

## ABSTRACT

Title of Dissertation: INTERLEUKIN-6 (IL6) GENOTYPE,  
PLASMA LIPOPROTEIN LIPIDS, AND  
THEIR RESPONSE TO EXERCISE  
TRAINING

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Substantial evidence indicates that plasma lipoprotein-lipid levels are related to cardiovascular disease, are highly variable among individuals, and have a strong genetic component. They are also modifiable with exercise training, and these responses are heritable. A small body of literature suggests an association between the interleukin-6 (IL6) –174G/C gene polymorphism and plasma lipoprotein-lipid levels such that the G allele may be associated with a more pro-atherogenic lipid profile than the C allele. We hypothesized that the IL6 –174G/C gene polymorphism would be associated with variation in plasma lipoprotein-lipid profiles at baseline and with their response to exercise training.

Sixty-five sedentary, healthy 50- to 75-year-old Caucasians were studied before and after 24 weeks of aerobic exercise training. Significant differences existed among genotype groups for change with exercise training in high-density lipoprotein (HDL)-C, HDL<sub>3</sub>-C, HDL<sub>5NMR</sub>-C, HDL<sub>4NMR</sub>-C, integrated HDL<sub>4,5NMR</sub>-C, and HDL<sub>size</sub>. For HDL-C,

the CC group increased significantly more than both the GG ( $7.0 \pm 1.3$  v.  $1.0 \pm 1.1$  mg/dL,  $p=0.001$ ) and the GC group ( $3.3 \pm 0.9$  mg/dL,  $p=0.02$ ). For HDL<sub>3</sub>-C, the CC group increased significantly more than both the GG ( $6.1 \pm 1.0$  v.  $0.9 \pm 0.9$ , mg/dL  $p<0.001$ ) and the GC group ( $2.5 \pm 0.7$  mg/dL,  $p=0.006$ ). For HDL<sub>5NMR</sub>-C, the GC group increased significantly less than both the CC and GG groups ( $0.0 \pm 0.7$  v.  $3.4 \pm 1.0$  mg/dL,  $p=0.02$  and  $1.4 \pm 0.8$  mg/dL,  $p=0.04$ ). For HDL<sub>4NMR</sub>-C, the GG group changed significantly differently from both the GC and CC groups with the GG group decreasing and the GC and CC groups increasing HDL<sub>4NMR</sub>-C ( $-0.4 \pm 1.1$  v.  $3.1 \pm 0.9$  mg/dL,  $p=0.02$  and v.  $3.2 \pm 1.3$  mg/dL,  $p=0.05$ , respectively). For integrated HDL<sub>4,5</sub>-C, the CC group increased significantly more than the GG group ( $6.5 \pm 1.6$  mg/dL v.  $1.0 \pm 1.3$  mg/dL,  $p=0.01$ ). For HDL<sub>size</sub>, the CC group increased significantly more than both the GG ( $0.3 \pm 0.1$  v.  $0.1 \pm 0.1$  nm,  $p=0.02$ ) and the GC groups ( $0.0 \pm 0.0$  nm,  $p=0.007$ ). These findings suggest that the IL6-174G/C polymorphism influences HDL-C and HDL-C subfraction changes with exercise training.

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RESPONSE TO EXERCISE TRAINING

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## INTRODUCTION

Currently the leading cause of morbidity and mortality in the developed world, cardiovascular (CV) disease will soon be the most critical health problem across the globe.<sup>137,190</sup> For over 30 years, plasma lipoprotein-lipids have been identified as major physiological contributors to the pathophysiology of atherosclerotic vascular disease, and they are highlighted as a major risk factor category for its development.<sup>190,196,201-203</sup> However, recent evidence suggests that inflammation may be the underlying mechanism that regulates the atherosclerotic process and potentially explains how the various CV disease risk factors influence each other and the development of CV disease.<sup>29,47,49,131,133,144,175,176,179,199,252</sup> This line of research suggests that various conditions and factors can result in the establishment of a chronic low grade inflammatory state, marked by elevated inflammatory activity that has a beneficial role in the innate immune response but, if present for prolonged periods of time, can become physiologically detrimental.<sup>80,105,106,252</sup> As the major mediator of this inflammatory activity, the inflammatory cytokine interleukin-6 (IL-6) may serve as the molecular link between metabolic, endothelial, and coagulatory CV disease risk factors, including alterations in lipid metabolism, dyslipidemia, and proatherogenic changes in lipoproteins, and the pathogenesis of atherosclerosis.<sup>80,105,106,133,135,252</sup>

