

# **Probiotic Supplementation, The Gut Microbiota, and Cardiovascular Health**

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## ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death in the United States<sup>1</sup>,  
<sup>2</sup>. Recently, the gut microbiota has been implicated in the pathophysiology and progression of CVD. Experimental evidence suggests that high fat feeding alters the functional composition of the gut microbiota (dysbiosis); leading to increased translocation of the pro-inflammatory, endotoxin, and increased production of the pro-atherogenic, trimethylamine-*N*-oxide (TMAO). Together, these changes are hypothesized to accelerate CVD progression. Conversely, administration of gut microbiota modulating agents, such as antibiotics and probiotics, attenuate high fat feeding induced CVD in rodent models. In humans, the capacity to produce TMAO following L-carnitine or phosphatidylcholine challenges is abolished after receiving broad spectrum antibiotics for a period of one week<sup>3,4</sup>. However, whether gut modulation over a longer period of time decreases fasting serum endotoxin, fasting plasma TMAO, and CVD risk in response to high fat feeding has been unexplored in humans. To address these issues we conducted a randomized, placebo controlled, parallel group designed, controlled feeding study in healthy, non-obese males receiving the multi-strain probiotic, VSL #3 (or placebo), while consuming a high fat diet for 4-weeks. First, we tested the hypothesis that VSL #3 would attenuate the rise in serum endotoxin and consequent arterial stiffening following high fat feeding in healthy, non-obese males. Second, we tested the hypothesis that VSL #3 would attenuate the rise in plasma TMAO concentrations following high fat feeding in healthy, non-obese males. In contrast to our first hypotheses, serum endotoxin concentrations and arterial stiffness did not change in response to high fat feeding or with

VSL#3 treatment. Interestingly, VSL #3 significantly attenuated the increase in body mass (+ 1.4±0.4 vs. +2.3±0.3 kg;  $P < 0.05$ ) and fat mass (+0.7±0.1 vs. + 1.4±0.3 kg;  $P < 0.05$ ) following high fat feeding compared to the placebo. In contrast to our second hypothesis, probiotic supplementation did not attenuate the rise in plasma TMAO following high fat feeding. Future studies are necessary to elucidate the mechanisms responsible for the prevention of body mass and fat mass gain with VSL#3 supplementation following high fat feeding. In addition, studies are needed to determine whether higher doses of VSL #3, other single or multispecies probiotics, prebiotics, or synbiotics attenuate the production of the proatherogenic, TMAO.

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## CHAPTER I

### Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States (U.S.) and its prevalence increases with advancing age<sup>1,2</sup>. It is estimated that 83.6 million people in the U.S. have CVD<sup>2</sup>. As such, there is a high economic cost (\$444 billion/year) associated with this disease<sup>3</sup>. Large-scale epidemiological studies have identified many risk factors in the development of CVD<sup>4-6</sup>, and many risk modifying interventions exist that have the potential to prevent CVD morbidity and mortality<sup>7</sup>. However, as currently delivered, estimations suggest that CVD prevention activities would actually increase CVD direct medical costs over the next 30 years<sup>8</sup>. The high individual and societal burden of CVD mandates simple, cost effective, and efficacious therapies to be explored.

Arterial stiffness and atherosclerosis play a key role in the pathophysiology of CVD. Stiffening of the arteries occurs progressively over time and is characterized by elastic fiber degradation and fibrotic material accumulation in medial layer of the large elastic arteries<sup>9</sup> (See appendix A for full review). Large artery stiffening creates gross hemodynamic changes that participates in end organ damage<sup>10</sup> and increase the risk for cardiovascular and all-cause mortality<sup>11</sup>. Atherosclerosis is a complex process that results in lipid and fibrous material accumulation in the arterial wall, which eventually leads to arterial stenosis or plaque rupture<sup>1,12</sup>. Importantly, atherosclerosis is the underlying cause of the majority of clinical cardiovascular events in westernized societies<sup>12</sup>.

The mechanisms responsible for the arterial stiffening and atherosclerosis are not fully elucidated, however inflammation appears to be important. For example, cross sectional studies indicate that circulating inflammatory markers such as soluble cluster of

differentiation 14 (sCD14), C-reactive protein (CRP), tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin 6 (IL-6) are strongly and independently associated with large artery stiffening in apparently healthy individuals<sup>13, 14</sup>. Furthermore, animal and cell culture models have identified endothelium-mediated inflammation and increased monocyte recruitment to the intimal wall as crucial initiating factors in atheroma formation<sup>15</sup>.

Recent animal and human studies have implicated the gut microbiota in CVD<sup>16-19</sup>. Specifically, the gut derived substances lipopolysaccharide (LPS or endotoxin) and trimethylamine *N*-oxide (TMAO) are hypothesized to participate in alternate vascular inflammatory pathways that accelerate CVD progression. The first mechanism relates to high fat diet induced deleterious changes in the gut microbial composition, a condition referred to as dysbiosis<sup>20, 21</sup>. Dietary induced dysbiosis leads to increased intestinal permeability, LPS translocation, and activation of systemic and local pro-inflammatory pathways<sup>21-23</sup>. This rise in systemic LPS is referred to as metabolic endotoxemia. Importantly, several lines of evidence implicate metabolic endotoxemia as a mediator in large artery stiffening and CVD. First, arterial stiffness is increased in healthy humans given a low dose of endotoxin, irrespective of atherosclerotic burden<sup>24</sup>. Second, cross sectional analyses have reported that plasma concentration of sCD14, a key adaptor molecule in LPS-TLR4 signaling, is associated with arterial stiffness independent of atherosclerotic disease and traditional CVD risk factors<sup>13</sup>. Finally, a large prospective study indicated that elevated plasma LPS at baseline is independently associated with incident atherosclerosis and CVD at follow up in apparently healthy individuals<sup>25</sup>. The second hypothesized mechanism of gut mediated CVD relates to the gut microflora's obligatory role in the metabolism of foods containing trimethylamine (TMA) structures

such as choline, phosphatidylcholine, and L-carnitine<sup>26, 27</sup>. High fat foods, such as eggs, milk, liver, and red meat have high quantities of choline, phosphatidylcholine, and L-carnitine<sup>28, 29</sup>. The gut microbial metabolism of these compounds in the intestine leads to the production of TMA<sup>30</sup>, which is readily absorbed into systemic circulation and oxidized by hepatic flavin monooxygenases (FMO) 3 to TMAO<sup>31</sup>. Importantly, TMAO is independently associated with incident major adverse cardiovascular events (MACE), even after accounting for traditional risk factors<sup>27</sup>. Furthermore, cross sectional and prospective data have shown that fasting plasma L-carnitine levels independently predict an increased risk for prevalent CVD and incident MACE, but only in individuals with concurrently high TMAO levels<sup>26</sup>.

The relationship between the gut microflora and CVD suggests that modification of the gut microbiota may be used as a therapeutic target for attenuating CVD risk and several lines of evidence support this postulate. First, selective modulation of the gut microbiota with pre- and probiotics during high fat feeding attenuates metabolic endotoxemia, overactivation of the immune system, and cardiometabolic diseases progression in rodent models<sup>16, 26, 30</sup>. Second, TMAO production and atherosclerosis progression is attenuated in Apoe -/- receiving an L-carnitine supplemented diet with antibiotics compared to mice receiving a L-carnitine supplemented diet alone<sup>26, 27</sup>. Finally in humans, the capacity to produce TMAO following L-carnitine or phosphatidylcholine challenges is virtually abolished after receiving broad spectrum antibiotics for one week<sup>16, 18</sup>. However, whether gut modulation over a longer period of time decreases fasting serum endotoxin, fasting plasma TMAO, and CVD risk in response high fat feeding has been unexplored in humans. To address these issues we conducted a

randomized, placebo controlled, parallel group designed controlled feeding study in healthy, non-obese males receiving either a placebo or a multi-strain probiotic (VSL #3) while consuming a high fat diet for 4-weeks. First, we tested the hypothesis that VSL #3 would attenuate the rise in serum endotoxin and consequent arterial stiffening following high fat feeding in healthy, non-obese males. Second, we tested the hypothesis that VSL #3 would attenuate the rise in plasma TMAO concentrations following high fat feeding in healthy, non-obese males. We chose to use probiotic supplementation since this therapy has been shown have many cardiovascular benefits<sup>31-33</sup>, and is able to alter TMAO production in a humanized microbiome mouse model<sup>34</sup>.

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## CHAPTER II

### Probiotic Supplementation and Arterial Stiffness Following High Fat Feeding

#### ABSTRACT

**BACKGROUND:** The purpose of this study was to test the hypothesis that a multi-strain probiotic, VSL #3, would attenuate an increase in arterial stiffness and serum endotoxin following 4 weeks of high fat feeding. In addition, we measured circulating pro-inflammatory cytokines, plasma lipids and lipoproteins, and body composition to further elucidate the effects of VSL #3 on the cardiovascular risk profile following high fat feeding. **METHODS:** Twenty non-obese (body mass index,  $23.7 \pm 0.6$  kg/m<sup>2</sup>) males between the ages 18-30 years volunteered to participate in the present study. Following a 2-week eucaloric control diet, subjects were randomized to either 2 sachets of VSL #3 (450 billion live bacteria per sachet) or 2 identical sachets of placebo (cornstarch) during the consumption of a hypercaloric (+1,000 kcal/day), high fat diet for four weeks. The diet consisted of 25% of total caloric intake supplied from saturated fat and a macronutrient composition of  $55 \pm 0.04$  % fat,  $30 \pm 0.04$  % carbohydrate, and  $15 \pm 0.01$  % protein. Measurements of aortic pulse wave velocity and  $\beta$ -stiffness index (carotid ultrasonography and applanation tonometry), serum endotoxin concentrations (Recombinant factor C endotoxin detection assay), high sensitivity C-reactive protein (ELISA), plasma lipids and lipoproteins (Nuclear Magnetic Resonance), and body composition (dual x-ray absorptiometry) were measured at baseline and following high fat feeding. **RESULTS:** Arterial stiffness, serum endotoxin concentrations, and high sensitivity C- reactive protein did not change following high fat feeding in either group. HDL cholesterol increased similarly between groups ( $+10.8 \pm 4.9$  and  $7.3 \pm 4.9$ mg/dL), but there were no significant changes in total cholesterol, triglycerides, LDL cholesterol, or

VLDL cholesterol following high fat feeding in either group. Interestingly, VSL #3 significantly attenuated the increase in body mass (+ 1.4±0.4 vs. +2.3±0.3 kg) and fat mass (+0.7±0.1 vs. + 1.4±0.3 kg) following high fat feeding compared to the placebo.

**CONCLUSIONS:** Arterial stiffness and endotoxin concentrations did not change in response to high fat feeding alone or with VSL#3 treatment in healthy, non-obese young (age, 22.7±0.16 year old) males. In addition, high fat feeding alone or with VSL# 3 did not influence plasma glucose, serum insulin or serum high sensitivity C-reactive protein concentrations, but increased HDL cholesterol similarly in both groups over time. Conversely, VSL#3 treatment attenuated body mass and fat mass gain following high fat feeding compared to placebo. We have previously reported that experimental weight gain (~5 kg) increases arterial stiffness in healthy, non-obese young males. Future studies are needed to test whether a threshold of body weight gain is necessary for arterial stiffening to occur. Furthermore, studies are necessary to elucidate the mechanisms responsible for the prevention of body mass and fat mass gain with VSL#3 supplementation during high fat feeding.

## **Introduction**

Cardiovascular disease (CVD) is the leading cause of death in the United States<sup>1</sup>. Large epidemiological studies have identified stiffening of the large elastic arteries as a mediator in the etiology and progression of CVD<sup>2, 3</sup>. Arterial stiffness is an independent predictor of coronary events and all-cause mortality<sup>4, 5</sup>. Importantly, numerous studies suggest that inflammation is associated with stiffening of the arteries in the cardiothoracic region<sup>6-9</sup>.

The gut microbiota is a large source of inflammatory agents. Gram-negative bacteria comprise approximately 70% of the total bacteria in the gut<sup>10</sup>.

Lipopolysaccharide (LPS or endotoxin) is the major glycolipid component of the outer membrane of gram negative bacteria<sup>11</sup> and can be transported from the gut to the systemic circulation through enterocytes or via chylomicrons<sup>12</sup>. Importantly, once in circulation, LPS initiates pro-inflammatory pathways by binding to its receptor, toll-like receptor- 4 (TLR4)<sup>13</sup>.

Experimental evidence suggests that energy surplus and/or a high-fat/westernized diet deleteriously changes the composition of the gut microbiota (dysbiosis) leading to increased intestinal permeability, systemically elevated LPS (metabolic endotoxemia), overactivation of pro-inflammatory pathways, and the development of cardiometabolic diseases<sup>14-16</sup>. Importantly, several lines of evidence implicate metabolic endotoxemia as a mediator in large artery stiffening and CVD. First, arterial stiffness is increased in healthy humans given a low dose of endotoxin, irrespective of atherosclerotic burden<sup>7</sup>. Second, cross sectional analyses have reported that plasma concentration of sCD14, a key adaptor molecule in LPS-TLR4 signaling, is associated with arterial stiffness independent of atherosclerotic disease and traditional CVD risk factors<sup>17</sup>. Finally, a large prospective study indicated that elevated plasma LPS at baseline is independently associated with incident atherosclerosis and CVD at follow up in apparently healthy individuals<sup>18</sup>.

Selective modulation of the gut microbiota with pre- and probiotics during high fat feeding attenuates metabolic endotoxemia, overactivation of the immune system, and cardiometabolic diseases in rodent models<sup>19-21</sup>. Probiotics populate the gut with live bacterial species that contribute to host cardiovascular health in many ways including, but not limited to, decreasing pro-inflammatory pathways<sup>22</sup>, improving lipid and lipoprotein profiles<sup>23</sup>, and reducing arterial blood pressure (BP)<sup>24</sup>. To our knowledge the potential

benefits of probiotic supplementation on arterial stiffness in response high fat feeding have not be studied in humans. Accordingly, we tested the hypothesis that 4-weeks of probiotic supplementation (VSL#3) would attenuate the increase in large artery stiffness induced by high fat feeding in non-obese, college aged males. We further hypothesized that probiotic supplementation would attenuate the rise in serum endotoxin and pro-inflammatory cytokines induced by high fat feeding.

### **Material and Methods**

#### ***Subjects***

A total of 20 non-obese (body mass index [BMI], 18- 30 kg/m<sup>2</sup>) young males (18-30 years old) volunteered and were determined to be eligible for the study from the 56 individuals who were screened. Subjects were weight stable ( $\pm$  2.5kg), sedentary to recreationally active ( $\leq$  2 days, 20 min/day of low-intensity physical activity), and abstained from antibiotic use for at least 6 months prior to study commencement. All subjects were normotensive (BP < 140/90 mm Hg), normoglycemic (fasting glucose < 100 mg/dL), normolipemic (total cholesterol <200 mg/dL, triglycerides <150 mg/dL), were not taking any medications or supplements (e.g., prebiotics/probiotics) that could influence variables at the time of the study, and were free from overt chronic diseases as determined by health history, urinalysis, and blood chemistry. In addition, subjects were excluded if their total daily fat consumption was  $\geq$  40% and/or their total daily saturated fat consumption was  $\geq$  15%. The Virginia Polytechnic and State University Institutional Review Board approved the study protocol. The nature, purpose, risks, and benefits of the study were explained before obtaining informed consent.

#### ***Experimental design***

This study utilized a double blind, placebo-controlled, parallel group design. Following screening measurements, all individuals participated in a 2 week lead-in diet

period, followed by baseline testing and randomization to one of two groups; VSL # 3 ([n=9], two packets a day; 450 billion live bacteria per packet) or placebo ([n=11], two identical packets a day of cornstarch) for 4 weeks of high fat feeding (see below). Subjects repeated baseline testing immediately following high fat feeding.

### ***Controlled feeding***

All diets were “standardized” to avoid any potential confounding due to inter-individual habitual dietary differences. Our research dietitian estimated energy requirements based on height, weight, age, and activity level using the Institutes of Medicine equation<sup>25</sup>. From this information, a 7-day cycle menu was constructed for each participant with the appropriate macronutrient and caloric content for each diet (lead-in and high fat) using Nutritionist Pro™ software (Axxya Systems, Stafford, TX). Food modules (250 kcal) with the same macronutrient composition as the lead-in diet were added or subtracted if weight trended > 1 kg down or up, respectively during this period. In addition, subjects were weighed each morning to ensure weight stability during the lead-in period and to track weight changes during high fat feeding.

Subjects consumed breakfast in the metabolic kitchen of the Department of Human Nutrition, Foods, and Exercise each day and were given a cooler with food for the remainder of the day upon finishing. Participants were instructed to only consume provided food for the duration of the study and were asked to report all non-study foods to research staff. Instructing subjects to return any uneaten food and all food containers unwashed ensured dietary compliance.

For the first two weeks of the study, all participants consumed a eucaloric lead-in diet (57±0.07 % carbohydrate, 28±0.1 % fat, 15±0.25 % protein) to reduce variability in

baseline measurements caused by inter-individual differences in habitual dietary patterns. Following the lead-in diet, all participants consumed a hypercaloric (+1000kcal/day surplus), high fat diet for 4 weeks. The diet consisted of 25% of total caloric intake supplied from saturated fatty acids (SFA) and a macronutrient composition of  $55\pm 0.07\%$  fat,  $30\pm 0.07\%$  carbohydrate, and  $15\pm 0.01\%$  protein. During the high fat feeding period, surplus calories were supplied in the form of a high fat, ice cream based shake (1,017 kcal, 15 g protein, 60 g carbohydrate, 81 g fat) which contained either two packets of VSL #3 or two identical packets of placebo (cornstarch). Each morning research staff delivered the shake to subjects and supervised its complete consumption.

### ***Experimental Testing***

Subjects began the study with pre-enrollment and dietary intake screening. Upon completion of screening, all subjects participated in the lead in diet for 2 weeks, which was immediately followed by baseline measures. Following baseline measures, subjects consumed the high fat diet for 4 weeks and completed the study with follow-up measures following high fat feeding. All testing took place at the Human Integrative Physiology Lab (HIPL) between the hours of 5:00 and 11:00am. Subjects remained 12 hours fasted (included caffeinated and alcoholic beverages), performed no vigorous physical activity for the prior 48 hours, and were free from acute illness for the prior two weeks.

### ***Measurements***

***Body mass and composition.*** Body mass was measured on a digital scale (Model 5002, Scale-Tronix, Inc.) accurate to  $\pm 0.1$  kg. Height was measured to the nearest 0.1cm using a stadiometer. Body composition was measured using dual-energy x-ray absorptiometry (DEXA; Lunar Prodigy Advance, GE Medical Systems, software version 8.10e).

**Brachial arterial pressure.** Brachial arterial pressure was measured as part of the pre-screening process and conformed strictly to AHA guidelines<sup>26</sup>. Briefly, subjects rested in a seated position for 10 minutes prior to automated sphygmomanometry (Pilot model 9200, Colin Instruments Corp.). Measurements were taken every three minutes until BP stability was reached ( $\pm 6$  mm Hg difference for both systolic and diastolic BP).

**Habitual dietary intake.** Habitual dietary intake was measured as part of the pre-screening process and was determined using detailed 4-day diet records. Subjects were instructed on the proper way to weigh and record food intake for 3 weekdays and 1 weekend day. Habitual dietary intake was analyzed with Nutrition Data System for Research (N-DSR) software (University of Minnesota) by a trained diet tech.

**Blood chemistry.** Plasma was collected in K<sub>3</sub> EDTA BD vacutainers™, and was immediately centrifuged at 4 C° for 15 minutes at 2500g. Serum was collected in silicone-coated BD vacutainers™, allowed to clot at room temperature for 15 minutes, and centrifuged at 4 C° for 15 minutes at 2500g.

Plasma triglycerides, very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) concentrations were determined by nuclear magnetic resonance (The Vantera® Clinical Analyzer) by a commercial laboratory (Liposcience, Raleigh NC). Total cholesterol and low-density lipoprotein (LDL) concentrations were determined by conventional enzymatic techniques by a commercial laboratory (Liposcience, Raleigh NC). Plasma glucose concentrations were determined with an YSI Stat Plus glucose analyzer (model 2300, Yellow Springs Instruments). Insulin and high sensitivity C-reactive protein (hsCRP) were determined via enzyme-linked immunosorbent assay (ELISA; ALPCO Diagnostics, Salem, NH & R&D Systems, Minneapolis, MN,

respectively). Serum endotoxin was determined using the PyroGene™ Recombinant Factor C Endotoxin Detection Assay (Lonza, Walkersville, MD) according to the manufactures instructions.

***Carotid-femoral pulse wave velocity.*** Carotid-femoral (C-F) pulse wave velocity (PWV) was our primary measure of arterial (aortic) stiffness and was obtained by serially measuring carotid and femoral artery waveforms using a high fidelity, non-invasive applanation tonometer (NIHem, Cardiovascular Engineering, Inc.) as previously described<sup>27,28</sup>. Briefly, subjects were studied in the supine position after ~10 minutes of rest. A semi-automated computed controlled device was used to auscultate brachial arterial pressure between 3-5 times at 2-minute intervals in order to obtain BP stability ( $\pm$  5 mm Hg difference for both systolic and diastolic blood pressure). Next, a high fidelity finger probe tonometer was used to obtain carotid, brachial, radial, and, femoral artery waveforms over 10-20 cardiac cycles. Arterial waveforms were then saved to a computer device for later analysis and body surface measurements were made from the suprasternal notch (SSN) to the carotid and femoral recording sites using a Gulick® tape measure and enlarged caliper, respectively.

Tonomtery waveforms were signal averaged and the electrocardiogram R wave served as a fiducial point<sup>28</sup>. BP values were over-read by an experienced reviewer and the average of the systolic and diastolic BPs were used to calibrate the peak and trough of the signal averaged brachial waveform. Brachial diastolic and mean arterial pressures were then used to calibrate the other arterial waveforms. C-F PWV was then calculated by dividing the travel distance (SSN to carotid recording site – SSN to the femoral recording

site) by the travel time obtained from the foot to foot of the signal-averaged carotid and femoral pulse waves.

