for NO production were measured on this subsample of subjects with endothelial dysfunction: $NO_2 + NO_3$ (NOx, the stable inert products of NO production), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and l-arginine. NOx concentrations of plasma were measured using the Griess assay after conversion of NO_3 to NO_2 with the NO_3 reductase enzyme, as described previously by Suto et al.[29]. Plasma l-arginine was measured by reverse-phase HPLC with precolumn derivatization

and fluorescent detection using modification of the ACCQ Tag system for amino acid analysis (Waters, Milford, MA). For the measurement of ADMA and SDMA, the reverse-phase HPLC with AccQ Tag method of Heresztyn et al. [30] was used with minor modifications.

The following pro-inflammatory markers were also measured on a subsample of subjects who presented with endothelial dysfunction: tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), and adiponectin. Commercially available radioimmunoassay assay (ELISA) kits were used (Quantikine HS, R&D systems, Minneapolis, MN) to measure plasma IL-6 and TNF-α. According to R&D systems detection limits are less than 0.094 and 0.18 pg/ml for IL-6 and TNF-α, respectively. Adiponectin levels were also measured by ELISA (Linco Laboratories, St. Charles, MI), and detection limits are less 0.78 ng/ml. High-sensitivity CRP concentrations were measured by radioimmunoassay (Life Diagnostics, Inc., West Chester, PA), and the minimum detectable concentration is estimated to be 0.1 mg/l.

Analysis of Data

Data were analyzed using SPSS 15.0 software for Windows (SPSS, Chicago, IL-USA). Descriptive data are expressed as the mean value ± standard deviation (SD). Between-group analyses were compared by independent sample t-tests and Mann-Whitney U tests, as appropriate. Test selection was based on evaluating the variables for normal distribution. Pearson's Product Moment correlation coefficients were calculated to determine significant relationships between variables. The determinants of FMD were assessed using univariate and multivariate linear regression analysis. Statistical significance was inferred at a two-tailed p-value of $<.05$.

RESULTS

Descriptive data for the subjects is provided in Table 1. Eighty two percent of these children presented with impaired endothelial function, and the majority of these overweight children had a family history of both heart disease and type 2 diabetes (Table 2). In addition, they presented with many risk factors for chronic disease themselves (Table 2).

Pearson Product Moment correlations were used to determine which variables were associated with FMD in all subjects. BMI (-.524, $p<.01$), WC ($r=-.479$, $p<.01$), HC ($r= -.430$, $p<.01$), SBP ($r= -.538$, $p<.01$) and DBP ($r= -.608$, $p<.01$) were all significantly correlated with FMD. These variables were entered into a multiple linear regression to evaluate significance and findings revealed an $r=.602$, $R^2=.362$, $p<.01$.

Inclusion into the study required subjects to be over the 85th percentile for BMI. Only three out of the 49 subjects were less than the 95th percentile for BMI, and 31 of the subjects were at the 98th percentile or higher. To determine whether the severity of obesity influenced risk, we used Pearson Product Moment Correlations to determine what other risk factors were associated with BMI in these overweight children. In addition to FMD, SBP (.469, $p<.01$), DBP (.413, $p<.01$), HDL (-.461, $p<.01$), and HOMA (.320, $p<.05$) were all significantly correlated with BMI.

Comparison of subjects with impaired endothelial function to those with normal function

Forty of the 49 subjects presented with endothelial dysfunction, those with normal function had a mean FMD response of 15.44 ± 4.60%, while those with impaired function had a mean FMD response of 3.40 ± 3.21% (Figure 1). Children with normal function (n=9) had lower BMI's (24.42 ± 3.73 to 29.44 ± 5.11, $p=.008$), lower diastolic blood pressures (63.78 ± 6.51 to

75.58 ± 8.77, p=.039) and lower systolic blood pressures (112.00 ± 8.77 to 119.48 ± 9.90, p=.000) compared to those with endothelial dysfunction (n=40) (Figure 2).

To determine what variables were associated with FMD in children with normal function (n=9) versus those with abnormal function (n=40), Pearson Product Moment correlations were again calculated. SBP was the only variable that was significantly ($p<.01$, $r= -.738$) correlated with FMD in those subjects with normal endothelial function. In children with endothelial dysfunction, BMI ($p<.05$, $r=-.402$), WC ($p<.01$, $r=-.449$), HC ($p<.05$, $r=-.346$), SBP ($p<.01$, $r=-.496$) and DBP ($p<.01$, $r=-.466$) were all significantly correlated with FMD. When the same variables were placed into a multiple regression, using the stepwise method, only SBP remained a significant predictor of FMD with $r=.505$, $R^2=.256$, $p<.001$.

In a subsample of subjects found to have impaired endothelial function, blood markers for NO production (n=34) and pro-inflammatory markers (n=23 for IL-6 and TNF-α, n=20 for CRP and adiponectin) were analyzed. All markers for NO production were within normal ranges when comparing results to other studies done on healthy individuals (both children and adults). Comparison of mean values from this study with other similar studies is presented in Table 3. All pro-inflammatory markers were found to be abnormal in comparison to other studies done in healthy individuals, and were found to be similar to values found in several studies of overweight children. CRP, TNF-α, and IL-6 were all elevated in comparison to studies in healthy individuals, while adiponectin levels were decreased, all of which are consistent with a pro-inflammatory state (Table 3). Pearson Product Moment correlations were used to determine if any of the anthropometric and physiologic variables were associated with the pro-inflammatory markers. BMI ($r=-.470$, $p=.037$), HDL ($r=.447$, $p=.048$), and HOMA ($-.727$, $p=.001$) were all

significantly correlated with adiponectin levels. HDL (-.416, p=.048) and IL-6 (-.447, p=.032) were all significantly correlated with CRP levels.

Comparison of subjects with the metabolic syndrome to those without metabolic syndrome

Subjects were further categorized into those who met the criteria for the metabolic syndrome versus those who did not. The presence of three or more of the following risk factors indicates the presence of the metabolic syndrome in children: HDL ≤ 40 mg/dl, TRIG ≥ 110 mg/dl, SBP or DBP greater than the 90th percentile, insulin resistance (defined as having a HOMA index of >3.0), fasting glucose ≥ 110 mg/dl , waist circumference > 90th percentile and a BMI greater than the 97th percentile[31-32]. Thirty-one of the 49 subjects presented with the metabolic syndrome. There were significant differences found between those with the metabolic syndrome compared to those without, and those differences are presented in Table 4. Those subjects with the metabolic syndrome had higher BMI, SBP, DBP, resting heart rates, TRIG, and HOMA, while they had lower HDL, aerobic capacities, FMD, and adiponectin values compared to those without the metabolic syndrome.

DISCUSSION

The most important finding of this study was that 82 percent of overweight children presented with markedly impaired FMD, and as BMI, blood pressure and degree of central obesity increased, a marked decrement in FMD was seen. In addition to the high incidence of impaired endothelial function in these overweight children, the majority of the subjects presented with multiple other risk factors for cardiovascular disease and diabetes, including insulin resistance, high blood pressure, and dyslipidemia. These results show a significant adverse

effect of worsening obesity on risk for both CVD and diabetes, underscoring the deleterious effect of increased BMI in this very young group of overweight children.

In addition to having impaired endothelial function, these overweight children presented with abnormal levels of all proinflammatory parameters measured. IL-6, CRP and TNF-α were all elevated, while adiponectin levels were lower than what other recent studies have found in healthy children (See Table 3). Until recently, adipose tissue was considered a metabolically inert tissue serving only as a depot for energy substrate and insulation. It is now widely recognized that adipose tissue is metabolically active. Many recent studies have been focused on the production of adipose derived cellular mediators, including those measured in the current study. Our study supports findings from other recent studies which have shown that in the presence of obesity, the balance between various inflammatory markers is altered such that the production of proinflammatory markers (such as TNF-α, IL-6 and CRP) from the adipose tissue is increased, while simultaneously fewer anti-inflammatory markers (such as adiponectin) are produced[33-34]. This unbalanced production of adipocytokines has been found to play a role in the development of both metabolic syndrome and cardiovascular disease[35-36].

In an attempt to understand the relationship between obesity and the inflammatory process, we analyzed the relationship between blood markers for inflammation with other anthropometric and physiologic variables measured in the study. Adiponectin levels were negatively associated with both BMI and level of insulin resistance, while adiponectin displayed a positive relationship with HDL. In studies completed on overweight adults, adiponectin levels have been reported to correlate inversely with BMI[37-38], and similar findings have been recently demonstrated in several different ethnic groups of overweight children[39-41]. Previous studies in children have also shown that adiponectin is a strong predictor of insulin sensititivity[39-41]. In our

study, adiponectin levels were inversely related to insulin sensitivity as measured by the HOMA index. The positive correlation found between adiponectin and HDL in the current study has been demonstrated previously in both adults and children[33,41-42]. While the mechanism by which adiponectin influences lipid metabolism remains unclear, the positive relationship seen between adiponectin and HDL values may result from increased lipoprotein lipase activity[33,42].

Thirty-one of the 49 participating subjects (63 percent) were classified as having the metabolic syndrome, according to the criteria used in the third National Health and Nutrition Examination Survey and World Health Organization. In a sample of U.S. adolescents who were included in NHANES III, the prevalence of the metabolic syndrome was found to be 6.8 percent among overweight and 28.7 percent among obese adolescents[31]. While the current study, has a much smaller number of subjects, the percent of children classified as having the metabolic syndrome is nearly twice that found in NHANES III study. Subjects classified as having the metabolic syndrome also had significantly higher resting heart rates, lower aerobic capacities, lower adiponectin levels, and they showed greater impairments in FMD responses than those subjects without the metabolic syndrome.

A limitation of the current study includes the possibility of selection bias in the recruitment process. All subjects were medically insured, and therefore socioeconomic status and other demographic characteristics may not be representative of the overweight population of West Virginia children at large.

Although coronary function was not assessed in the present study, other investigators have demonstrated that arterial endothelial function in a peripheral vessel such as the brachial artery can be used as a surrogate of coronary endothelial function[43]. Due to ethical considerations, we did not administer nitroglycerine, which is commonly used to assess

endothelium-independent vasodilation. Because of this limitation, we can only assume that there was no impairment in endothelium independent dilation.

We did not include normal-weight children as controls to determine whether there was a significant different between normal-weight and over-weight children with respect to endothelial dysfunction at baseline. However, the values of FMD peak in our study population are similar to those of overweight children in other studies[9,11,44], which demonstrated decreased function in overweight children compared to normal-weight controls.

Pro-inflammatory markers were only assessed on a sub-sample of children who presented with endothelial dysfunction. While all values were found to be abnormal in these children, we did not analyze these markers in those children who had normal endothelial function, therefore no relationship was seen between level of endothelial function and markers for inflammation. Other studies that include comparisons between normal- and overweight children have shown that abnormal levels of these markers seen in an obese state do impose deleterious effects on the vascular walls, leading to impaired endothelial function[45-46].

While recruitment criteria for this study included children greater than the 85th percentile for BMI, only 3 out of the 49 children in the study were below the 95th percentile for BMI and 31 of the subjects were at the 98th percentile or higher. Therefore, while we were attempting to examine a variety of BMI percentiles which would classify children as both at-risk and overweight according to the CDC Clinical Growth Charts, the vast majority of the children were extremely overweight. We have shown that degree of overweight does correlate with cardiovascular risk, demonstrating that even modest weight loss in these children may have significant effect on their overall health risk.

In conclusion, the majority of overweight children presented with impaired endothelial function in association with several other risk factors for both heart disease and type 2 diabetes. FMD was inversely related to BMI. In children found to have impaired endothelial function, pro-inflammatory markers (IL-6, CRP, TNF-α, and adiponectin) were all abnormal compared to reported recent studies on healthy subjects. These data indicate that a substantial percentage of overweight children are at a significantly heightened risk for the future development of both CVD and diabetes. The high prevalence of both endothelial dysfunction and the metabolic syndrome in these subjects emphasizes the need for effective preventive and intervention strategies to improve the health profiles of children in the United States.

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Table 1: Baseline Demographic Data, Cardiovascular and Laboratory Values

	N	Mean	SD
Age (years)	49	10.22	1.76
Height (centimeters)	49	147.87	11.60
Weight (kilograms)	49	63.55	18.32
BMI (kg/ht^2)	49	28.52	5.24
BMI Percentile	49	97.77	2.81
Waist Circumference (inches)	49	35.54	5.36
Hip Circumference (inches)	49	37.90	4.56
Systolic Blood Pressure (mmHg)	49	118.10	10.05
Diastolic Blood Pressure (mmHg)	49	73.41	9.53
Total Cholesterol (mg/dl)	49	167.24	28.37
LDL Cholesterol (mg/dl)	49	109.16	23.42
HDL Cholesterol (mg/dl)	49	36.41	8.00
Triglycerides (mg/dl)	48	103.77	61.90
Glucose (mg/dl)	48	91.43	5.77
Insulin (μU/L)	46	15.86	11.14
NOx (μM)	34	21.58	10.62
L-arginine (μM)	34	39.97	14.77
ADMA (μM)	34	.43	.16
SDMA (μM)	34	.33	.13
TNF-α (pg/ml)	23	11.23	5.10
IL-6 (pg/ml)	23	3.88	2.50
CRP (mg/L)	20	3.61	2.84
Adiponectin (μg/ml)	20	8.88	2.60

Table 2: Risk Profile of Subjects

Risk Factor	# of Subjects that Presented with Risk Factor	% of Subjects that Presented with Risk Factor
Family history of CVD	40	82%
Family history of Diabetes	20	40%
Family history of Dyslipidemia	38	77%
Family history of Obesity	45	92%
Elevated Systolic Blood Pressure	24	49%
Elevated Diastolic Blood Pressure	18	37%
Low HDL	33	67%
Elevated LDL	24	49%
Elevated Insulin	12	24%
Impaired Endothelial Function	40	82%

Figure 1: Flow-mediated dilation of those with normal endothelial function compared to those with abnormal function. Values represent means and SD.