IL-6 is a multifunctional cytokine secreted by a variety of cell types including lymphocytes, macrophages, monocytes, endothelial cells, adipocytes, and skeletal muscle.<sup>80,82,162,239</sup> Possessing autocrine, paracrine, and endocrine functions, it acts as a major regulator of the hepatic acute phase response and plays a role in coagulatory

status, endothelial function, energy regulation, and lipid metabolism.<sup>42,56,136,161,162,170,224,238</sup> These biological activities result in increased levels of circulating acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA), soluble adhesion molecules, plasminogen-activator-1 (PAI-1), and fibrinogen, decreased nitric oxide secretion, increased basal glucose uptake, decreased insulin sensitivity, increased TG and non esterified FFA levels, and decreased HDL-C levels.<sup>136,162,239,252,253</sup> IL-6 also acts to stimulate the hypothalamic-pituitary-adrenal axis, induce the secretion of vasopressin and growth hormone, and suppress the thyroid axis.<sup>162,242,252</sup> However, due to such initiation factors as chronic low-grade infection, obesity, environmental or emotional stress, or genetic programming, IL-6 can become chronically elevated, resulting in the prolonged activation of these biological activities.<sup>105,252</sup> This dysregulation in IL-6 levels, irrespective of cause or magnitude, could then turn the initially beneficial responses into deleterious conditions.<sup>44,47-50,206,243,252</sup>

Genetic factors affecting IL6 gene transcription and expression are one potential influence on IL-6 production.<sup>53,252</sup> Several potentially functional promoter polymorphisms have been identified in the IL6 gene 5' flanking region that may influence IL6 gene transcription and thus IL-6 production, including the -597G/A, -572 G/C, -373A<sub>n</sub>T<sub>n</sub>, and -174G/C promoter polymorphisms.<sup>23,51-53,57,98,146,225,232,242</sup> Whether these single nucleotide polymorphisms (SNPs) alone or in combinations are responsible for alterations in IL6 gene function is unclear.<sup>23,51-53,57,98,146,225,232,242</sup> However, previous work supports strong linkage disequilibrium among these polymorphisms, gives estimates for 94% of the haplotype combinations at these loci,

and supports the use of the –174G/C promoter polymorphism as a marker for the most frequent of those haplotypes.<sup>23,51-53,57,98,146,225,232,242</sup> Additionally, research has shown associations between the –174G/C polymorphism, IL-6 levels, and various health related phenotypes, including bone mineral density, insulin sensitivity, and plasma lipoprotein lipid levels.<sup>44,46,49,50,93,127,138,226,242,243</sup> For example, Fernandez-Real et al.<sup>46</sup> showed an association between IL-6 levels and dyslipidemia in which the –174 G allele showed a trend toward higher IL-6 levels and was associated with increased triglycerides (TG), very low-density lipoprotein triglycerides (VLDL-TG), and apolipoprotein B (apo B) and decreased high-density lipoprotein (HDL)<sub>2</sub>-C. Stouthard et al.<sup>226</sup> found an association between IL-6 and plasma free fatty acids. Epidemiological studies have also shown associations between elevated IL-6 levels and lipid abnormalities in which IL-6 was associated with increased TG in men with CV disease risk factors and with high TG and low HDL-C in healthy 100–106 year olds.<sup>9,133</sup> Additionally, since inflammation is known to induce pro-atherogenic changes in lipoproteins and lipid metabolism and IL-6 is the primary mediator of the inflammatory response, it is biologically plausible that genetic variation in IL6, resulting in dysregulation of IL-6 levels, may be linked to alterations in lipid metabolism.<sup>68,86,105,106</sup> Thus, the literature supports further investigation of the IL6 -174G/C gene polymorphism in regard to IL-6 and various health phenotypes, including lipid metabolism and plasma lipoprotein-lipids.

In addition to genetic variation, IL6 transcription rates, mRNA, and IL-6 release are influenced by acute exercise.<sup>40-43,84,88,89,97,103,104,151,153,154,167-172,193,218,219,221-224,238</sup> The effect of exercise on the changes in IL6 gene expression and IL-6 release

are influenced by the mode, intensity, and duration of the exercise.<sup>42,154,172</sup> For example, IL-6 levels have been shown to increase 100-fold following a marathon and to be augmented in states of low muscular glycogen.<sup>103,104,152</sup> The primary source of the increase in IL-6 with exercise is the contracting skeletal muscle with the release stimulated by the muscle contraction itself, low intramuscular glycogen levels, or changes in energy turnover.<sup>104,168,171,172</sup> Other organs, including adipose tissue, also release IL-6 during exercise.<sup>129,218</sup> Thus, acting as an energy sensor and signaling molecule between the contracting muscle and other organs, the IL-6 protein appears to play a role in energy regulation during exercise as it functions to maintain glucose availability and mediate the mobilization of free fatty acids.<sup>129,170-172,218,224</sup> In terms of exercise training, little data exists on the IL-6 response. However, some evidence suggests that endurance exercise training may attenuate the IL-6 response to exercise in CHF patients and healthy adults.<sup>3,13,59,177,235</sup> In terms of the interactive effects of endurance exercise training and IL6 genotype on IL-6 levels and subsequently on plasma lipoprotein-lipids, we know of no investigations that have looked at that gene\*environment interaction.