***β-Stiffness index.*** β-Stiffness index, a relatively BP independent index of carotid artery stiffness, was measured using an ultrasound unit (Sonos 7500, Phillips Medical Systems) equipped with a high-resolution linear array transducer (3-11 MHz) and applanation tonometry (NIHem, Cardiovascular Engineering, Inc.) as previously described<sup>29</sup>. Briefly, after resting in the supine position for ~20 minutes, longitudinal B mode images of the cephalic portion of left common carotid artery, 1-2 cm proximal to the carotid bulb, were obtained over 15 cardiac cycles by placing the transducer at a 90° angle over the artery. When clear visibility of the near and fall walls was obtained, the images were stored on an optical disk for offline quantification. The maximal and minimal carotid artery diameters of 3 consecutive cardiac cycles were acquired with commercially available software (Vascular Research Tools 5, Medical Imaging Applications, LLC). Carotid artery BP was acquired from applanation tonometry of the carotid artery and brachial artery auscultation as described above. β-Stiffness index was calculated as:  $\beta = \ln(P_1/P_0)/((D_1-D_0)/D_0)$ , where  $P_1$  represents carotid artery systolic pressure,  $P_0$  represents carotid artery diastolic pressure,  $D_1$  represents the maximal diameter recorded during systole, and  $D_0$  represents the minimal diameter recorded during diastole.

***Statistical Analysis.*** Repeated measures analysis of variance was used to examine the between and within group differences in arterial stiffness and other dependent variables from baseline (post-lead in diet) to follow-up testing. A Tukey's post hoc analysis was used for multiple comparisons. hsCRP concentrations were not normally distributed and as such were log transformed. Independent T-tests were used to compare the magnitude

of change in dependent variables between groups. All of the data are expressed as means  $\pm$  standard error (SE). The significance level was set *a priori* at  $\alpha=.05$ .

## Results

Subjects' characteristics at baseline and following high fat feeding are shown in Table 1. There were no differences in age, weight, body mass index (BMI), body fat percentage, fat mass, and fat-free mass (all  $p > 0.05$ ) at baseline. In addition, supine resting brachial systolic BP (SBP), brachial diastolic BP (DBP), brachial pulse pressure (PP), carotid SBP, carotid DBP, carotid PP, and heart rate were not significantly different between groups at baseline.

Following high fat feeding, there was a significant increase in body mass ( $+2.3\pm 0.3$  and  $+1.4\pm 0.4$  kg) in the placebo and VSL groups, respectively. BMI ( $+ 0.7\pm 0.1$  and  $+ 0.4\pm 0.1$  kg/m<sup>2</sup>), body fat percentage ( $+ 1.2\pm 0.4$  and  $0.5\pm 0.2$  %), fat mass ( $+ 1.4\pm 0.3$  and  $+0.7\pm 0.1$  kg), and fat free mass ( $+ 0.9\pm 0.2$  and  $0.7\pm 0.4$  kg) significantly increased in the placebo and VSL groups, respectively. Importantly, the magnitude of change in BMI ( $+ 0.7\pm 0.1$  vs.  $+ 0.4\pm 0.1$  kg/m<sup>2</sup>), body mass ( $+2.3\pm 0.3$  vs.  $1.4\pm 0.4$  kg), and fat mass ( $+ 1.4\pm 0.3$  vs.  $+0.7\pm 0.1$  kg) was significantly greater in the placebo compared with the VSL group, respectively. There were no changes in supine resting brachial SBP, brachial DBP, brachial PP, carotid SBP, carotid DBP, carotid PP, and heart rate following high fat feeding in either group (all  $p > 0.05$ ).

Dietary intake for the lead-in and high fat diet periods is shown in Table 2. Subjects consumed significantly greater total energy ( $3947\pm 79$  vs.  $2903\pm 78$  kcal/day) as fat ( $256\pm 5$  vs.  $98\pm 4$  g/day), SFA ( $140\pm 2$  vs.  $28\pm 1$  g/day) and protein ( $125\pm 3$  vs.  $108\pm 3$  g/day) when consuming the high fat diet compared to the consumption of the lead-in diet.

Conversely, subjects consumed significantly lower total carbohydrate ( $282\pm 6$  vs.  $408\pm 11$  g/day), total fiber ( $14\pm 1$  vs.  $18\pm 1$  g/day), and the relative amount fiber per 1000kcal/day ( $3.5\pm 1$  vs.  $6.2\pm 1$  g/1000kcal/day) when consuming the high fat diet compared to the consumption of the lead-in diet.

Circulating metabolic and cardiovascular risk factors before and after the intervention are shown in Table 3. There were no differences in metabolic and cardiovascular risk factors at baseline (all  $p > 0.05$ ). Following high fat feeding there was a significant increase in HDL cholesterol ( $+10.8\pm 4.9$  and  $7.3\pm 4.9$ mg/dL) in the placebo and VSL groups, however this change was not significantly different between groups. In addition, total cholesterol trended to increase over time in both groups ( $+21\pm 11.8$  and  $9\pm 8.7$ ,  $P=0.056$ ), but there were no differences in VLDL, LDL, and triglyceride concentrations following high fat feeding (all  $p > 0.05$ ).

CF-PWV and  $\beta$ -stiffness index before and after the intervention are shown in Figure 1. There were no differences in CF-PWV and  $\beta$ -stiffness index at baseline and following high fat feeding (all  $p > 0.05$ ).

### **Discussion**

The major findings from the present study are that arterial stiffness and endotoxin concentrations did not change following high fat feeding alone or with VSL# 3 treatment. In addition, high fat feeding alone or with VSL# 3 did not influence plasma glucose, serum insulin or serum hsCRP concentrations, but increased HDL cholesterol similarly in both groups over time. Interestingly, VSL#3 treatment attenuated body mass and fat mass gain compared with placebo following high fat feeding.

Our current findings are inconsistent with our previous observation that experimental weight gain increases arterial stiffness and brachial SBP in healthy college aged, non-obese males<sup>30</sup>. Importantly, the current dietary intervention only increased total body weight by ~2.0kg, while our previous overfeeding trial resulted in an ~ 5.0kg body weight gain. It is possible that a threshold of body weight gain is necessary for arterial stiffening to occur. Future studies are needed to test this hypothesis.

Observational data suggest that individuals with greater energy intake have higher circulating LPS concentrations when compared to their age and weight matched counterparts<sup>16</sup>. Conversely, overfeeding and weight gain increases postprandial, but not fasting concentrations of LPS in healthy, non-obese males<sup>31</sup>. Our findings are consistent with the observation that overfeeding and modest weight gain does not induce fasting endotoxemia in healthy, non-obese men. We did not assess postprandial endotoxin concentrations in the present study.

It has been shown that there is an increase in HDL cholesterol, but not LDL cholesterol or serum triglycerides have following short-term experimental weight gain in healthy, young, sedentary subjects<sup>32</sup>. Our current findings are consistent with these reports.

Our current findings are consistent with the observation that probiotic supplementation reduces total fat mass during eucaloric, controlled feeding in overweight humans<sup>33</sup>. Our novel findings extend these observations by demonstrating that probiotic supplementation attenuates body mass and fat mass gain during high fat feeding.

The mechanisms mediating the prevention of body mass and fat mass gains with VSL # 3 are unclear, however a few possibilities exist. For example, the administration of

*Lactobacillus plantarum*, a species also present in VSL#3, results in a significant reduction in body mass and adipose tissue mass in diet-induced obese mice through the production of *trans*-10, *cis*-12 conjugated linoleic acid<sup>34</sup>. In addition, there is evidence to suggest that probiotic supplementation reduces adipose tissue size through a reduction in lipid absorption<sup>35</sup>, an increase in angiopoietin-like protein-4 (inhibitor of lipoprotein lipase)<sup>36</sup>, and an increase in sympathetic nervous system activity in white and brown adipose tissue<sup>37</sup>.

There are some limitations of the present study that should be discussed. First, findings in our sample population of healthy, college aged non-obese males may not be generalizable to the general population. Second, we did not assess physical activity during the intervention. Therefore, we cannot rule out the possibility of an increase in physical activity or non-exercise activity thermogenesis in the VSL #3 group during high fat feeding. However, we should emphasize that all the participants were sedentary and instructed not to change their current level of habitual physical activity. Finally, high fat feeding did not increase arterial stiffness and serum endotoxin concentrations in the present study. Therefore, we cannot rule out the possibility that VSL#3 might influence arterial stiffness or endotoxin concentrations with more significant body mass gain or in obese individuals.

In conclusion, the major findings from the present study are that arterial stiffness and endotoxin concentrations did not change with the high fat diet or with VSL#3 treatment. In addition, high fat feeding alone or with VSL# 3 did not influence plasma glucose, serum insulin or serum hsCRP concentrations, but increased HDL cholesterol similarly in both groups over time. Interestingly, VSL#3 treatment attenuated the increase

in body mass and fat mass gain with high fat diet. Future studies are needed to test the hypothesis that a threshold of body weight gain may be necessary for arterial stiffening to occur. Furthermore, future studies are necessary to elucidate the mechanisms responsible for the VSL #3 effect to attenuate body mass and body fat gain during high fat feeding.

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## **Disclosures**

None.

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Table 1: Subject characteristics at baseline (PRE) and after the high fat diet (POST).

Variable	Placebo (N=11)		VSL#3 (N=9)	
	PRE	POST	PRE	POST
Age, yr	22.9±1	--	22.4±1.1	--
Body weight, kg	74.7±2.6	77±2.6	73.7±3.9	75.1±4.3*‡
Body mass index, kg/m <sup>2</sup>	23.2±0.5	23.9±0.5	24.5±1.1	24.9±1.2*‡
Body Fat, %	18.3±2.5	19.5±2.3	21±2.4	21.7±2.4*
Total Fat Mass, kg	13.6±1.9	15±1.8	16.3±3	16.9±3*‡
Fat Free Mass, kg	58.3±2.8	59.2±2.7	55.1±1.6	55.8±1.8*
Brachial SBP, mm Hg	118±3.2	121±3.3	115±2.6	115±2.6
Brachial DBP, mm Hg	60±2	64±2.3	55±1.5	56±3.9
Brachial PP, mm Hg	60±3.3	59±2.6	58±2.2	61±3.9
Carotid SBP, mm Hg	103±3.6	106±5.3	96±6	98±6.2
Carotid DBP, mm Hg	59±2	62±2.3	57±3.7	56±3.9
Carotid PP, mm Hg	45±3.5	46±5	40±3.8	44±5
Heart rate, bpm	59±4	63±3	60±3	64±5

Values expressed as mean ± SE. SBP=Systolic Blood Pressure; DBP=Diastolic Blood Pressure; PP= Pulse Pressure; BPM= beats per minute. \* P<0.05 Time Effect; ‡ P< 0.05 Interaction Effect

Table 2: Dietary intake for the lead-in and high fat diets.

n=20	Energy, kcal/day	Protein, g/day	CHO, g/day	Fat, g/day	SFA, g/day	Fiber, g/day	Fiber, g/1000kcal/day
Lead-in Diet	2903±78	108±3	408±11	98±4	28±1	18±1	6.2±1
High Fat Diet	3947±79*	125±3*	282±6*	256±5*	140±2*	14±1*	3.5±1*

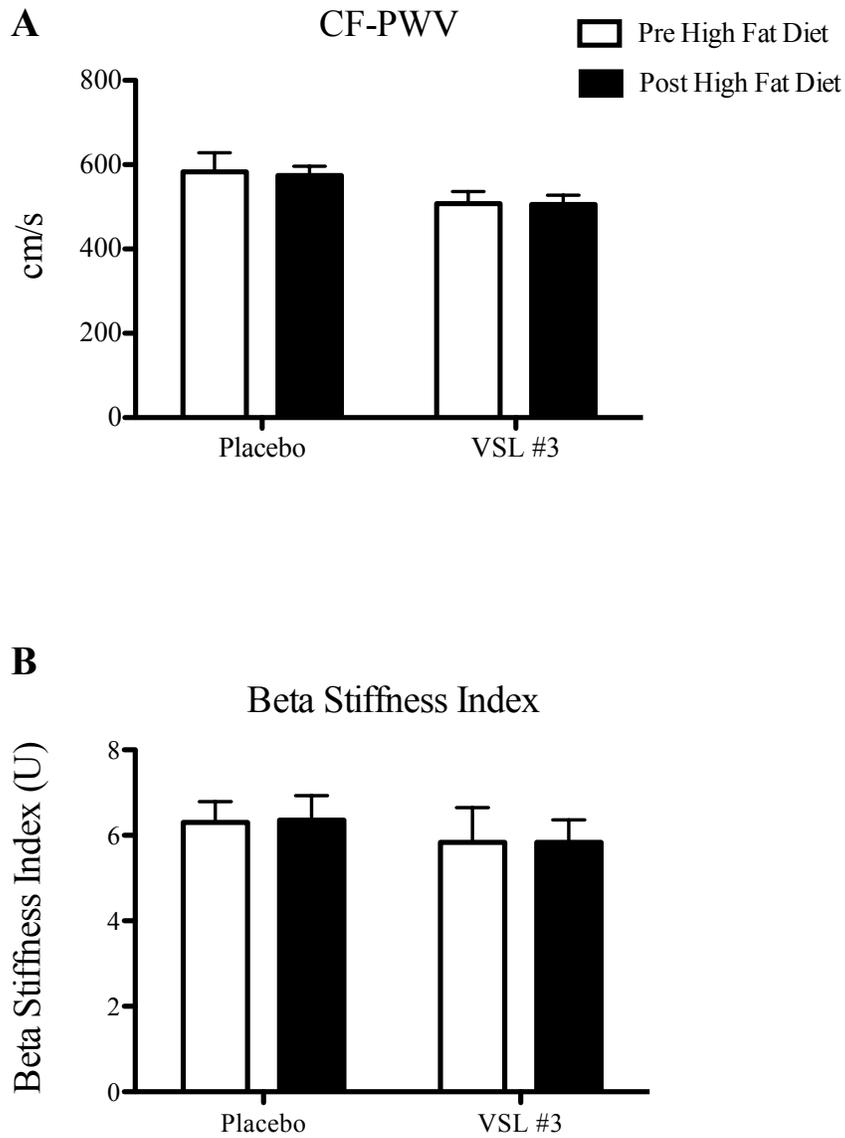
Values expressed as mean ± SE. CHO= carbohydrates; SFA= saturated fatty acids\*P<0.05 Time Effect.

Table 3: Cardiometabolic risk factors at baseline (PRE) and after the high fat diet (POST)

Variable	Placebo (N=11)		VSL #3 (N=9)	
	PRE	POST	PRE	POST
Triglycerides, mg/dL	103.6±8.3	102.6±6.1	90.5±9.8	102.3±6.9
Total cholesterol, mg/dL	158±7.6	179±6.4	140.6±7.2	149.6±8.2#
HDL, mg/dL	53.7±2.8	64.5±4.7	49.2±2.85	56.6±2.8**
LDL, mg/dL	95.8±6.6	103.9±6.1	85.4±6.2	85.8±8.5
VLDL, mg/dL	72.7±9.3	66.7±5.6	62.6±9	72.4±7.1
Glucose, mg/dL	79±3.8	81±2.2	81.8±2.6	81.5±4
Insulin, pg/dL	4±0.7	4±0.8	5.5±1.5	6.8±1.9
Endotoxin, EU/mL	1.4±0.4	1.6±0.4	2±0.5	1.7±0.2
hsCRP†, ng/mL	6.2±0.3	6.8±0.3	6.6±0.3	6.7±0.3

Values expressed as mean ± SE. HDL= high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein; hsCRP= High Sensitivity C-reactive protein. \*P<0.05 Time Effect, \*\*P<0.01 Time Effect; #P=0.056 Time Effect. †=log transformed.

**Figure 1**



**FIGURE LEGEND**

**Figure 1:** Carotid femoral PWV (A) and  $\beta$ -stiffness Index (B) before and after high fat feeding with placebo or VSL #3 treatment. Values expressed as mean  $\pm$ SE

## CHAPTER III

### Probiotic Supplementation and Trimethylamine-N-Oxide Production Following High Fat Feeding

#### ABSTRACT

**BACKGROUND:** The purpose of this study was to test the hypothesis that the multi-strain probiotic, VSL #3, would attenuate the increase in fasting plasma trimethylamine-*N*-oxide (TMAO) following high fat feeding in healthy, non-obese males.

**METHODS:** Nineteen healthy, non-obese (body mass index,  $23.7 \pm 0.6$  kg/m<sup>2</sup>) males between the ages 18-30 years volunteered to participate in the present study. Following a 2-week eucaloric control diet, subjects were randomized to either 2 sachets of VSL #3 (450 billion live bacteria per sachet) or 2 identical sachets of placebo (cornstarch) during the consumption of a hypercaloric (+1,000 kcal/day), high fat diet for four weeks. The macronutrient composition of the high fat diet was  $55 \pm 0.04$  % fat,  $30 \pm 0.04$  % carbohydrate, and  $15 \pm 0.01$  % protein. In addition, the diet contained  $70 \pm 2$  mg/day of L-carnitine,  $181 \pm 7$  mg/day of phosphatidylcholine, and  $300 \pm 9$  mg/day of choline. Plasma TMAO, choline, and betaine were measured with ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) at baseline and following high fat feeding. **RESULTS:** Plasma TMAO significantly increased in both the placebo and VSL # 3 treatment groups, however the magnitude of change in plasma TMAO was not statistically different between them. Plasma choline and betaine concentrations did not increase following high fat feeding in either group. **CONCLUSIONS:** High fat feeding increases plasma TMAO healthy non-obese, young (age,  $22.4 \pm 0.16$  years old) males. Importantly, VSL # 3 treatment does not appear to influence plasma TMAO concentrations following high fat feeding. Future studies are needed to determine whether

higher doses of VSL #3, other single or multispecies probiotics, prebiotics, or synbiotics attenuate the production of the proatherogenic, TMAO.

## **Introduction**

Cardiovascular disease (CVD) is leading cause of death in the United States<sup>1</sup>. High fat/westernized diets have been linked to CVD<sup>2, 3</sup>. It is generally presumed that the high saturated fat and cholesterol content present in high fat foods is responsible for increased CVD risk<sup>4</sup>. However, recent studies have provided evidence of other components present in high fat foods that contribute to the pathophysiology of atherosclerosis and CVD risk<sup>5-7</sup>. These studies indicate that the gut microbiota plays an obligatory role in the metabolism of nutrients containing trimethylamine (TMA) structures such as L-carnitine, phosphatidylcholine, and choline<sup>6, 7</sup>. The metabolism of these compounds leads to the production of TMA<sup>8</sup>, which is then readily absorbed into systemic circulation and oxidized by hepatic flavin monooxygenases (FMO) 3 to trimethylamine-*N*-oxide (TMAO)<sup>9</sup>. Importantly, TMAO is independently associated with incident major adverse cardiovascular events (MACE), even after accounting for traditional risk factors<sup>6</sup>. Furthermore, cross sectional and prospective studies indicate that fasting plasma L-carnitine levels independently predict an increased risk for prevalent CVD and incident MACE, but only in individuals with concurrently high TMAO levels<sup>7</sup>.

Atherosclerosis is accelerated in Apoe -/- mice fed a normal chow diet supplemented with L-carnitine or choline compared to animals on a standard chow diet<sup>6, 7</sup>. Mice on these diets are characterized as having elevated plasma TMAO, reduced cholesterol transport, and increased “forward” cholesterol transport. Conversely, mice administered broad spectrum antibiotics while on these diets produce significantly less

TMAO and are protected from pro-atherogenic changes<sup>6, 7</sup>. Furthermore, humans receiving an acute L-carnitine or phosphatidylcholine challenge produce significantly greater plasma TMAO in the postprandial period when compared to fasting. In addition, gut flora suppression with broad spectrum antibiotics, administered over a one week period, abolishes the increase in TMAO in response to these meal challenges<sup>5, 7</sup>. Interestingly, probiotic supplementation has been shown to alter liver concentrations of TMAO in a humanized microbiome mouse model<sup>10</sup>. However, whether gut modulation over a longer period of time decreases fasting plasma TMAO in response high fat feeding has been unexplored in humans. Accordingly, we hypothesized that probiotic supplementation (VSL#3) would attenuate the increase in plasma TMAO concentrations induced by high fat feeding.

## **Material and Methods**

### ***Subjects***

Nineteen non-obese (body mass index [BMI], 18- 30 kg/m<sup>2</sup>) college aged males (18-30 years old) who were included in a larger study examining the effects of probiotics on cardiometabolic health comprised the study sample. We excluded females to eliminate confounding due the transient trimethylaminuria (elevated urinary TMA) that has been reported to occur during menstruation<sup>11</sup>. Subjects were weight stable ( $\pm$  2.5kg), sedentary to recreationally active ( $\leq$  2 days, 20 min/day of low-intensity physical activity), and abstained from antibiotic use for at least 6 months prior to study commencement. All subjects were normotensive (BP < 140/90 mm Hg), normoglycemic (fasting glucose < 100 mg/dL), normolipemic (total cholesterol <200 mg/dL, triglycerides <150 mg/dL), were not taking any medications or supplements (e.g., prebiotics/probiotics) that could influence variables at the time of the study, and were free from overt chronic diseases as

determined by health history, urinalysis, and blood chemistry. In addition, subjects were excluded if their total daily fat consumption was  $\geq 40\%$  and/or their total daily saturated fat consumption was  $\geq 15\%$ . The Virginia Polytechnic and State University Institutional Review Board approved the study protocol. The nature, purpose, risks, and benefits of the study were explained before obtaining informed consent.

### ***Experimental design***

This study utilized a double blind, placebo-controlled, parallel group design. Following screening measurements, all individuals participated in a 2 week lead-in diet period, followed by baseline testing and randomization to one of two groups; VSL # 3 ([n=9], two packets a day; 450 billion live bacteria per packet) or placebo ([n=10], two identical packets a day of cornstarch) for 4 weeks of high fat feeding (see below). Subjects repeated baseline testing immediately following high fat feeding.