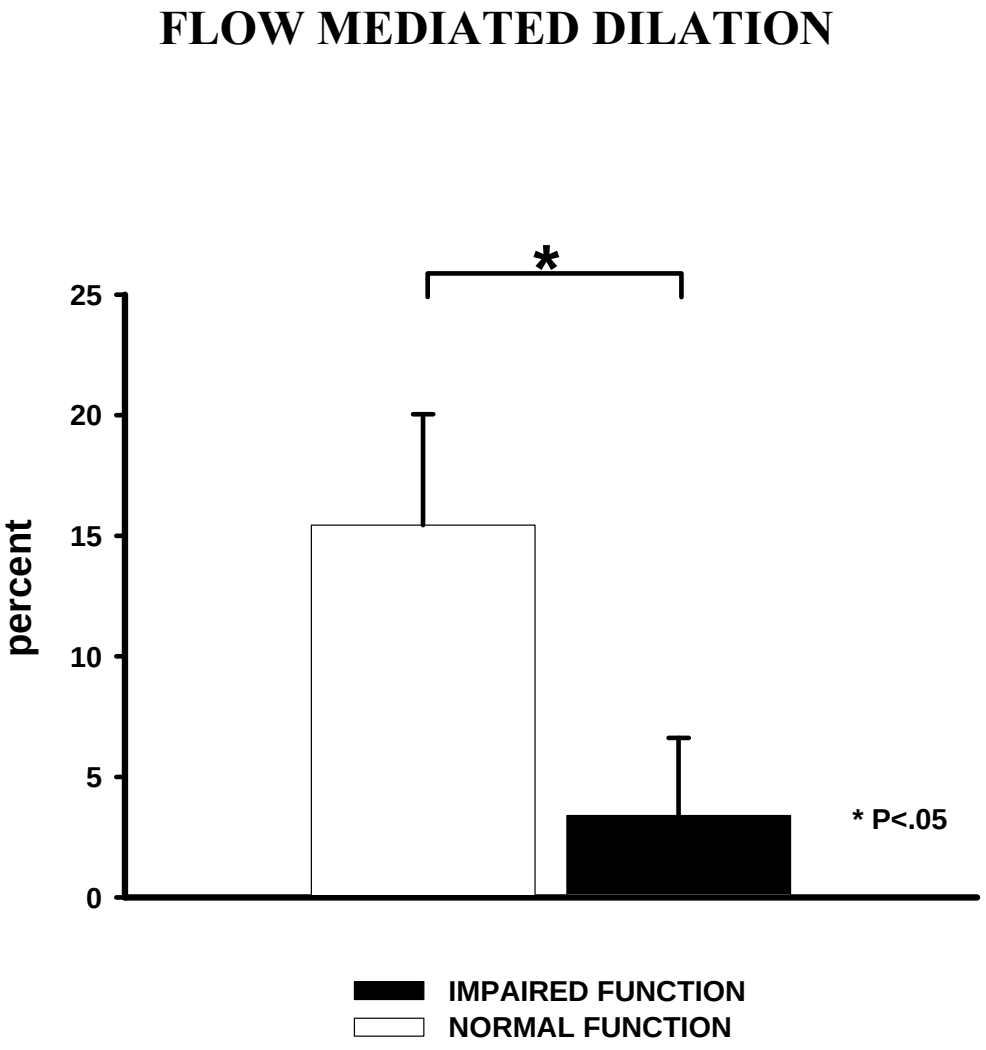

Table 3: Comparison of Blood Markers Values with other Recent Studies

Blood Marker	Ages	Range	Reference	Our Study
HOMA	9-12 years (Obese had abnormal Insulin)	**Lean:** 2.7 ± 2.6 (boys) 3.1 ± 1.1 (girls) **Obese:** 4.9±2.1 (boys) 6.2 ± 0.3 (girls)	Krekoukia, et al. (2006)	3.41 ± 2.1
		Lean: 1.29 ± .07 (SE) **Obese:** 1.80 ± 0.11 (SE)	Valle M et al. (2005)	
CRP (mg/L)	9-12 years	**Lean:** 0.78 ± 0.34 (boys) 1.30 ± 1.48 (girls) **Obese:** 2.33 ± 1.72 (boys) 2.73 ± 2.08 (girls)	Krekoukia, et al. (2006)	3.61± 2.84
	mean=10.9 yrs	**Obese:** 4.2 ± 0.8	Kelly AS, et al. (2004)	
	11-16 years	**Lean:** 1.24 ± 0.44 **Obese:** 4.84 ± 6.31	Meyer AA et al. (2006)	
	6-9 years	**Lean:** 0.92 ± 0.22 **Obese:** 1.67 ± 0.16	Valle M et al. (2005)	
IL-6 (pg/mL)	9-12 years	**Lean:**1.86 ± 1.42 (boys) .072 ± 0.70 (girls) **Obese:** 1.21 ± 0.50 (boys) 1.12 ± 0.45 (girls)	Krekoukia, et al. (2006)	3.88 ± 2.50
	6-9 years	**Lean:** 1.81 ± 0.24 **Obese:** 2.32± 0.23	Valle M et al. (2005)	
	mean=15	**Lean:** 2.73 ± 0.26 **Obese:**3.16 ± 0.26	Sinaiko (2005)	
Adiponectin (µg/ml)	6-9 years	**Lean:** 11.58 ± 0.63 **Obese:** 9.64 ± 0.49	Valle M et al. (2005)	8.88 ± 2.60
	Mean=15	**Lean:** 15.2 ± 6.2 **Obese:** 11.7 ± 6.1	Sinaiko (2005)	
TNF-α (pg/ml)	Mean =15	**Lean:** 5.90 ± 0.83 **Obese:** 6.56 ± 0.83	Sinaiko (2005)	11.23 ± 5.10
L-arginine (µmol/l)	Mean=12 years	**Healthy Controls:** Range, 80-120	Bennett-Richards et al. (2002)	39.97 ± 14.78
	Pediatric Population	94 ± 29	Morris et al. (2004)	
ADMA (µmol/l)	Mean=8.3 years	**Healthy Controls:** 0.10 ± 0.01	Goonasekera et al. (1997)	.44 ± .16
	Adults	**Healthy Controls:** 0.73 ± 0.06	Fleck at al(2001)	
SDMA (µmol/l)	Mean=8.3 years	**Healthy Control:** 1.18 ± 0.06	Goonasekera et al. (1997)	.33 ± .13
	Adults	**Healthy Controls:** 0.5 ± 0.04	Fleck et al. (2001)	
NOx	Mean=6.9 years	**Healthy Controls:** 11.9 ± 5.9	Goonasekera, Shah, Rees and Dillon (1997)	21.58 ± 10.62
	Adolescents	Range: 15.6-92.8	Choi JW (2004)	
	Adults, age 61	28 ± 3 (SE)	Schmidt RJ et al. (Baylis)	

Figure 2: BMI and blood pressure difference between those with normal endothelial function compared to those with impaired endothelial function. Values represent means and SD.

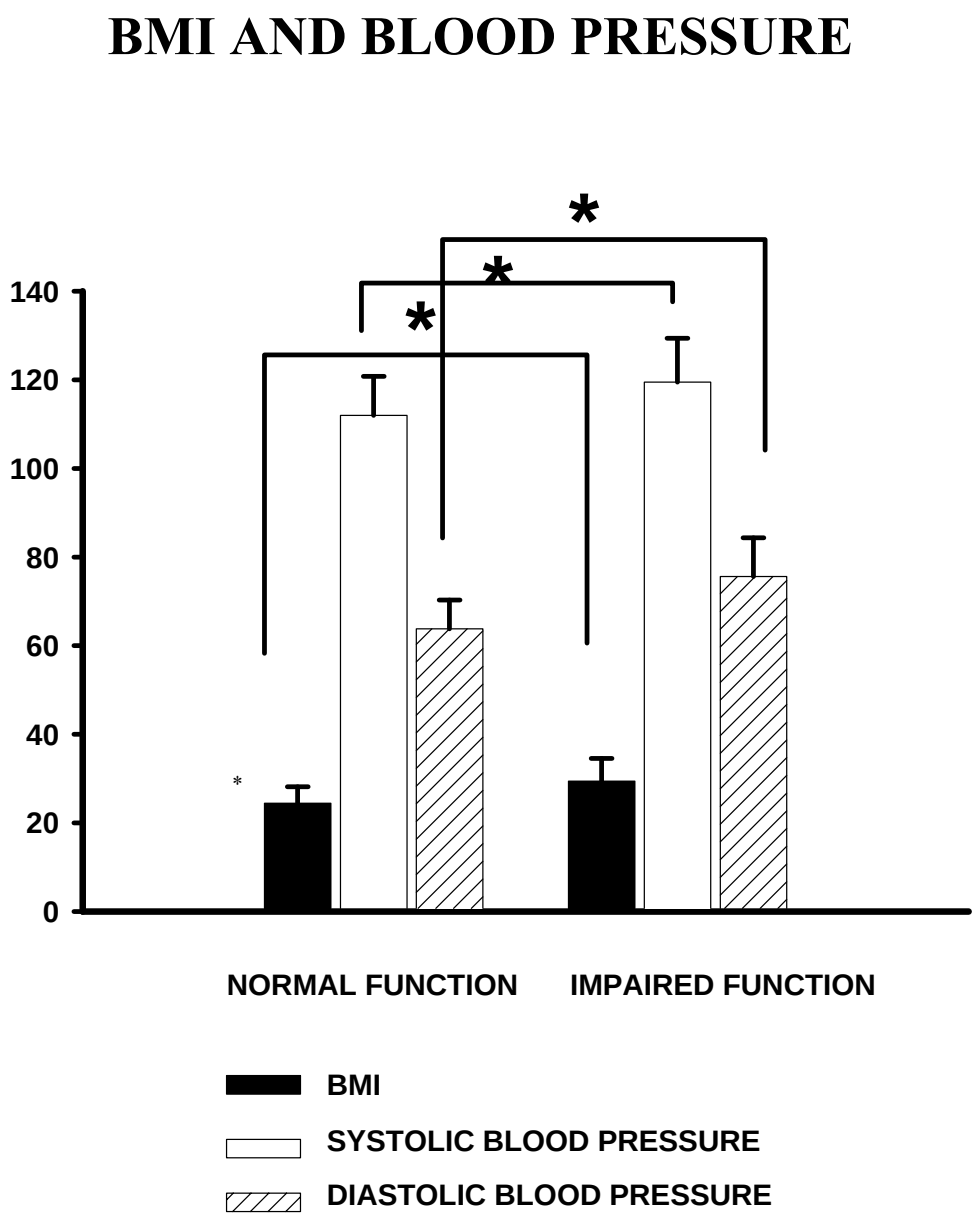

Table 4: Comparison of Physiologic Variables in Children With and Without Metabolic Syndrome

	Metabolic Syndrome Present		Metabolic Syndrome Absent	
	Mean	SD	Mean	SD
Age (years)*	10.54	1.67	9.39	1.80
BMI**	30.09	3.68	23.57	3.81
WC (inches)**	36.88	3.44	23.57	3.81
HC (inches)**	38.83	3.39	33.59	3.68
SBP (mmHg)**	120.55	9.24	111.60	7.49
DBP (mmHg)**	76.16	8.84	66.13	7.46
Resting HR (beats/min)**	80.9	10.08	70.53	6.52
HDL (mg/dl)**	34.67	6.85	41.67	8.52
TRIG (mg/dl)**	126.55	65.20	57.87	17.13
Relative VO2 (ml/kg/min)*	25.18	4.93	29.12	5.82
FMD (% dilation)*	4.54	5.39	8.33	6.27
Adiponectin (μmol/l)**	8.11	1.99	11.58	2.82

*p<.05, **p<.01

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ARTICLE 2:
Effects of an Aerobic Exercise Intervention Using Dance Dance Revolution on Endothelial Function and Other Risk Factors in Overweight Children

ABSTRACT

Objectives To assess whether an aerobic exercise intervention using a total body video game (Dance Dance Revolution^{TM} [DDR]) is effective in improving endothelial dysfunction and other cardiovascular risk factors in overweight children and to explore possible mechanisms for change.

Study design Thirty-five overweight children (BMI $\geq 85^{th}$ percentile, mean age 10.21 ± 1.67 years, 17 females) with documented endothelial dysfunction were assessed for flow-mediated dilation (FMD) of the brachial artery, lipids, insulin, glucose, $NO_2 + NO_3$ (NOx, the stable inert products of NO production), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), l-arginine, aerobic fitness, waist (WC) and hip circumference (HC), and blood pressure. In a subsample, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), and adiponectin were also assessed. Subjects were randomly assigned assigned to either 12-weeks of aerobic exercise (EX) using DDR or to a non-exercising delayed-treatment control group (DTC).

Results Baseline BMI (r= -.370, p<.05); WC (r= -.409, p<.05), resting SBP (r= -.488, p<.01), resting DBP (r= -.473, p<.01), ADMA (r=.411, p<.05), and SDMA (r=.635, p<.05) were all correlated with FMD. There were no baseline differences between EX and DTC groups. Change scores indicated that the EX had a significant improvement in FMD (5.56 ± 5.04 % compared to .263 ± 4.54 %, p=.008), exercise time on the graded exercise test (53.59 ± 91.54 compared to -12.83 ± 68.10 seconds, p=.025), mean arterial pressure (-5.62 ± 7.03 compared to -1.44 ± 2.16 mmHg, p=.05), weight (2.01± 3.38 compared to 5.35 ± 3.97 pounds, p=.017) and relative VO2

(2.38 ± 3.91 compared to -1.23 ± 3.18 mg/kg/min, p=.005) compared to the DTC group. The EX was further divided into those who had an improvement in FMD to greater than eight percent post-intervention (responders) and those who did not achieve FMD of eight percent post-intervention (non-responders). A significant baseline difference existed between the responders and non-responders in both low-density lipoprotein (LDL,p=.021) and total cholesterol (TC, p=.036) values. An ANCOVA (using LDL and TC as covariates) revealed significant differences between the groups from pre- to post-intervention in NOx (non-responders decreased from 22.12 ± 3.52 μmol/l to 17.60 ± 2.61 μmol/l and the responders increased from 21.58 ± 2.85 μmol/l to 23.16 ± 2.11 μmol/l , p=.018) and adiponectin (the nonresponders decreased from 10.56 ± 1.47 μg/ml to 10.13 ± 1.32 μg/ml and the responders increased from 7.80 ± 1.34 μg/ml to 8.43 ± 1.21 μg/ml, p=.03).

Conclusions In overweight children with endothelial dysfunction, 12 weeks of aerobic exercise using DDR improved FMD, aerobic fitness, and mean arterial pressure, and this improvement occurred without the subsequent improvement in inflammatory markers or markers for NO production. An improvement in FMD to greater than eight percent was seen in only 13 of the EX subjects. When comparing the responders to non-responders, the non-responders had significantly higher LDL and TC values at baseline. When both these variables were controlled for, improvements in both NO production and the inflammatory profile were seen in the responders compared to the non-responders, indicating an important role of elevated lipids on vascular function. The comprehensive results of this study document the need to assess the complex relationships between obesity, endothelial function, inflammation, exercise intensity and duration, and gender in a larger sample of overweight children.

INTRODUCTION

The prevalence of childhood obesity has increased rapidly over the last three decades, and is now at epidemic proportions with more than 22 percent of children in the United States classified as overweight[1]. Childhood obesity strongly relates to early development of atherosclerosis and type II diabetes[2]. Arterial endothelial dysfunction is an early abnormality present in both disease processes, and is a marker of damage prior to plaque formation[3]. Several studies have identified impaired flow-mediated dilation (FMD), as a validated measure of vascular endothelial function[4], in overweight children[5-7]. A chronic proinflammatory state associated with obesity, insulin resistance, and presence of the metabolic syndrome generated by genetics, lifestyle factors or a combination of both, may contribute to the endothelial dysfunction seen in these obese individuals[8-9].

Lack of physical activity is instrumental in the development of CVD and may prove to be the intermediate between obesity, inflammation, insulin resistance and premature atherosclerosis[10]. Exercise has been shown to improve vascular function in overweight children, with or without concurrent weight loss[5-7]. Shear stress mediated upregulation of nitric oxide (NO)-synthase expression, resulting from increased blood flow across the endothelium, decreased free radical degradation of NO[11], and increased production of superoxide dismutases are the two most likely mechanisms responsible for the improvement in endothelial function associated with increased physical activity[5]. In addition to the impact exercise has on vascular function, a number of recent studies have also shown that aerobically fit individuals tend to have a better profile of inflammatory markers[12-14].

Lack of time, body consciousness (lower levels of self-esteem and self-confidence), and various environmental barriers (e.g., lack of equipment, access to exercise facility) are among the

most common barriers to physical activity in overweight children[15-16]. Previous studies examining the effects of exercise on the cardiovascular profiles of overweight children have not taken these perceived barriers to exercise into consideration, and have taken place in a structured laboratory environment where bicycle ergometry or circuit training are used as the mode of exercise[5-7]. In an attempt to overcome some of these known barriers an innovative home-based aerobic exercise program, using an interactive video game, Dance Dance Revolution (DDR), was chosen as the exercise modality for this project. The purpose of this study was to determine whether Dance Dance Revolution™ (DDR) was effective in improving endothelial dysfunction and other cardiovascular risk factors in overweight children and to explore possible mechanisms for change in vascular function.