In contrast to IL-6 response, endurance exercise training has been shown to produce favorable modifications in plasma lipoprotein-lipid levels.<sup>1,35,64,109,115-117,214,233</sup> Additionally, studies show that both baseline plasma lipoprotein lipid levels and their response to exercise training are influenced by genetic variation at specific lipid metabolism related gene loci.<sup>70,73,74,190,249</sup> For example, in a meta-analysis of 52 exercise training studies involving training protocols of > 12 weeks, Leon et al.<sup>116,117</sup> found an average increase of 4.6% in HDL-C, an average reduction of 3.7% in TG,

and an average reduction of 5.0% in low-density lipoprotein (LDL)-C. Similarly, Kraus et al.<sup>109</sup> recently found that a relatively high amount of regular exercise significantly improved overall lipoprotein profiles, including an increase in total HDL concentration, large HDL particle concentration, and average size of HDL particles, even without clinically significant weight loss. Halverstadt et al.<sup>74</sup> found that genetic variation at a specific locus on the lipid metabolism related endothelial lipase gene was associated with inter-individual variability in HDL C and its subfractions and their responses to exercise training. Thus, combined with heritability estimates of 0.64 for total cholesterol (TC), 0.67 for LDL-C, 0.42 for HDL-C levels, and .25 to .38 for the general lipid response to exercise training, these studies support the role of exercise training in improving plasma lipoprotein lipid profiles and demonstrate that the variability in lipid levels and response to training is partially accounted for by a genetic component.<sup>75,79,91,174,181</sup>

With IL-6 having a very short biphasic half-life (initial 3 minute and then second 55 minute clearance), marked diurnal variability (low in the morning and high at bedtime), and large within and between individual variation (30% and 44%, respectively), accurate and consistent measurement is difficult.<sup>12,26,30,33,132,166,215,216</sup> However, C-reactive protein (CRP) has been identified in the literature as a surrogate measure for plasma IL-6 levels as it is more stable (long-term stability in storage and half-life of 15 to 19 hours), lacks diurnal variability, is reproducible, and is regulated by IL-6.<sup>12,26,30,33,132,166,215,216</sup> Additionally, while inconclusive as to which allele is associated with higher levels of CRP, evidence does suggest an association of the IL6

–174G/C polymorphism with CRP levels.<sup>51,107,241</sup> Consequently, CRP levels can be used as a surrogate marker for plasma IL-6 levels.

Thus, with research demonstrating the potential impact of genetic variation and the interaction of genetic variation with exercise training on IL-6 and plasma-lipoprotein lipid levels, we hypothesized that the IL6 –174G/C gene polymorphism would be associated with variation in plasma lipoprotein-lipid profiles and their response to exercise training.

## PURPOSE OF THE STUDY

The purpose of this investigation was to determine if the IL6 -174G/C gene polymorphism was associated with plasma lipoprotein lipids and their subfractions at baseline and with their responses to exercise training.

### Hypotheses

1. IL6 genotype will influence plasma lipoprotein lipid levels such that carriers of the G allele will demonstrate a more pro-atherogenic baseline lipid profile (higher TC, TG, LDL-C, VLDL-TG, HDL<sub>2</sub>NMR-C and integrated HDL<sub>1,2</sub>NMR-C and lower HDL<sub>5</sub>NMR-C, HDL<sub>4</sub>NMR-C, HDL<sub>3</sub>NMR-C and integrated HDL<sub>3,4,5</sub>NMR-C and HDL<sub>4,5</sub>NMR-C) and will have higher baseline levels of CRP versus non-carriers of the G allele.
2. Carriers of the IL6 -174G allele will demonstrate less favorable changes in lipid profiles (less reduction or no change in TC, TG, LDL-C, VLDL-TG, HDL<sub>2</sub>NMR-C and integrated HDL<sub>1,2</sub>NMR-C and less of an increase or no change in HDL<sub>5</sub>NMR-C, HDL<sub>4</sub>NMR-C, HDL<sub>3</sub>NMR-C and integrated HDL<sub>3,4,5</sub>NMR-C and HDL<sub>4,5</sub>NMR-C) with exercise training as compared to non-carriers of the G allele.



## METHODS

### Subjects

Sixty-five healthy sedentary men and women were recruited for this study from the College Park, MD and Washington, D.C. metropolitan area. Subjects underwent a telephone screening to determine their eligibility and interest. The University of Maryland College Park Institutional Review Board approved the study, and written informed consent was obtained on all subjects. All procedures were followed in accordance with institutional guidelines. Eligibility for the study required subjects to be healthy, sedentary (regular aerobic exercise  $\leq 2$  times/wk and  $< 20$  min/session, sedentary occupation), 50-75 years old, not on lipid- or glucose-lowering medications, normotensive or hypertension controlled (BP  $< 160/99$  mmHg) with medications not affecting lipid metabolism (no thiazides,  $\alpha$ - or  $\beta$ -blockers, etc), non-diabetic, non-smoking, no history of CV disease, body mass index (BMI)  $< 37$  kg/m<sup>2</sup>, and not have any other medical conditions that would preclude vigorous exercise. All female participants were required to be  $> 2$  years past menopause and agree to maintain their hormone replacement therapy (HRT) status (on or not on HRT) constant for the duration of the study.