### ***Controlled feeding***

All diets were controlled to avoid any potential confounding due to inter-individual habitual dietary differences. Our research dietitian estimated energy requirements based on height, weight, age, and activity level using the Institutes of Medicine equation<sup>12</sup>. From this information, a 7-day cycle menu was constructed for each participant with the appropriate macronutrient and caloric content for each diet (lead-in and high fat) using Nutritionist Pro™ software (Axxya Systems, Stafford, TX). Food modules (250 kcal) with the same macronutrient composition as the lead-in diet were added or subtracted if weight changed  $> 1$  kg. In addition, subjects were weighed each morning during the lead-in period and during high fat feeding.

Subjects consumed breakfast in the metabolic kitchen of the Department of Human Nutrition, Foods, and Exercise each day and were given a cooler with food for the remainder of the day upon finishing. Participants were instructed to only consume provided food for the duration of the study and were asked to report all non-study foods to research staff. Instructing subjects to return any uneaten food and all food containers unwashed ensured dietary compliance.

For the first two weeks of the study, all participants consumed a eucaloric lead-in diet ( $57\pm 0.07\%$  carbohydrate,  $28\pm 0.11\%$  fat,  $15\pm 0.26\%$  protein) to reduce variability in baseline measurements caused by inter-individual differences in habitual dietary patterns. Following the lead-in diet, all participants consumed a hypercaloric ( $+1000\text{kcal/day}$  surplus), high fat diet for 4 weeks. The diet consisted of 25% of total caloric intake supplied from saturated fatty acids (SFA) and a macronutrient composition of  $55\pm 0.04\%$  fat,  $30\pm 0.04\%$  carbohydrate, and  $15\pm 0.01\%$  protein. In addition, the diet contained  $70\pm 2\text{ mg/day}$  of L-carnitine,  $181\pm 7\text{ mg/day}$  of phosphatidylcholine, and  $300\pm 9\text{ mg/day}$  of choline. Quantities of L-carnitine, phosphatidylcholine, and choline were estimated from gram quantities of diet foods using previously published conversion tables<sup>13-15</sup>.

During the high fat feeding period, surplus calories were supplied in the form of a high fat, ice cream based shake (1,017 kcal, 15 g protein, 60 g carbohydrate, 81 g fat) which contained either two packets of VSL #3 or two identical packets of placebo (cornstarch). Each morning research staff delivered the shake to subjects and supervised its complete consumption.

### ***Experimental Testing***

Subjects began the study with pre-enrollment and dietary intake screening. Upon completion of screening, all subjects participated in the lead in diet for 2 weeks, which was immediately following by baseline measures. Following baseline measures, subjects consumed the high fat diet for 4 weeks and completed the study with follow-up measures following high fat feeding. All testing took place at the Human Integrative Physiology Lab (HIPL) between the hours of 5:00 and 11:00am. Subjects remained 12 hours fasted (included caffeinated and alcoholic beverages), performed no vigorous physical activity for the prior 48 hours, and were free from acute illness for the prior two weeks.

### ***Measurements***

***Body mass and height.*** Body mass was measured on a digital scale (Model 5002, Scale-Tronix, Inc.) accurate to  $\pm 0.1$  kg. Height was measured to the nearest 0.1cm using a stadiometer.

***Brachial arterial pressure.*** Brachial arterial pressure was measured as part of the screening process and conformed strictly to American Heart Association guidelines<sup>16</sup>. Briefly, subjects rested in a seated position for 10 minutes prior to automated sphygmomanometry (Pilot model 9200, Colin Instruments Corp.). Measurements were taken every three minutes until BP stability was reached ( $\pm 6$  mm Hg difference for both systolic and diastolic BP).

***Habitual dietary intake.*** Habitual dietary intake was measured as part of the pre-screening process and was determined using detailed 4-day diet records. Subjects were instructed on the proper way to weigh and record food intake for 3 weekdays and 1

weekend day. Habitual dietary intake was analyzed with Nutrition Data System for Research (N-DSR) software (University of Minnesota) by a trained diet tech.

***Quantification of plasma TMAO, choline, and betaine.*** Fasting plasma concentrations of TMAO, choline, and betaine were quantified by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using the stable isotope dilution method against internal standards as described previously by Kirsch et al<sup>17</sup>.

TMAO, choline chloride, betaine chloride, betaine-d9 chloride, and choline-d9 chloride standards were obtained from Sigma (St. Louis, MO). TMAO-d9 standard was obtained from Cambridge Isotope Laboratories (Tewksbury, MA). A stock solution of the 3 internal standards (IS) (25.5, 26.8 and 28.0  $\mu\text{M}$  for betaine-d9, choline-d9, and TMAO-d9, respectively) was prepared in water and stored at  $-20^{\circ}\text{C}$ . Immediately prior to sample preparation, the IS were diluted 100x in acetonitrile. Following dilution, 300 $\mu\text{L}$  of the IS was combined with 25 $\mu\text{L}$  of plasma and was vigorously vortex (30 seconds) to remove analytes. Samples were then centrifuged at 17,000 g for 3 minutes at  $21^{\circ}\text{C}$  and the resultant supernatant was then vacuum filtered into HPLC vials and analyzed immediately by UPLC-MS/MS. UPLC-MS/MS analyses were carried out using a Waters Acquity UPLC system coupled to a Waters TQD triple quadrupole mass spectrometer equipped with MassLynx software (Waters, Milford, MA).

***Statistical Analysis.*** Repeated measures analysis of variance was used to examine the between and within group differences in TMAO production and other dependent variables from baseline (post-lead in diet) to follow-up testing. A Tukey's post hoc analysis was used for multiple comparisons. TMAO concentrations were not normally distributed and as such were log transformed. Independent T-tests were used to compare

the magnitude of change in dependent variables between groups. All of the data are expressed as means  $\pm$  standard error (SE). The significance level was set *a priori* at  $\alpha=0.05$ .

## Results

Dietary intake for the lead-in and high fat diet periods is shown in Table 1. Subjects consumed significantly greater total energy ( $3942\pm 83$  vs.  $2989\pm 82$  kcal/day), total fat ( $255\pm 5$  vs.  $98\pm 4$  g/day), SFA ( $140\pm 2$  vs.  $28\pm 1$  g/day), protein ( $125\pm 3$  vs.  $108\pm 3$  g/day), L-carnitine ( $70\pm 2$  vs.  $52\pm 2$  mg/day), phosphatidylcholine ( $181\pm 7$  vs.  $116\pm 4$  mg/day), and choline ( $300\pm 9$  vs.  $224\pm 12$  mg/day) when consuming the high fat diet compared to the consumption of the lead-in diet. Conversely, subjects consumed significantly lower total carbohydrate ( $282\pm 6$  vs.  $407\pm 11$  g/day), and total fiber ( $14\pm 1$  vs.  $18\pm 1$  g/day), and the relative amount fiber per 1000kcal/day ( $3.5\pm 1$  vs.  $6.2\pm 1$  g/1000kcal/day) when consuming the high fat diet compared to the consumption of the lead-in diet.

There were no differences in age, weight, and body mass index (BMI) (all  $p > 0.05$ ) at baseline. As we have previously reported, there was a significant increase in body mass and BMI following the high fat diet in both groups. In addition, the magnitude of change in body mass and BMI was significantly greater in the placebo compared with the VSL group, respectively (*data not shown*).

Fasting plasma concentrations of TMAO, choline, and betaine at baseline and following high fat feeding are shown in are shown in Figure 1. There were no differences in TMAO, choline, and betaine in the groups at baseline ( $p > 0.05$ ). Plasma TMAO significantly increased in both groups following high fat feeding, however the magnitude

of change in TMAO was not significantly different between them (Figure 1 A). There were no significant changes in the plasma concentrations of choline and betaine following high fat feeding in either group (Figure 1 B-C).

### Discussion

The main finding from the present study is that, in contrast to our hypothesis, probiotics supplementation did not attenuate the rise in plasma TMAO following high fat feeding. In addition, neither high fat feeding nor VSL #3 treatment influenced plasma concentrations of choline or betaine.

Stella et al.<sup>18</sup> reported that consumption of a diet high in red meat (420g/day) for 15 days increased urinary TMAO in non-obese, healthy males. Our results extend these findings by demonstrating that a high fat diet increases plasma TMAO in non-obese, healthy college aged males. The clinical significance this elevation in plasma TMAO ( $2.59 \pm 0.45$  vs.  $3.96 \pm 0.72$   $\mu\text{M}$ ; raw values) is not clear at this time. However, elevated fasting plasma TMAO concentrations are associated with prevalent coronary artery disease severity and the incidence of MACE in middle-aged and older individuals<sup>6</sup>. Future investigation of the effect of an acute increase in plasma TMAO on cardiovascular risk following high fat feeding is necessary.

In contrast to our hypothesis, probiotic supplementation did not attenuate the increase in plasma TMAO following high fat feeding. Proportions of several bacterial genera have been associated with plasma TMAO such as, *peptostreptococcaceae incertae sedis* and *clostridium*<sup>5</sup>. It is possible that VSL #3 did not alter the proportions of bacterial species within these genera. Future studies are needed to determine which bacterial

species metabolize L-carnitine, phosphatidylcholine, and choline to TMA so better therapies can be tailored.

There are some limitations of the present study that should be discussed. First, findings in our sample population of healthy, non-obese males may not be generalizable to the general population. Second, we did not measure biomarkers of cardiovascular risk in the current study. Therefore, we cannot make any conclusions regarding whether elevations in plasma TMAO following high fat feeding influence cardiovascular risk. Finally, we did not assess postprandial elevations in plasma TMAO following high fat feeding. It has been shown the gut modulation with antibiotics suppresses postprandial TMAO elevations following an L-carnitine meal challenge<sup>7</sup>. Therefore, we cannot rule out the possibility that VSL#3 might influence postprandial plasma TMAO elevations following an L-carnitine challenge.

In summary, our findings indicate that high fat feeding increases plasma TMAO in healthy non-obese, college-aged males. Interestingly, high fat feeding did not influence plasma choline or betaine. Importantly, VSL # 3 treatment did not appear to influence plasma TMAO concentrations following high fat feeding. Future studies are needed to determine whether higher doses of VSL #3, other single or multispecies probiotics, prebiotics, or synbiotics attenuate the production of the proatherogenic, TMAO.

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## **Disclosures**

None.

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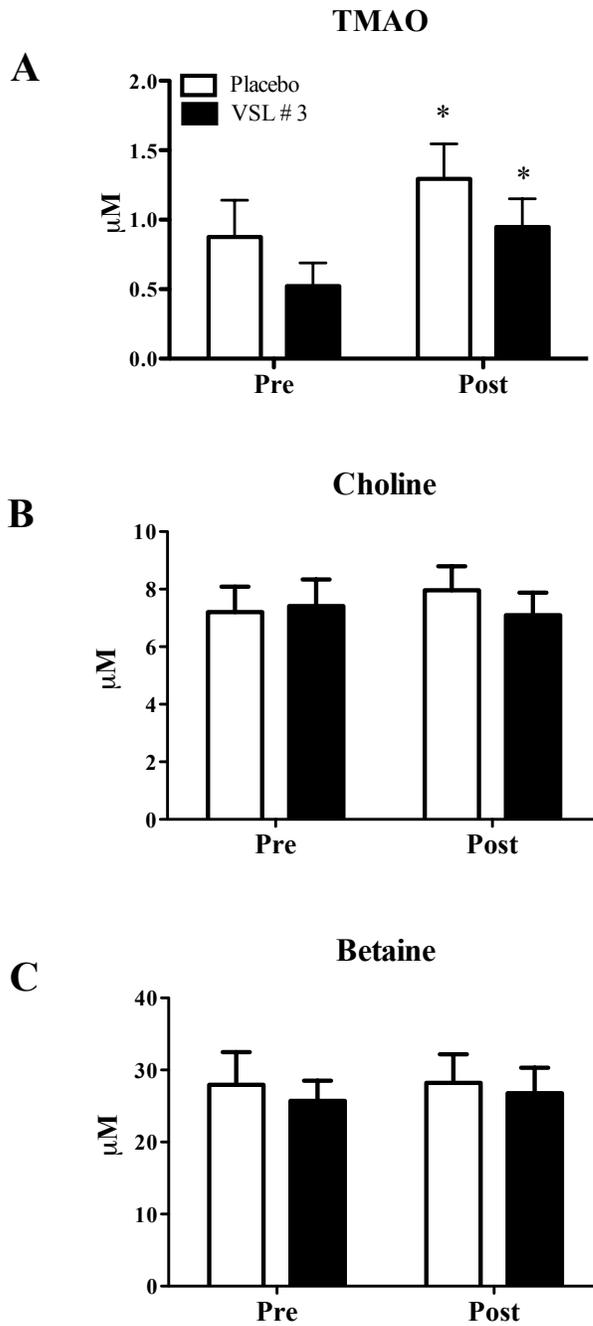
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Table 1: Dietary intake for the lead-in and high fat diets.

n=19	Energy kcal/day	Protein, g/day	CHO, g/day	Fat, g/day	SFA, g/day	Fiber, g/day	Fiber, g/1000kcal /day	Choline mg/day	PC mg/day	LC mg/day
Lead-in Diet	2898±82	108±3	407±1	98±4	28±1	18±1	6.2±1	224±12	116±4	52±2
High Fat Diet	3942±83*	125±3*	282±6*	255±5*	140±2*	14±1*	3.5±1*	300±9**	181±7**	70±2**

Values expressed as mean  $\pm$  SE. CHO= carbohydrates; SFA= saturated fatty acids;  
 PC=phosphatidylcholine; LC=L-carnitine. \*P<0.05 Time Effect; \*\*P<0.01 Time Effect

**Figure 1**



**FIGURE LEGEND**

**Figure 1:** (A) Plasma TMAO, (B) choline, and (C) betaine before and after high fat feeding with placebo or VSL #3 treatment. Values expressed as mean  $\pm$  SE.

## **CHAPTER IV**

### **Conclusions and Future Directions**

The main findings from our first study are that arterial stiffness and endotoxin concentrations did not change with high fat feeding or with VSL#3 treatment. Interestingly, VSL#3 treatment attenuated the increase in body mass and fat mass gain following high fat feeding.

We have previously shown that arterial stiffness increases in healthy, non-obese males with experimental weight gain of  $\sim 5 \text{ kg}^1$ . In the current study, experimental weight gain of  $\sim 2\text{kg}$  did not induce arterial stiffening in this population. Therefore, it is possible that we did not induce adequate weight gain to observe changes in arterial stiffness and thus cannot rule out the possibility that VSL # 3 influences arterial stiffness with more significant weight gain. Future studies are needed to test this hypothesis.

Serum endotoxin was not increased following high fat feeding in our study, therefore the effect of VSL # 3 on serum endotoxin cannot be ruled out. Elevated fasting serum endotoxin is associated with CVD disease severity in congestive heart failure (CHF)<sup>2</sup> and chronic kidney disease (CKD) patients<sup>3</sup>. Importantly, individuals with these disease states have increased aortic stiffening compared to age-matched, healthy controls<sup>4, 5</sup>. Therefore, these diseased populations may be better models to determine the efficacy of VSL # 3 on reducing serum endotoxin levels and arterial stiffness. Future studies in these populations are needed to test elucidate the effects of VSL # 3 on serum endotoxin and arterial stiffness.

Herein, we demonstrate that probiotic supplementation attenuates body mass and fat mass gain following high fat feeding. However, we did not measure fecal bacteria or explore potential mechanisms mediating body and fat mass attenuation. Therefore, future

studies that examine fecal bacteria taxonomic ranks and determine the mechanism(s) of body and fat mass attenuation following high fat feeding are imperative.

The main finding from our second study is that plasma TMAO increased similarly in the placebo and VSL # 3 treatment groups following high fat feeding in healthy, non-obese males. However, gut microbiota modulation with antibiotics has been shown to attenuate postprandial increases in plasma TMAO following an L-carnitine or phosphatidylcholine challenge in humans<sup>6</sup>. We did not assess postprandial TMAO concentrations in the present study. Therefore, we cannot rule out the possibility that VSL#3 may influence postprandial plasma TMAO elevations following an L-carnitine challenge. Future study is needed to assess the efficacy of VSL # 3 treatment of postprandial TMAO production in response to high fat feeding.

Fasting plasma TMAO has been independently associated with increased risk of prevalent coronary artery disease and increase risk of major adverse cardiovascular events<sup>7, 8</sup>. We did not measure biomarkers of cardiovascular risk in the current study. Therefore, we cannot make any conclusions regarding whether elevations in plasma TMAO following high fat feeding influence the cardiovascular system deleteriously. Future study is needed to determine the relationship between the changes in TMAO to the changes in cardiovascular risk biomarkers.

In conclusion, the major findings presented herein are that high fat feeding, with or without VSL# 3 treatment, does not influence serum endotoxin or arterial stiffness in healthy, non-obese males. In addition, we demonstrate that VSL # 3 does not attenuate the increase in plasma TMAO following high fat feeding in healthy, non-obese males. Interestingly, we observed attenuation of body and fat mass gain with VSL # 3 treatment

compared to the placebo following the high fat diet. Future studies are needed to determine the mechanisms responsible for the body mass and body fat mass gain attenuation following high fat feeding. In addition, studies are needed to determine whether higher doses of VSL#3, other single or multispecies probiotics, prebiotics, or synbiotics attenuate the production of the proatherogenic, TMAO.

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## APPENDICES

### APPENDIX A: Arterial Stiffness Review of Literature

#### A. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States (U.S.) and its prevalence increases with advancing age<sup>1,2</sup>. It is estimated that 83.6 million people in the U.S. have CVD<sup>2</sup>. As such, there is a high economic cost (\$414 billion) associated with this disease<sup>1</sup>. In the past decade, large-scale epidemiological studies have identified a key role of large artery stiffening in the etiology CVD<sup>3,4</sup>.

Stiffening of the large elastic arteries, in particular the aorta is central to the aging process. However, aortic stiffening is associated with end organ damage<sup>5</sup>, CVD risk factors, and atherosclerotic disease<sup>6-8</sup>. The measure of aortic stiffening can be used as a powerful biomarker of CVD risk and has the ability to independently predict fatal and non-fatal coronary events<sup>3,9</sup>. Therefore, strategies that attenuate the progression of age-related arterial stiffening are important for CVD risk reduction and prevention. The purpose of this review is to provide an up to date summary of the etiology and therapies of age-related arterial stiffening.

#### B. Arterial Structure and Function

##### Arterial Structure

The structure of the arterial system determines its function. Structurally, the arterial wall is composed of three zones or regions: the tunics intima, media, and adventitia<sup>10</sup>. The innermost layer, the intima, is composed of a single layer of endothelial cells and is supported by smooth muscle cells. This layer is separated from the media by the internal elastic lamina. The media is largely composed of elastic fibers and is

interspersed with collagen and smooth muscle cells<sup>10</sup>. The outermost layer, the adventitia, is mainly composed of collagen and fibroblasts<sup>10, 11</sup>. These three layers are continuous, but vary histologically throughout the arterial tree.

The arterial system can be sub-divided into two systems based on differing fibrous material composition. 1) The large elastic arteries (e.g., aorta, carotid) are characterized as having a high elastin: collagen ratio, while the more distal 2) muscular arteries (e.g., femoral, popliteal) are characterized as having a low elastin: collagen ratio. A high elastin: collagen ratio represents greater elasticity of an arterial segment, while a low elastin: collagen ratio represents greater stiffness<sup>10</sup>. Elasticity can be defined as “the ability of a body or material to return to its previous shape after a deforming force is released”, while stiffness is defined as “the resistance offered by an elastic body to deformation”<sup>12</sup>. In addition, as arteries move distally from the heart, the internal diameter of the lumen decreases. A decrease in luminal diameter also contributes to the greater stiffness of the peripheral compared to the central arteries<sup>12</sup>.

### **Arterial Function**

The arterial system functions as a transport and buffering system. The delivery of oxygen rich blood, nutrients, metabolites, and hormones to the tissues relates to transport and this function is largely performed by the muscular arteries and arterioles<sup>10, 13</sup>. Conversely, the buffering function is determined by the large elastic arteries which possess the ability to dissipate pulsatile flow generated by the cyclically beating heart<sup>10, 13</sup>.

As the heart contracts during systole, a forward pulse wave is generated that travels relatively slowly towards the periphery. The elastic structures of the aorta and

other large arteries buffer this pulsatile flow (energy) through their capacity to distend during systole and return to their resting diameter in diastole. The distention and relaxation of these arteries also provides the conversion of intermittent flow into an almost continuous stream throughout the entire cardiac cycle. The stiffness of the aorta largely determines to the amplitude and velocity of the forward wave. Therefore, under normal circumstances, the forward wave amplitude and pulse wave velocity (PWV) are relatively low given the high elastin: collagen ratio of the aorta<sup>5</sup>.

Structural differences between elastic and muscular arteries allow for the forward pulse wave to be partially reflected and amplified as it propagates distally. Partially reflected waves summate from sites of impedance (e.g., bifurcations) and create an aggregate retrograde wave that travels towards the aorta. The timing of the aggregate reflective wave is important since the majority of coronary perfusion occurs in diastole. Therefore, the return the reflective wave in late systole--early diastole produces a favorable secondary diastolic pressure rise that enhances coronary perfusion<sup>5, 10</sup>. Wave reflection also contributes to the dampening of pulsatile energy since partial wave reflection means less pulsatile energy is transmitted to the microcirculation. Furthermore, reflective waves contribute to pressure amplification in the periphery due to reflective sites being closer to the forward wave as the pulse travels distally. The amplification of the forward pressure wave allows for lower central pressures and maintenance of a driving pressure in the periphery. Lower central pressures will contribute to lower afterload and myocardial oxygen demand.