METHODS

Subjects and screening measures

Thirty-five overweight (body mass index [BMI] ≥ 85th percentile for age and gender, 17 females) subjects between the ages of seven to12 years were recruited for the study. Subjects had to be insured by West Virginia Public Employee Insurance Agency or Mountain State Blue Cross/Blue Shield to be eligible. Recruitment was accomplished through website and newsletter advertisements of the participating insurers. A telephone interview was used to screen children for the study, which included a parental report of height and weight, so that an estimate of current BMI could be calculated. All participants and a parent/guardian provided informed assent/consent prior to study participation. Participation in the study was voluntary and subjects were free to withdraw at any time. The research protocol was approved by the Institutional Review Board for the Protection of Human Subjects at West Virginia University.

This study was a component of a larger study on overweight children. Criteria for inclusion in this study required that subjects have impaired flow-mediated dilation (FMD), defined as having less than an eight percent FMD response to five minutes of blood flow occlusion in the brachial artery.

Study Design

Upon entrance into the study, subjects were randomly assigned to a 12-week exercise (EX, n=23) or delayed treatment control (DTC, n=12) group. DTC subjects were instructed to maintain current levels of physical activity for the initial 12-week period after which they received the 12-week exercise intervention. All experimental variables were measured at baseline and 12-weeks in both groups.

During the 12-week study period, subjects in both groups were asked to fill out daily physical activity logs. Parents were requested not to modify the dietary behaviors of their children during the 12-week study period. Parents were asked to complete three-day diet diaries for their children during the first week (including two weekdays and one weekend day) of the study and for three days during week 12. Diet diaries confirmed that no significant changes to dietary intake were made during the study period.

Anthropometric Measurements and Questionnaires

Body weight (pounds) and height (inches) were measured, and BMI was calculated using the following equation, BMI=[weight in pounds/(height in inches)^2*703][17] . Waist circumference was measured in inches at the smallest area of the waist, and hip circumference was measured in inches at the largest area of the hips and buttocks. A waist/hip ratio was

calculated. Health history was obtained via interview and family history of CVD, diabetes, dyslipidemia and obesity were also recorded. Tanner growth staging for secondary sexual development was estimated by parental completion of the Pubertal Development Scale[18].

Vascular Testing

Participants arrived at the Human Performance Laboratory at West Virginia University Health Sciences Center after a 12 hour fast and after abstaining from exercise and caffeine for 24 hours. Upon arrival, subjects were instructed to rest for 15 minutes. Electrodes were placed on the subject's chest to monitor heart rate by electrocardiogram and a resting blood pressure was taken in the subject's left arm. A pediatric-sized blood pressure (with a 10-inch extension) cuff was placed loosely around the top portion of the subject's right arm. A pediatric cuff was used to ensure that the width of the cuff would allow ample space for measurement. Brachial artery resting diameter was assessed by ultrasound using a 10-MHz high-frequency linear array probe (ATL UM-9 HDL Ultrasound System), approximately five to ten centimeters proximal to the elbow. When a clear picture of the brachial artery was obtained, the arm was marked, so that subsequent measurements could be repeated at the same location. Subjects were asked to lie quietly for an additional ten minutes. Another resting scan was taken after the 10-minute rest period, and the blood pressure cuff was immediately inflated to 50 mmHg above resting systolic blood pressure and remained at this pressure for five minutes. A third scan was taken beginning ten seconds before cuff removal and was continued for 90 seconds after the cuff was removed. The procedure produced minimal discomfort and was well tolerated by all subjects.

All ultrasound scans were performed by a single trained vascular sonographer. Images were stored on a VHS tape for off-line analysis. The arterial diameter was measured at a fixed

distance from an anatomic marker. Measurements were taken from the anterior to the posterior `m' line at end diastole, incident with the R wave on a continuously recorded ECG.

A second trained, blinded observer repeated the measurement of vessel diameters from the VHS tapes. The inter-observer coefficient of variation for brachial artery diameter was 2.17 ± 1.97%. The correlation coefficient between paired measurements was .973 ($p<.01$) for diameter.

Assessment of Blood Chemistry

A fasting blood sample was collected to determine concentrations of lipids (total cholesterol TC, high-density lipoprotein cholesterol [HDL], and triglycerides), insulin, and glucose. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation[19]. Plasma glucose and lipid levels were measured with the use of an Autoanalyzer (SYNCHRON LX Systems, Beckman Coulter, Inc., Fullerton, CA). Plasma insulin levels were quantified using an immunoassay system, by (Warde Medical Laboratory, Ann Arbor, Michigan). Insulin sensitivity was calculated using the homeostasis model assessment (HOMA)[20], a mathematical estimate of insulin sensitivity based on fasting glucose and insulin concentrations. The HOMA index yields an equation where insulin resistance = [fasting insulin (μIU/ml)*fasting glucose (mmol/l)]/22.5.

An additional tube of blood was drawn to assess markers of NO production and inflammation (inflammatory markers were measured on a sub-sample of subjects). Fasting plasma samples were aliquotted and snap frozen at -80º C after centrifugation (2500 rpm, 15 minutes). Baseline and 12-week samples were run simultaneously on the same plate. Samples were thawed immediately before analysis.

The following markers of NO production were measured: $NO_2 + NO_3$ (NOx, the stable inert products of NO production), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and l-arginine. NOx concentrations in plasma were measured using the Griess assay after conversion of NO_3 to NO_2 with the NO_3 reductase enzyme, as described previously by Suto et al. Plasma l-arginine was measured by reverse-phase HPLC with precolumn derivatization and fluorescent detection using modification of the ACCQ Tag system for amino acid analysis (Waters, Milford, MA). ADMA and SDMA levels were measured in plasma using reverse-phase HPLC with the Waters AccQ-Fluor fluorescent reagent kit. Methodology used was adapted from Heresztyn et al., 2004[30].

The following pro-inflammatory markers were measured on a subsample of subjects: tumor necrosis factor alpha (TNF-α, n=23), interleukin-6 (IL-6, n-23), C-reactive protein (CRP, n=20), and adiponectin (n=20). Commercially available radioimmunoassay assay (ELISA) kits (Quantikine HS, R&D systems, Minneapolis, MN) were used to measure plasma IL-6 and TNF-α. According to R&D systems detection limits are less than 0.094 and 0.18 pg/ml for IL-6 and TNF-α, respectively. Adiponectin levels were also measured by ELISA (Linco Laboratories, St. Charles, MI); detection limits for these kits were less 0.78 ng/ml. High-sensitivity CRP concentrations were measured by radioimmunoassay (Life Diagnostics, Inc., West Chester, PA), and the minimum detectable concentration is estimated to be 0.1 mg/l.

Assessment of Aerobic Exercise Capacity

Aerobic fitness was quantified as peak oxygen uptake (VO_2peak), and was assessed with a ramped intensity protocol on a stationary cycle ergometer, starting at 0 Watts and increasing 15 Watts every minute until exhaustion. The subjects were required to maintain a pedaling speed of

50 revolutions per minute (rpm) throughout the test. Expired oxygen and carbon dioxide concentrations and volumes were collected and analyzed using a MedGraphics metabolic cart (MedGraphics, St. Paul, Minn).

Electrodes were placed in the standard position for a four-lead ECG, and a resting blood pressure was measured prior to the exercise test. Heart rate was continuously monitored. Subjects were familiarized with the ergometer and taught how to estimate perceived exertion prior to beginning the test. Subjects were asked to give a rating of perceived exertion every two minutes using the ten-point Child's Effort Rating Table (CERT). CERT is similar to the Rating of Perceived Exertion Scale (RPE) that is used with adults. It differs from the RPE scale in that it has fewer possible responses, a range of numbers one to ten, which is more familiar to children than six to 20[31]. Blood pressure was measured and recorded every minute of the test and for five minutes following cessation of the exercise. The test was stopped when the subject could no longer maintain a pedaling speed of 50 rpm or if the subject asked to discontinue.

Aerobic Exercise Training Protocol

DTC subjects were instructed to maintain current levels of physical activity throughout the 12-week study period. The EX subjects attended a training session on a home-based aerobic exercise intervention using DDR, a total-body active video game. EX subjects were required to exercise using DDR five days per week. The progression of the exercise protocol over the 12 weeks is depicted in Table 1.

Subjects from both groups were asked to record daily physical activity by means of a self-report weekly physical activity log. All subjects wore a pedometer seven days per week, and were asked to record accumulated daily steps as well daily screen-time (watching TV,

playing non-active video games, or using the computer for non-homework activities). The EX subjects were asked to record daily DDR use and the numbers of steps they took while playing DDR. Subjects were instructed to place their activity logs in a pre-addressed envelope and mail them to the investigators at the end of each week.

Analysis of Data

Descriptive data were computed for variables of interest and were expressed as mean values ± standard deviation (SD) unless specified otherwise. Between group analyses were compared by Mann Whitney and independent t-tests, as appropriate. Test selection was based on evaluating the variables for normal distribution. Univariate associations between the study variables were analyzed by calculating the Pearson's correlation coefficients (r). To assess differences between EX and DTC groups following the exercise treatment, change scores were calculated (post-intervention value minus pre-intervention value) for each variable. Independent t-tests or Mann-Whitney U tests were completed to distinguish differences between groups in relation to change scores. The EX group was further subdivided into two groups based on whether they reached a FMD greater than eight percent after the 12 week exercise program (responders) and those that did not (non-responders). Baseline between group analyses were compared by Mann Whitney and independent t-tests, as appropriate. Significant differences were found between the two groups at baseline; therefore an analysis of covariance was used to determine whether there were differences between the groups following the exercise treatment.

Statistical analyses were performed using SPSS 15.0 software for Windows (SPSS for Windows; Chicago, IL-USA). Results are expressed as mean ± standard deviation (SD). Statistical significance was inferred at a two-tailed p-value of $< .05$.

RESULTS

Baseline Measurements

In addition to impaired endothelial function, the majority of the subjects had other risk factors for both CVD and type 2 diabetes (Table 2). At baseline, 31 (88.6 percent) of the subjects presented with a clustering of these risk factors and were classified as having Metabolic Syndrome (MS), according to the modified pediatric criteria . Plasma levels of all pro-inflammatory markers measured at baseline in the current study (CRP, IL-6, TNF-α, and adiponectin) were markedly abnormal compared to previous studies conducted in healthy, normal-weight pediatric subjects[21-24]. In addition, plasma levels of l-arginine were markedly lower than those previously reported in healthy pediatric subjects[25-26].

There were no baseline differences between the exercise and delayed-treatment control group for any of the measured descriptive variables. Baseline characteristics of the subjects are provided in Table 2. The mean FMD was 3.99 ± 3.14% for EX and was 2.69 ± 3.61% for DTC. Baseline and post-intervention data for both the EX and DTC groups are presented in Table 3.

Because improvement in FMD was the main outcome of the study, factors that predicted FMD were determined. When EX and DTC groups were combined at baseline, the following variables were found to be significantly correlated with FMD: BMI ($r=-.370$, $p<.05$); WC ($r=-.409$, $p<.05$), resting SBP ($r=-.488$, $p<.01$), resting DBP ($r=-.473$, $p<.01$), ADMA ($r=.411$, $p<.05$), and SDMA ($r=.635$, $p<.05$). When all variables were placed into a multiple regression, enter method, significance was achieved with an $r=.624$, $R^2=.390$, $p<.039$. When these same variables were placed into a stepwise multiple regression, only SBP remained a significant predictor of FMD at baseline, with a $r=.502$, $R^2=.252$, $p<.003$.

Compliance to Exercise Intervention

Compliance was analyzed two ways: 1) number of days DDR was played each week 2) number of days DDR was played each week with the minimum number of songs completed. Seventy-five percent of the subjects in the EX group were compliant (defined as exercising for at least five days per week), and an additional 15 percent were categorized as somewhat compliant (defined as exercising for at least three days per week) when looking at numbers of days DDR was played each week. When incorporating required songs per week into compliance, 68 percent of EX group were compliant (defined as exercising five days per week for the required number of songs) and an additional 14 percent were categorized as somewhat compliant (defined as exercising for at least three days per week for the required number of songs). When comparing step-count information from the pedometers between the EX and DTC groups, neither total weekly steps or total steps taken over the 12-week intervention period differed between the two groups.

Effects of Exercise Intervention: EX Group compared to DTC Group

To determine whether there were differences between the EX and DTC groups after the exercise intervention, change scores were calculated (post-intervention value minus baseline value) for each variable. The EX had a significant improvement in FMD (Figure 1) (5.56 ± 5.04 % compared to .263 ± 4.54 %, p=.008), exercise time on the graded exercise test (53.59 ± 91.54 compared to -12.83 ± 68.10 seconds, p=.025), mean arterial pressure (-5.62 ± 7.03 compared to -1.44 ± 2.16 mmHG, p=.05), weight (2.01± 3.38 compared to 5.35 ± 3.97 pounds, p=.017) and relative VO2 (2.38 ± 3.91 compared to -1.23 ± 3.18 mg/kg/min, p=.005) compared to the DTC group (Figure 2). The change scores for height did not differ between the groups,

while the change scores in weight did, indicating that although the exercise group did not show a significant change in BMI from baseline to 12 weeks, they gained less weight than the control group over the intervention period.

Responders compared to Non-responders:

Thirteen of the subjects in the EX group responded to the exercise intervention with a post-intervention FMD greater than eight percent while the other ten subjects did not achieve a FMD of eight percent (figure 3a). Therefore, subjects in the EX group were further categorized into two groups: responders (those that achieved an FMD of eight percent or greater post-intervention) and non-responders (those that did not achieve a FMD of eight percent post-intervention). Compliance to exercise did not differ between the groups in total number of days DDR was played over the entire 12-week intervention period. In fact, the non-responders played DDR for significantly more days in weeks 10 (5.90 ± 1.19 days compared to 3.50 ± 2.81 days, p=.021) and 11 (5.60 ± 1.35 days compared to 3.41 ± 2.81 days, p=.031) than the responders. Pedometer data showed that there were significant differences between the non-responders and responders in both total number of steps taken over the 12 week period (708,611 ± 395,868 steps compared to 359,513 ± 190,599 steps, p=.011) and total number of steps taken while playing DDR (169,563 ± 66,299 steps compared to 105,963 ± 48,174 steps, p=.025), with the non-responders taking significantly more steps in both categories.

In addition to the difference in FMD and compliance to exercise between the groups, there was a significant difference at baseline between the responders and non-responders in both LDL (p=.021) and TC (p=.036) values. The responders had a mean LDL value of 99.54 ± 22.01

mg/dl and a mean TC of 157.46 ± 17.65 mg/dl, while the non-responders had a mean LDL value of 126.30 ± 29.46 mg/dl and a mean TC value of 185.90 ± 4.25 mg/dl (figure 3b).

Gender distribution between the groups was similar, with five females and five males in the non-responders group and six females and seven males in the responders group.

Change scores indicated that the non-responders had greater decreases in the following variables compared to the responders: resting diastolic blood pressure (-9.10 ± 7.57 mmHg compared to 1.23 ± 9.39 mmHg respectively, p=.008), mean arterial pressure (-8.4 ± 4.19 mmHg compared to -1.13 ± 8.66 mmHg respectively, p=.042), and BMI (-2.68 ± 4.92 compared to -.165 ± 1.06 respectively, p=.006).