### Screening

Screening Visit 1: Medical histories were reviewed and BMI confirmed to be  $< 37$  kg/m<sup>2</sup>. A fasted blood sample was drawn for genotyping and plasma lipoprotein-lipid profile analysis. Subjects had to have  $\geq$  one National Cholesterol Education Program lipid abnormality (cholesterol  $> 200$  mg/dL, HDL-C  $< 40$  mg/dL, TG  $> 200$  mg/dL); total cholesterol and low density lipoprotein cholesterol (LDL-C)

must have been < 90<sup>th</sup> and HDL-C > 20<sup>th</sup> percentile for their age and gender to exclude familial hypercholesterolemia. Subjects had DNA isolated from peripheral monocytes<sup>134</sup> and the DNA typed at the IL6 –174G/C locus (see Genotyping).

Screening Visit 2: Each subject had fasting blood samples drawn and underwent a two-hour 75-g oral glucose tolerance test to assess diabetes status.<sup>2</sup> For study inclusion, subjects had to have fasting glucose levels < 126 mg/dL and 2-hour glucose levels < 200 mg/dL.

Screening Visit 3: Subjects qualified to this point underwent a physical and cardiovascular (CV) examination by a physician to detect CV, pulmonary, or other chronic diseases that would preclude exercise testing or training.<sup>6</sup> They underwent a Bruce maximal treadmill exercise test to ensure they had no evidence of overt CV disease.<sup>22</sup> Blood pressure, heart rate, and ECG were recorded before the test, at the end of every exercise stage, and every other minute for 6 minutes after exercise. The test was terminated when the subject could no longer continue or CV signs or symptoms occurred.<sup>6</sup> Subjects had to have <2 mV ST-segment depression and no CV signs or symptoms during this test to be included in the study.<sup>6</sup>

### **Dietary Stabilization**

All subjects underwent six weeks of dietary instruction (twice a week, one hour per session, for a total of 12 hours) with a registered dietician on the American Heart Association Step 1 diet.<sup>111</sup> Subjects were required to follow this prescribed diet and be weight stable for > 3 weeks prior to undergoing baseline testing.

## Baseline Testing

To analyze conventional plasma lipoprotein lipid levels, subjects had fasted samples drawn on two separate occasions, with the values averaged. If these measures differed by > 10%, a third separate measurement was included in the average. Plasma TG and cholesterol levels were analyzed in a Centers for Disease Control (CDC) certified lab using a CDC standardized Hitachi 717 autoanalyzer.<sup>4,211</sup> HDL-C was measured after precipitation with dextran sulfate<sup>245</sup>, and LDL-C was calculated using the Friedewald equation.<sup>55</sup> HDL<sub>2</sub>-C and HDL<sub>3</sub>-C were separated using a second high-molecular weight dextran sulfate precipitation with HDL<sub>3</sub>-C measured and HDL<sub>2</sub>-C calculated.<sup>58</sup>

Nuclear magnetic resonance (NMR) techniques (LipoScience, Raleigh, NC) also were used to determine plasma lipoprotein lipids, using techniques previously standardized and validated against conventional methods of separation and analysis.<sup>159,190,192</sup> NMR measures were made on a single blood sample that was frozen at -80° C. Baseline and final test samples were analyzed at the same time. Rough equivalence of NMR and conventional HDL-C, LDL-C, and VLDL TG measures are as follows: HDL<sub>1NMR</sub>-C ~ HDL<sub>3c</sub>-C; HDL<sub>2NMR</sub>-C ~ HDL<sub>3b</sub>-C; HDL<sub>3NMR</sub>-C ~ HDL<sub>3a</sub>-C; HDL<sub>4NMR</sub>-C ~ HDL<sub>2a</sub>-C; and HDL<sub>5NMR</sub>-C ~ HDL<sub>2b</sub>-C; LDL<sub>3NMR</sub>-C ~ LDLI, LDLIIa, and LDLIIb; LDL<sub>2NMR</sub>-C ~ LDLIIIa and LDLIIIb; LDL<sub>1NMR</sub>-C ~ LDLIVa and LDLIVb; V<sub>5</sub>, V<sub>6</sub> ~ large VLDL; V<sub>3</sub>, V<sub>4</sub> ~ intermediate VLDL; and V<sub>1</sub>, V<sub>2</sub> ~ small VLDL.<sup>95,155-158,247</sup> Coefficients of variation for standard lipid panel variables are < 3%, for VLDL, LDL, and HDL particle concentrations are

$\leq 4\%$ , and for individual subclasses contributing to total VLDL, LDL, and HDL are  $< 10\%$ .<sup>95,155-158,247</sup>

Similar to lipid analysis, the CRP blood sampling required subjects to have had no alcohol for the previous 24 hours, no exercise for 24 – 36 hours, and no infections the previous week. CRP was measured by enzyme-linked immunosorbent assay system (CRP measured at minimum detectable level of 0.35 ng/ml and inter-assay coefficient of variation of 3-7%)(Alpha Diagnostic International, San Antonio, TX). Blood samples were drawn, processed, and frozen at  $-80^{\circ}\text{C}$  until assayed. Baseline and final samples were analyzed in the same assay run in order to prevent any inter-assay variation.