### **C. Aging and large artery stiffening**

#### **Arterial structural changes with age**

Age is the main determinant of large artery stiffness<sup>14, 15</sup>. While the large elastic arteries stiffen with age, the more peripheral muscular arteries are protected from this derangement<sup>10, 14, 16</sup>. Over time the relative content of elastin decreases, while the content of collagen and mucopolysaccharide increases<sup>7, 17, 18</sup>. In addition, elastin fibers fragment, the elastic lamellae becomes disorganized, and collagen fibers become cross-linked<sup>10</sup>. Together these changes lead to an increase in arterial stiffness, as the mechanical load of the cardiac cycle will be born by the stiffer collagenous fibers. Furthermore, an age-related increase in intima media thickness (IMT) will contribute to arterial stiffening since a thicker vessel made out of the same material as a less thick vessel will require more pressure to distend the same amount<sup>19, 20</sup>. Finally, the lumen of large arteries progressively dilates with age<sup>21</sup> and also contributes to arterial stiffening according to LaPlace's law<sup>22</sup>.

Indeed, these structural changes disrupt the functionality of the arterial system, leading to hemodynamic changes that affect multiple tissues/organs. Importantly, although arterial stiffening is a process of normative aging, it can be accelerated by traditional cardiovascular risk factors<sup>23</sup>, obesity<sup>24</sup>, inflammation<sup>25</sup>, genetic predisposition<sup>26</sup>, and environmental factors<sup>27</sup> (see below).

### **Hemodynamic changes with age**

Distinct hemodynamic changes occur before the age of 50 and after the age of 60. Before the age of 50, mean arterial pressure (MAP) rises, producing increases in systolic blood pressure (SBP) and diastolic blood pressure (DBP). Since the stress – strain relationship of the elastic arteries is non-linear; the increase in MAP drives a modest increase in aortic (a)PWV<sup>14</sup>. In addition, the increase in SBP and DBP slightly reduces

peripheral pulse pressure (pPP; brachial SBP-DBP). However, central pulse pressure (cPP) increases due to the premature arrival of the reflective wave in early systole as opposed to in late systole- early diastole<sup>28</sup>. The reflective wave arriving in systole increases central pressure and is expressed as the augmentation index (AI). Thus, AI is the product of augmented pressure (Ap) over cPP (Ap/cPP).

After the age of 60, a hemodynamic shift occurs, in which MAP reaches a maximum and begins to fall, while pPP reaches a minimum and thereafter begins to rise dramatically. The stiffening of the aorta and large arteries results in greater forward wave magnitude and aPWV. The accompanying lack of stiffening in the periphery leads to “impedance matching” and therefore shifts reflective sites distally<sup>14</sup>. The resultant is an increase in cPP despite a decrease in AI<sup>14, 29</sup>.

Importantly, these hemodynamic changes have clinical implications, with the increase in aPWV and cPP seemingly the most deleterious to end organs. cPP and aPWV are associated with isolated systolic hypertension<sup>30</sup> (ISH), myocardial ischemia<sup>31</sup>, left ventricular hypertrophy<sup>32</sup>, atrial fibrillation<sup>9, 33</sup>, stroke<sup>34, 35</sup>, cognitive impairments<sup>36, 37</sup>, and kidney failure<sup>38</sup>. As such, aPWV independently predicts the incidence of a first cardiac event<sup>3</sup>, cardiovascular mortality<sup>4, 9</sup> and all-cause mortality<sup>4, 39</sup>.

#### **D. Hypertension, large artery stiffening, and small artery remodeling:**

Sustained increases in blood pressure lead to higher arterial stiffness<sup>23, 40</sup>. The association between arterial stiffness and HTN is due to the non-linear elasticity of arteries. The manifestations of an increase in blood pressure are increased aortic impedance, PWV, and reflective pulse wave speed and magnitude. Together these changes further increase central and peripheral systolic and pulse pressures<sup>10</sup>.

Consequently, untreated HTN may accelerate arterial stiffening<sup>41</sup>, which may lead to a vicious cycle of further arterial stiffening and HTN. Although the bidirectional relationship between arterial stiffness and HTN is evident, the temporal relationship between these variables remains unclear<sup>42-45</sup>.

A reduction in blood pressure with antihypertensive agents reduces arterial stiffness. However, the arteries of treated hypertensive patients may remain stiffer than aged-matched normotensive controls, indicating arterial remodeling occurs due to HTN<sup>10</sup>. Several lines of evidence support this assertion. First, an increase in blood pressure can stimulate elastin fragmentation<sup>46</sup>, collagen production<sup>47</sup>, and vascular smooth muscle cell (VSMC) growth<sup>48, 49</sup> in experimental models. Secondly, hypertensive individuals present with greater aortic degeneration and dilation<sup>50</sup>, and greater cross sectional area and thickness of the elastic arteries compared to age matched normotensive controls<sup>51</sup>. Finally, there is strong evidence that resistance arteries undergo inward remodeling during essential hypertension<sup>51, 52</sup>, thus contributing to increased total peripheral resistance, MAP, and subsequently PWV.

#### **E. Overweight and Obesity on Stiffness**

Numerous reports suggest that overweight and obesity are associated with arterial stiffening independently of age, BP, and other traditional cardiovascular risk factors<sup>53-56</sup>. In fact, when matched for age, aPWV is ~ 50 cm/s higher in obese compared with non-obese individuals---an increase in aPWV that is equivalent to the effect of 5-10 years of aging<sup>24</sup>. The measure of central adiposity or direct imaging of visceral adipose tissue (VAT) shows stronger associations with arterial stiffening than other anthropometric measures<sup>56-58</sup>. For example, waist circumference and visceral adiposity are strongly

associated with aPWV, independent of traditional risk factors, most anthropometric measures, and total and peripheral fat stores<sup>57-59</sup>. In addition, VAT is correlated with some measures of vascular function and arterial stiffness in a large population of individuals, after adjusting for BMI and traditional cardiovascular risk factors<sup>60</sup>.

The relationship between adiposity and vascular properties has been described even in children and teenagers suggesting that neither age nor long term adiposity are prerequisite for arterial stiffening<sup>24, 61</sup>. Conversely, a few recent cross-sectional studies argue that body fat percentage, as measured bioelectrical impedance, is negatively correlated to aPWV prior to the age of 20<sup>62, 63</sup>. Importantly, the majority of cross-sectional and large scale prospective studies have described a relationship between the accumulation of adiposity and the acceleration of arterial stiffening across the lifespan; of which is strengthened by measuring central adipose stores<sup>24, 57, 61, 64, 65</sup>.

Unfortunately, the mechanisms by which visceral adiposity causes and/or contributes to arterial stiffness are poorly understood; however oxidative stress and inflammation seem to play an important role. VAT secretes a plethora of adipokines<sup>66</sup> and components of the renin angiotensin-aldosterone system (RAAS)<sup>67</sup>, which have been shown to directly or indirectly lead to inflammation and reactive oxygen species (ROS) production in the vasculature<sup>68, 69</sup>. Of particular to the stiffening process, adipose tissue secretes interleukin (IL) 6, tumor necrosis factor alpha (TNF $\alpha$ ), angiotensin (Ang) II, aldosterone, and matrix metalloproteinases (MMPs)<sup>66</sup>. Furthermore, total adiposity is associated with elevated sympathetic nervous activity to the kidney and heart<sup>70</sup>, while visceral adiposity is associated with elevated muscle sympathetic nerve activity<sup>71</sup>; together further elevating RAAS components in the systemic circulation. In addition, visceral and total adiposity

are associated with hyperinsulinemia<sup>72</sup>, hyperglycemia<sup>73</sup>, insulin resistance<sup>74</sup>, HTN<sup>75</sup> and endothelial dysfunction<sup>76</sup>; all of which have been shown to contribute to arterial stiffness<sup>7</sup>.

Interestingly, changes in arterial stiffness are related to changes in weight over time, and the presence of overt obesity does not seem to be a prerequisite for significant changes in arterial stiffness to occur<sup>64</sup>. In fact, findings from a study performed in our laboratory showed that intentional weight gain of ~ 5kg in healthy, non-obese men increased arterial stiffness and decreased arterial compliance over a 6-8 week experimental weight gain period. In addition, those individuals above the median in visceral fat gain had a significantly greater increase in arterial stiffness compared to those below the median<sup>77</sup>. Taken together, the above summarized suggests that visceral adiposity, more than subcutaneous adiposity, is a phenotype independently associated with arterial stiffness in young and older adults. Therefore, targeting visceral adiposity depots as part of risk reduction therapy may attenuate the acceleration of arterial aging, especially in high-risk individuals.

#### **F. Mechanisms of large artery stiffening**

Morphological and cellular alterations lead to functional (measured) stiffening of the large elastic arteries. Many factors, which work through independent and shared mechanisms, participate in the stiffening process. These factors include: alterations in hemodynamic forces, the neuro-hormonal milieu, glucose homeostasis, lipid metabolism, immune function and activity, environmental stimuli, and genetic phenotype. Indeed, disease states that further alter these factors, such as HTN can accelerate normal vascular stiffening<sup>78, 79</sup>. The following discussion focuses on the mechanisms hypothesized to

contribute to age-related arterial stiffening, but also addresses risk factors leading to these mechanisms for clarity.

### **Fatigue failure of elastin and large artery stiffening**

In 1976, O'Rourke theorized that the age-related elastin fragmentation and degeneration within the human aorta and other large elastic arteries was due to the fatiguing effects of repeated cyclic stress<sup>80</sup>. This theory was based on engineering principles regarding the behavior of any inanimate object exposed to flexing and stretching forces<sup>10</sup>. When these principles are applied to natural rubber at a stretch approximate to what the human aorta experiences per heart beat (~ 10% stretch) it is predicted that  $10^9$  cycles will lead to elastic fiber fracture<sup>81, 82</sup>. These principles may be applied to elastin since this protein is one of the most inert in the human body with a half-life of more than 40 years in man<sup>83, 84</sup>. Therefore, it is predicted that  $10^9$  cycles at 10% stretch are achieved within 25-40 years of life in the proximal aorta at a heart rate of 60-70 beats per minute<sup>50</sup>. Furthermore, when stretch is < 5%, which is approximate to the more distal muscular arteries, fracture is not expected within this same span. Thus, this theory also supports the relative protection of the peripheral muscular arteries from elastin fragmentation<sup>14, 85</sup>.

There remains little evidence in humans that arterial elastin fractures/fragments in response to repeated cyclic stretch. One study performed on purified pig aortas, exposed to different levels of mechanical stretch and cycle number provided evidence that cyclic loading to elastin causes structural changes and failure<sup>20</sup>. This study also reported that specimens subjected to greater stretch fatigued with a reduced number of stretch cycles, therefore somewhat substantiating the theory of accelerated elastin fragmentation due to

increased distending pressure and stretch<sup>86</sup>. Along these lines, individuals with consistently elevated heart rate and arterial blood pressure have accelerated age-related stiffening<sup>42</sup>.

Although attractive, this theory assumes that the elastin is not exposed to the vast cellular and chemical environment of the body. Many other cellular and hormonal factors participate in accelerated elastin degradation and arterial stiffening during elevated autonomic nervous system activity. Therefore it is challenging to determine the sole contribution of fatigue-failure in regards to elastin fragmentation *in vivo*. Future studies, possibly with the use of mechanical paced hearts in animal models, are needed to fully elucidate and substantiate the theory of fatigue-failure of elastin and its role in large artery stiffening.

#### **Matrix metalloproteinases and large artery stiffening:**

In adult animals, the turnover of elastin and collagen is a relatively slow<sup>83</sup>. However, acceleration of elastin and collagen degradation and fragmentation occurs during vascular inflammation<sup>87</sup>. Matrix metalloproteinase (MMPs) are collagenolytic and elastinolytic enzymes found in polymorphonuclear (PMN) cells, monocytes/macrophages<sup>88</sup>, and VSMC cells<sup>89</sup> and play a fundamental role in the degradation of vascular extracellular matrix (ECM)<sup>87</sup>. Under normal homeostatic conditions various activators and inhibitors regulate MMPs activity<sup>90</sup>, however as arteries age or undergo pathological changes the activity of MMPs increase while the activity of their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs), decrease<sup>7</sup>. Many mediators perturb the balance between MMPs and their inhibitors such as mechanical stress<sup>91</sup>, ROS<sup>92</sup>, growth factors, and pro-inflammatory proteins<sup>93</sup>. In addition,

fragments of elastin degradation can serve as chemotactic agents, which further enhance MMP activity.

Increased MMP9 and MMP2 activity is associated with destruction of the elastic laminae of arteries and aneurysm formation in animal models<sup>94</sup>. In addition, MMP2 may contribute to the hyperpermeability of the intimal basement membrane that allows transmigration of circulating VSMC to the intima<sup>95</sup>. In humans, increased total serum elastase and MMP9 levels are associated with increased aPWV in individuals with ISH and in normotensive participants even after adjusting for potential confounders<sup>96, 97</sup>.

In rodents, many of the age associated intimal and matrix changes are delayed by chronic administration of ACE inhibitors<sup>98</sup>. However, treatments targeting MMPs in humans are complicated due to MMPs widespread expression and contradictory roles in many pathophysiological processes<sup>99, 100</sup>. Nevertheless, the translation of findings from rodent to humans deserves further study.

#### **Advanced glycation end products and large artery stiffening:**

Advanced glycation end products (AGEs) are formed by a complicated, non-enzymatic, and irreversible process that links reducing sugar groups to proteins, lipids, and nucleic acids<sup>101</sup>. Glycation and AGE formation occurs in a time dependent fashion, however AGE formation is enhanced in carbonyl-enriched environments such as diabetes and end stage renal disease<sup>102</sup>. In addition, AGE formation can be accelerated in HTN<sup>103</sup>. Accumulation of AGE inside the vascular wall leads to collagen cross linking resulting in a stiffer protein that is resistant to regulatory turnover<sup>104</sup>. Indeed, the levels of AGE in the aorta of diabetics and control subjects are positively correlated to its stiffness<sup>103</sup>.

Soluble AGEs also have deleterious consequences to the vasculature. Soluble AGEs have been shown to activate stress signaling and pro-inflammatory responses by binding to their receptors, receptors of AGEs (RAGEs). RAGE signaling leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and the increased expression of pro-inflammatory proteins and adhesion molecules, oxidative stress, and transmigration of leukocytes across the endothelial barrier<sup>105, 106</sup>. In turn, NFκB promotes the expression of RAGE, thus leading to a feed forward loop and further enhancing inflammation and ROS production<sup>107, 108</sup>. Not surprisingly, circulating levels of soluble AGE in hypertensive, diabetic, and non-diabetic individuals have been related to aortic stiffness<sup>109, 110</sup>. Intriguingly, Alagebrium (formerly known as ALT-711) has been shown to break down AGEs *in vitro* and *in vivo*<sup>111</sup> and reduce aPWV and PP in elderly subjects with stiff arteries<sup>112</sup>. Unfortunately, the use of Alagebrium as a destiffening therapy has been largely underexplored in clinical trials and its efficacy as an agent to reduce age-related arterial stiffening merits future study.

### **Endothelial dysfunction and large artery stiffening:**

Age is also associated with endothelial dysfunction<sup>113</sup>. Under normal conditions the healthy endothelium elegantly regulates vasomotor tone by balancing the production of vasodilators and vasoconstrictors in response to a variety of stimuli<sup>114</sup>. The endothelium also contributes to the inhibition of atherosclerotic plaque formation by participating in anti-inflammatory and antioxidant functions<sup>115</sup>. However the endothelium becomes disrupted as a function of time, or through mechanical and/or inflammatory stress, rendering it dysfunctional and leading to a loss or attenuation of its beneficial functions.

Alterations in vasomotor tone and endothelial function have been shown to influence elastic and muscular artery stiffness<sup>116, 117</sup>. For example, it has been shown that intravenous (I.V.) administration of nitric oxide (NO) decreases aPWV<sup>118</sup>, while I.V. administration of endothelin (ET) 1 increases aPWV, independent of changes in BP<sup>119</sup>. In addition, percent flow mediated dilation (FMD) of the brachial artery is negatively correlated to cPP and positively correlated with proximal aortic compliance in patients with or without coronary artery disease (CAD)<sup>120</sup>. On the other hand, recent evidence suggests that large artery stiffening may lead to endothelial disturbances<sup>121-123</sup>. As such, large artery stiffening may lead to endothelial disturbances, which in turn may contribute to further arterial stiffening.

#### **Inflammation, oxidative stress, and large artery stiffening:**

It has been demonstrated that inflammation and oxidative stress are important in the development of arterial stiffness and endothelial dysfunction<sup>7, 124</sup>. Cross sectional and randomized control designed studies have demonstrated that pro-inflammatory cytokines (e.g., CRP, IL-6, TNF $\alpha$ ) are strongly and independently associated with aPWV during acute inflammation<sup>25</sup>, low-grade inflammation<sup>125-127</sup>, and chronic inflammatory disease states<sup>128</sup>. In addition, the extent of systemic inflammation has been correlated to arterial stiffness and cPP in apparently healthy individuals<sup>129-131</sup>. Furthermore, inflammation appears to mediate the severity of arterial stiffness in disease states such as the metabolic syndrome and hypercholesterolemia<sup>125, 127</sup>.

The aged human aortic wall presents a pro-inflammatory profile (macrophages, mast cells, macrophage chemoattractant protein [MCP] 1)<sup>124</sup>. In addition, Ang II and its signaling molecules are increased within the arterial wall of older individuals<sup>124</sup>. Ang II

signaling reduces the bioavailability of NO and increases the production of ROS<sup>132</sup>, both of which contribute to endothelial dysfunction and inflammation<sup>133</sup>. Ang II induced ROS production stimulates nuclear translocation of NFκB via an nicotinamide adenine dinucleotide phosphate (NADPH) dependent mechanism, leading to the translation of MMPs and pro-inflammatory cytokines including MCP1, TNFα and IL6, as well as the activation of transforming growth factor beta (TGFβ)<sup>124</sup>. NFκB also encodes genes for NADPH oxidase, which stimulates vascular ROS. ROS production leads to an upregulation of the Ang II receptor, AT1 and NFκB, thus creating a positive feedback loop for perpetual inflammation and oxidative stress in the vasculature<sup>133, 134</sup>. Furthermore, the activation of MCP1 has been demonstrated to lead to further vascular inflammation and remodeling through increased secretion of MMPs, increased expression of adhesion molecules, and increased VSMC migration<sup>135</sup>.

Ang II is not the sole mechanism mediating the relationship between systemic inflammation and arterial stiffness. Numerous studies have demonstrated a strong relationship between CRP and arterial stiffness<sup>25, 126-129, 136</sup>. CRP can be produced in the liver via IL-6 stimulation or locally by VSMCs during vascular inflammation. CRP plays an active role in promoting vascular inflammation and endothelial dysfunction by up regulating AT1<sup>137</sup>, ROS, and stimulating the activation of VSMC<sup>138</sup>. In addition, CRP has a direct pro-inflammatory effect on endothelial cells and induces the secretion of MCP1, adhesion molecules, and E-selectin<sup>139</sup>. Therefore, CRP- induced vascular inflammation may contribute to increased arterial stiffness by leading to vascular fibrosis, endothelial dysfunction, and VSMC proliferation. Taken together, the above support the hypothesis

that inflammation and oxidative stress are important in the etiology of vascular aging in middle-aged and older adults.

**Lipids, lipoproteins, and large artery stiffening:**

In large epidemiological studies elevated triglycerides (TAG)<sup>14, 23, 140</sup>, oxidized low-density lipoprotein (oxLDL)<sup>141</sup> and altered lipoprotein ratios<sup>142</sup> are independently related to indices of arterial stiffness. In addition, LDL-C and HDL-C (inversely) are correlated with arterial stiffness in univariate models<sup>140</sup>.

Studies in patients with familial hypercholesterolemia have reported an increase in aortic, carotid, and radial stiffness compared to age matched healthy controls<sup>143, 144</sup>. In contrast, the relationship between serum lipids and lipoproteins and arterial stiffness is less clear adults without familial hypercholesterolemia<sup>127, 145</sup>. However, the consistent reduction in aortic stiffness observed in both hyperlipidemic and normolipidemic individuals receiving statins strengthens the association between aortic stiffening and serum lipids and lipoprotein fractions<sup>146-148</sup>. For example, Orr et al.<sup>146</sup> reported that the magnitude of reduction in LDL cholesterol following 12 weeks of atorvastatin therapy was related to the magnitude of reduction in arterial stiffness indices in overweight and obese middle aged and older adults with both normal and abnormal serum lipid and lipoprotein levels.

A number of mechanisms may explain the relationship between dyslipidemia and arterial stiffness. Specifically, lipoprotein fractions have many effects on the arterial wall, which may eventually lead to arterial stiffening. For example, oxLDL has been reported to stimulate collagen synthesis in arterial smooth muscle cells<sup>149</sup> and to promote intimal thickening in animal models<sup>150</sup>. In addition, oxLDL increases the expression of MMPs in

macrophages<sup>151</sup> and endothelial cells<sup>152</sup>, thus potentially leading to medial and ECM remodeling and subsequent arterial wall stiffening. Furthermore, it has been reported that individuals with high and borderline high LDL-C<sup>153</sup> and hypertriglycemia<sup>154</sup> have impaired forearm blood flow responses to acetylcholine and flow mediated dilation, respectively. This decrement in endothelium dependent dilation may be due to oxLDL, which impairs NO bioactivity through promotion superoxide anion formation<sup>155</sup>. Apart from oxLDL, evidence suggests that ET-1 partially mediates the observed vasoconstrictor tone in hypercholesterolemic patients<sup>156</sup>. Lastly, vascular inflammation may play a role in cholesterol mediated vascular remodeling. Pirro et al<sup>127</sup> reported that CRP mediates the relationship between elevated aPWV and serum lipids in newly diagnosed hypercholesterolemic patients.

#### **Medial calcification and large artery stiffening:**

As humans age the incidence of vascular calcification increases steadily<sup>157</sup>. The process of calcification is evident in the intimal and medial layers. Atherosclerosis is related to intimal calcification and arteriosclerosis is related to medial calcification. As such, focus will be on medial rather than intimal calcinosis. Considering that the elastic lamina is calcified, the term “medial elastocalcinosis” is used below.