Because there was a significant difference in TC and LDL between the groups at baseline, a repeated measures ANCOVA was used to compare changes between and within groups from baseline to 12 weeks. There was a significant difference in NOx values between the groups from pre- to post-intervention when covaried by both TC (p=.018) and LDL (p=.018), with the non-responders decreasing from 22.12 ± 3.52 μmol/l to 17.60 ± 2.61 μmol/l and the responders increasing from 21.58 ± 2.85 μmol/l to 23.16 ± 2.11 μmol/l (expressed as mean ± standard error), figure 4a. This difference remained when analyzing NOx alone and when normalizing NOx by creatinine. There was also a significant difference in adiponectin values between the groups from pre- to post-intervention when covaried by TC (p=.043) and LDL (p=.030), with the nonresponders decreasing from 10.56 ± 1.47 μg/ml to 10.13 ± 1.32 μg/ml and the responders increasing from 7.80 ± 1.34 μg/ml to 8.43 ± 1.21 μg/ml (expressed as mean ± standard error), Figure 4b. Triglyceride levels were also significantly different between the groups when covaried by both TC (p=.002) and LDL (p=.026), with the nonresponders

increasing from 93.47 ± 15.93 mg/dl to 94.55 ± 14.21 mg/dl and the responders decreasing from 106.61 ± 14.22 mg/dl to 96.82 ± 12.69 mg/dl (expressed as mean ± standard error).

Pearson Moment Correlations were used to determine which variables post-intervention were significantly associated with FMD post-intervention in the EX group only. Both LDL(r= -.479, p=.024) and TC (r=-.477, p=.025) were significantly correlated with post FMD. When placed into a stepwise multiple regression, LDL remained the only predictor, R-.479, r2=.230, p=.024.

Gender Data:

Differences between genders at baseline and as a result of exercise intervention

Eighteen male subjects and 17 female subjects participated in the study. Significant differences between genders were found at baseline. Females had a higher tanner stage (2.3 ± .61 compared to 1.78 ± .65, p=.01), lower SBP (115.00 ± 7.11 compared to 121.27 ± 9.69 mmHg, p=.037), lower mean blood flow (252.31 ± 103.41 compared to 341.54 ± 111.72 ml/min, p=.02) and peak blood flow (444.93 ± 169.99 compared to 596.74 ± 158.75 ml/min, p=.01) than males. Female exercisers had a significantly greater reduction in SBP (-6.71 ± 6.44 – vs 3.22 ± 9.46 mmHg, p=.05) compared to the males exercisers.

By Gender-Results of Exercise Intervention: EX compared to DTC

In female subjects there were significant differences in change scores between the exercise and DTC control groups in regards to IL-6 (p=.048, n=11), with the EX group decreasing from 3.78 ± 1.88 pg/ml to 2.63 ± 1.41 pg/ml and the DTC increasing from 3.23 ± 1.66 pg/ml to 4.15 ± 1.80 pg/ml. The change scores for relative peak VO2 (p=.02, n=17) in the

females subjects was also different between the EX group (26.15 ± 4.76 to 28.22 ± 4.72 ml/kg/min) and the DTC group (26.83 ± 3.14 to 24.78 ± 2.74 ml/kg/min). Change scores also indicated that the EX group (138.80 ± 34.90 to 141.38 ± 34.68 pounds) gained significantly less weight (p=.009, n=17) than the DTC (142.02 ± 14.59 to 149.01 ± 15.46 pounds). Change scores in the female EX group compared to the DTC for DBP (p=.062, n=17), FMD (p=.060, n=17) and exercise time (p=.062, n=17) showed a trend toward significance.

In the male subjects, the only significant difference in change scores between the EX (38.44 ± 15.68 to 32.08 ± 10.07 μM) and the DTC (39.20 ± 10.81 to 49.58 ± 12.25 μM) groups was for l-arginine (p=.027, n=17).

DISCUSSION:

The principle finding of this study is that 12 weeks of aerobic exercise, using DDR, significantly improved endothelium-dependent vasodilation of the brachial artery in overweight subjects compared to overweight control subjects in a delayed treatment group. Flow-mediated dilation of the brachial artery is largely NO-dependent, and exercise interventions have been shown to improve NO-dependent vascular function in adults with coronary artery disease, congestive heart failure, hypercholesterolemia, and diabetes[27-30]. This improved vascular function is likely due to the generalized impact of vessel wall shear stress, up-regulation of eNOS[32], and bioavailability of NO. Several recent studies[5-7,24] have documented that exercise also improves vascular function in overweight children, and this study is consistent with these findings. Watts et al.[5] showed that an impairment was evident in overweight children as young as six years of age. The children in the current study ranged in age from seven to 12 years of

age, and in agreement with Watts et al.[5], vascular dysfunction was evident in overweight children as young as seven years.

In addition to an improvement in vascular function, the exercise subjects also showed an improvement in mean arterial pressure, aerobic capacity, exercise time, and gained less weight than the control group. Watts et al.[5] also showed improvement in aerobic capacity similar to the current findings, and Meyer et al.[24] were also able to elicit a reduction in both body weight and blood pressure through exercise. Our study adds to the limited evidence that increased physical activity decreases weight gain and improves other health outcomes in overweight children and adolescents.

Due to ethical considerations, we did not administer nitroglycerine, which is commonly used to assess endothelium-independent vasodilation. Because of this limitation, we can only assume that the improvement in FMD as a result of a 12-week exercise intervention was endothelium-dependent.

While an improvement in FMD was elicited in the exercise group compared to the control group, other expected changes (e.g., improvements in lipids, improvement in pro-inflammatory markers) as a result of the exercise intervention were not apparent. Therefore, we further subdivided the EX group by post-intervention FMD scores to determine why the intervention had a greater impact on FMD in some EX subjects compared to others. Thirteen of the exercising subjects achieved a post-intervention FMD greater than eight percent (responders) while the other ten did not (non-responders). This apparent difference in vascular response to exercise could be the result of the non-responders not adhering to the exercise protocol as well as the responders. However, when compliance was measured by total days, total songs or time over the intervention period, there was no difference between the two groups. Compliance was then

further analyzed on a weekly basis, and the non-responders were found to play DDR for significantly more days in weeks ten and 11 than the responders. Although this is self-reported data and cannot be confirmed, it appears that the non-responders exercised as much or more than the responders. This is further supported by the fact that total steps taken over the intervention period as measured by pedometer counts and time on the graded exercise test post-intervention were significantly higher in the non-responders compared to the responders.

MAP, DBP and BMI were significantly less in the non-responders compared to the responders after exercise, which may indicate that the responders exercised at a greater intensity. Although we did not directly measure exercise intensity, the subjects recorded pedometer counts during DDR exercise periods. The non-responders took 63,600 more steps during DDR exercise periods over the 12-week intervention period. Since the subjects exercised for a similar amount of time, this suggests that the non-responders performed exercise at a higher intensity. In a recent study, Goto et al.[33] demonstrated that 12 weeks of moderate exercise, but not mild or high intensity exercise, augmented endothelium-dependent vasodilation in healthy adult male subjects. The high-intensity exercise was shown to increase the plasma concentration of 8-hydroxy-2'-deoxyguanosine and malondialdehyde-modified low density lipoprotein, both markers of oxidative stress. Bergholm et al.[34] reported that three months of high intensity training using running as the exercise modality, reduced endothelium-dependent function, and that this reduction was significantly correlated with improvement aerobic capacity. In addition, these researchers found that neither LDL cholesterol nor LDL particle size changed significantly during the intervention period. Taken together, these studies suggest that high-intensity exercise induces oxidative stress, and can adversely effect vascular function.

Both at baseline and post-intervention, LDL and TC were significantly higher in the non-responders compared to the responders. At post-intervention, LDL and TC were the only variables significantly correlated (inversely) with FMD when including all exercising subjects (combination of both the responders and non-responders). Al-Benna et al.[35] also found a significant relationship between LDL cholesterol levels and endothelium-dependent vasodilation in adult subjects with known coronary artery disease. Similar findings were reported in a study of 119 healthy subjects in which the significant predictors of endothelium-dependent vasodilation were age, total cholesterol, and LDL cholesterol[36]. Oxidized LDL has been shown to interfere with the formation of NO and to inactivate NO directly[37]. Further oxidized LDL has been shown to be cytoxic to endothelial cells[37]. Endothelial cells exposed for prolonged periods of time to LDL or oxidized LDL exhibit decreased NO production and a parallel increase in superoxide brought about through the uncoupling of eNOS[38]. Although we did not measure oxidized LDL directly in the current study, LDL and oxidized LDL are highly correlated.

Significant differences found between groups in NOx values from pre- to post-intervention, revealed that responders had higher NOx values than non-responders at post-intervention. These results indicate that elevated LDL levels may alter nitric oxide bioavailability resulting in a deleterious effect on FMD. It was notable that there were no differences in plasma arginine, ADMA or the arginine:ADMA ratio, which regulates NO production. Also, although oxidative stress has been reported to increase ADMA via both increased synthesis and decreases catabolism[40-41], there were no differences between the responders and non-responders (presumed to have increased oxidative stress).

A significant difference was found between the groups in adiponectin levels from pre- to post-intervention when covaried by both TC and LDL, with the responders having higher

adiponectin levels post-intervention. These results support the argument that adiponectin helps modulate endothelial function. Previous studies have shown that adiponectin levels affect vascular function by stimulating the production of NO[42] and by suppressing adhesion molecule expression in vascular endothelial cells[43]. Several clinical studies have shown that hypoadiponectinemia correlated with endothelial dysfunction[44-47]. Adiponectin circulates in plasma in three forms: a trimer, a heximer, and a multimeric high-molecular weight form. There is some controversy in the literature regarding which form is biologically active. In the present study we measured total adiponectin levels, and our results support the work of Okui et al.[48] that showed an independent relationship between total adiponectin concentrations and endothelium-dependent vasodilation.

Because there were several significant differences between genders at baseline, analyses were conducted on each gender separately. A significant reduction in IL-6 was observed in the girls who exercised compared to the girls who did not. Some studies have shown that regular exercise decreases levels of IL-6 in adults[49-50]. Data on the effect of aerobic exercise on IL-6 values in children is very limited, but Nassis et al.[51] reported that IL-6 levels remained unchanged in a group of obese girls who were aerobically trained three days per week for a 12 week period. These girls did not lose weight. In the current study, the children exercised five days per week and there was a significant reduction in weight gain in our study. Gallisstil et al.[52] had children exercise three times per day for a three week period, and participate in a weight reduction program. They reported improved body composition induced by diet and physical activity resulted in decreased levels of IL-6 in obese children. It is possible that both decreased weight gain and physical activity are necessary to elicit a subsequent decrease in IL-6 levels in overweight females. It is unclear whether exercise alone may improve IL-6 levels without

weight loss in obese girls. In our study girls in the exercise group gained less weight than those in the DTC group.

When looking at male subjects only, the only difference in change scores found between the EX and the DTC groups was that the DTC had an increased L-arginine concentration at post-intervention compared to baseline, while the EX group had a decreased level. NO concentrations can be affected by the availability of the precursor molecule, l-arginine. The decreased levels seen in the EX group compared to the DTC group may be due to the fact that shear stress increases the velocity of the l-arginine transmembrane transport system and/or increased utilization. Parnell et al.[53] examined the effect of exercise on the l-arginine transport rate in patients with congestive heart failure, and documented that exercise training increases L-arginine transport, while at the same time there was a reduction in plasma L-arginine levels from pre- to post-intervention[53]. L-arginine levels of both the DTC and EX subjects were considerably lower than those reported for normal weight, healthy adult and children subjects[26,54].

There are several important factors that make this study unique compared to other exercise studies in overweight children. First, we utilized DDR as the exercise mode, whereas the other studies used more traditional modes of exercise such as bicycle ergometry and circuit regimens in laboratory settings. Our exercise intervention was home-based making it possible for children in rural areas without access to exercise facilities to participate. This was also the first study in children to examine the effect of exercise on multiple pro-inflammatory markers.

Future studies will be required to assess the effect of exercise intensity while playing DDR on endothelial function and markers for oxidative stress. The DDR software allows progression from low to high intensity, therefore future studies should require subjects to document level of play along with a more accurate measurement of intensity through the use of

accelerometers instead of pedometers. The addition of accelerometers in future studies will also be important due to the fact that DDR uses both a forward-backward and left-right movement. The accuracy of the pedometers detecting the movement in the left-right plane is questionable. The addition of accelerometers will allow for a more accurate measurement of exercise intensity as well as a more stringent measurement of exercise compliance.

We did not show a significant difference in steps taken between the EX and DTC over the intervention period. Future studies should assess physical activity levels of both groups prior to the intervention period. The need for a pre-intervention assessment of activity level is two-fold: 1) we were unable to determine whether the EX and DTC group differed in daily steps taken prior to the intervention period, even though we determined that the groups did not differ in regards to aerobic capacity; and 2) we can not determine if the mere act of wearing the pedometer in the DTC group had an intervention-effect in itself.

Several studies have shown that oxidized LDL not only interferes with the formation of NO, but that it can also directly inactivate NO[32,37]. While LDL levels are strongly correlated with oxidized LDL values, we did not directly measure oxidized LDL. Due to the fact that LDL and TC were the only distinguishing factors between the responders and non-responders, future studies should include the direct measurement of oxidized LDL to determine its effect on NO production and its relationship to other pro-inflammatory markers in obese children.

In conclusion, the current study shows that an innovative home-based exercise intervention does elicit improvement in endothelial function of overweight children without concurrent improvement in inflammatory markers or NO production. Based on the comprehensive results of this study, further work should be initiated using a larger sample of

overweight children to assess the complex relationships between obesity, endothelial function, inflammation, exercise intensity and duration, and gender.

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Table 1: Aerobic Exercise Training Protocol

Week of Intervention	Required Exercise Time (minutes)	Required Number of Songs
1	**10**	**8**
2	**15**	**12**
3	**20**	**16**
4	**25**	**20**
5-12	**30**	**24**

Table 2: Health Profile of Subjects at Baseline

Risk Factor	# of Subjects that Presented with Risk Factor	% of Subjects that Presented with Risk Factor
Family History of Heart Disease	29	83%
Family History of Diabetes	25	71%
Family History of Obesity	32	91%
Family History of Dyslipidemia	27	77%
Elevated SBP	19	54%
Elevated DBP	17	49%
Low HDL	23	66%
Elevated LDL	18	51%
Elevated Insulin	8	23%

Table 3: Descriptives, Exercise and Delayed Treatment Control Groups Pre- and Post-Intervention

	EX Pre	SD	EX Post	SD	DTC Pre	SD	DTC Post	SD
Height (cm)	148.9	10.4	150.3	10.4	147.1	7.5	149.1	7.0
Weight (kg)	62.5	15.3	63.4	15.5	69.5	17.0	71.9	16.6
BMI	27.9	4.8	27.8	4.7	31.8	5.0	32.1	4.9
WC (in)	34.7	4.8	34.4	4.6	37.4	5.4	36.6	7.0
HC (in)	36.9	3.9	36.4	3.5	39.3	4.6	38.5	5.7
SBP (mmHg)	117.7	9.1	111.4	8.0	119.2	9.0	114.2	5.7
DBP (mmHg)	74.1	9.0	70.9	8.4	76.7	5.7	71.5	7.5
MAP(mmHg)	90.9	6.3	84.4	7.3	88.7	8.3	85.7	6.4
HR (beats/min)	77.4	9.5	76.8	10.0	81.6	9.8	77.8	9.0
TC (mg/dl)	169.8	32.8	160.81	31.6	163.4	24.6	159.0	19.4
LDL (mg/dl)	111.2	28.3	105.8	27.9	109.0	20.4	106.1	16.7
HDL (mg/dl)	37.0	8.9	36.0	8.2	35.6	7.0	35.6	6.4
Trig (mg/dl)	98.3	61.7	98.4	51.3	94.5	44.3	96.8	37.4
Gluc (mg/dl)	91.0	5.8	91.4	4.4	89.6	4.1	91.6	2.8
Insulin (µU/L)	14.9	8.8	14.2	9.5	16.1	11.4	22.1	21.1
HOMA	3.4	2.1	3.2	2.1	3.8	3.4	4.9	4.7
RelVO2 (ml/kg/min)	27.1	5.4	29.5	4.5	25.6	5.4	24.3	4.8
AbVO2 (ml/min)	1724.0	514.7	1778.9	372.0	1655.6	253.3	1695.1	264.5
Ex Time (s)	490.8	137.6	540.3	103.9	454.9	109.7	442.1	114.7
Bas Diam (cm)	.313	.05	.32	.04	.328	.03	.33	.035
FMD (%)	4.0	3.2	9.6	4.7	2.7	3.6	3.0	4.0
L-arginine (µM)	39.5	15.5	37.1	10.7	40.9	13.9	43.5	13.2
ADMA (µM)	.41	.13	.41	.14	.49	.21	.49	.19
SDMA (µM)	.32	.11	.31	.11	.36	.17	.38	.14
NOx (µM)	21.8	10.6	20.9	7.1	21.2	11.2	28.4	22.5
CRP (µg/ml)	3.1	2.9	2.6	7.1	4.7	2.7	4.8	2.6
IL-6 (pg/ml)	3.6	2.3	2.5	1.2	4.6	3.0	4.3	2.5
TNF-α (pg/ml)	11.3	4.8	11.2	4.5	11.1	6.1	14.1	5.6
Adiponectin (µg/ml)	9.07	2.89	9.16	2.73	8.51	2.10	8.50	2.29

Figure 1: FMD pre- and post-intervention, EX vs. DTC. Values represent mean and SD.