Body composition was analyzed using dual-energy x-ray absorptiometry (DPX-L; Lunar Corp, Madison, WI).<sup>142</sup> All subjects underwent a second maximal treadmill exercise test to assess  $\text{VO}_2\text{max}$  as an index of CV fitness.<sup>31</sup> This test started at 70% of the peak heart rate achieved on the subject's screening exercise test and treadmill grade was increased by 2% every 2 minutes.<sup>31</sup> Blood pressure, heart rate, and ECG was monitored and the test terminated when the subject could no longer continue.  $\text{VO}_2$  was measured continuously and directly using a customized metabolic system (Marquette Respiratory Mass Spectrometer, Rayfield Mixing Chamber, VMM Ventilatory Turbine) throughout the test and standard criteria were used to determine if a true  $\text{VO}_2\text{max}$  had been achieved (no further increase in oxygen uptake with increased work rate ( $<150\text{ ml/min}$ ), exceeding age-predicted maximal heart rate, or achieving a respiratory exchange ratio of greater than 1.15).<sup>6,208</sup>  $\text{VO}_2\text{max}$  was

measured to derive valid exercise prescriptions for the exercise training intervention and to quantify generalized CV training adaptations.

## Genotyping

### *IL6 –174G/C Promoter Polymorphism*

High molecular weight DNA was isolated from peripheral monocytes<sup>134</sup> and genotyped for the –174 G to C promoter polymorphism of the IL6 gene (genotype groups: G/G, G/C, or C/C) using fluorescence polarization (FP).<sup>204</sup>

### *Haplotype Information*

Several polymorphisms have been identified in the 5' flanking region of the IL6 gene that have been shown to be in at least partial linkage disequilibrium and to affect IL6 gene transcription and IL-6 protein production.<sup>23,51-53,57,98,146,225,232,242</sup>

These polymorphisms have been studied in relation to CVD and include the –597G/A, –572 G/C, –373A<sub>n</sub>T<sub>n</sub>, and –174G/C promoter polymorphisms.<sup>23,51-</sup>

<sup>53,57,98,146,225,232,242</sup> Of these, the associations among the three biallelic polymorphisms have been most studied (–597G/A, –572 G/C, and –174G/C) with the –373 repeat polymorphism being less studied.<sup>23,51-53,57,98,146,225,232,242</sup> Based on previous work,

estimates of the frequency of the most common haplotype groups are as follows<sup>57,232</sup>:

<b>Haplotype:</b>	<b>-597</b>	<b>-572</b>	<b>-174</b>	<b>GGG</b>	<b>AGC</b>	<b>GCG</b>	<b>GGC</b>	<b>AGG</b>
Frequency (%)				54	40	5	0.6	0.3

Thus, knowing the –174G/C genotype allows for the estimation of 94% of the haplotype combinations for these three loci (GGG 54%; AGC 40%). With only 65

subjects, typing of additional loci would only identify 4 additional subjects beyond the 61 identified by the -174G/C site.

Additionally, these three biallelic loci have been shown to be in very strong linkage disequilibrium (LD) with each other (-597 and -572:  $D = -1$ ; -174 and -597:  $D = +0.99$ ; -174 and -572:  $D = -1.00$ ;  $p < 0.001$ ).<sup>57</sup> This strong LD further supports the use of the -174 loci as a marker for the most frequent haplotypes in which the typing of the additional SNPs would provide no additional information beyond that provided by the -174 locus.

The estimated haplotype frequencies for all four of the polymorphisms are as follows<sup>232</sup>:

**Haplotype Frequency at Sites -597, -572, -373A<sub>n</sub>T<sub>n</sub>, -174G/C:**

AG8/12C (36%)	GG10/11G (24%)	GG9/11G(18%)	GG10/10G(12%)
GC10/10G(6%)	AG8/12G(1%)	GG10/11G(1%)	GG9/11C(1%)

The -373 A<sub>n</sub>T<sub>n</sub> run polymorphism has been shown to be in strong linkage disequilibrium with the -174G/C polymorphism<sup>236</sup> and associated with the biallelic haplotypes as follows: the A8T12 polymorphism with the AGC haplotype, the A10T10 with the GCG haplotype, and the A9T11 with the GGC haplotype.<sup>23,53,232</sup> However, since the effect of the -373 A<sub>n</sub>T<sub>n</sub> polymorphism on IL6 gene expression and transcription appears to be cell type specific, its influence is not yet completely understood, and all experiments have been conducted in vitro, no conclusions regarding its influence on human IL6 gene expression can be drawn from the literature and suggest that genotyping this site would provide no additional information to this study.<sup>23,53,232</sup>

The present investigation will study only the –174G/C promoter polymorphism based on 1) previous work supporting the allelic association among the 5' flanking region polymorphisms and providing estimates for 94% of the haplotypes at the –597G/A, –572G/C, and –174G/C loci, 2) results suggesting the –174G/C polymorphism as a marker for the most frequent of these haplotypes, and 3) evidence showing associations between the –174G/C polymorphism and specific health phenotypes.<sup>44,46,50-53,57,138,146,232,236,243</sup> The genotyping and analysis of the additional SNPs would provide no additional information above that found from analyzing the –174 G/C site alone, particularly in light of this study having such a modest sample size.<sup>51,52,57,57,232</sup>