Age related medial elastocalcinosis is accelerated during disease states such as HTN, chronic kidney disease, and conditions of hyperglycemia (diabetes mellitus)<sup>158</sup>. Although through slightly different mechanisms, vascular mineralization is mediated through VSMC that express an osteogenic phenotype. VSMC are transformed into an osteogenic phenotype in response to stress stimuli, including mechanical stress<sup>158</sup>, inflammatory cytokines, oxidized lipids, and mineral imbalance<sup>159</sup>. The secretion of microvesicles,

encapsulating osteoid-like matrix proteins that are able to calcify, is a major hallmark of this phenotypic transformation. These osteoid like matrix proteins bind to elastin fibers and mineralize, thus creating a stiffer tissue. Whether or not calcification occurs, however, is determined by the balance between promoters and inhibitors of calcification<sup>160</sup>.

Experimental models of drug induced medial arterial elastocalcinosis in rodents have reported a strong relationship between the extent of aortic calcification and accelerated aPWV, ISH, and left ventricular hypertrophy<sup>161, 162</sup>. Imaging studies in humans report an independent relationship between aortic calcification and aPWV in healthy middle-aged<sup>163</sup> and older adults<sup>157</sup>. In addition, the extent of aortic calcification in HTN, type 2 diabetes (T2D), and end-stage renal disease is positively correlated with arterial stiffness indices<sup>157, 164, 165</sup>. Unfortunately, in human studies, the use of non-invasive imaging techniques limits the distinction between intimal and medial calcinosis in these studies. Furthermore, the cross-sectional nature of these studies does not allow for determination of a cause and effect relationship between medial elastocalcinosis and arterial stiffening.

### **Sympathetic nerve activity and large artery stiffening:**

The sympathetic nervous system (SNS) plays a key role in maintaining cardiovascular homeostasis<sup>166</sup>. Overactivation of this system is associated with tachycardia, muscular artery vasoconstriction, vascular wall remodeling, and chronically elevated BP<sup>167</sup>. Since these factors influence arterial stiffening, it has been hypothesized that elevated SNS activity plays a role in the stiffening process. Several lines of evidence support this hypothesis. First, cross-sectional<sup>168, 169</sup> studies suggest that muscle sympathetic nerve activity (MSNA) independently predicts CFPWV, and is positively

associated with AI in men. Secondly, longitudinal studies<sup>42, 170</sup> report that elevated HR at baseline is associated to increased CFPWV and baPWV at follow-up. Finally, the removal of adrenergic tone significantly during anesthesia increases radial and femoral artery distensibility<sup>171</sup>.

Several possible mechanism exist that may explain the relationship between elevated SNS activity and arterial stiffening. First, elevated SNS activity may alter the material stiffness of the arterial wall via a chronic elevation of BP<sup>172</sup>. Second, epinephrine release and RAAS activation can promote VSMC hypertrophy and fibrosis<sup>173, 174</sup>. Lastly, the increased load and oscillatory shear stress associated with tachycardia may promote collagen synthesis, thus stiffening the arterial wall<sup>175</sup>.

#### **Genetics and large artery stiffening:**

Large genome-wide studies have demonstrated genetic involvement in the pathophysiology of arterial stiffening. The Bogalusa Heart study<sup>176</sup> was the first family study to demonstrate that adolescents with a parental history of myocardial infarction or diabetes had greater carotid artery stiffness compared to their age matched counterparts without a family history for these diseases. Other offspring studies have since reported a similar relationship between family history for HTN and elevated AI<sup>177</sup>. Recently, large genome-wide linkage studies have reported moderate to substantial heritability estimates ranging from 0.21-0.66 for key measures of arterial stiffness<sup>178</sup>. In addition, these studies have indicated distinct chromosomal regions of significant or suggestive linkages for PP, CFPWV, and forward and reflected pressure wave properties. For example, Mitchell et al.<sup>179</sup> indentified four suggestive linkage regions for CFPWV in chromosomes 2,7, 13, and 15.

Many studies have explored the contribution of gene polymorphisms in the pathophysiology of arterial stiffness. Candidate genes polymorphisms of the RAAS, elastin, fibrillin-1, MMPs, eNOS, adhesion molecules, collagen type 1A, ET1 and its receptors (ET<sub>A</sub>R & ET<sub>B</sub>R), and  $\beta_2$ -adrenoreceptor demonstrate moderate associations with indices of arterial stiffness<sup>178, 180</sup>. In addition, gene expression profiling studies have identified a relationship between aortic stiffness and many genes related to the aortic cytoskeleton<sup>181, 182</sup>. Taken together, there appears to be a genetic contribution to the etiology of age-related arterial stiffening. Unfortunately, these studies and others have failed to identify new molecule determinants and pathways involved in age-related arterial stiffening. Therefore, the therapeutic potential of targeting these gene candidates is limited and future study is needed to address these issues.

#### **Lifestyle and large artery stiffening:**

Lifestyle choices strongly influence many chronic ailments and diseases. In a similar fashion, age-related arterial stiffening is influenced by lifestyle decisions. Poor dietary behaviors (e.g., caffeine consumption, high saturated fat diet, sodium intake, excessive alcohol consumption), smoking, and physical inactivity are among the most studied lifestyle factors that influence large artery stiffening.

***Caffeine consumption.*** Caffeine consumption is widely popular in the U.S. with 54% of adults over 18 years old reporting drinking caffeine in the form of coffee everyday (*National Coffee Drinking Trends 2010, National Coffee Association*). The effect of caffeine consumption on cardiovascular health is not well defined<sup>183, 184</sup>, however the potent acute pressor effect of caffeine is clear<sup>185</sup>. Caffeine consumption increases both central and peripheral blood pressure but the response is more pronounced

centrally<sup>186, 187</sup>. In addition, caffeine consumption (in beverage or pill form) has been shown to increase aPWV and AI in normotensive and hypertensive individuals<sup>187, 188</sup>. Interestingly, Mahmud and Feely<sup>186</sup> showed that changes in aPWV remain significant after accounting for changes in SBP at 30 and 60 minutes post caffeine consumption, suggesting a BP independent effect of caffeine on large artery hemodynamics.

The effects of chronic caffeine consumption on large artery stiffness and hemodynamics are less studied. Cross-sectional analyses<sup>189, 190</sup> have shown a positive association between chronic coffee consumption and increased aPWV and wave reflection in apparently healthy adults. Peripheral artery vasoconstriction through antagonism of adenosine and the release of catecholamines may mediate caffeine induced hemodynamic changes<sup>191</sup>.

***Dietary fats.*** Recently, a prospective analysis from the Caerphilly study showed that higher baseline saturated fatty acid (SFA) consumption was associated with higher aPWV after a 17.8-year follow-up. This same study reported that higher baseline polyunsaturated (PUFA) consumption was associated with lower aPWV over this same time period<sup>192</sup>. However, not all longitudinal studies support these findings<sup>193</sup>. In contrast, many intervention studies do not report an association between dietary fat composition and changes in arterial stiffness, especially when weight loss is apparent<sup>194-196</sup>. The discrepancy between longitudinal and intervention studies is unclear, however time differences between studies may play a role. At present, the relationship between dietary fat consumption and arterial stiffness is not well characterized and future investigations are needed.

***Dietary sodium.*** Cross sectional analyses<sup>197, 198</sup> have reported lower age-related arterial stiffness and BP in those with lower dietary sodium intake compared to those with normal intake. Importantly, the relationship between lower sodium intake and lower CFPWV remained in these studies after controlling for BP. A randomized, crossover designed study<sup>199</sup> reported that a dietary intervention which manipulated dietary sodium to levels comparable to the average consumption in Westernized societies increased CFPWV, AI, SBP, and DBP when compared to a low sodium arm. Conversely, Gates et al.<sup>200</sup> reported an improvement in carotid arterial compliance following sodium restriction in older men and women with stage 1 hypertension. Interestingly, changes in arterial hemodynamics were apparent within 1-2 weeks in the above dietary interventions.

Both BP dependent and independent mechanisms are hypothesized to contribute to arterial stiffening with excess sodium intake. Studies have shown a causal link between high salt intake and elevated BP<sup>201</sup>. An increase in BP will lead to increased arterial stiffness. Conversely, in a BP independent fashion, sodium can increase medial layer thickness by stimulating VSMC hypertrophy and collagen production<sup>202, 203</sup>. In addition, sodium intake may reduce endothelial function through ROS production and the activation of natural inhibitors of eNOS<sup>204</sup>. Together, medial thickening and endothelial dysfunction will contribute to arterial stiffening.

***Smoking.*** The effects of acute and chronic smoking on arterial stiffening have been extensively studied. Smokers and non-smokers show an increase in central and peripheral arterial stiffness after acute exposure to cigarette or cigar smoke<sup>205, 206</sup>. Interestingly, current smokers appear to have an exacerbated stiffening response after acute exposure to tobacco<sup>207</sup>. Interestingly, passive exposure to tobacco smoke may

increase arterial stiffening<sup>208</sup>. However, the chronic effects of smoking on PWV are unclear. With some<sup>209, 210</sup>, but not all<sup>211, 212</sup> reporting an elevation in central and peripheral PWV in chronic smokers compared to non-smokers.

Smoking may exert its stiffening effect through other mechanistic pathways. For example, smoking has been associated with oxidative stress, inflammation, endothelial dysfunction, and HTN<sup>213</sup>. In addition, smoking has been associated with kidney damage, leading to a decrement in glomerular filtration rate<sup>214</sup>. Importantly, mild kidney dysfunction is associated with collagen and calcium deposition in the arterial walls<sup>215</sup>. Taken together, the risk of accelerated age-related arterial due to smoking is great, and even acute and passive exposure to tobacco leads to a reduction in central and peripheral artery elasticity.

***Alcohol consumption.*** Epidemiological studies demonstrate a J- or U-shaped association between alcohol consumption and CVD risk in men and women<sup>216, 217</sup>. That is, low-moderate alcohol consumption is associated with lower CVD risk, while excessive alcohol consumption is associated with elevated CVD risk when compared to non-drinkers. In accordance, most studies report a J-shaped association between alcohol consumption and PWV, wave reflections, and central aortic blood pressure<sup>210, 218-220</sup>.

The mechanisms involved in the J-association between alcohol consumption and arterial stiffness remains unclear, however a few possibilities exist. Light-moderate alcohol consumption has been associated with increased HDL-C<sup>221</sup> and a reduction in pro-inflammatory proteins<sup>222</sup>. The reduction in pro-inflammatory pathways may improve insulin sensitivity and endothelial function, thus leading to improvements in arterial stiffening and central hemodynamics<sup>220</sup>. In contrast, more than moderate alcohol

consumption has been shown to increase BP possibly through ethanol's influence on SNS and RAAS activation<sup>223</sup>. In addition, ethanol's ability to activate matrix proteases, increase oxidative stress, and activate of NFκB in VSMC are other potential mechanisms by which excessive alcohol consumption may accelerate arterial stiffening<sup>224</sup>.

**Physical inactivity.** Sedentary behavior independently predicts greater cardiometabolic and mortality risk<sup>225, 226</sup>. Cross-sectional data suggests that habitual physical activity is inversely associated with arterial stiffness in healthy adults<sup>227, 228</sup>. In hypertensive subjects, indices of arterial stiffness were positively correlated with inactive minutes and negatively correlated with minutes spent carrying out physical activity<sup>229</sup>. Interestingly, a recent observational study<sup>230</sup> reported that overall weekend sitting time was correlated to arterial stiffness indices, even when accounting for physical activity time and cardiorespiratory fitness. These findings suggest that prolonged sitting time, even in individuals who meet guideline recommendations for physical activity, can accelerate age-dependent arterial stiffness. However, the cross sectional nature of these studies prevents inference of causation. On the other hand, studies that model physical inactivity through bed rest allow for a clearer understanding of how sedentary behavior affects vascular structure and function. Short-term (5 days) bed rest has been shown to decrease brachial artery FMD and increase central AI without influencing CFPWV<sup>231</sup>.

The relationship between physical inactivity and arterial stiffening is not completely understood, however a few possibilities exist. Bed rest studies and other physical inactivity models in humans have demonstrated that prolonged physical inactivity can lead to increased oxidative stress<sup>232</sup>, imbalanced vasoconstrictor (e.g., ET1 and Ang II) hormone action<sup>233</sup>, and development of insulin resistance<sup>234</sup>. Furthermore,

prolonged physical inactivity can lead to structural and functions changes in the vasculature such as reduced conduit artery diameter, inward remodeling of conduit vessels and decreased resistance vessel reactive hyperemia<sup>235</sup>. Indeed these factors, along with the aforementioned, can promote and/or accelerate age-related arterial stiffening.

### **G. Therapies of age-related large artery stiffening**

It is evident that many mechanisms contribute, albeit at different magnitudes, to pathophysiology of age-related stiffening of the large, elastic arteries. “Destiffening” therapies target one or more of these mechanisms and often include lifestyle modification, and/or pharmaceutical agents. These therapies have been shown to be efficacious destiffening options in various populations, however a degree of therapeutic resistance is evident among certain disease states<sup>236</sup>. The combination of pharmaceutical agents is common and often superior to monotherapy<sup>237</sup>, however the combination of pharmaceutical and lifestyle modification therapy is largely unexplored and deserves for study.

#### **Lifestyle modification as a therapy for large artery stiffening**

*Weight loss.* Weight loss in overweight and obese individuals via a caloric restriction, physical activity, or a combination of these therapies has been shown to reduce total body mass, BMI, total adiposity, and central adiposity. Most intervention studies have reported improvements in arterial stiffness with weight loss and many mechanisms exist that may explain these improvements<sup>238-241</sup>.

One potential mechanism of reduced arterial stiffness with weight loss is a reduction in the pro-inflammatory milieu. Specifically, most intervention trails report a reduction in CRP<sup>238, 242-245</sup>. In addition, reductions in IL6, TNF $\alpha$ , T- lymphocyte number, and the cell

surface expression of monocyte adhesion molecules is observed following weight loss<sup>244, 246</sup>. Other possible mechanisms mediating improvements in arterial stiffening after weight loss include: improvements glucose metabolism<sup>244</sup>, improvement in central and peripheral hemodynamics<sup>238</sup>, beneficial changes in serum lipids and lipoproteins<sup>239, 241</sup>, and improvements in endothelial function<sup>247, 248</sup>. Importantly, the magnitude of total weight loss appears to be important.

***Aerobic and resistance training.*** Cross-sectional analyses have reported less pronounced arterial aging in middle-aged and older adults who participate in regular endurance exercise compared to their age-matched sedentary peers<sup>249, 250</sup>. It has even been reported in some cases that arterial stiffness is not different between endurance trained middle-aged and older adults when compared to sedentary young adults<sup>228</sup>. However, these findings have not been consistently reported<sup>250, 251</sup>. In addition, moderate intensity aerobic exercise interventions have been shown to be efficacious destiffening therapies in normal weight, sedentary middle-aged and older men and women<sup>250, 251</sup>. Interestingly, improvements in arterial stiffness occur independently from changes total in body mass, adiposity, arterial BP, plasma lipoproteins, fasting glucose, and fasting insulin.

It has been shown that overweight men and women with ISH are resistant to improvements in large artery compliance after completing 8 weeks of moderate intensity aerobic exercise<sup>236</sup>. In addition, overweight middle-aged and older postmenopausal women with elevated SBP have shown a similar resistance to improvements in large arterial compliance following 3-months of moderate intensity aerobic activity<sup>252</sup>.

Therefore, the comorbid conditions of HTN and obesity may lead to a resistance in arterial stiffening improvements following an aerobic exercise intervention.

In contrast to aerobic exercise interventions, some<sup>253, 254</sup>, but not all<sup>255, 256</sup> intervention trials have been reported an increase central arterial stiffness in healthy men following several months of resistance training. Discrepancy between studies may be attributed to moderate vs. high intensity training programs. A recent meta-analysis<sup>257</sup> reported only high intensity resistance training to be related to increased arterial stiffness. Importantly, the addition of endurance exercise training to resistance training appears to prevent central artery stiffening that occurs with resistance training alone<sup>258, 259</sup>.

The benefits of aerobic exercise on age-related large artery stiffening are not fully elucidated. However, improved endothelial function<sup>260</sup>, a reduction in oxidative stress<sup>261</sup>, and a decrease in adventitial fibrosis and AGEs<sup>262</sup> are thought to play a key role. Conversely, increased efferent SNS tone and significantly elevated arterial BP are hypothesized to contribute to the stiffening process associated with resistance training<sup>257, 262</sup>.

**Sodium restriction.** Cross-sectional studies<sup>197, 198</sup> have reported lower age-related arterial stiffness and BP in those with lower dietary sodium intake compared to those with normal dietary sodium intake. The observed reduction in arterial stiffening in these individuals appears to be partly independent of BP reduction. Similarly, some<sup>200, 252, 263</sup>, but not all<sup>264, 265</sup> intervention trials have reported a significant reduction in brachial SBP and arterial stiffening following sodium restricted diets when compared to normal dietary sodium intake. Importantly, the magnitude of reduction in brachial SBP following these interventions correlates strongly with the magnitude of change in arterial stiffness<sup>200, 252</sup>.

Conversely, sodium restriction improves  $\beta$ -stiffness following a short-term intervention in stage 1 hypertensives<sup>200</sup>. Notably,  $\beta$ -stiffness is a relatively BP independent measure of arterial stiffness.

Unfortunately the mechanisms responsible for the improvements in large artery stiffness following sodium restriction are largely unexplored. It is plausible to assume that lower dietary sodium intake has the opposite effects of higher sodium intake, however future study is needed to test this hypothesis.

***Smoking cessation.*** Observational data suggest that smoking cessation improves central<sup>266</sup> and peripheral arterial stiffening<sup>267</sup>. In addition, smoking cessation of > 10 years appears to reverse the deleterious effects of smoking on arterial stiffness. In contrast, intervention trials have only reported improvements in central Aix<sup>268, 269</sup>, central BP<sup>270</sup>, baPWV<sup>270</sup>, and small artery compliance<sup>267, 271</sup> following smoking cessation. Importantly, complete reversal of smoking induced stiffness is not observed in these studies. Discrepancy between observational and intervention studies may be due to the relatively short duration of the intervention trials (4 weeks- 2 years). It is possible that a longer duration is necessary for restoration of smoking induced damage to large elastic arteries occur.

The mechanisms responsible for improvements in arterial stiffening following smoking cessation are unclear, but improvements in oxidative stress, inflammation, and endothelial function are thought to play a central role<sup>272, 273</sup>.

### **Pharmaceutical interventions as a therapy for large artery stiffening**

A decrease in arterial BP pressure will decrease arterial stiffness<sup>10</sup>. Not surprisingly, antihypertensive therapy leads to a reduction in arterial stiffness in non-

obese and obese hypertensives<sup>274, 275</sup>. Importantly, these improvements are independent of pharmacological treatment. However, certain drug combinations may be more effective than mono-therapy or other drug combinations at improving central BP and aPWV in hypertensive individuals<sup>237, 276</sup>. Interestingly, drugs that inhibit components of the RAAS seem to improve large artery compliance independently of changes in BP<sup>6, 79, 277</sup>.

**Statins.** 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) improve serum lipid and lipoprotein levels and have many pleiotropic effects on the vasculature<sup>7, 278</sup>. Most<sup>146, 148, 279-281</sup>, but not all<sup>282</sup> intervention trials report improvements in large artery stiffness following statin treatment in normolipidemic and hyperlipidemic individuals. Improvements in serum lipids and lipoproteins, oxidative stress, and inflammation may mediate the improvements in large artery stiffness following statin therapy<sup>278, 283</sup>.

**Combination therapy.** It is evident that lifestyle modification and pharmacological therapies are efficacious destiffening agents. Therefore, it is likely that the combination of these therapies would have an additive or synergistic effect on arterial stiffening and arterial BP. Unfortunately, not many intervention trials have explored this hypothesis, and the results from these studies have been inconsistent<sup>284, 285</sup>. Blumenthal et al.<sup>285</sup> reported significantly greater reductions in aPWV and brachial arterial BP in overweight and obese adults with elevated BP when combining a weight loss diet with a structured aerobic exercise program compared to controls. Conversely, Werner et al.<sup>284</sup> reported similar reductions in  $\beta$ -stiffness and brachial arterial BP between lifestyle modification, nebivolol, and combined therapy (lifestyle + nebivolol) groups in a similar

population. Discrepancy between studies may be due to differences in the amount of weight loss, aerobic exercise intensity, and magnitude of sodium restriction. Indeed, future study is needed given the paucity of research on the issue.

### **E. Conclusion**

Arterial stiffening is an age-dependent process, however its progression can be accelerated in the presence of mechanical, biological, and/or environmental stressors. These stressors act through independent and dependent mechanisms that alter vascular structure and function. Although the mechanisms of stiffening are not fully elucidated, inflammation and oxidative stress appear to be important. Along these lines, the most effective lifestyle and pharmacological therapies either directly or indirectly attenuate inflammation and oxidative stress. It is likely that the combination of these therapies would have an additive or synergistic effect on arterial destiffening, however this hypothesis is largely underexplored and deserves future study. Taken together, arterial stiffening and its manifestations strongly influence cardiovascular disease risk, end organ damage, and consequently all-cause mortality. Therefore, arterial stiffening will continue to be a public health issue in our aging nation unless strategies are implemented to interfere with its progression.