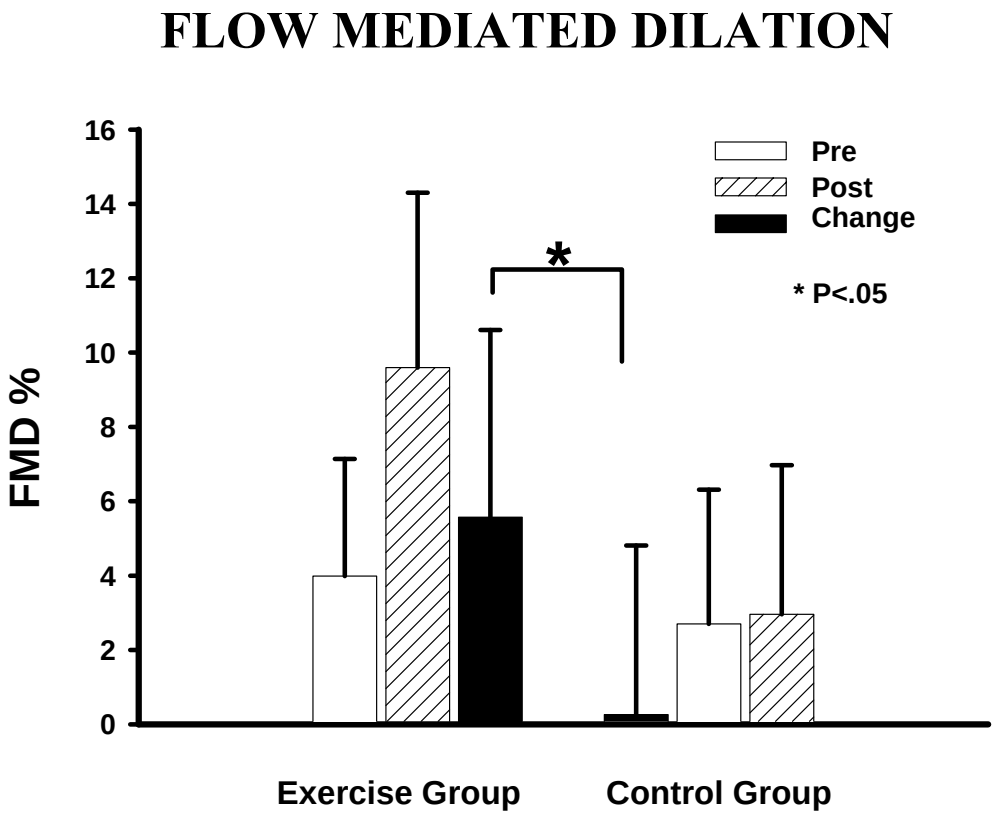


Figure 2: Changes score comparison between exercise group and control group. Values represent means and SD. Significant difference between groups were found, p<.05.

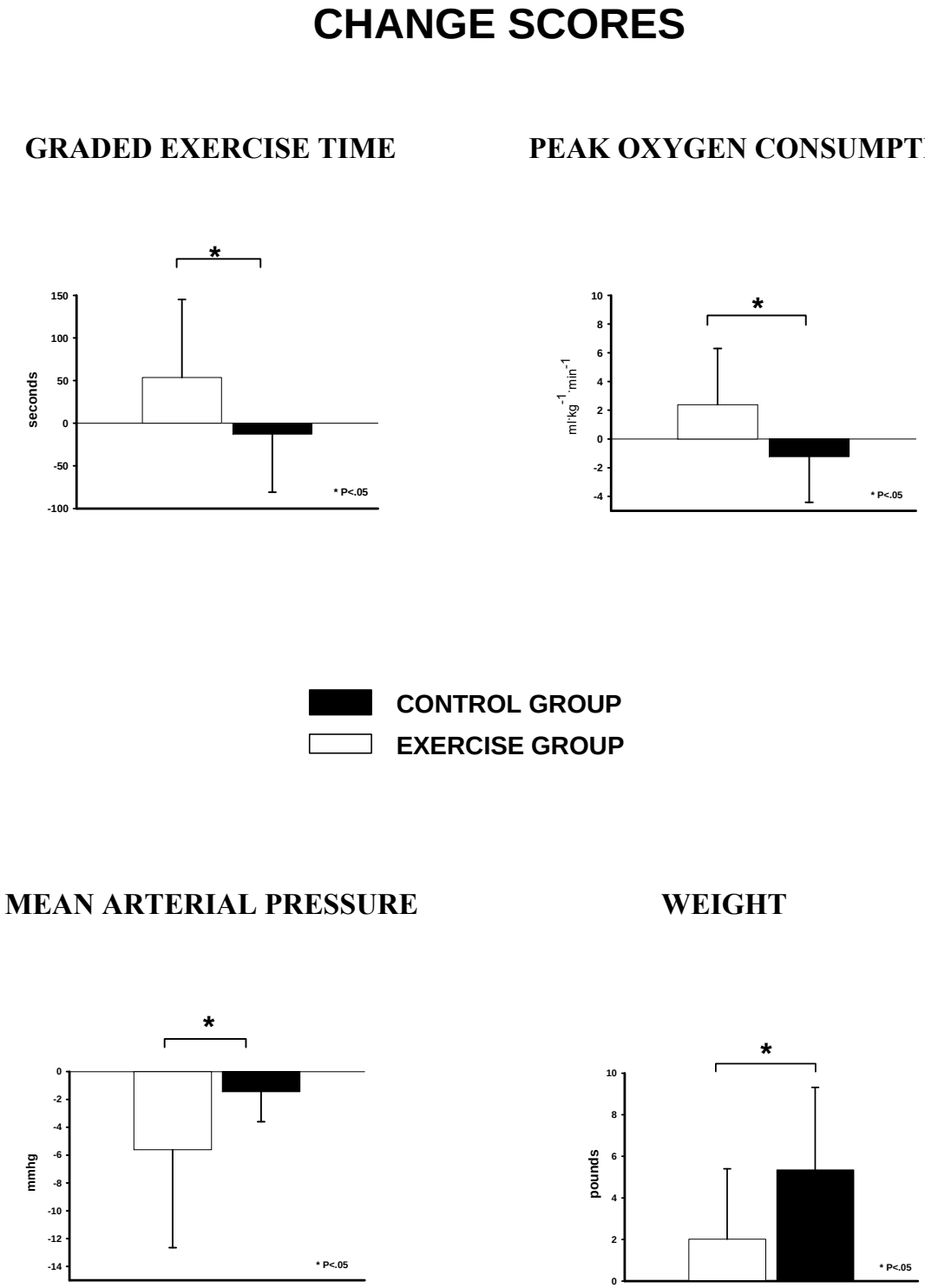


Figure 3a. FMD of non-responders and responders pre- and post-intervention. Values represent means and SD.

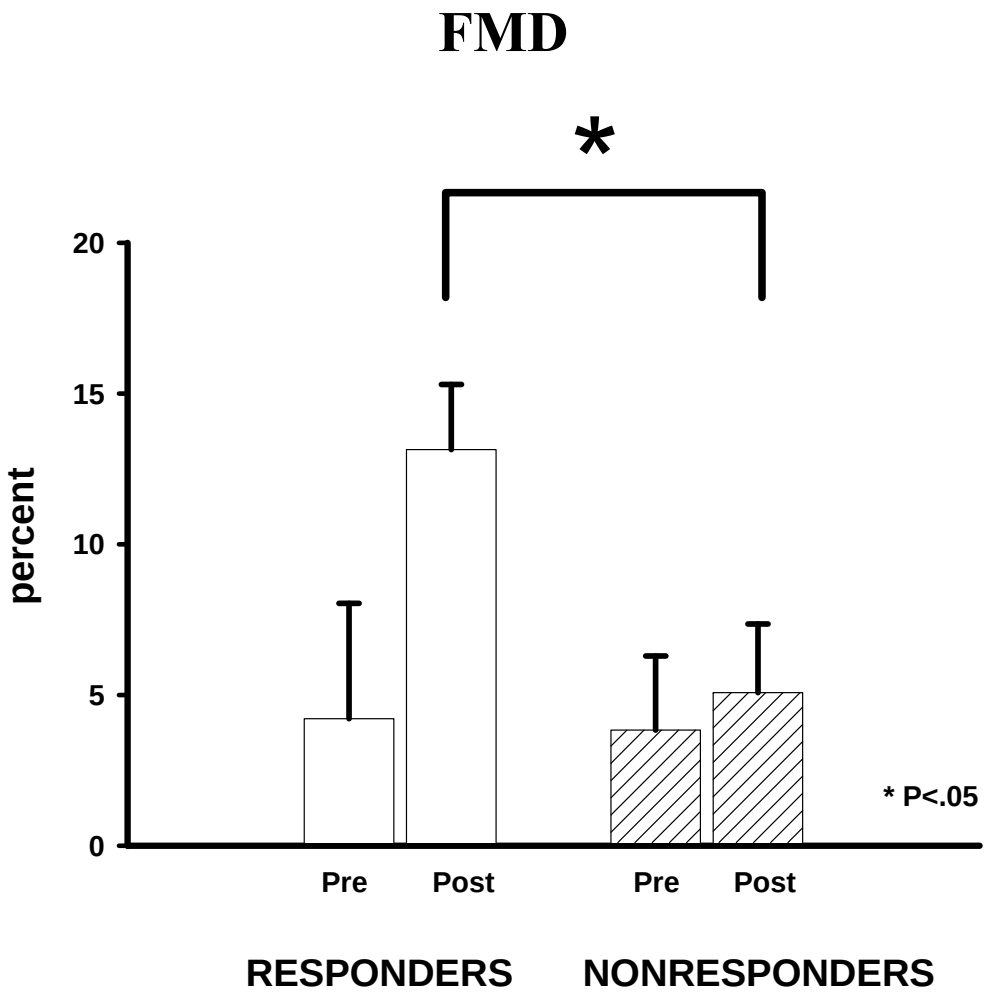


Figure 3b. Lipids of non-responders and responders at pre-intervention. Values represent means and SD.

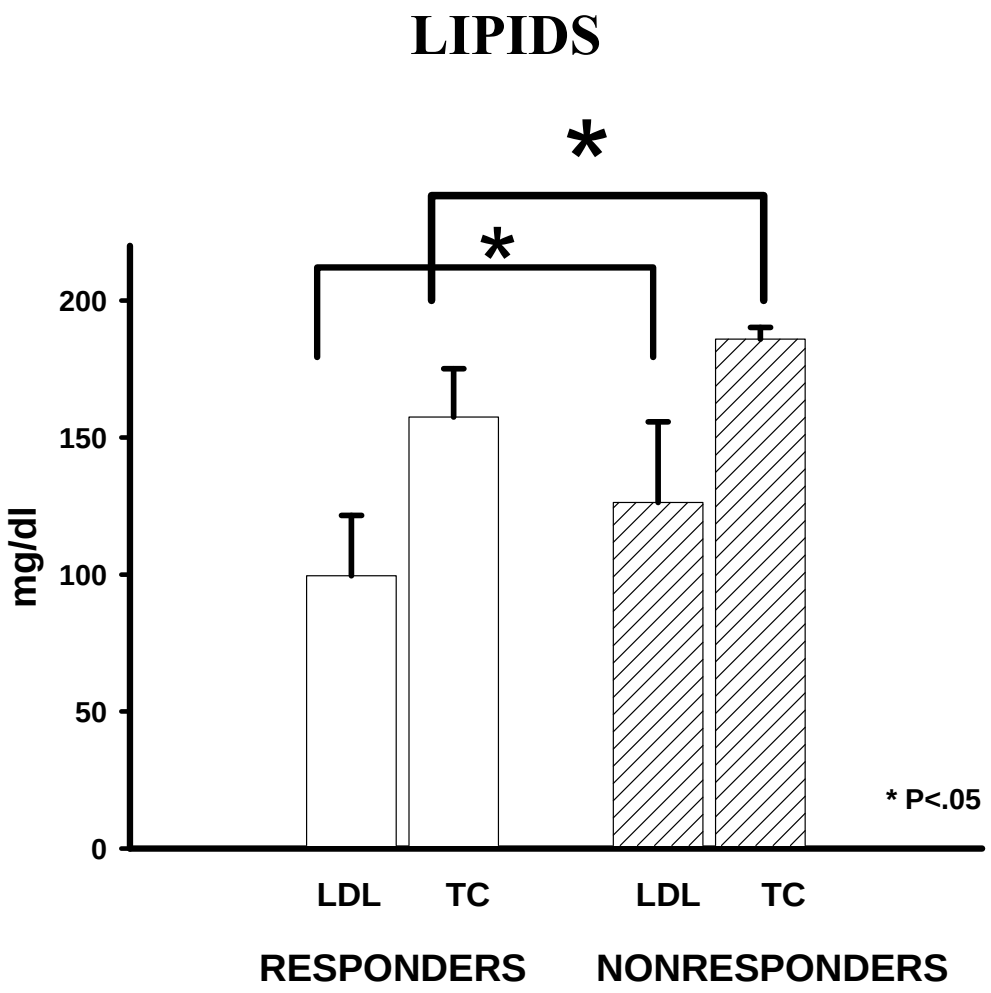


Figure 4a: Comparison of NOx estimated means of responders vs. non-responders using LDL and TC as covariates. Significant differences were found between groups, p<.05.