### **Exercise Training**

Subjects underwent 24 weeks of supervised endurance exercise training consisting of 3 sessions/wk. Heart rate monitors were used to assess training intensity and ensure that subjects trained at a heart rate corresponding to the appropriate intensity (% of  $\text{VO}_{2\text{max}}$ ). Training began with 20 minutes at 50%  $\text{VO}_{2\text{max}}$  and progressed to 70%  $\text{VO}_{2\text{max}}$  for 40 minutes where it remained for the final 14 weeks. Subjects added a weekend lower intensity 45-60 minute exercise session during weeks 12-24. Inclusion in the final analyses required subjects to have completed  $\geq$  75% of training sessions. To ensure dietary compliance, subjects completed diet records periodically throughout the exercise training.

## **Final Testing**

After completing exercise training, all subjects completed the same tests as at baseline. All blood samples were drawn 24-36 hours after a usual exercise training session.

## **Statistical Procedures**

Data are presented as adjusted mean  $\pm$  SE. Chi square tests were used to determine gender and hormone replacement therapy (HRT) status frequency differences among genotype groups and to determine if genotype frequencies deviated from the expected Hardy-Weinberg equilibrium. Baseline subject characteristics were compared between the IL6 GG, GC, and CC genotype groups using analysis of variance (ANOVA). Where significant differences existed among groups, post-hoc tests were performed. Analysis of covariance (ANCOVA) was used to compare baseline plasma lipoprotein-lipids and CRP levels among genotype groups with age, gender, HRT status, and body fat percentage as covariates. ANCOVA was used to compare plasma lipoprotein-lipid and CRP level changes with exercise training among genotype groups with age, gender, HRT status, change in body fat, and baseline value of the outcome variable as covariates. Change with exercise training was calculated as the final outcome measure value minus the baseline outcome measure value (F-B). Paired t-tests were used to analyze whole group and within genotype group changes in outcome measures with exercise training. Sample size varied according to outcome measure as complete data were not available for all subjects. The sample size for the entire group was 65 for conventional lipids and 52 for NMR plasma lipoprotein-lipid measures. Sample size



by genotype group was as follows: GG genotype group was 20 for conventional lipids and 17 for NMR measured lipids; GC genotype group was 30 for conventional lipids and 24 for NMR measured lipids; CC genotype group was 15 for conventional lipids and 11 for NMR measured lipids. For CRP measures, sample size for the entire group was 29 for baseline and change with training measures. By genotype group, sample size for baseline and change with training values was 10 for the GG group, 12 for the GC group, and 7 for the CC group.

With recent evidence suggesting that the current methods available for adjusting family-wise alpha error rate in gene association studies involving multiple phenotypes do not take into account the correlation among variables or allow only for conclusions to be made about multivariate composites of variables rather than specific variables, no adjustment of the p-value per test was made.<sup>5</sup> Some protection against the relevance of family-wise alpha error rate is provided by the fact that the study hypotheses were made *a priori* based on specific functional relationships suggested between IL-6 and baseline blood lipids and blood lipid response to exercise training.<sup>32,46,74,105,106</sup> Additionally, no multiple comparison tests were performed among genotype groups unless a significant genotype effect was found in the ANCOVA model, thus providing a protected test.

A developing argument among researchers looking at gene\*environment interactions involves the handling of potential confounding variables in the statistical analysis of the data.<sup>10,229,230,244</sup> Many studies adjust for potential confounding factors with known biological influence on the gene product and/or outcome measure by including them in a statistical model as covariates without investigating whether a

significant gene\*covariate interaction exists. Another approach supports the inclusion of covariates only if a significant gene\*covariate interaction exists for the outcome variable. Analyses in the present study included covariates in the ANCOVA based on known biological influence of those covariates on both the gene product and the outcome measures.<sup>18,24,39,54,63,100,140,147,173,178,190,227</sup> However, to explore the argument of only including covariates in a model when there is a significant gene\*covariate interaction, a modified general linear backwards elimination model was used to test for significant interactions between IL6 genotype and each covariate included in the main ANCOVA analysis. This analysis was performed for a representative group of the outcome variables (TC, TG, LDL-C, VLDL<sub>6</sub>, HDL-C, HDL<sub>2</sub>-C, HDL<sub>3</sub>-C, HDL<sub>5NMR</sub>-C, HDL<sub>4NMR</sub>-C, and integrated HDL<sub>4,5NMR</sub>-C). The full model included all covariates, genotype, and all covariate\*genotype interactions. The model was reduced by eliminating the least significant interaction term. All covariates and the IL6 genotype term remained in the model as they were not eligible for removal. The procedure was stopped when the significance level of all remaining interaction terms was greater than  $p=0.05$ . If a significant gene\*covariate interaction term remained in the model, it was reported. For a covariate that was a continuous variable, regression analysis was used to plot the relationship between the covariate and outcome variable by genotype at the mean values of the remaining covariates. For a covariate that was a categorical variable, the least squares means for the genotype were reported (adjusted for any unequal replication in the categorical variable).