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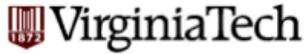
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## Appendix B: Approved Institutional Review Board Research Protocol



### Institutional Review Board Research Protocol

Once complete, upload this form as a Word document to the IRB Protocol Management System: <https://secure.research.vt.edu/irb>

#### Section 1: General Information

##### 1.1 DO ANY OF THE INVESTIGATORS OF THIS PROJECT HAVE A REPORTABLE CONFLICT OF INTEREST? (<http://www.irb.vt.edu/pages/researchers.htm#conflict>)

- No  
 Yes, explain:

##### 1.2 WILL THIS RESEARCH INVOLVE COLLABORATION WITH ANOTHER INSTITUTION?

- No, go to question 1.3  
 Yes, answer questions within table

IF YES
Provide the name of the institution [for institutions located overseas, please also provide name of country]: <b>Heart Specialists of Southwest Virginia</b>
Indicate the status of this research project with the other institution's IRB: <input type="checkbox"/> Pending approval <input type="checkbox"/> Approved <input checked="" type="checkbox"/> Other institution does not have a human subject protections review board <input type="checkbox"/> Other, explain: <input type="text"/>
Will the collaborating institution(s) be engaged in the research? ( <a href="http://www.hhs.gov/ohrp/policy/engage08.html">http://www.hhs.gov/ohrp/policy/engage08.html</a> ) <input type="checkbox"/> No <input checked="" type="checkbox"/> Yes
Will Virginia Tech's IRB review all human subject research activities involved with this project? <input type="checkbox"/> No, provide the name of the primary institution: <input type="text"/> <input checked="" type="checkbox"/> Yes  <i>Note: primary institution = primary recipient of the grant or main coordinating center</i>

##### 1.3 IS THIS RESEARCH FUNDED?

- No, go to question 1.4  
 Yes, answer questions within table

IF YES
Provide the name of the sponsor [if NIH, specify department]: <b>VSL Pharmaceuticals Inc.</b>
Is this project receiving federal funds? <input checked="" type="checkbox"/> No <input type="checkbox"/> Yes  If yes,

**Does the grant application, OSP proposal, or “statement of work” related to this project include activities involving human subjects that are not covered within this IRB application?**

No, all human subject activities are covered in this IRB application

Yes, however these activities will be covered in future VT IRB applications, these activities include: \_\_\_\_\_

Yes, however these activities have been covered in past VT IRB applications, the IRB number(s) are as follows: \_\_\_\_\_

Yes, however these activities have been or will be reviewed by another institution’s IRB, the name of this institution is as follows: \_\_\_\_\_

Other, explain: \_\_\_\_\_

**Is Virginia Tech the primary awardee or the coordinating center of this grant?**

No, provide the name of the primary institution: \_\_\_\_\_

Yes

**1.4 DOES THIS STUDY INVOLVE CONFIDENTIAL OR PROPRIETARY INFORMATION (OTHER THAN HUMAN SUBJECT CONFIDENTIAL INFORMATION), OR INFORMATION RESTRICTED FOR NATIONAL SECURITY OR OTHER REASONS BY A U.S. GOVERNMENT AGENCY?**

*For example – government / industry proprietary or confidential trade secret information*

- No
- Yes, describe: \_\_\_\_\_

**1.5 DOES THIS STUDY INVOLVE SHIPPING ANY TANGIBLE ITEM, BIOLOGICAL OR SELECT AGENT OUTSIDE THE U.S.?**

- No
- Yes

**Section 2: Justification**

**2.1 DESCRIBE THE BACKGROUND, PURPOSE, AND ANTICIPATED FINDINGS OF THIS STUDY:**

Sixty-two percent of the American population exceeds the recommended guidelines for total lipid intake while fifty-nine percent exceeds the guidelines for saturated fatty acid (SFA) intake. Consumption of a high fat, Westernized diet is largely responsible for the prevalence of obesity and type II diabetes among Americans. Saturated fatty acid consumption is positively and significantly correlated to body mass index (BMI) and it has long been established that consumption of SFA is closely correlated to skeletal muscle insulin resistance and metabolic disease. Replacing five percent of energy from SFA consumption with energy from polyunsaturated fatty acids (PUFA) reduces the risk of type II diabetes by 35%. The mechanism(s) underlying the connection between high saturated fat intake and disease are not entirely understood.

In response to short term, high fat feeding, some, but not all individuals are able to up shift energy metabolism to burn off excess fat, a phenomenon now known as metabolic flexibility. Since skeletal muscle comprises approximately 30-50% of an individual's body mass and therefore accounts for a significant contribution to overall energy metabolism, most of the changes occurring in response to high fat feeding are occurring in muscle. However, the ability of skeletal muscle to facilitate this shift varies significantly between individuals and this variation may be the reason why some people gain weight following short term high fat feeding and some people don't. In fact, many obese individuals contain a metabolic defect within their skeletal muscle that reduces their capacity to shift substrate metabolism. While this defect is more prevalent in obese individuals, it is unclear whether this is a cause or effect of the metabolic syndrome. Following a year after weight loss surgery, previously morbidly obese individuals retained impaired skeletal muscle metabolic flexibility despite significant weight loss. Studies have also documented impaired metabolic flexibility in previously obese women as compared to weight-matched controls. These findings

suggest that some individuals are predisposed to excessive lipid storage in response to elevated consumption of dietary fats.

Growing evidence suggests that fatty acids induce insulin resistance through a mechanism involving chronic low-grade stimulation of the immune system. Exposing skeletal muscle to saturated fatty acids increases plasma levels and peripheral tissue accumulation of the inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) in vitro and in vivo. In addition, excessive consumption of SFA increases gut production and circulating levels of lipopolysaccharide (LPS), a ligand for toll-like receptor 4, an integral component of the immune system. Once activated, TLR4 initiates a signaling cascade that results in liberation of nuclear factor kappa B (NF-kB), allowing it to translocate to the cellular nucleus where it stimulates the activation of pro-inflammatory pathways. Data from our own laboratory has previously shown that SFA's heighten LPS-stimulated TLR4 activation of the immune system in cell culture and animal models. Furthermore, protein and mRNA expression of TLR4 is significantly increased in skeletal muscle of obese and insulin resistant individuals suggesting a link between high saturated fat intake and inflammation in skeletal muscle and disease. Our own data has shown that short term high fat feeding (5 days) results in increased skeletal muscle inflammation that is associated with defective substrate metabolism. Individuals that were metabolically flexible did not have increased skeletal muscle inflammation following high fat feeding, however those that were not able to respond to the diet became "inflamed." However, whether abnormal metabolism is caused by inflammation or vice versa is not known. As stated above, recent evidence has indicated that the gut and the gut microbiome may play a significant role in the development of metabolic disease. In mice, high fat feeding results in an alteration of the gut microbiome, which allows for increased energy extraction from the diet as well as potentially increasing inflammation through low-grade endotoxemia. However, whether alterations in the gut microbiome following high fat feeding can affect skeletal muscle is not known. In the current study, we propose to test whether alteration of the gut microbiome through probiotic supplementation can alter the effects of high saturated fat feeding on substrate metabolism and inflammation in skeletal muscle.

Probiotics are cultures of beneficial bacteria that are normally present in a healthy digestive tract. Increasing evidence shows that the activity of probiotic bacteria in the human GI tract plays an important role in the dietary management of metabolic health. Probiotic bacteria, specifically the Lactobacilli and Bifidobacteria species, have been recognized as potential therapeutic agents for over a century but only recently has attempts been made to use probiotics to promote health. This renewed interest in the use of probiotics is in part due to the recent publication of numerous clinical studies demonstrating the use of probiotics is associated with a reduction in chronic inflammation in a number of conditions. In addition, there has been a considerable expansion in the commercial availability of probiotics in yogurt, capsule and powder form. However, it is unknown whether probiotic use can alter the effects of saturated fat feeding on skeletal muscle substrate metabolism and inflammation. Therefore the current study will test whether supplementation of VSL#3, a probiotic supplement, can alter the effects of 4 weeks of high saturated fat feeding on skeletal muscle substrate metabolism and inflammation. We expect that individuals supplemented with VLS#3 for weeks will not develop skeletal muscle inflammation or abnormal substrate metabolism while on a high fat diet. Because vascular health is also impacted by inflammation, a secondary aim will be to determine if individuals supplemented with VSL#3 will be protected from the development of arterial stiffening while on a high fat diet.

## 2.2 EXPLAIN WHAT THE RESEARCH TEAM PLANS TO DO WITH THE STUDY RESULTS:

*For example - publish or use for dissertation*

The results will be published in peer reviewed journals. The study may be used for dissertation research as well.

## Section 3: Recruitment

### 3.1 DESCRIBE THE SUBJECT POOL, INCLUDING INCLUSION AND EXCLUSION CRITERIA AND NUMBER OF SUBJECTS:

*Examples of inclusion/exclusion criteria - gender, age, health status, ethnicity*

13.2 Non-smoking males between the ages of 18-40 who do not have diabetes or high cholesterol as assessed by medical history and blood tests with body mass index less than 30 kg/m<sup>2</sup> and blood pressure less than 140/90mmHg. Subjects will be weight stable (+/-2.5 kg) for the last 6 months. Subjects will be sedentary to recreationally active (less than 2 d/wk, 20 min/d). Subjects will have a total cholesterol that is less than 200 mg/dl, and a triglyceride concentration of less than 150 mg/dl. Subjects will have no past or current history of coronary heart disease, stroke or major cardiovascular disease events, respiratory diseases, endocrine or metabolic, neurological, or hematological disorders that would compromise the study or the health of the subject. Subjects will also have no evidence of liver diseases or persistent unexplained elevation in transaminases. Subjects should not be on any medication that could influence the outcome of the study including cholesterol lowering medication (including fibrin acid derivatives and niacin), erythromycin, immunosuppressive drugs, or antioxidant/ supplements or taken antibiotics in the last month. Subjects should not have food allergies, allergy to silicon dioxide, or lidocaine/bipivucaine. Subjects should not have had recent surgery, or history of alcohol or drug abuse. Females will not be included in this study because many of the variables we are measuring change during the menstrual cycle and performing measurements during the early follicular phase, for example, is impractical for our initial study on this topic. More importantly, we do not yet know how gut bacteria fluctuate during the menstrual cycle in females. This will need to be determined before an intervention such as proposed is performed. Parenthetically, we have had very few females volunteer for similar studies in the past. Thus, future studies with special efforts to oversample females will be necessary.

**3.2 WILL EXISTING RECORDS BE USED TO IDENTIFY AND CONTACT / RECRUIT SUBJECTS?**

*Examples of existing records - directories, class roster, university records, educational records*

- No, go to question 3.3
- Yes, answer questions within table



IF YES	
Are these records private or public?	
<input type="checkbox"/> Public	
<input type="checkbox"/> Private, describe the researcher's privilege to the records:	
Will student, faculty, and/or staff records or contact information be requested from the University?	
<input type="checkbox"/> No	
<input type="checkbox"/> Yes, visit the following link for further information: <a href="http://www.policies.vt.edu/index.php">http://www.policies.vt.edu/index.php</a> (policy no. 2010)	

**3.3 DESCRIBE RECRUITMENT METHODS, INCLUDING HOW THE STUDY WILL BE ADVERTISED OR INTRODUCED TO SUBJECTS:**

Subjects will be recruited through advertisement. We anticipate recruiting through posted fliers, emails, and internet surveys.

**3.4 PROVIDE AN EXPLANATION FOR CHOOSING THIS POPULATION:**

*Note: the IRB must ensure that the risks and benefits of participating in a study are distributed equitably among the general population and that a specific population is not targeted because of ease of recruitment.*

Recruiting through the general population. We are recruiting men ages 18-40 years of age of all races and ethnic backgrounds. We are not targeting a specific population.

**Section 4: Consent Process**

For more information about consent process and consent forms visit the following link: <http://www.irb.vt.edu/pages/consent.htm>

*If feasible, researchers are advised and may be required to obtain signed consent from each participant unless obtaining signatures leads to an increase of risk (e.g., the only record linking the subject and the research would be the consent document)*

and the principal risk would be potential harm resulting in a breach of confidentiality). Signed consent is typically not required for low risk questionnaires (consent is implied) unless audio/video recording or an in-person interview is involved. If researchers will not be obtaining signed consent, participants must, in most cases, be supplied with consent information in a different format (e.g., in recruitment document, at the beginning of survey instrument, read to participant over the phone, information sheet physically or verbally provided to participant).

**4.1 CHECK ALL OF THE FOLLOWING THAT APPLY TO THIS STUDY'S CONSENT PROCESS:**

- Verbal consent will be obtained from participants
- Written/signed consent will be obtained from participants
- Consent will be implied from the return of completed questionnaire. Note: The IRB recommends providing consent information in a recruitment document or at the beginning of the questionnaire (if the study only involves implied consent, skip to Section 5 below)
- Other, describe:

**4.2 PROVIDE A GENERAL DESCRIPTION OF THE PROCESS THE RESEARCH TEAM WILL USE TO OBTAIN AND MAINTAIN INFORMED CONSENT:**

Those who respond will be told the general plan for the study and asked to complete a brief online screening to confirm basic eligibility requirements (e.g., age, body mass index, medications). Those still interested and eligible to participate will be invited to a group or individual session to hear the details of participation and potential risks. They will be given a chance to ask any questions. Those still interested will receive a copy of the informed consent to take home with them to read and consider further. Those who return this signed document will proceed with screening and testing.

**4.3 WHO, FROM THE RESEARCH TEAM, WILL BE OVERSEEING THE PROCESS AND OBTAINING CONSENT FROM SUBJECTS?**

Kevin Davy Ph. D. will be responsible for this and all aspects of the study.

**4.4 WHERE WILL THE CONSENT PROCESS TAKE PLACE?**

Human Integrative Physiology Laboratory and the metabolic kitchen/laboratory in Wallace Hall on Virginia Tech Campus

**4.5 DURING WHAT POINT IN THE STUDY PROCESS WILL CONSENTING OCCUR?**

*Note: unless waived by the IRB, participants must be consented before completing any study procedure, including screening questionnaires.*

In the initial contact with the subject the study will be explained to them and they will receive a copy of the informed consent.

**4.6 IF APPLICABLE, DESCRIBE HOW THE RESEARCHERS WILL GIVE SUBJECTS AMPLE TIME TO REVIEW THE CONSENT DOCUMENT BEFORE SIGNING:**

*Note: typically applicable for complex studies, studies involving more than one session, or studies involving more of a risk to subjects.*

- Subjects will be allowed to take a copy of the informed consent home with them to review. They will return at a later date with the consent to ensure they have had enough time to review the consent and have any questions answered.
- Not applicable

**Section 5: Procedures**

**5.1 PROVIDE A STEP-BY-STEP THOROUGH EXPLANATION OF ALL STUDY PROCEDURES EXPECTED FROM STUDY PARTICIPANTS, INCLUDING TIME COMMITMENT & LOCATION:**

+

Subjects are being asked to participate in a study involving a high fat diet with or without VSL supplementation. As part of their participation, subjects will undergo a two week habitual diet lead-in period that will be followed by a four week high fat diet feeding where subjects will either be randomized to VSL supplementation or placebo. During the two week of the lead-in period, subjects will be provided with all of their meals, which will be similar in composition and amount to their habitual diet. After completion of the 2 week habitual diet lead-in period, subjects will be randomized to one of two groups: high fat diet with VSL supplementation and high fat diet without VSL supplementation. During the four week high fat diet period, subjects will be provided with all of their meals that will contain 1000 kcals more than their habitual diet. The composition of the "extra" calories will be 50-60% of calories as fat, 20-30% as carbohydrate, and 10-20% as protein. Subjects will be provided all of their meals throughout the study. Food will be purchased and/or prepared and packaged for subjects to take home with them in the metabolic kitchen in the Department of Human Nutrition, Foods, and Exercise. Uneaten items will be returned and weighed. Subjects will be asked to return to Wallace Hall once daily during all of the feedings periods for the food to be weighed, receive more food, and turn in any uneaten food to be weighed. Subjects will also be weighed daily and asked to report any intake of food not provided to them. During the second week of the lead-in period and the 4 week high fat feeding period subjects will be asked to come to Wallace Hall each morning to eat breakfast and pick up the two remaining meals for the day.

**Testing Sessions in War Memorial Hall**

Participants will participate in all testing sessions (except session 1) on three occasions, once before the two week controlled/ habitual diet feeding period, once immediately following the two week controlled/ habitual diet feeding period, and once immediately following the four week high fat feeding period.

**Session One: (Approximately 1.5 hour)**

**Overnight Fast:** Subjects will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food they eat or by the normal digestion process.

**Medical History:** Subjects will be asked to complete a medical history questionnaire, which will be used to screen for health problems or any reason and individual should be excluded from the study.

**Resting Blood Pressure and Heart Rate.** Blood pressure measurements will be made under quiet, comfortable ambient laboratory conditions via mercury sphygmomanometry. Measurements will conform strictly to American Heart Association guidelines. Heart rate will be determined from a standard electrocardiographic signal.

**Physical Activity Questionnaire:** Subjects will be asked to fill out a questionnaire concerning their previous physical activity level.

**Blood Draw:** A small needle will be inserted into the subjects arm to draw blood (approximately 3 table spoons). The blood will be used to measure fasting glucose, insulin, lipids, and other factors that may affect a subjects health.

**Urine Test.** A small cup of urine will be collected to measure electrolytes, glucose, protein, pH, and blood cells.

**Session 2: (Approximately 1 hr)**

**Overnight Fast:** Subjects will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food they eat or by the normal digestion process.

**Urine Test.** A small cup of urine will be collected to measure byproducts of metabolism that may change as a result of the probiotic supplement.

**Body Mass and Composition.** Body weight will be measured on a digital scale accurate to +0.01 kg. Height will be measured with a standard stadiometer. Percent body fat and fat-free mass will be measured in all subjects using dual-energy x-ray absorptiometry (DEXA) (Prodigy Advance, GE Healthcare).

**Resting Blood Pressure and Heart Rate.** Blood pressure measurements will be made under quiet, comfortable ambient laboratory conditions via mercury sphygmomanometry. Measurements will conform strictly to American Heart Association guidelines. Heart rate will be determined from a standard electrocardiographic signal.

**Arterial Stiffness: Carotid Ultrasonography:** Common carotid artery diameters will be measured from the images obtained from an ultrasound unit (HP Sonos 7500, Phillips Medical Systems) equipped with a high resolution linear array transducer.

**Applanation Tonometry:** The carotid, brachial, radial and femoral artery pressure waveform and amplitude will be obtained using a finger tip probe incorporating a high fidelity strain gauge transducer.

### **Session 3: (Approximately 4.5 hours)**

**Overnight Fast:** Subjects will be asked to avoid eating 12 hours prior to this visit so that the test results will not be influenced by the food they eat or by the normal digestion process.

**Skeletal Muscle Biopsy.** This procedure is used to sample a small amount of muscle (~450 mg) from the Vastus Lateralis. The actual biopsy site will be on the top of thigh, midway between the knee and hip. Volunteers will be placed in the supine position and the skin will be cleansed with an iodine-type solution (Providine or Betadine). If the subject is allergic to iodine, chlorhexadine will be used. A sterile drape will be placed over the area and the skin, fat tissue, and skeletal muscle fascia will be anesthetized by injecting a local anesthetic (lidocaine/bupivacaine) into the area. If the subject is allergic to either lidocaine or bupivacaine, s/he will not be allowed to take part in the study. Then, a small incision (about 1/4 of an inch) will be made in the skin. A needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle. Some suction may be applied to the other end of the needle to help remove the muscle. After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleansed with saline. The biopsy site will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE wrap. The participant will be encouraged to leave the ACE wrap on for at least 10-15 minutes. This procedure will be performed by the principal investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) of the study. Subjects will be provided with instructions on how to care for the biopsy site as well as what to look for if a problem were to occur. We will measure factors which are involved in metabolism or contribute to inflammation in these samples. This test will take place at either Dr Jose Rivero's medical office in Christiansburg or the Human Integrative Physiology Laboratory at Virginia Tech (228 War Memorial Hall). Directions will be provided.

**Intravenous Glucose Tolerance Test (IVGTT):** Two small plastic tubes (catheters) will be placed in each of two arm veins (different arms). The test involves injecting small amounts of glucose (0.3mg/kg body weight) and insulin (0.03 units/kg body weight) into your veins and blood stream (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) approximately 28 times over a 3 hour period. A registered nurse will be present to perform this test with the assistance of investigators.

**Infection/Inflammation Questionnaire:** Subjects will be asked to complete a questionnaire about any recent illnesses or infections that they may have had in the prior month.

### **Take Home Tests:**

**Food records:** Subjects will be asked to record all the food that they eat during a four day period. They will also be asked to record all of the food they eat during the baseline or lead-in period during which they consume their typical diet.

**Stool Collection:** Participant will be given the stool collection container, commode hat, and a Zip loc bag. They will also be given a stool collection cooler and freezer pack. Study ID number and collection date will be written on the collection container along with the assessment point (e.g., baseline, post-lead in, or post intervention). Subjects will be instructed to collect one fecal sample in the day prior to or day of their scheduled lab visit. After securely covering the sample with the lid, the subject should place the sample (not hat) in the Zip loc bag, seal it, and place it in the cooler with an ice pack. Upon return, sample will be verified for proper labeling information and placed in the freezer.

**5.2 DESCRIBE HOW DATA WILL BE COLLECTED AND RECORDED:**

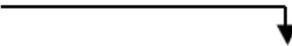


Study data will be collected on data sheets (see attached) and manually entered into a database (excel format).

**5.3 DOES THE PROJECT INVOLVE ONLINE RESEARCH ACTIVITIES (INCLUDES ENROLLMENT, RECRUITMENT, SURVEYS)?**

View the "Policy for Online Research Data Collection Activities Involving Human Subjects" at <http://www.irb.vt.edu/documents/onlinepolicy.pdf>

- No, go to question 6.1
- Yes, answer questions within table



**IF YES**

**Identify the service / program that will be used:**

- [www.survey.vt.edu](http://www.survey.vt.edu), go to question 6.1
- Blackboard, go to question 6.1
- Center for Survey Research, go to question 6.1
- Other

**IF OTHER:**

Name of service / program:

URL:

This service is...

- Included on the list found at: <http://www.irb.vt.edu/pages/validated.htm>
- Approved by VT IT Security
- An external service with proper SSL or similar encryption (https://) on the login (if applicable) and all other data collection pages.
- None of the above (note: only permissible if this is a collaborative project in which VT individuals are only responsible for data analysis, consulting, or recruitment)

**Section 6: Risks and Benefits**

**6.1 WHAT ARE THE POTENTIAL RISKS (E.G., EMOTIONAL, PHYSICAL, SOCIAL, LEGAL, ECONOMIC, OR DIGNITY) TO STUDY PARTICIPANTS?**

**The potential risks include the following:**

- **VSL#3 Probiotic Supplement:** VSL#3 has been shown to be a safe. However, there is small risk of flatulence, bloating, and/or a change in bowel habits while taking VSL#3. Individuals should not be in the study if you are allergic to silicon dioxide, a food additive used to absorb moisture during the packaging and storage of VSL#3 and the placebo.