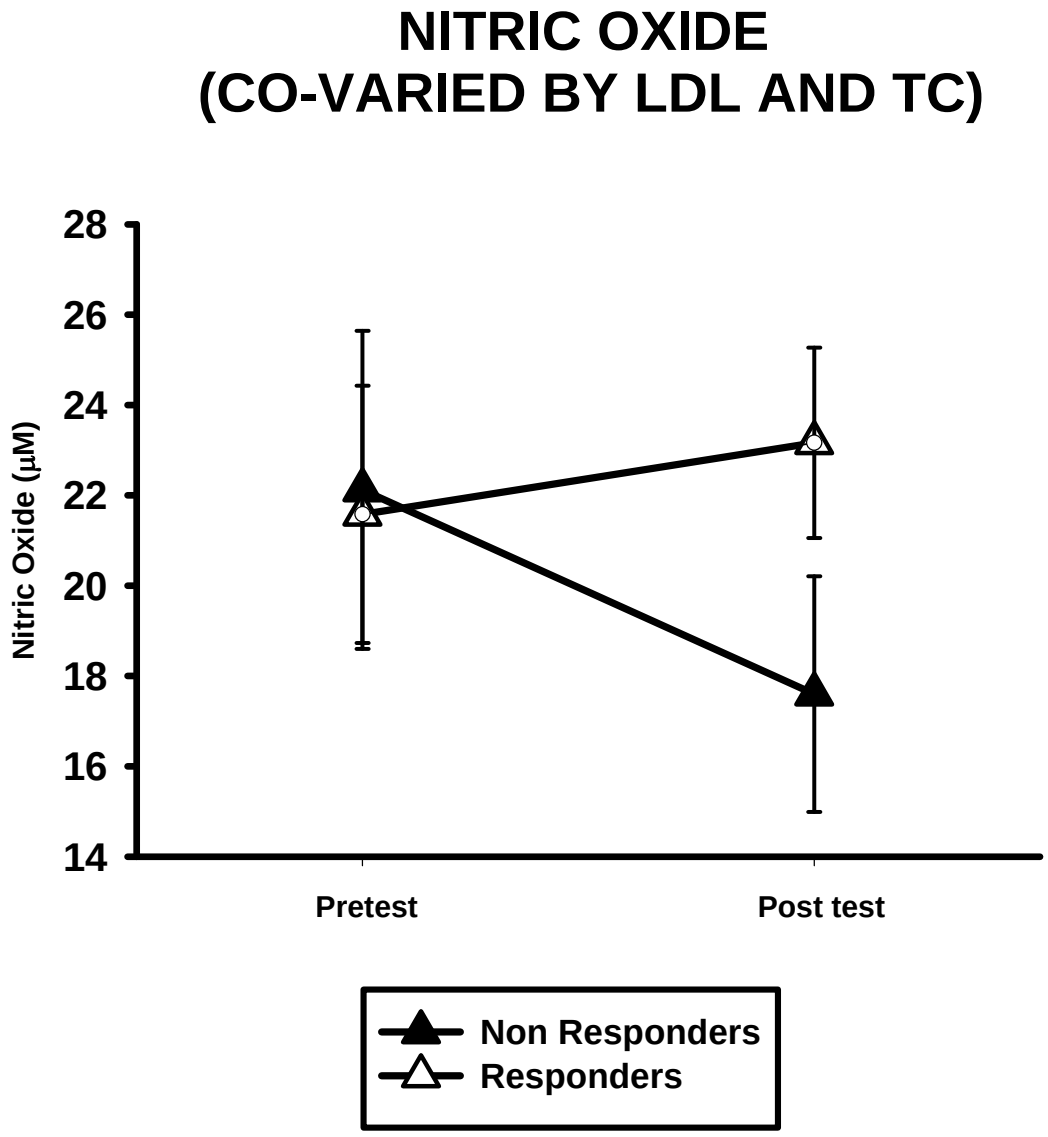
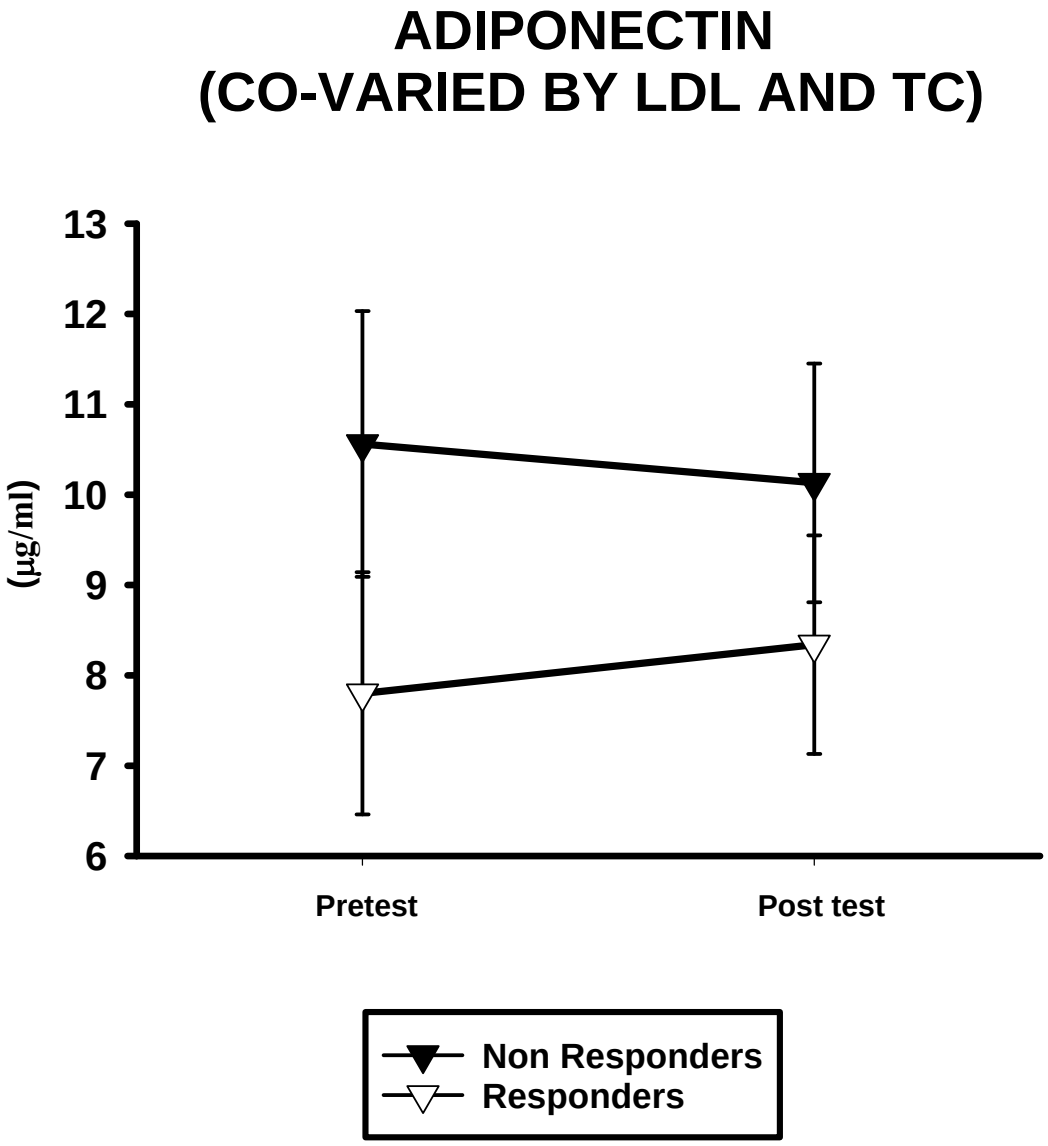


Figure 4b: Comparison of adiponectin estimated means of responders vs. non-responders using LDL and TC as covariates. Significant differences were found between groups, p<.05.



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GENERAL RESULTS:

Specific Aim #3:

Establish that flow mediated tissue oxygenation can be used as a clinical marker for endothelial function.

This specific aim determined whether the change in forearm tissue oxygen saturation after five minutes of blood flow occlusion was directly related to FMD of the brachial artery in seven to12 year old overweight children. The difference in oxygen saturation normalized by mean blood flow was calculated by: subtracting oxygen saturation at 60 seconds post occlusion, normalized by the ten-second post occlusion mean blood flow from baseline oxygen saturation, normalized by baseline mean blood flow. The same data were calculated normalizing by peak blood flow. The difference in vessel diameter normalized by mean blood flow was calculated by: subtracting 60 second post occlusion diameter, normalized by ten-second post occlusion mean blood flow from baseline diameter, normalized by baseline mean blood flow. The data were also calculated normalizing by peak blood flow.

Pearson correlations were run to determine how each of these calculated variables correlated with FMD and with one another.

- FMD was correlated with difference in oxygen saturation normalized by mean blood flow ($r=-.342$, $p=.036$) and difference in oxygen saturation normalized by peak blood flow ($r=-.517$, $p=.001$).
- The difference in vessel diameter normalized by mean blood flow was correlated with difference in oxygen saturation normalized by mean blood flow ($r=.657$, $p=.000$).
- The difference in vessel diameter normalized by peak blood flow was correlated with difference in oxygen saturation normalized by peak blood flow ($r=.617$, $p=.000$), humidity ($r=-.404$, $p=.018$), HC ($r=.349$*, $p=.040$), and resting SBP ($r=.378$, $p=.016$). When these variables were entered into a linear regression using the ENTER method significance was achieved with an $r=.686$ and $R^2=.470$, $p=.002$. A stepwise regression was then completed, and the only variable that remained in the equation was difference in oxygen saturation normalized by peak blood flow with an $r=.616$ and $R^2=.380$, $p=.000$.

Variables that were correlated with FMD at baseline in these overweight children were determined: age, BMI, WC, HC, tanner stage, resting SBP, resting DBP, difference in oxygen saturation normalized by mean blood flow, difference in oxygen saturation normalized by peak blood flow, and oxygen saturation past max at 31, 35,38,42,45,49, and 52 seconds. Past max values were oxygen saturation measurements taken every three seconds after occlusion and included those children who had not reached their max oxygen value by that time period.

- Difference in oxygen saturation normalized by peak blood flow and difference in oxygen saturation normalized by mean blood flow were highly correlated with one another, therefore only difference in oxygen saturation normalized by peak blood flow was entered into the linear regression to predict FMD.
- The oxygen saturation past max values were highly correlated with one another, therefore past max 38 was selected to be placed into a linear regression because it demonstrated the strongest correlation with FMD, r=.584, p=.003, and still retained an n=23.

To determine whether any variables associated with NIRS measurement were able to predict FMD, linear regressions were used. When all the variables (age, BMI, WC, HC, tanner stage, resting SBP, resting DBP, difference in oxygen saturation normalized by peak blood flow and past max 38 were placed into a linear regression using the Enter Method, significance was achieved (p=.043) with an r=.968 and R^2= .937. Past max 38 was then taken out of the linear regression due to the fact that only those that had not reached max had a value for this variable (n=23) and therefore not all subjects were entered. When past max 38 was removed, significance was lost using the Enter Method. A stepwise linear regression was then conducted, using .05/.10 as the inclusion/exclusion criteria. Past max 38 and WC were the only variables that remained significant, with an r=.911 and R^2= .830, p=.000, which again indicates the importance of using past max 38.

To assess whether oxygen saturation could be used as a screening tool only or whether it can be used to see effectiveness of an intervention, Pearson correlations on both pre- and post-intervention values were calculated using subjects in the EX group only.

The following variables correlated with FMD pre-intervention:

- BMI (r=-.545, p=.007)
- WC (r=-.550, p=.021)
- Resting SBP (r=-.655, p=.001)
- Resting DBP (r=-.722, p=.000)
- Difference in vessel diameter normalized by peak blood flow (r=-.494, p=.027)
- Difference in oxygen saturation normalized by mean blood flow (r=-.634, p=.004)
- Difference in oxygen saturation normalized by peak blood flow (r=-.738, p=.000).
- Various past max oxygen saturation values also correlated with FMD, however past max 38 was selected (r=.809, p=.003) because it was the most highly correlated while keeping (r= .809, p=.003) while keeping a fair number of subjects (n=11).

Because the difference in oxygen saturation normalized by mean blood flow and difference in oxygen saturation normalized by peak flow were highly correlated, only the difference in oxygen saturation normalized by peak blood flow was used in the regression equations. Significance was achieved with a r=.950 and R^2=.903, p=.034, when an ENTER method linear regression was conducted. When the stepwise method was used with these same variables, only past max 38 and WC remained significant predictors of FMD, with a r=.911 and R^2=.803, p=.000.

The only variables that correlated with FMD post-intervention were the difference in vessel diameter normalized by mean blood flow, (r=-.499, p=.030), TC (r= -.477, p=.025), and LDL (r= -.479, p=.024). Therefore it was determined that the regression equations used to predict FMD baseline were not appropriate to use post-intervention. While none of the post-intervention oxygen saturation values were associated with FMD, the difference in vessel

diameter normalized by peak blood flow post-intervention was highly correlated with difference in oxygen saturation normalized by peak blood flow post-intervention (r=.884, p=.000).

GENERAL DISCUSSION:

Specific Aim 1:

Establishing the prevalence of endothelial dysfunction in children at risk for obesity or children who are obese.

Working hypothesis: Obesity will cause impaired endothelial function in children seven-12 years of age.

Eighty-two percent of the overweight children (40 out of 49) in this study presented with endothelial dysfunction, as defined as having a FMD response of less than eight percent. This study is the first study that determined the number of children over the 85th percentile for BMI who presented with endothelial dysfunction. Vascular endothelial function has been implicated as one of the key determinants of early atherogenesis. Endothelial function was assessed in the current study by examining endothelial-dependent vasodilation of the brachial artery in response to a hyperemic-induced increase in shear stress, a method commonly used in both adults and children.

There is increasing evidence that obesity is associated with changes in endothelial health in childhood. Soluble adhesion molecules (CAMs, including ICAM-1 and VCAM-1)[1-2] and selectins (specifically E-selectin)[2] have been found to be elevated in obese children. While the pathophysiology is not completely understood, upregulation of CAMs is thought to play a pivotal role during the earliest phases of atherogenesis by allowing monocyte and leukocyte adhesion to the endothelial cell surface[1]. Results from a study conducted by Tounian et al.[3], showed that

carotid distensibility as well as brachial artery FMD were significantly lower in severely obese children compared to normal-weight controls. Impairment in endothelial function has been corroborated in several other studies[4-7]. While all of these studies have found impaired function when comparing obese children to normal-weight children, none have reported the percent of overweight children that present with this impairment.

In addition to the high incidence of impaired endothelial function amongst these overweight children, multiple other risk factors for cardiovascular disease and diabetes were also reported in these children. Forty-nine percent had elevated systolic blood pressure, 37 percent had elevated diastolic blood pressure, 67 percent had low HDL levels, 29 percent had elevated LDL levels, and 24 percent had elevated insulin levels. These findings are consistent with many other studies in obese children that have studied the associations of obesity with other cardiovascular risk factors [3,6,8-9].

Increased levels of inflammatory markers have also been documented in childhood obesity. In the past, adipose tissue was thought to be metabolically inert. Adipose tissue is now widely recognized as metabolically active tissue that produces various cellular mediators. In a sub-sample of those subjects that presented with endothelial dysfunction, plasma levels of the following pro-inflammatory markers were measured: TNF-α, IL-6, CRP and adiponectin. All markers measured were found to be abnormal compared to levels found in healthy normal-weight children[5,9-12]. This study supports other recent studies which have shown that with obesity, the balance between various inflammatory markers is altered. More specifically proinflammatory markers such as TNF-α, IL-6 and CRP are increased, while the plasma level of the anti-inflammatory marker, adiponectin, is decreased[13-14]. While all values were found to be abnormal in children presenting with endothelial dysfunction, we did not analyze these markers

in those children that had normal endothelial function. Therefore we were unable to determine whether there was a relationship between level of endothelial function and any of the markers for inflammation.

Specific Aim 2:

Evaluating the effectiveness of an exercise intervention to improve endothelial function.

Working hypothesis: Twelve weeks of moderate intensity aerobic exercise will improve endothelial function in overweight children seven to 12 years of age, and the improvement in endothelial function will be directly related to the improvement in aerobic capacity.

Those children that presented with endothelial dysfunction were entered into the second phase of the study that examined the effects of a 12-week aerobic exercise intervention using DDR on endothelial function and other risk factors for both CVD and diabetes. To determine the effects of the exercise intervention, 35 subjects were randomly assigned to either an exercise group (EX, n=23) or delayed-treatment control group (DTC, n=12).

The most important finding of this study is that 12 weeks of aerobic exercise, using DDR, significantly improved endothelium-dependent vasodilation of the brachial artery in the EX group compared to the DTC group. A direct shear-stress mediated effect of NO availability is the plausible cause for improvement in vascular function as a result of exercise[15]. Others have previously demonstrated this improvement in vascular function with exercise in overweight children [4-6]. The current finding of improved vascular function in conjunction with previous studies is important because of the need to identify non-pharmacologic interventions aimed at improving both vascular health and other risk factors in obese individuals.