Data analysis was limited to Caucasians due to the relatively small number of African American participants and their reported rare allele frequency of 0.08.<sup>94</sup> Logarithmic transformation was performed on the change in HDL<sub>5NMR</sub>-C due to variance inequalities. Statistical significance was set at  $p \leq 0.05$ . Statistical procedures were analyzed using SPSS 11.0.1 (SPSS, Inc., Chicago, IL) and SAS 8e (Carey, NC) software.

## RESULTS

IL6 allele and genotype frequencies did not differ significantly from Hardy-Weinberg expectations (Table 1) and were similar to those reported recently.<sup>23,53,94</sup> Age, number of women and men, and percentage of women on HRT did not differ among genotype groups (Table 2).

Baseline age, body weight, total body fat, and  $VO_{2max}$  did not differ among IL6 genotype groups (Table 2). No significant differences existed among genotype groups for baseline CRP levels, conventional lipid measures, or NMR-measured lipids (Tables 3-8). However, while CRP levels were not significantly different among genotype groups ( $p=0.4$ ), the G allele carriers showed higher CRP levels (GG =  $2.8 \pm 0.7$  mg/L and GC =  $2.9 \pm 0.7$  mg/L) and noncarriers of the G allele lower CRP levels (CC =  $1.5 \pm 0.9$  mg/L) (Table 3).

With exercise training, total body fat decreased and  $VO_{2max}$  increased similarly among genotype groups (Table 2). Within group weight change with exercise training was not significant for the CC group ( $-0.1 \pm 0.7$  kg) but was significant for the GG ( $-2.0 \pm 0.5$  kg,  $p<0.001$ ) and GC ( $-0.9 \pm 0.4$  kg,  $p=0.03$ ) groups. Among genotype groups, the CC group reduction in body weight was significantly less than the GG group ( $p=0.05$ ), but the GC group reduction was not significantly different from either the GG or the CC groups. Significant differences existed among genotype groups for change with exercise training in HDL-C, HDL<sub>3</sub>-C, HDL<sub>5NMR</sub>-C, HDL<sub>4NMR</sub>-C, integrated HDL<sub>4,5NMR</sub>-C, and HDL<sub>size</sub> (Tables 4,5, and 9). For HDL-C, the CC group increased significantly more than both the GG ( $7.0 \pm 1.3$  v.  $1.0 \pm 1.1$  mg/dL,  $p=0.001$ ) and the GC group ( $3.3 \pm 0.9$  mg/dL,  $p=0.02$ ). For

HDL<sub>3</sub>-C, the CC group increased significantly more than both the GG ( $6.1 \pm 1.0$  v.  $0.9 \pm 0.9$  mg/dL,  $p<0.001$ ) and the GC group ( $2.5 \pm 0.7$  mg/dL,  $p=0.006$ ). For HDL<sub>5NMR</sub>-C, the change with training for the CC group was significantly greater than the GC group ( $3.4 \pm 1.0$  v.  $0.0 \pm 0.7$  mg/dL,  $p=0.02$ ). The GG group also had a significantly greater increase in HDL<sub>5NMR</sub>-C than the GC group ( $1.4 \pm 0.8$  v.  $0.0 \pm 0.7$  mg/dL,  $p=0.04$ ) (Table 9). For HDL<sub>4NMR</sub>-C, the CC group changed significantly differently from the GG group with the CC group increasing and the GG group decreasing HDL<sub>4NMR</sub>-C ( $3.2 \pm 1.3$  v.  $-0.4 \pm 1.1$  mg/dL,  $p=0.05$ ). The GC group also differed significantly from the GG group with the GC group increasing HDL<sub>4NMR</sub>-C ( $3.1 \pm 0.9$  v.  $-0.4 \pm 1.1$  mg/dL,  $p=0.05$ ) (Table 9). For integrated HDL<sub>4,5NMR</sub>-C, the CC group increased significantly more than the GG group ( $6.5 \pm 1.6$  v.  $1.0 \pm 1.3$  mg/dL,  $p=0.01$ ) (Table 9). For HDL<sub>size</sub>, the CC group increased significantly more than both the GG ( $0.3 \pm 0.1$  v.  $0.1 \pm 0.1$  mg/dL,  $p=0.02$ ) and GC groups ( $0.0 \pm 0.0$  mg/dL,  $p=0.007$ ) (Table 9).

No significant differences were evident among genotype groups for change with exercise training in CRP levels (Table 3), in TC, LDL-C, TG, or HDL<sub>2</sub>-C measures (Table 4), or change in various lipoprotein lipid subfractions (intermediate-density lipoproteins (IDL), LDL<sub>1-3</sub>-C, VLDL<sub>1-6</sub>-TG, HDL<sub>3NMR</sub>-C, HDL<sub>2NMR</sub>-C, HDL<sub>345NMR</sub>-C, HDL<sub>12NMR</sub>-C, VLDL<sub>size</sub>, LDL<sub>size</sub>, and LDL<sub>[particle]</sub>) (Tables 5-8).