**Blood Draw:** There is some pain and discomfort that may be experienced when the catheter is inserted in the vein. There may be pain and/or bruising at the place on the arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200 (0.005%), while the risk of infection or significant blood loss is 1 in 1000 (0.001%). There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed.

**HIV/AIDS:** Blood from a subject will be tested for HIV if one of the study investigators are exposed. There will not be any cost to the subject. The results will be sent to the individuals primary care physician or the study medical director, Dr. Jose Rivero. He/she will discuss the results and provide referral for further evaluation or counseling if the results are positive. The results will remain confidential.

**Arterial Stiffness:** There are no known risks associated with this procedure.

**DEXA Scan:** The amount of radiation that subjects will receive in the DEXA exam (combined with the CT scan) is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount subjects will receive is equal to 1/20 of a chest x-ray. The more radiation an individual receives over the course of their lifetime, the more likely that individual's risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks, however the exact increase in such risk is not known.

**Muscle Biopsy:** For the muscle biopsy, there may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but the subject should not feel significant discomfort during the actual biopsy procedure. There is a small risk of bleeding during the procedure but this will be minimized by placing immediate pressure over the incision site. Bruising in the area of the biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately to limit this potential and its accompanying tenderness. There is a slight risk of infection at the biopsy site, however subjects will be required to return to the lab within 5 days following the biopsy to have the site checked to ensure proper healing.

Subjects will be shown pictures of a typical biopsy scar. It will also be explained that the pictures are just one example of scarring and that individuals will scar differently.

**Intravenous Glucose Tolerance Test:** Because this procedure requires the placement of a catheter in an arm vein, the risks here are identical to that stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. We will be monitoring your blood sugar frequently and can usually anticipate this before your blood sugar drops too low. If this happens, orange juice (with table sugar) or some other simple carbohydrate containing food will be provided. We will monitor the individuals glucose until it returns to normal. A registered nurse will perform the test with the assistance of the investigators.

**Weight gain:** There is a risk of weight gain of approximately 3 to 4 pounds. Some subjects may gain a less and some may gain more. Upon completion of the study subjects will meet with a registered dietician who will provide them with a weight loss program to restore them to their original weight. NOTE: We have done this successfully in the past at Virginia Tech (IRB# WG\_05-457).

It is not possible to identify all potential risks. However, the study doctors and staff will take all possible safeguards to minimize any known and potential risks to their well being. All of the procedures are will established and used routinely in the investigators laboratory.

## 6.2 EXPLAIN THE STUDY'S EFFORTS TO REDUCE POTENTIAL RISKS TO SUBJECTS:



**Blood Draw:** Dr. Kevin Davy, a registered nurse, or a trained technician will perform all blood draws. Aseptic conditions will be followed during all of the procedures. Universal precautions will be taken in collection and handling of all blood samples. Subjects will be told that their blood will be analyzed for presence of HIV if an experimenter is exposed to their blood.

**Muscle Biopsy:** The muscle biopsies will be performed by a trained investigator or technician under the supervision of Dr Jose Rivero. The possible risk involved with the biopsies are minimized by having trained individuals use aseptic techniques. In addition, subjects will be asked to return to lab within 5 days following the biopsy in order to ensure proper healing.

## 6.3 WHAT ARE THE DIRECT OR INDIRECT ANTICIPATED BENEFITS TO STUDY PARTICIPANTS AND/OR SOCIETY?

There are no direct benefits of participation. Subjects will receive health information including blood pressure, fasting glucose, insulin, and lipids, and body composition.

## Section 7: Full Board Assessment

### 7.1 DOES THE RESEARCH INVOLVE MICROWAVES/X-RAYS, OR GENERAL ANESTHESIA OR SEDATION?

- No  
 Yes

### 7.2 DO RESEARCH ACTIVITIES INVOLVE PRISONERS, PREGNANT WOMEN, FETUSES, HUMAN IN VITRO FERTILIZATION, OR MENTALLY DISABLED PERSONS?

- No, go to question 7.3  
 Yes, answer questions within table

IF YES	
This research involves:	
<input type="checkbox"/> Prisoners	<input type="checkbox"/> Human in vitro fertilization
<input type="checkbox"/> Pregnant women	<input type="checkbox"/> Fetuses
<input type="checkbox"/> Mentally disabled persons	

### 7.3 DOES THIS STUDY INVOLVE MORE THAN MINIMAL RISK TO STUDY PARTICIPANTS?

*Minimal risk means that the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily activities or during the performance of routine physical or psychological examinations or tests. Examples of research involving greater than minimal risk include collecting data about abuse or illegal activities. Note: if the project qualifies for Exempt review (<http://www.irb.vt.edu/pages/categories.htm>), it will not need to go to the Full Board.*

- No  
 Yes

IF YOU ANSWERED "YES" TO ANY ONE OF THE ABOVE QUESTIONS, 7.1, 7.2, OR 7.3, THE BOARD MAY REVIEW THE PROJECT'S APPLICATION MATERIALS AT ITS MONTHLY MEETING. VIEW THE FOLLOWING LINK FOR DEADLINES AND ADDITIONAL INFORMATION: <http://www.irb.vt.edu/pages/deadlines.htm>

## Section 8: Confidentiality / Anonymity

For more information about confidentiality and anonymity visit the following link: <http://www.irb.vt.edu/pages/confidentiality.htm>

### 8.1 WILL PERSONALLY IDENTIFYING STUDY RESULTS OR DATA BE RELEASED TO ANYONE OUTSIDE OF THE RESEARCH TEAM?

*For example – to the funding agency or outside data analyst, or participants identified in publications with individual consent*

- No  
 Yes, to whom will identifying data be released?

### 8.2 WILL ANY STUDY FILES CONTAIN PARTICIPANT IDENTIFYING INFORMATION (E.G., NAME, CONTACT INFORMATION, VIDEO/AUDIO RECORDINGS)?

*Note: if collecting signatures on a consent form, select "Yes."*

- No, go to question 8.3  
 Yes, answer questions within table

IF YES
Describe if/how the study will utilize study codes: <input type="text"/>
If applicable, where will the key [i.e., linked code and identifying information document (for instance, John Doe = study ID 001)] be stored and who will have access? <input type="text"/>
<i>Note: the key should be stored separately from subjects' completed data documents and accessibility should be limited.</i>
<i>The IRB strongly suggests and may require that all data documents (e.g., questionnaire responses, interview responses, etc.) do not include or request identifying information (e.g., name, contact information, etc.) from participants. If you need to link subjects' identifying information to subjects' data documents, use a study ID/code on all data documents.</i>

### 8.3 WHERE WILL DATA BE STORED?

*Examples of data - questionnaire, interview responses, downloaded online survey data, observation recordings, biological samples*

They will be stored in a locked cabinet in the human integrative physiology laboratory and Dr. Hulvers laboratory/office in ILSB, which is also locked.
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### 8.4 WHO WILL HAVE ACCESS TO STUDY DATA?

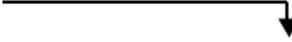
Investigators and graduate students involved in the study.
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### 8.5 DESCRIBE THE PLANS FOR RETAINING OR DESTROYING THE STUDY DATA

De-identified data may be kept indefinitely
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### 8.6 DOES THIS STUDY REQUEST INFORMATION FROM PARTICIPANTS REGARDING ILLEGAL BEHAVIOR?

- No, go to question 9.1  
 Yes, answer questions within table



IF YES
Does the study plan to obtain a Certificate of Confidentiality?
<input type="checkbox"/> No <input type="checkbox"/> Yes (Note: participants must be fully informed of the conditions of the Certificate of Confidentiality within the consent process and form)
<i>For more information about Certificates of Confidentiality, visit the following link:</i> <a href="http://www.irb.vt.edu/pages/coc.htm">http://www.irb.vt.edu/pages/coc.htm</a>

## Section 9: Compensation

For more information about compensating subjects, visit the following link: <http://www.irb.vt.edu/pages/compensation.htm>

### 9.1 WILL SUBJECTS BE COMPENSATED FOR THEIR PARTICIPATION?

- No, go to question 10.1

Yes, answer questions within table

**IF YES**

What is the amount of compensation? **\$200**

Will compensation be prorated?

Yes, please describe: **The total amount of compensation is \$500.00. Subjects will be compensated \$150 for completing each muscle biopsy during the three session 2 visits which take place before and after the baseline period and following the high fat diet (\$450 total). Additional compensation of \$50 will be provided for successful completion of high fat diet period.**

No, explain why and clarify whether subjects will receive full compensation if they withdraw from the study?

*Unless justified by the researcher, compensation should be prorated based on duration of study participation. Payment must not be contingent upon completion of study procedures. In other words, even if the subject decides to withdraw from the study, he/she should be compensated, at least partially, based on what study procedures he/she has completed.*

## Section 10: Audio / Video Recording

For more information about audio/video recording participants, visit the following link: <http://www.irb.vt.edu/pages/recordings.htm>

### 10.1 WILL YOUR STUDY INVOLVE VIDEO AND/OR AUDIO RECORDING?

No, go to question 11.1

Yes, answer questions within table

**IF YES**

This project involves:

- Audio recordings only
- Video recordings only
- Both video and audio recordings

Provide compelling justification for the use of audio/video recording:

How will data within the recordings be retrieved / transcribed?

How and where will recordings (e.g., tapes, digital data, data backups) be stored to ensure security?

Who will have access to the recordings?

Who will transcribe the recordings?

When will the recordings be erased / destroyed?

## Section 11: Research Involving Students

### 11.1 DOES THIS PROJECT INCLUDE STUDENTS AS PARTICIPANTS?

- No, go to question 12.1
- Yes, answer questions within table

**IF YES**

**Does this study involve conducting research with students of the researcher?**

- No
- Yes, describe safeguards the study will implement to protect against coercion or undue influence for participation: \_\_\_\_\_

*Note: if it is feasible to use students from a class of students not under the instruction of the researcher, the IRB recommends and may require doing so.*

**Will the study need to access student records (e.g., SAT, GPA, or GRE scores)?**

- No
- Yes

**11.2 DOES THIS PROJECT INCLUDE ELEMENTARY, JUNIOR, OR HIGH SCHOOL STUDENTS?**

- No, go to question 11.3
- Yes, answer questions within table

**IF YES**

**Will study procedures be completed during school hours?**

- No
- Yes

**If yes,**

**Students not included in the study may view other students' involvement with the research during school time as unfair. Address this issue and how the study will reduce this outcome:** \_\_\_\_\_

**Missing out on regular class time or seeing other students participate may influence a student's decision to participate. Address how the study will reduce this outcome:** \_\_\_\_\_

**Is the school's approval letter(s) attached to this submission?**

- Yes
- No, project involves Montgomery County Public Schools (MCPS)
- No, explain why: \_\_\_\_\_

*You will need to obtain school approval (if involving MCPS, click here: <http://www.irb.vt.edu/pages/mcps.htm>). Approval is typically granted by the superintendent, principal, and classroom teacher (in that order). Approval by an individual teacher is insufficient. School approval, in the form of a letter or a memorandum should accompany the approval request to the IRB.*

**11.3 DOES THIS PROJECT INCLUDE COLLEGE STUDENTS?**

- No, go to question 12.1
- Yes, answer questions within table

**IF YES**

**Some college students might be minors. Indicate whether these minors will be included in the research or**

**actively excluded:**

Included

Actively excluded, describe how the study will ensure that minors will not be included: **Date of Birth**

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**Will extra credit be offered to subjects?**

No

Yes

**If yes,**

**What will be offered to subjects as an equal alternative to receiving extra credit without participating in this study?** \_\_\_\_\_

**Include a description of the extra credit (e.g., amount) to be provided within question 9.1 ("IF YES" table)**

## Section 12: Research Involving Minors

### 12.1 DOES THIS PROJECT INVOLVE MINORS (UNDER THE AGE OF 18 IN VIRGINIA)?

*Note: age constituting a minor may differ in other States.*

- No, go to question 13.1
- Yes, answer questions within table



IF YES
<p><b>Does the project reasonably pose a risk of reports of current threats of abuse and/or suicide?</b></p> <p><input type="checkbox"/> No</p> <p><input type="checkbox"/> Yes, thoroughly explain how the study will react to such reports: _____</p> <p><i>Note: subjects and parents must be fully informed of the fact that researchers must report threats of suicide or suspected/reported abuse to the appropriate authorities within the Confidentiality section of the Consent, Assent, and/or Permission documents.</i></p>
<p><b>Are you requesting a waiver of parental permission (i.e., parent uninformed of child's involvement)?</b></p> <p><input type="checkbox"/> No, <b>both</b> parents/guardians will provide their permission, if possible.</p> <p><input type="checkbox"/> No, <b>only one</b> parent/guardian will provide permission.</p> <p><input type="checkbox"/> Yes, describe below how your research meets <b>all</b> of the following criteria (A-D):</p> <p>Criteria A - The research involves no more than minimal risk to the subjects: _____</p> <p>Criteria B - The waiver will not adversely affect the rights and welfare of the subjects: _____</p> <p>Criteria C - The research could not practicably be carried out without the waiver: _____</p> <p>Criteria D - (Optional) Parents will be provided with additional pertinent information after participation: _____</p>
<p><b>Is it possible that minor research participants will reach the legal age of consent (18 in Virginia) while enrolled in this study?</b></p> <p><input type="checkbox"/> No</p> <p><input type="checkbox"/> Yes, will the investigators seek and obtain the legally effective informed consent (in place of the minors' previously provided assent and parents' permission) for the now-adult subjects for any ongoing interactions with the subjects, or analysis of subjects' data? If yes, explain how: _____</p> <p><i>For more information about minors reaching legal age during enrollment, visit the following link:</i>  <a href="http://www.irb.vt.edu/pages/assent.htm">http://www.irb.vt.edu/pages/assent.htm</a></p> <p><i>The procedure for obtaining assent from minors and permission from the minor's guardian(s) must be described</i></p>

in Section 4 (Consent Process) of this form.

### Section 13: Research Involving Deception

For more information about involving deception in research and for assistance with developing your debriefing form, visit our website at <http://www.irb.vt.edu/pages/deception.htm>

#### 13.1 DOES THIS PROJECT INVOLVE DECEPTION?

- No, go to question 14.1
- Yes, answer questions within table

IF YES
Describe the deception: [REDACTED]
Why is the use of deception necessary for this project? [REDACTED]
Describe the debriefing process: [REDACTED]
<b>Provide an explanation of how the study meets <u>all</u> the following criteria (A-D) for an alteration of consent:</b> Criteria A - The research involves no more than minimal risk to the subjects: [REDACTED] Criteria B - The alteration will not adversely affect the rights and welfare of the subjects: [REDACTED] Criteria C - The research could not practicably be carried out without the alteration: [REDACTED] Criteria D - (Optional) Subjects will be provided with additional pertinent information after participation (i.e., debriefing for studies involving deception): [REDACTED]
<i>By nature, studies involving deception cannot provide subjects with a complete description of the study during the consent process; therefore, the IRB must allow (by granting an alteration of consent) a consent process which does not include, or which alters, some or all of the elements of informed consent.</i>
<i>The IRB requests that the researcher use the title "Information Sheet" instead of "Consent Form" on the document used to obtain subjects' signatures to participate in the research. This will adequately reflect the fact that the subject cannot fully consent to the research without the researcher fully disclosing the true intent of the research.</i>

### Section 14: Research Involving Existing Data

#### 14.1 WILL THIS PROJECT INVOLVE THE COLLECTION OR STUDY/ANALYSIS OF EXISTING DATA DOCUMENTS, RECORDS, PATHOLOGICAL SPECIMENS, OR DIAGNOSTIC SPECIMENS?

*Please note: it is not considered existing data if a researcher transfers to Virginia Tech from another institution and will be conducting data analysis of an on-going study.*

- No, you are finished with the application
- Yes, answer questions within table

IF YES
From where does the existing data originate? [REDACTED]

<p><b>Provide a detailed description of the existing data that will be collected or studied/analyzed:</b> _____</p>
<p><b>Is the source of the data public?</b>  <input type="checkbox"/> No, continue with the next question  <input type="checkbox"/> Yes, you are finished with this application</p>
<p><b>Will any individual associated with this project (internal or external) have access to or be provided with existing data containing information which would enable the identification of subjects:</b></p> <ul style="list-style-type: none"> <li>▪ <b>Directly</b> (e.g., by name, phone number, address, email address, social security number, student ID number), or</li> <li>▪ <b>Indirectly through study codes</b> even if the researcher or research team does not have access to the master list linking study codes to identifiable information such as name, student ID number, etc or</li> <li>▪ <b>Indirectly through the use of information that could reasonably be used in combination to identify an individual</b> (e.g., demographics)</li> </ul> <p><input type="checkbox"/> No, collected/analyzed data will be completely de-identified  <input type="checkbox"/> Yes,</p> <p><b>If yes,</b></p> <p style="padding-left: 40px;"><i>Research will not qualify for exempt review; therefore, if feasible, written consent must be obtained from individuals whose data will be collected / analyzed, unless this requirement is waived by the IRB.</i></p> <p><b>Will written/signed or verbal consent be obtained from participants prior to the analysis of collected data?</b> <input checked="" type="checkbox"/> Yes, signed consent will be obtained _____</p>

*This research protocol represents a contract between all research personnel associated with the project, the University, and federal government; therefore, must be followed accordingly and kept current.*

*Proposed modifications must be approved by the IRB prior to implementation except where necessary to eliminate apparent immediate hazards to the human subjects.*

*Do not begin human subjects activities until you receive an IRB approval letter via email.*

*It is the Principal Investigator's responsibility to ensure all members of the research team who interact with research subjects, or collect or handle human subjects data have completed human subjects protection training prior to interacting with subjects, or handling or collecting the data.*

-----END-----

## **Appendix C: Informed Consent For Subjects**

### **Informed Consent for Participants of Investigative Projects Department of Human Nutrition, Foods and Exercise *Virginia Tech***

**TITLE:**                    **High Fat Feeding, Gut Microflora, and Skeletal Muscle Substrate Metabolism**

**INVESTIGATORS:**        **Kevin P. Davy, Ph.D.**  
**Mathew W. Hulver, Ph.D.**  
**Madlyn I. Frisard, Ph.D.**  
**Brenda M. Davy, Ph.D., R.D.**

**MEDICAL DIRECTOR:**   **Jose Rivero, M.D.**

**Sponsor:** **VSL Pharmaceuticals, Inc.**

#### ***PURPOSE:***

Too much fat in the diet is a risk for diabetes and cardiovascular disease. Too much fat in the diet can increase inflammation (a response of your immune system to defend your body against harmful substances) and change metabolism (how the body gets energy from food in the diet). Probiotics are good bacteria found in the intestines. Probiotic supplements can be found in grocery stores and health food stores. They have been used to help with some gastrointestinal disorders such as irritable bowel syndrome. Probiotics have also been shown to reduce inflammation. We do not know if probiotic supplements improve metabolism in muscle. The first purpose of this study is to determine if a probiotic supplement change the effects of eating a high fat diet on skeletal muscle metabolism. Inflammation can also influence the health of your blood vessels. Therefore, a second purpose is to see if probiotics reduce the effects of a high fat diet on the function of your blood vessels. Thirty males will be included in this study.

#### ***METHODS:***

You are being asked to be involved in a study that involves eating a diet similar to your usual diet for two weeks. You will then follow a 4-week high fat diet with or without a probiotic supplement. VSL#3 is a probiotic supplement that is made of probiotic bacteria. The placebo powder contains maltose (a type of sugar). You will be asked to take 2 packets (4.4 grams each) of either the VSL#3 or placebo powder with juice or water in the morning and another 2 packets in the evening. VSL#3 should be stored in

your refrigerator and not in your freezer. You should not mix VSL#3 with hot beverages or food or keep in clothes pockets close to your body. If you want to take the VSL#3 or placebo away from home, you should store it in the cooler that we will provide. If you agree to be involved in this study you will first have to fill out a health history questionnaire. The additional tests are described below under Session 1. Your results may be discussed with the study medical director to determine if you can be a subject. You may be able to be a subject if you are between 18 and 40 years of age. Your body mass index (a measure of obesity) must be less than or equal to 30. If you smoke or have high blood pressure, heart disease or diabetes then you cannot be in this study. You will not be able to participate if your cholesterol is too high or have other health problems that would make it unsafe for you to be in the study. Your body mass index (a measure of fatness) must be less than 30 kg/m<sup>2</sup>. You will not be able to participate if you have lost or gained more than 5 pounds in the last 6 months or exercise three or more times a week at a moderate to hard level (e.g., exercise that causes you to breathe hard and sweat). If you use any medication or nutritional supplements that might influence the study variables or have taken antibiotics in the last month then you will not be able to be in this study. You will not be able to be a subject if you have an allergy to lidocaine or bipivacaine, have food allergies (for example, gluten allergy), or are allergic to silicon dioxide (a food additive used to reduce moisture in the VSL#3 or placebo packet). The amount of silicon dioxide is less than 0.1 % (or 0.44 grams) of each 4.4 gram packet.

During the first two-week period you will be given all your food. This food will have the same number of calories you usually eat. The food given to you will have 55% of the calories from carbohydrates, 30% from fat, and 15% from protein. After this 2 week period, you will be randomized (a process similar to flipping a coin) to either a high fat diet with VSL#3 probiotic supplement powder or high fat diet with a placebo (sugar powder).

You will be provided all of your food during this period so that 50-60% of all the calories you eat come from fat. In addition, your calorie intake will be increased by 1000 calories during the 4-week high fat diet portion of the study. Although most subjects will gain weight approximately 3-4 pounds during the study, some may gain less and some may gain more. However, you will meet with a registered dietitian upon completion of the study to help you lose the weight you gained through diet and exercise.