In addition to the improvement seen in endothelial function, the exercise training also resulted in significant improvements in aerobic capacity, exercise time, mean arterial pressure and weight gain. These findings add to the limited evidence that increased physical activity facilitates weight loss and other health outcomes in overweight children and adolescents[4,12].

While our findings are similar to recently published articles that show the beneficial effects of exercise in overweight or obese children, there are some distinct differences that make the current study unique. Our study used DDR which is a total-body video game as the mode of aerobic exercise used as the intervention. Children's screentime (television, playing video games, and free-time computer use) has been consistently associated with low levels of physical activity[16]. Weekly screentime for children is as high as 55 hours/week[17]. While several programs have tried to limit children's screentime and replace it with physically active behaviors, children have been resistant to these changes[18]. By using an active video game as an alternative to sedentary screentime, the reluctance to be physically active was overcome.

Over the 12-week exercise intervention period, subjects were compliant 75 percent of the time. The high compliance rate may be due to the fact that the current study tried to take into consideration common barriers to physical activity among overweight children when designing the exercise protocol (lack of time, body consciousness, and various environmental barriers)[19-20]. Unlike previous studies where interventions were done in a structured laboratory setting using either bicycle ergometry or circuit-training as the mode of exercise, our study was home-based and provided subjects with all necessary equipment. Because of the home-based nature of the exercise intervention, subjects were recruited statewide.

While we did see an improvement in both FMD and aerobic capacity, the change scores for both variables were not directly associated. However, change in exercise time on the bicycle

ergometer test was significantly associated with FMD (r=.356, p=.039). The exercise time on the bicycle ergometer test of the DTC subjects actually decreased from baseline to post-intervention, while the EX group improved their time. This may be an indication that the EX group became more efficient, and that they were able to exercise for a longer period of time, at a lower VO_2 than at the time of the baseline exercise test.

An improvement in inflammatory markers was also expected to be seen in conjunction with the improvement in endothelial function. As was mentioned previously, all inflammatory markers measured in the current study were abnormal compared to studies that have examined markers of inflammation in healthy normal-weight children. It has been suggested that all markers for inflammation measured in the current study are associated directly with percent body fat[21-22], and that a reduction in body fat results in a subsequent improvement in an individual's inflammatory profile. Body composition was not measured in the current study, and therefore we can not assess whether the lack of improvement was due to the lack of improvement in percent body fat. We also expected to see an improvement in markers of NO production that corresponded with the improvement in endothelial function, however no improvements were seen in any of the markers measured; NOx, l-arginine, ADMA or SDMA.

To seek an explanation for the lack of improvement in markers of inflammation and NO production, groups were further divided in two ways: 1) by gender and 2) by those in the EX that achieved eight percent FMD compared to those that did not. The discussions of these results are in summarized on pages 86-90.

Specific Aim 3:

Establish that flow mediated tissue oxygenation can be used as a clinical marker for endothelial function

Working hypothesis: The increase in muscle tissue oxygenation in the forearm after five minutes of blood flow occlusion will be directly related to flow mediated dilation of the brachial artery.

This portion of the study was conducted to determine whether change in forearm tissue oxygenation saturation after five minutes of blood flow occlusion would be related to FMD of the brachial artery. Pearson correlations were run to determine what study variables, including those determined from NIRS, were associated with FMD. In a previous pilot study conducted on adults, it was determined that O2 saturation and artery diameter needed to be normalized by bloodflow. Therefore, both variables were also normalized by blood flow in the current study as well. FMD was found to be correlated with BMI, waist circumference, resting blood pressure, difference in diameter normalized by peak blood flow, difference in oxygen saturation normalized by both mean and peak blood flow, and various past max oxygen saturation values (past max values were oxygen saturation values taken every three seconds after occlusion and included values for those children only that had not yet met their maximum oxygen saturation value). When all variables were places into a linear regression using the ENTER method, significance was achieved with an r=.950 and R2=.903. However when placed into a stepwise linear regression, the past max oxygen saturation value at 38 seconds post occlusion and waist circumference were the only variables that remained significant predictors of FMD.

These results indicate that FMD can be predicted in overweight children using NIRS, however the past oxygen saturation value at 38 seconds post occlusion is very important in the

prediction equation, and only 23 of the children at 38 seconds post occlusion had not reached their maximum values. In these 23 children, FMD varied greatly and ranged from 0 to 21.05 percent allowing for a strong linear relationship to be established. When this variables was removed from the regression using the ENTER method, significance was lost. Past max seemed to influence the r value most likely due to the fact that as more subjects were eliminated, a greater number of the subjects with normal endothelial function were retained, and therefore the correlation became stronger because there was a more equal distribution over the spectrum of possible FMD values.

At post-intervention, no NIRS variables were correlated with FMD. However, difference in vessel diameter normalized by peak blood flow post-intervention was highly correlated with difference in oxygen saturation normalized by peak blood flow post-intervention (r=.884, p=.000). These variables were also correlated at baseline. This relationship is similar to that found in the pilot study on healthy young adults. After the exercise intervention (only those children with abnormal values at baseline were included in the exercise portion of the study) there was a wide range of values for FMD since some children were control subjects and half of the subjects in the EX responded to the treatment and half did not. This suggests that a larger study, including children with normal values at baseline, might yield even stronger correlations between difference in oxygen saturation normalized by peak blood flow and difference in vessel diameter normalized by peak blood flow.

NIRS methodology is particularly suited for studies of the microcirculation, since spectroscopy principles dictate that the NIRS signal is derived predominantly from hemoglobin in small arterioles, capillaries and venules. This is the case, because light waves emitted into larger vessels (arteries and veins) is almost completely absorbed since the quantity of blood

(water) is so large[23]. Several studies employing different methods of microvascular responses to hyperemia have shown that the function of microvessels does not correlate with those of larger conduit vessels[24-26]. Shamim-Uzzaman et al.[24] compared microvascular endothelial function by measuring skin blood flow with laser Doppler flowmetry and brachial artery function using FMD. Laser Doppler flowmetry technique uses transdermal delivery of selective endothelium-dependent vasodilators or reactive hyperemia to estimate endothelial function in the microcirculation[27]. As with studies using FMD of the brachial artery, laser Doppler flowmetry studies have shown impaired microvascular responses in subjects with classic risk factors of CVD[28]. Results of their study indicated that there was no significant relationship between the two techniques. These authors state that this finding is consistent with emerging data indicating that regulators of microvascular function are not NO dependent[27,29], and it is this difference that resulted in a lack of association. When NIRS was used as a surrogate of microvessel function, there was no association between FMD and oxygen saturation values. There was, however, an association between the absolute change in vessel diameter from baseline to 60 seconds post occlusion and the absolute change in oxygen saturation values from baseline to 60 seconds post occlusion when both variables were normalized by blood flow. There were no associations found between any of the markers for NO production with any of the NIRS variables in the current study, which may indicate that microcirculation function is not NO-mediated, which is in agreement with other recent studies on the microcirculation.

FUTURE STUDIES:

Interpretation of the results of the current study raises some interesting questions that deserve further examination. BMI was used as a surrogate for body composition in the current study. While BMI has been shown to be highly correlated with total body fat and percent body

fat[30-31] in children, BMI and its relationship to fat and fat-free components are somewhat complicated by growth and maturation levels. Although our data indicated that FMD improved without changes in gross measures of body composition (BMI and waist/hip circumferences), we are unable to determine whether there were regional changes in fat or lean mass in response to the exercise intervention. A measurement of body composition such as dual x-ray absorptiometry (DEXA) would provide an accurate measurement of both fat and lean mass as well as distribution. While we demonstrated that all markers for inflammation were increased in these overweight children, we were not able to relate values of these markers to percent body fat at baseline or whether changes in body composition as a result of the exercise-intervention resulted in a change of any of these markers. Circulating levels of IL-6, TNF-α and adiponectin levels have all been shown to be related to body fat percent in adults[32-33]. This relationship is not clearly defined in children, and therefore warrants further investigation to help understand the mechanisms that not only lead to a state of chronic inflammation in overweight children, but relate mechanisms of increased levels of these markers to vascular damage, and assess whether exercise has any effect in improving inflammatory profiles of overweight children with or without changes in body composition.

Pubertal status (Tanner Stage) was determined via the use of a validated questionnaire that the parents completed. At baseline, there was a significant difference between genders in Tanner Stage, with girls having a higher Tanner Stage than the boys. Sex hormone levels were not directly measured in the current study. There was no difference in FMD at baseline and no difference seen in the impact of the exercise training on FMD responses between the male EX subjects (3.97 ± 3.36% to 9.11 ± 3.51%) and the female EX subjects (4.01 ± 3.06% to 10.04 ± 5.82%). It has been hypothesized that estrogen may be cardioprotective, and measurement of

estrogen levels in future studies may determine if this protective effect of estrogen is seen in children. There were also differences in the effects of the exercise intervention on inflammatory markers between genders. In female subjects only, IL-6 levels were decreased as a result of the exercise intervention. L-arginine levels increased as a result of the exercise intervention only in the male subjects. Measuring both estrogen and testosterone levels in these children, may give some insight to these gender-related differences observed.

When examining the EX subjects only, there were subjects that achieved an FMD of eight percent or greater (responders) post-intervention while others did not (non-responders). Differences between these groups were therefore assessed. While compliance to exercise would seem a likely explanation of why some subjects improved FMD while other did not, there was no difference in compliance when looking at the total intervention period. Surprisingly, the non-responders played DDR for significantly more days in week ten (5.90 ± 1.19 days compared to 3.50 ± 2.81 days, p=.021) and week 11 (5.60 ± 1.35 days compared to 3.41 ± 2.81 days, p=.031) than the responders. Pedometer data showed that there were significant differences between the non-responders and responders in both total number of steps taken over the 12 week period (708,611 ± 395,868 steps compared to 359,513 ± 190,599 steps, p=.011) and total number of steps taken while playing DDR (169,563 ± 66,299 steps compared to 105,963 ± 48,174 steps, p=.025), with the non-responders taking significantly more steps in both categories. These findings indicate that there may have been a difference in exercise intensity between the two groups. Exercise intensity has been shown to affect vascular function (refer to pages 92-93) in several studies in adults with high intensity exercise adversely affecting FMD. Exercise intensity was not measured in the current study, and these findings indicate the need to measure intensity in subsequent studies. The DDR software allows for the measurement of exercise intensity in

two ways: 1) players progress from a beginner mode to a heavy mode as they master the game 2) when using work-out mode a player is able to track calories burned, step-counts, and other workout parameters. Future studies should use these aspects of the video game to assess exercise intensity and other outcomes by having the subject record daily workouts on memory cards. In addition studies that incorporate different training intensities to determine the effect of exercise intensity on vascular function in overweight children are also needed.

The non-responders differed from the responders in that they had higher total cholesterol and LDL values both at baseline and post-intervention. Several studies using non-invasive methods, such as high-resolution ultrasound, have demonstrated endothelial dysfunction in hypercholesterolemic children and adolescents[34-36]. In a study looking at the effects of statin therapy on endothelial dysfunction in children with familial hypercholesterolemia, de Jongh et al.[36] showed that short-term statin therapy was successful in improving endothelial function in these children. These results indicate that in children with elevated cholesterol levels, improvements in lipid profiles may be necessary to result in improved vascular function. While children with extremely high cholesterol values would warrant pharmacologic treatment based on the NCEP guidelines, lifestyle changes to improve lipid profiles would be recommended for those children with only moderately elevated cholesterol values. When comparing the responders to the non-responders, only 30 percent of the responders had LDL values above 110 mg/dl while 80 percent of the non-responders had LDL values above 110 mg/dl at both baseline and post-intervention. Because the non-responders showed greater improvement in other risk factors for CVD and were as compliant to the exercise intervention as the responders, these results may indicate the need to lower lipid levels to see subsequent improvement in vascular function. This may be able to be achieved by a longer exercise-intervention, an exercise

intervention with longer exercise session durations, or a combination of an exercise intervention with a dietary component. In a recent study by Woo et al.[37], improvement in endothelial function was greater in a group of children that received diet plus exercise as opposed to diet alone. In addition to this marked difference in improved vascular function, these authors also demonstrated a greater improvement in LDL values of the combined exercise plus dietary intervention as well.

The non-responders showed greater improvements in blood pressure and BMI than the responders. It is not known whether marked improvement in endothelial function versus improvement in other classic risk factors have more of an impact on cardiovascular and overall health risk. A longitudinal study to assess the long-term effect would provide insight into which factors prove more important in health outcomes of these children as adults.

Normal-weight control subjects were not used in the current study. There are very few studies that examined the levels of markers for inflammation in normal weight children (refer to page 67, Table 3), and no current studies of the effects of an exercise intervention on markers for inflammation in normal-weight children. Future studies incorporating normal-weight children will allow researchers to establish normal ranges for inflammatory markers in normal-weight compared to overweight children and the effects that exercise has on both populations.

There is also a need to conduct future studies with normal-weight children to examine the efficacy of NIRS to assess vascular function in children. Results from the current study indicated that values of those children that had not yet met maximum oxygen saturation values at 38 seconds post-occlusion (pastmax 38) were associated with FMD. There were only 24 out of the 49 subjects that had a pastmax 38 value. In these 24 children there was a wide range of values for FMD ranging from 0 to 21.05 percent allowing for a linear relationship. Additional

studies including a greater number of children with normal endothelial function, may give more insight into the possible relationship between conduit vessel function and microvessel function.

Larger well-controlled studies are warranted to focus on the exercise effects on various study outcomes (i.e. inflammatory markers, FMD, insulin sensitivity) with and without weight-loss. Larger numbers of subjects would also allow additional classification of subjects, i.e. gender-specific responder and non-responder groups.

The current study showed that a 12-week exercise intervention where subjects were all asked to exercise the same amount (five days per week for at least 30 minutes) was effective in improving FMD in overweight children. Testing was completed at baseline and post-intervention at 12 weeks. Future studies using more frequent testing and variable exercise protocols may help determine whether exercise has dose- and duration-dependent effects on FMD and other study outcomes.

Exercise compliance data showed that compliance was very good for the first six weeks of the study, then dropped during the seventh through the ninth week of the study, and then again increased during weeks eight through 12. Weekly phone-coaching sessions were conducted to encourage compliance throughout the study period. More intensive coaching or giving subjects incentives may prove successful in increasing compliance in future studies.

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General Results and Discussion

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APPENDIX 1: Recruitment Ad

These arrows might change the direction of your child's life.

Concerned about your child's weight?
Does your child need more exercise?
Does your child enjoy video games?
If you answered yes to these questions, you might want to enroll your child in PEIA's ***Games for Health*** *Pilot Project!*

What is it?
A pilot research project for children, ages 7-12 who are overweight or at risk of being overweight conducted at West Virginia University and sponsored by PEIA. This program involves the use of an interactive video game that makes physical activity FUN! The purpose of the project is to determine the effects of a home-based exercise program on cardiovascular risk factors.

How Does it Work?
Qualified children will be provided the training and software to participate. You must be willing to travel to the Human Performance Lab at West Virginia University for three evaluation sessions over a six month period. Children can play the game in the privacy of their own homes. Parents must provide consent and be willing to answer questionnaires about family history, demographics, and lifestyle issues.

What Will it Cost?
There is no cost to you other than time and minimal travel expenses.

Benefits?
Will potentially decrease subject's cardiovascular risk. Upon completion of the study, subjects will keep pedometer, video game software, and game pad.