Blood samples and muscle biopsies will be taken at three time points during the 6 week study, once in the beginning of the study, once immediately following the 2 weeks of eating a diet similar to your normal, habitual diet period, and once immediately following the 4-week high fat diet. You will also be required to obtain stool sample the day prior to your laboratory visit and bring it with you so we can measure how the VSL#3 influences

the bacteria in your intestine. You will need to come to the laboratory each day during the initial two weeks and the 4-week high fat diet period to have your bodyweight measured as well as to pick up your food and return any uneaten foods from the previous day.

There will be about 50 visits if you participate in the study. This will require approximately 25 hours of time commitment. The real number and order of visits will depend on your and the study staffs schedule. In addition, the order may differ from the order of appearance in this document. You will undergo Session 1 one time and session 2 and 3 three times (before and after 2 weeks of eating your typical diet and again following the high fat diet).

Session 1: Approximate time required: 1.5 hour

**(You will complete this session only one time at the beginning of the study)**

- **Overnight Fast:** You will have to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat.
- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time. Your body weight will be measured on a bathroom scale. Your height will be measured with a kind of ruler. Your waist, hip, and neck circumference will be measured using a measuring tape.
- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Physical Activity Questionnaire:** You will need to answer questions so we estimate your physical activity level. This will take about 15 minutes.
- **Urine Test:** You will be asked to urinate in a small cup that we provide to you. We will measure the amount of sodium and other electrolytes, glucose, protein, pH and whether there are blood cells present to determine whether it is safe for you to participate in the study.
- **Blood Draw:** A small needle will be put in your arm to draw blood (about 3 tablespoons). We will measure glucose, cholesterol, and other factors to determine if you can be a subject.

**Session 2: Approximate time required: 1 hour**

*(You are being asked to complete this session three times; before and after the 2 week habitual diet period and once following the 4 week high fat diet trial).*

- **Overnight Fast:** You will have to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat.
- **Urine Test:** You will be asked to urinate in a small cup that we provide to you. We will measure the byproducts of metabolism that may change after you take the probiotic supplement.
- **Body Weight and Composition:** These tests are to measure your body weight and body fat. Your body weight will be measured without shoes on a hospital scale. Then you will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 5 minutes and there is no pain associated with the procedure. This procedure will be performed once at the beginning of the study and a second time at the end of the study. Your weight and height will also be measured at this time.
- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Arterial Stiffness:** To measure arterial stiffness, the blood flow and diameter in the arteries in your chest, neck and leg will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a “TV-like” screen. A mobile hand unit used will be pressed gently against an artery in your neck and leg.

*Session 3: Approximate time required: 4.5 hours*

*(You are being asked to complete this session three times; before and after the 2 week habitual diet period and once following the 4 week high fat diet trial).*

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine-containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.
- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.
- **Physical Activity Questionnaire:** You will be asked a series of questions to estimate your usual physical activity level, which will require about 15 minutes to complete.

- **Muscle Biopsy:** You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medication (such as Advil, Motrin, Celebrex, or Vioxx, or other medication or anything that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 450 mg) from below the skin on your thigh. The actual biopsy site will be on the top of either the right or left leg between your knee and hip.

This procedure will be performed by a study investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) who has been trained to perform the biopsy. A Physician or a Nurse will be on site and available if needed, but may not be present during the procedure itself. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine, which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bipivacaine) into the area with a small needle. If you are allergic to lidocaine or bipivacaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle (about 450 mg). Some suction may be applied to the other end of the needle to help remove the muscle.

After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors which contribute to inflammation. The biopsy will take place at either the Human Integrative Physiology Laboratory (228 War Memorial Hall) or Dr. Jose Rivero's medical office in Christiansburg. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

*You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.*

- **Intravenous Glucose Tolerance Test (IVGTT).** You will need to fast 12 hours prior to your visit to the lab. Two small plastic tubes (intravenous catheters) will be placed in each of two arm veins (different arms) and about 3 tablespoons of blood will be taken to measure hormones or proteins that influence your metabolism and cardiovascular system. We will then inject a small amount of glucose (0.3 mg/kg body weight) and insulin (0.03 unit/kg body weight) into your veins (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) about 28 times over a 3-hour period. A

registered nurse will be present to perform this test with the assistance of investigators.

### **Take-Home Tests**

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day). You will also be asked to keep track of the food you eat for the 5 days you are in the habitual-diet phase of the study.
- **Stool Collection:** You will be asked to collect a stool sample to bring to the laboratory on three occasions (each time you come in for session two).

Collection Instructions:

- Collect stool sample on the day prior to, or day of, your scheduled visit.
- Place plastic stool hat onto toilet seat for stool collection.
- Tightly close (seal) the white container and put in the clear plastic bag (zip lock), which was provided. Close the bag tightly.
- Place a freezer pack in the cooler with your sample, and return the items to the research study staff.

### ***SUMMARY OF SUBJECT RESPONSIBILITIES***

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiment.
- Follow all participant instructions for each session.
- Collect your stool samples and follow the instructions for returning in the cooler provided.
- Record any food you eat that has not been provided by the investigators.
- Return any uneaten food that has been provided by the investigators.
- Follow physical activity instructions provided by the investigators.
- Carefully read the instructions on consuming any food provided to you.

## ***RISKS OF PARTICIPATION***

- **VSL#3 Probiotic Supplement:** VSL#3 has been shown to be a safe. There is small risk of flatulence (gas), bloating, and/or a change in bowel habits while taking VSL#3. You should not be in the study if you are allergic to silicon dioxide, a food additive used to absorb moisture during the packaging and storage of VSL#3 and the placebo.
- **Catheter and Blood Draw:** Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in catheter placement and blood draws will minimize these risks.
- **Intravenous Glucose Tolerance Test:** Because this procedure requires the placement of a catheter in an arm vein, the risks here are identical to that stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. We will be monitoring your blood sugar frequently and can usually anticipate this before your blood sugar drops too low. If this happens, orange juice (with table sugar) or some other simple carbohydrate containing food will be given to you. We will monitor your glucose until it returns to normal. A registered nurse will perform the test with the assistance of the investigators.
- **HIV/AIDS:** Your blood will be tested for the presence of HIV if one of the study investigators is exposed to your blood. There will not be any cost to you for this test. The results will be sent to your primary care physician or the study medical director, Dr. Jose Rivero, if you do not have a primary care physician. He/she will discuss them with you and provide you with the necessary referral for further evaluation and/or counseling if your results are positive. The results of your test will remain confidential.
- **Muscle Biopsies:** If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are

applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before or during the procedures. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. These risks are minimized by having a trained individual perform the procedure. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring. Individuals with darker skin (e.g., African Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

- DEXA Scan: The amount of radiation that you will receive in the DEXA exam (combined with the CT scan) is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however the exact increase in such risk is not known.
- Arterial Stiffness: There are no known risks associated with these procedures.
- Weight gain: It is expected that you will gain 3-4 pounds during the study but some individuals will gain less and some will gain more. You should know that your weight can fluctuate 1-2 pounds over the course of 1-2 days even without changing your diet. At the end of the study you will meet with a dietitian and receive instructions on how to modify your diet and increase your physical activity to return to your original body weight.
- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.

- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

### **BENEFITS OF PARTICIPATION**

Your participation will provide you with:

- Information on your body composition.
- Information on your blood pressure, cholesterol and glucose tolerance
- A primary goal of this study is to obtain generalizable medical knowledge related to the benefits of this probiotic supplement.

### **COMPENSATION**

You will be compensated \$100 for completing each muscle biopsy. Muscle biopsies will be performed during the three session 3 visits and will be performed before and after your typical diet and again after the high fat diet (\$300 total). You can receive an additional \$200 for your participation in the high fat feeding period. Total compensation for your participation in the study is \$500.

### **CONFIDENTIALITY**

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by a code, without anything to identify you by name. In the event that any of your tests indicate a problem, your results may be shared with the medical director, Dr. Rivero, and your personal physician.

### **FREEDOM TO WITHDRAW**

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain an adequate muscle sample or other measurements that are necessary for the study. All of the sessions and measurements are required components.

### **INJURY DURING PARTICIPATION IN THIS STUDY**

Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if injured as a result of your participation in this study. Any expenses that you incur including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

### **APPROVAL OF RESEARCH**

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

**SUBJECT PERMISSION**

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:

- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: 540-230-0486
- Chairman, Institutional Review Board for Research Involving Human Subjects:

**David Moore, Associate Vice President for Research (540) 231-4991**

Name of Subject (please print) \_\_\_\_\_

Signature of Subject \_\_\_\_\_ Date \_\_\_\_\_

Appendix D: Health History Questionnaire

Virginia Tech  
Department of Human Nutrition, Foods, and Exercise

HEALTH HISTORY QUESTIONNAIRE

STUDY \_\_\_\_\_

DATE \_\_\_\_\_

SUBJECT ID # \_\_\_\_\_

PLEASE PRINT

1. Address:

City: \_\_\_\_\_

State: \_\_\_\_\_

Zip Code \_\_\_\_\_

Home Phone: \_\_\_\_\_

Work Phone: \_\_\_\_\_

E-mail address: \_\_\_\_\_

Emergency Contact: \_\_\_\_\_

Phone: \_\_\_\_\_

Relation to you: \_\_\_\_\_

2. Employer: \_\_\_\_\_

Occupation: \_\_\_\_\_

3. Date of Birth: \_\_\_\_\_

Age: \_\_\_\_\_

Sex: \_\_\_\_\_

Race and/or Ethnic Origin

American Indian or Alaskan Native  Asian or Pacific Islander  Black, not of Hispanic Origin

Hispanic

White, not of Hispanic Origin

Other

4. GENERAL MEDICAL HISTORY

Do you have any current medical conditions? YES

NO

If Yes, please explain:

Are you allergic to any medications? YES

NO

If Yes, please explain:

Have you had any major illnesses in the past? YES  NO   
If Yes, please explain:

Have you ever been hospitalized or had surgery? YES  NO   
If Yes, please explain: (include date and type of surgery, if possible)

Are you currently taking any medications or supplements, including aspirin, hormone replacement therapy, or other over-the-counter products?

YES  NO   
If Yes, please explain:

<u>Medication/Supplement</u>	<u>Reason</u>	<u>Times taken per Day</u>	<u>Taken for</u>	<u>how long?</u>
------------------------------	---------------	----------------------------	------------------	------------------

Have you ever had an EKG? YES  NO   
If Yes, please explain:

Have you been diagnosed with diabetes? YES  NO   
If Yes, please explain:

Age at diagnosis

5. **FAMILY HISTORY**

Age (if alive)	Age of Death	Cause of Death
----------------	--------------	----------------

Father

Mother

Brothers/Sisters

Do you have a family history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

YES	NO	Relation	Age at Diagnosis
a.	High blood pressure	<input type="checkbox"/>	<input type="checkbox"/>
b.	Heart Attack	<input type="checkbox"/>	<input type="checkbox"/>
c.	Coronary bypass surgery	<input type="checkbox"/>	<input type="checkbox"/>
d.	Stroke	<input type="checkbox"/>	<input type="checkbox"/>
e.	Diabetes	<input type="checkbox"/>	<input type="checkbox"/>
f.	Obesity	<input type="checkbox"/>	<input type="checkbox"/>

**6. TOBACCO/ALCOHOL HISTORY** (check one)

**CURRENT TOBACCO**

**USE**

(if applicable)

None	<input type="checkbox"/>	
Quit	<input type="checkbox"/>	(when)
Cigarette	<input type="checkbox"/>	
Cigar	<input type="checkbox"/>	
Pipe	<input type="checkbox"/>	
Chew Tobacco	<input type="checkbox"/>	
Snuff	<input type="checkbox"/>	

**# per day**

Cigarette  
Cigar  
Pipe  
Chew Tobacco  
Snuff

Total years of tobacco use \_\_\_\_\_

Do you consume alcohol? Drinks per day \_\_\_\_\_ Drinks per week \_\_\_\_\_

**7. CARDIORESPIRATORY/METABOLIC HISTORY**

**YES**

**NO**

Are you presently diagnosed with heart disease?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have any history of heart disease?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have a heart murmur?	<input type="checkbox"/>	<input type="checkbox"/>
Occasional chest pain or pressure?	<input type="checkbox"/>	<input type="checkbox"/>
Chest pain or pressure on exertion?	<input type="checkbox"/>	<input type="checkbox"/>
Episodes of fainting?	<input type="checkbox"/>	<input type="checkbox"/>
Daily coughing?	<input type="checkbox"/>	<input type="checkbox"/>
High blood pressure?	<input type="checkbox"/>	<input type="checkbox"/>
Shortness of breath?		
At rest?	<input type="checkbox"/>	<input type="checkbox"/>
lying down?	<input type="checkbox"/>	<input type="checkbox"/>
After 2 flights of stairs?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have asthma?	<input type="checkbox"/>	<input type="checkbox"/>

Do you have a history of bleeding disorders?

**YES** **NO**

Do you have a history of problems with blood clotting?

Do you have high cholesterol? Or, low good (HDL) cholesterol?

Do you have thyroid problems?

***If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.***

**8. MUSCULOSKELETAL HISTORY**

**YES** **NO**

Any current muscle injury or illness?

Any muscle injuries in the past?

Do you experience muscle pain at rest?

Do you experience muscle pain on exertion?

Any current bone or joint (including spinal) injuries?

Any previous bone or joint (including spinal) injuries?

Do you ever experience painful joints?

Do you ever experience swollen joints?

Do you ever experience edema (fluid build up)?

Do you have pain in your legs when you walk?

***If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.***

**9. NUTRITIONAL HABITS**

Have you ever dieted? YES  NO

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES  NO

If YES, please describe the diet:

a). Name (if applicable):

b). Prescribed by a Physician/nutritionist? YES  NO

c). Have you lost weight? YES  NO

d). Duration of diet

What was your weight 24 months ago? \_\_\_\_\_ 12 months ago? \_\_\_\_\_ 6 months ago?  
\_\_\_\_\_

Have you dieted other than in the past 12 months? YES  NO

If YES, please answer the following:

a). How many times have you dieted?

b). How old were you?

c). Weight loss (amount)?

***You may be asked to complete a more detailed diet survey if you are volunteering for a research study.***

**10. PHYSICAL ACTIVITY SURVEY**

Compared to a year ago, how much regular physical activity do you get? (Check one)

Much less

Somewhat less

About the same

Somewhat more

Much more

Have you been exercising regularly for the past three months? YES  NO

If YES, what type of exercise do you regularly participate in? (check those that apply)

Days per week (1=easy, 10=very hard)	Minutes per session	Intensity
Walking	<input type="checkbox"/>	
Running	<input type="checkbox"/>	
Cycling	<input type="checkbox"/>	
Swimming	<input type="checkbox"/>	
Aerobics	<input type="checkbox"/>	
Weight Training	<input type="checkbox"/>	
Martial Arts	<input type="checkbox"/>	
Other (describe)		

**You may be asked to complete a more detailed diet survey if you are volunteering for a research study.**

**11. OBSTETRIC/GYNECOLOGICAL HISTORY**

**YES**                      **NO**

Do you have a normal menstrual cycle (1 menses each ~1 month)?

If no, please indicate frequency \_\_\_\_\_

Do you take any kind of contraceptive (oral, injectable, implant)?

If yes, please indicate type and name \_\_\_\_\_

How many full term pregnancies have you had? \_\_\_\_\_ How long ago was your more recent pregnancy? \_\_\_\_\_

Have long since you have last breast fed? \_\_\_\_\_

**12. SLEEP HISTORY**

**YES**                      **NO**

Do you snore?

**Don't Know**

Snoring loudness

- Loud as breathing
- Loud as talking
- Louder than talking
- Very loud. Can be heard in nearby rooms.

Snoring frequency

- Almost every day
- 3-4 times per week
- 1-2 times per week
- 1-2 times per month

Never or almost never

Does your snoring bother other people?

Has anyone told you that you quit breathing during your sleep?

How often have your breathing pauses been noticed?

- Almost every day
- 3-4 times per week
- 1-2 times per week
- 1-2 times per month
- Never or almost never

Are you tired after sleeping?

Almost every day

- 3-4 times per week
- 1-2 times per week
- 1-2 times per month
- Never or almost never

Are you tired during waketime?

- Almost every day
- 3-4 times per week
- 1-2 times per week
- 1-2 times per month
- Never or almost never

Have you ever fallen asleep while driving?

- Almost every day
- 3-4 times per week
- 1-2 times per week
- 1-2 times per month
- Never or almost never

### Sleepiness Assessment

0 (zero) = would never doze off

1 (one) = slight chance of dozing

2 (two) = moderate chance of dozing

3 (three) = high chance of dozing

#### Situation

#### Chance of Dozing

Sitting and reading

—

Watching TV

—

Sitting, inactive in a public place (e.g., a theatre or meeting)

—

As a passenger in a car for an hour without a break

—

Lying down to rest in the afternoon when circumstances permit

—

Sitting quietly after lunch without alcohol

—

Sitting and talking to someone

—

In a car, while stopped for a few minutes in traffic

—

13. **EDUCATION**

Please check the highest degree obtained:

- Grade School
- Junior High
- High School
- College Degree
- Master's Degree
- Doctorate

14. **FAMILY PHYSICIAN**

Name:

Address:

Phone:

Reviewer: \_\_\_\_\_  
Print Name                      Signature

Date:

## Appendix E: Physical Activity Questionnaire

### Godin Leisure-Time Exercise Questionnaire

#### INSTRUCTIONS

In this excerpt from the Godin Leisure-Time Exercise Questionnaire, the individual is asked to complete a self-explanatory, brief four-item query of usual leisure-time exercise habits.

#### CALCULATIONS

For the first question, weekly frequencies of strenuous, moderate, and light activities are multiplied by nine, five, and three, respectively. Total weekly leisure activity is calculated in arbitrary units by summing the products of the separate components, as shown in the following formula:

$$\text{Weekly leisure activity score} = (9 \times \text{Strenuous}) + (5 \times \text{Moderate}) + (3 \times \text{Light})$$

The second question is used to calculate the frequency of weekly leisure-time activities pursued "long enough to work up a sweat" (see questionnaire).

#### EXAMPLE

Strenuous = 3 times/wk

Moderate = 6 times/wk

Light = 14 times/wk

$$\text{Total leisure activity score} = (9 \times 3) + (5 \times 6) + (3 \times 14) = 27 + 30 + 42 = 99$$

Godin, G., Shephard, R. J.. (1997) [Godin Leisure-Time Exercise Questionnaire](#). *Medicine and Science in Sports and Exercise*. 29 June Supplement: S36-S38.

### Godin Leisure-Time Exercise Questionnaire

1. During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number).

	<b>Times Per Week</b>
<p><b>a) STRENUOUS EXERCISE</b>  <b>(HEART BEATS RAPIDLY)</b>            (e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)</p>	<hr style="border: 0; border-top: 1px solid black; width: 100%;"/>
<p><b>b) MODERATE EXERCISE</b>  <b>(NOT EXHAUSTING)</b>            (e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)</p>	<hr style="border: 0; border-top: 1px solid black; width: 100%;"/>
<p><b>c) MILD EXERCISE</b>  <b>(MINIMAL EFFORT)</b>            (e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)</p>	<hr style="border: 0; border-top: 1px solid black; width: 100%;"/>

2. During a typical **7-Day period** (a week), in your leisure time, how often do you engage in any regular activity **long enough to work up a sweat** (heart beats rapidly)?

OFTEN	SOMETIMES	NEVER/RARELY
1. <input type="checkbox"/>	2. <input type="checkbox"/>	3. <input type="checkbox"/>

## Appendix F: Infection and Inflammation Questionnaire

### INFECTION/INFLAMMATION QUESTIONNAIRE

Evaluator Script: I would like you to think if you had a cold, the flu, a dental infection or other infection during the past month. I am going to ask you about some symptoms that may have accompanied those types of conditions.

1) Did you have a cold, the flu, a dental infection or other infection in the past month?

Yes       No       Refused       Don't Know

If yes,  Within 1 week     2 weeks prior     3 weeks prior     4 weeks prior

In the prior month did you experience any of the following symptoms? [Note to examiner: If symptom was present, the timing of symptom onset and resolution (# days) prior to interview is recorded. If symptom is still present on the day of interview, place 0 in "Resolved \_\_\_ days ago".]

2) Did you feel feverish or have a fever?       Yes  No

If Yes, Symptom Started \_\_\_ days ago. Resolved \_\_\_ days ago.

Did you take your temperature?       Yes  No

3) Chills?       Yes       No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

4) Sore throat ?       Yes       No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

5) Coughing?       Yes       No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

6) Sputum?  Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

7) Sneezing?  Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

8) Runny nose or nasal congestion?  Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

If Yes to (5), (6), (7), or (8). Do you have seasonal allergies?  Yes  No

Do you have a chronic lung or sinus condition?  Yes  No

If Yes, are these symptoms typical for your chronic lung or sinus condition?

Yes  No

9) Ear pain or discharge?  Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

10) Run down feeling or achy muscles you feel may have been due to a cold or flu?

Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

11) Tooth/Gum pain?  Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

If Yes, did you seek dental care?  Yes  No

If Yes, did a Dentist find a cavity or other dental infection?  Yes  No

12) Mouth/gum ( Y N ), Skin ( Y N ), or Joint ( Y N ) redness or swelling?

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

13) Skin infection?  Yes  No

If Yes, Started \_\_\_ days ago. Resolved \_\_\_ days ago.

14) Nausea/Vomiting? ( )Yes ( )No

If Yes, Started\_\_\_\_days ago. Resolved\_\_\_\_days ago.

15) Diarrhea? ( )Yes ( )No

If Yes, Started\_\_\_\_days ago. Resolved\_\_\_\_days ago.

16) Pain upon urination or urgency? ( )Yes ( )No

If Yes, Started\_\_\_\_days ago. Resolved\_\_\_\_days ago.

17) Cloudy discolored urine? ( )Yes ( )No

Urinalysis showing evidence of infection? ( )Yes ( )No

If Yes, Started\_\_\_\_days ago. Resolved\_\_\_\_days ago.

18) Did you seek medical care for any sort of cold, flu, or infection in the prior month?

( )Yes ( )No

If yes, diagnosis given

19) Did you take any over the counter or prescription medications for a cold, flu, or any infection in the prior month?

( )Yes ( )No

If yes, names of medication