This study is conducted by the following investigators:
Emily S. Murphy, MS, Linda Carson, EdD, Rachel Yeater, PhD, Guyton Hornsby, PhD, William Neal, MD, Irma Ullrich, MD, and Dave Donley, MS

For more information contact:
Emily S. Murphy, MS at (304)293-0767

APPENDIX 2:Health History Form

General Information:

Date_____ Personal Physician _____
Name _____ Age _____ Date of Birth _____
Address _____
Home Phone _____ Office Phone _____ SS# _____
Occupation _____ Employer_____
Marital Status _____ Education _____
Height _____ Weight _____
BMI _____ Waist Circumference _____

Medical/Surgical History (mark x if answer yes)

Have you ever had:
____ rheumatic heart disease
____ heart murmur
____ high blood pressure
____ gout
____ varicose veins
____ injuries to back, neck, etc.
____ lung disease
____ epilepsy
____ diabetes
____ heart disease
____ heart surgery
____ kidney disease
____ stomach ulcers
____ arthritis
____ cardiac catherization
____ strokes
____ thyroid problems
____ dizziness or fainting spells
____ nervous or emotional problems
____ allergies
____ phlebitis
____ cardiac arrest
____ hospitalizations

Have you recently had:
____ irregular heart beat
____ chest pressure
____ shortness of breath
____ heart fluttering
____ cough on exertion
____ back pain
____ swollen, stiff, and painful joints
____ difficulty sleeping
____ fatigue
____ calf pain when exercising
____ nervousness
____ fainting
____ swollen ankles, legs, etc.
____ Other problems: _____
_____
_____
_____
_____
_____
_____
_____
_____

Medications:

Drug	Dosage	Times/Day	How Long

Family History:

Have any of your relatives had:	Age	Relative
____ heart attacks	_____	_____
____ high blood pressure	_____	_____
____ high blood fats	_____	_____
____ obesity	_____	_____
____ stroke	_____	_____
____ other heart disease	_____	_____
____ diabetes	_____	_____

Smoking:

	Yes	No
Do you smoke?	_____	_____
Have you ever?	_____	_____

Cigarettes, pipe, cigar _____
What age did you start _____
What age did you stop _____
If you stopped, when? _____
Why? _____

Stress: Rate yourself in relation to tension:
____ Usually stressed _____ Relaxed,but occasionally tense ____ tense more often than relaxed ____ very tense

Rate the amount of stress in your job:
_____ little _____ average _____ above average _____ Severe

Nutrition:
Rate your diet in relation to fat content:
_____ low fat _____ average fat _____ above average fat _____ high fat

Do you consume alcohol?
Yes _____ No _____
What kind? _____
Average amount per week? _____

Cholesterol:
What is your cholesterol values ? TC _____ HDL_____ LDL_____ Trig _____

Physical Fitness: How often do you engage in VIGOROUS activities for 20 minutes or more ?

	Never	Seldom	1-2 times/wk	3-6 times/wk	Every day
Running or jogging	_____	_____	_____	_____	_____
Hiking or outdoor walking	_____	_____	_____	_____	_____
Bicycling	_____	_____	_____	_____	_____
Swimming	_____	_____	_____	_____	_____
Aerobics	_____	_____	_____	_____	_____
Vigorous gardening	_____	_____	_____	_____	_____
Dancing	_____	_____	_____	_____	_____
Other (specify) _____	_____	_____	_____	_____	_____

How physically fit do you feel at the present time?
____ Unfit _____ Below Average _____ Average _____ Above Average _____ Very fit

_____
Interviewer

APPENDIX 3: Tanner Staging Questionnaires (Male and Female)

Parent Questionnaire: Boy's Tanner Stage (NICHD Study of Early Child Care and Youth Development, 2001)

1. Would you say that your son's growth spurt (in height) has started yet? (a growth spurt is defined as growth in height that is faster than usual.)
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

2. Would you say that growth of his underarm and pubic hair has started yet?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

3. Have you noticed any changes in his skin, especially pimples?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

4. Have you noticed a deepening of his voice?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

5. Has he started to grow hair on his face?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

6. Compared with other boys his age, would you say your son's physical development is:
    1. Much earlier than the other boys
    2. Somewhat earlier than the other boys
    3. About the same as the other boys
    4. Somewhat later than the other boys
    5. Much later than the other boys

Parent Questionnaire: Girl's Tanner Stage
(NICHD Study of Early Child Care and Youth Development, 2001)

1. Would you say that your daughter's growth spurt (in height) has started yet? (a growth spurt is defined as growth in height that is faster than usual.)
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

2. Would you say that growth of her underarm and pubic hair has started yet?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

3. Would you say that her breast have started to grow?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

4. Have you noticed any changes in her skin, especially pimples?
    1. No
    2. Yes, barely
    3. Yes, definitely
    4. Development completed
    5. Don't know

5. Has she had her first menstrual period? Yes No

    If yes, how old was she when she had her first period? _____ years _____months

6. Compared with other girls her age, would you say your daughter's physical development is:
    1. Much earlier than the other girls
    2. Somewhat earlier than the other girls
    3. About the same as the other girls
    4. Somewhat later than the other girls
    5. Much later than the other girls

APPENDIX 4: Exercise Thought Questionnaire

EXERCISE THOUGHTS QUESTIONNAIRE (ETQ) (Kendierski and Johnson 1993)

We are interested in thoughts you may have had about exercising during the past week. Below is a list of thoughts that people sometimes have when they consider whether or not to exercise. It is important that you read each thought carefully. Then, next to each thought, please indicate how frequently you had that thought during the past week. Use the following scale:

1	2	3	4	5
Not at all	Sometimes	Moderately often	Often	All the time

___1. I'm too tired to exercise
___2. I need to sleep.
___3. I would rather get some sleep.
___4. There are more important things I have to do.
___5. I'm too busy.
___6. I haven't got time.
___7. It's not that important right now.
___8. I'd rather relax.
___9. I'd rather watch TV.
___10. I'd rather socialize.
___11. I'd rather do something else.
___12. I have social obligations.
___13. I don't feel good enough to exercise.
___14. Exercising will only make me more tired.
___15. It will take a lot of energy.
___16. It will take too long.
___17. I'm just not motivated enough to exercise.
___18. I don't feel like exercising.
___19. I'll make it up later.
___20. I'll do it tomorrow.
___21. I'll do it later.
___22. I'll work out extra hard tomorrow.
___23. I'll cut down on eating instead.
___24. Missing one day won't make that much of a difference.
___25. I can afford to miss one day.

APPENDIX 5: Food/Beverage Record

3-Day Food/Beverage Record

Day of the Week:_____

Time of day	Food/Beverage/Water	Amount/Serving Size

FOOD AND BEVERAGE RECORD

GOAL: **To record all food and beverages for 3 days at the beginning of the study and once again at the end of the study.**

REMINDERS:

- **Choose 2 weekdays and 1 weekend day**
- **Record all food and beverages consumed (including water)**
- **Break down foods into parts; for example, a ham sandwich with mayonnaise and cheese with a glass of milk would look like this:**

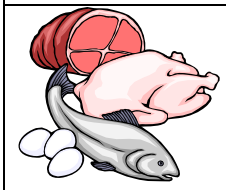
Time of day	Food/Beverage/Water	Amount/Serving Size
12:00	*Ham*	*1 slice*
	Bread	*2 slices*
	Mayonnaise	*1 tablespoon*
	Cheese	*1 slice*
	1% milk	*8 oz. (1 cup)*

WHAT COUNTS AS ONE SERVING?

- **1 piece of fruit**
- **¾ cup juice**
- **½ cup canned fruit**

- **½ cup raw or cooked vegetables**
- **1 cup leafy raw vegetables**

- **1 cup milk or yogurt**
- **1 ½ - 2 ounces of cheese**



- **2 ½ - 3 ounces of cooked meat, poultry or fish**
- **1 cup cooked beans**
- **2 eggs**
- **5 tablespoons of peanut butter**

- **1 slice of bread**
- **½ cup cooked rice or pasta**
- **1 ounce ready to eat cereal**
- **½ cup cooked cereal**

- **1 slice of cake**
- **2 cookies**
- **1 small muffin**
- **½ can of soda**
- **1 cup of popcorn**
- **1 small bag of snack food**

APPENDIX 6: Weekly Activity Log Sample

SAMPLE - Week #1 Activity Log

Date:	Sunday 02/13/05	Monday 02/14/05	Tuesday 02/15/05	Wednesday 02/16/05	Thursday 02/17/05	Friday 02/18/05	Saturday 02/19/05
Total STEPS Today:	3398	2348	3150	4509	5054	3877	2987
Total ACTIVE Time Today:	0:20:35	1:05:22	0:25:09	1:00:07	2:05:15	0:23:13	0:46:34
Amount of Screen time: (Other than DDR, e.g. TV, computer, other video games)	2 hours	1 hour	1 ½ hours	15 minutes	¾ hour	0	3 hours
Other activities:	Went to Church	Piano lesson, 30 minutes	Homework 1 hour	Homework 20 minutes	Homework 30 minutes	Went to movie with friends	Went to the mall for 1 hour

My Name:_____

Call 1-800-_____ for help or support!

APPENDIX 7: My DDR Calendar

MY DDR CALENDAR

	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
WEEK 1	Food/Beverage Log DDR – 8 songs	Food/Beverage DDR – 8 songs	Food/Beverage DDR – 8 songs	DDR – 8 songs	DDR – 8 songs	DDR – 8 songs	DDR – 8 songs Phone Coach
WEEK 2	MAIL WEEK 1 + Food/Beverage Log DDR – 12 songs	DDR – 12 songs	DDR – 12 songs	DDR – 12 songs	DDR – 12 songs	DDR – 12 songs	DDR – 12 songs Phone Coach
WEEK 3	MAIL WEEK 2 DDR – 16 songs	DDR – 16 songs	DDR – 16 songs	DDR – 16 songs	DDR – 16 songs	DDR – 16 songs	DDR – 16 songs Phone Coach
WEEK 4	MAIL WEEK 3 DDR – 20 songs	DDR – 20 songs	DDR – 20 songs	DDR – 20 songs	DDR – 20 songs	DDR – 20 songs	DDR – 20 songs Phone Coach
WEEK 5	MAIL WEEK 4 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 6	MAIL WEEK 5 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 7	MAIL WEEK 6 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 8	MAIL WEEK 7 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 9	MAIL WEEK 8 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 10	MAIL WEEK 9 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 11	MAIL WEEK 10 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	DDR – 24 songs Phone Coach
WEEK 12	MAIL WEEK 11 DDR – 24 songs	DDR – 24 songs	DDR – 24 songs	Food/Beverage DDR – 24 songs	Food/Beverage DDR – 24 songs	Food/Beverage DDR – 24 songs	MAIL WEEK 12 + Food/Beverage Log DDR – 24 songs

APPENDIX 8: Training Protocol

Games for Health – DDR Training Protocol

PREPARATION:

- Assemble pad
- Remove hand controller from PS2 box
- Assemble file folder with all necessary hand outs, pen/pencil
- Pedometer
- Digital Camera
- Games for Health DVD (hook up DVD player to TV FIRST)
- Do not hook up pads to game console – let the subject do that

OVERVIEW:

- 1 hour in length
- parent involved fully in all aspects of training
- by appointment, in Choosy Room
- consequences for non-compliance – expectation for completing all 12 weeks, if two consecutive weeks are missed (e.g. sending in logs), then out of study, equipment returned (I have not mentioned this – I like the positive approach, and assume it won't be necessary!)

ACTIVITIES:

- Introductions – explain the purpose – **to get healthy!** Give summary of training session – what is going to happen – complete **CONTACT INFORMATION SHEET** (pink)
- **TAKE PICTURE** with CHOOSY – explain who Choosy is, if necessary, and explain why the picture is necessary (e.g. so phone coach will know exactly who he/she is talking to).
- Discuss the phone coaching protocol, discuss good time for phone calls, how often they will occur, they can call the hotline if needed if any difficulties. I provide my office number and email, writing it on inside of folder.
- **PEDOMETER** training – explain what it does (let the subject figure out how to open it), how to wear it, DO NOT SHAKE IT (that is dishonest and damaging to the pedometer!), describe both settings – **STEP** count and **ACTIVE** time; reset, and go for a short walk, and see how many steps have been recorded; leave pedometer on for the rest of the session.
- **Show GAMES FOR HEALTH DVD,** answer any questions, emphasize that the dancers in the video have been playing for a long time.
- Record the number of steps **BEFORE DDR** on the worksheet (ivory color)
- DDR training (pads will be pre-assembled for subjects)
    - Equipment set up and operation - subject/parent repeats the process until they are confident – if necessary (most subjects will be familiar with video game set-up)
    - Use of DDR – can use Getting Started sheet here if necessary.
        - Settings – go over OPTIONS MODE and GAME MODE only
        - Selection of difficulty level – in OPTIONS, set this to BEGINNER
        - Selection of song

    - Backing up previous screen or stopping a song
    - Strategies for play – not returning to middle of pad
    - Give enough time for parent and child to become familiar with game controls, game play, etc.
    - Explain that for the first week, the subject must do **8 SONGS in GAME MODE**, and he/she must develop a strategy to keep track of how many songs are completed (e.g. paper and pencil by the TV).
    - **CHECK PEDOMETER**, record the number of steps, do one song, then do the practice calculation on the **WORKSHEET**
- Discuss the workout protocol for each week – use **DDR CALENDAR**
- Discuss how to record data for each day of the week – use **TRAINING LOG** sample and recording sheets, explain screen time, when to enter data, answer any questions.
- Show the Getting Started sheet – they can refer to this if having problems getting to the GAME MODE
- Show the FAQ's sheet – check this if they have any questions. If the FAQ sheet does not answer their question, have them call us. Refer to **WEBSITES**, and if they wish to get another Red Octane Pad.
- Food/Beverage Record – explain how to use this, and when to collect information, when to send in recording sheets; emphasize that they do not need to change their diet.
- Review what they are to do in upcoming week, when to mail their logs, etc.
- Closure – all present swear the PLEDGE (holding up the right hand) – "I promise to give my best effort"
- Be sure that the subject leaves with a folder (with envelopes and DVD), a game, a pad and a Game Unit, if necessary.

APPENDIX 9: Directions on DDR Use

Getting Started: DDR FOR PLAYSTATION 2

Once you have the PlayStation correctly hooked up to your TV, insert the DDR CD into the PlayStation, close the cover, and press the RESET button. The game should start automatically. Follow these steps to properly set up the game for you workout:

1. The game will prompt you to insert a memory card (red screen). Touch the left arrow once (to select YES, continue), then press X.
2. Press X when the instruction screen comes up.
3. When the MODE SELECT screen comes up, press the BACK arrow until OPTIONS lights up, then press X.
4. Press the BACK arrow until GAME OPTIONS lights up, then press X.
5. Press the BACK arrow until MAX STAGE lights up, then press the RIGHT arrow once until "4" is displayed.
6. Press BACK arrow until GAME OVER lights up, then press RIGHT arrow until END OF MUSIC lights up.
7. Press BACK arrow until DEFAULT DIFFICULTY lights up, then press the left arrow once so that BEGINNER is displayed.
8. Press the BACK arrow until EXIT lights up, then press X.
9. Press BACK arrow until EXIT lights up, and press X.
10. You should see the MODE SELECT screen again. Press the UP arrow until GAME MODE lights up, then press X.
11. Select SINGLE, press X.
12. Select CHARACTER, press X.
13. Select MUSIC by pressing LEFT arrow 13 times until the song "HIGHS OFF U" shows in the box, press X.
14. Press X once again, and the song begins.
15. After the song, the word CLEARED comes across the screen, and then the GRADE screen is displayed showing your grade (A, B, C, D, or E).
16. Press X to continue
17. Press X to select the same song for the 2nd stage.
18. Continue through to the 4th stage.
19. After STAGE 4, continue to press X to reset the game. You do not have to reset the OPTIONS unless you turn the game OFF.
20. If you need to re-start the game at any time, press and hold START.