The gene\*covariate interaction analysis resulted in two significant interactions for one outcome measure. The outcome was change in integrated HDL<sub>4,5NMR</sub>-C, and the interactions were genotype\*gender-HRT status ( $p=0.03$ ) (Figure 1) and genotype\*change in body fat (%) ( $p=0.01$ ) (Figure 2). Plot estimates were based on

least squares means at AGE = 57.71154, change in body fat (%) = -1.18269, and  
baseline HDL<sub>4,5NMR</sub>-C = 15.64038.

Table 1. IL-6 –174 G/C promoter polymorphism allele and genotype frequencies

-174 G/C	Allele		Genotype Frequency			$\chi$
	G	C	GG	GC	CC	
Total Study	0.54	0.46	0.31 (20)	0.46 (30)	0.23 (15)	$\chi=.33$ p=.56
CRP Sample	G	C	GG	GC	CC	
	0.55	0.45	0.34 (10)	0.41 (12)	0.24 (7)	$\chi=.77$ p=.38
NMR Lipid Sample	G	C	GG	GC	CC	
	0.56	0.44	0.33 (17)	0.46 (24)	0.21 (11)	$\chi=.22$ p=.64

Numbers in parentheses are actual sample sizes (n).

Table 2. Subject characteristics: baseline and change with exercise training

Characteristics	Total	IL6 -174G/C Genotype Group			P
		GG	GC	CC	
Age (yrs)	57.9 ± 0.7	57.9 ± 1.3	57.7 ± 1.0	58.5 ± 1.5	NS
Female/Male	41/24	11/9	20/10	10/5	NS
% Females on HRT	54% (22)	55% (6)	55% (11)	50% (5)	NS
Weight (kg)					
Baseline	80.0 ± 2.0	81.6 ± 4.3	78.7 ± 1.0	78.4 ± 3.3	NS
Change	-1.1 ± 0.3*	-2.0 ± 0.5*	-0.9 ± 0.4*	-0.1 ± 0.7†	0.05
Body fat (%)					
Baseline	37.0 ± 1.1	36.5 ± 2.1	36.7 ± 1.4	38.1 ± 2.9	NS
Change	-1.3 ± 0.2*	-2.0 ± 0.4*	-0.8 ± 0.3*	-1.4 ± 0.6*	NS
VO <sub>2</sub> max (L/min)					
Baseline	2.0 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	1.9 ± 0.1	NS
Change	0.3 ± 0.0*	0.3 ± 0.0*	0.3 ± 0.0*	0.2 ± 0.0*	NS
VO <sub>2</sub> max (mL/kg/min)					
Baseline	25.0 ± 0.6	25.4 ± 1.3	25.2 ± 0.7	24.1 ± 1.3	NS
Change	3.9 ± 0.3*	4.9 ± 0.7*	3.5 ± 0.5*	3.2 ± 0.4*	NS

Values are expressed as adjusted means ± SE except for frequency data.

Sample size: total =65; GG=20; GC=30; CC=15.

\* Indicates significant change within group after training p<0.01.

P = statistical probability for the difference among genotype groups.

† = CC significantly different from GG.

Change = Change with exercise training (F-B).



Table 3. CRP measures: baseline and change with exercise training

	Total	IL6 Genotype Group			P
		GG	GC	CC	
CRP (mg/L)					
Baseline	2.4 ± 0.4 (n=29)	2.8 ± 0.7 (n=10)	2.9 ± 0.7 (n=12)	1.5 ± 0.9 (n=7)	0.43
Change	-0.1 ± 0.3 (n=28)	-0.2 ± 0.5 (n=10)	-0.4 ± 0.4 (n=12)	0.2 ± 0.6 (n=6)	0.73

Values are expressed as adjusted means ± SE.

\* Indicates significant change within group after training  $p < 0.05$ .

P = statistical probability for the difference among genotype groups.

Change = Change with exercise training (F-B).

Table 4. Conventional lipids: baseline and change with exercise training

	Total	IL6 Genotype Group			
		GG	GC	CC	P
Total-C					
Baseline	211.1 ± 4.4	207.9 ± 7.6	209.6 ± 6.2	215.9 ± 8.8	NS
Change	-0.6 ± 2.5	-7.7 ± 4.4	- 1.1 ± 3.6	6.9 ± 5.2	NS
LDL-C					
Baseline	131.7 ± 3.7	124.6 ± 6.4	130.9 ± 5.2	139.7 ± 7.4	NS
Change	0.2 ± 2.4	-6.2 ± 4.3	2.1 ± 3.5	4.7 ± 5.1	NS
Triglycerides					
Baseline	157.0 ± 9.2	167.6 ± 16.0	153.6 ± 13.1	149.7 ± 18.5	NS
Change	-12.2 ± 4.8*	-7.0 ± 8.4	-13.7 ± 6.8*	-16.0 ± 9.8	NS
HDL-C					
Baseline	47.7 ± 1.7	49.9 ± 3.0	45.8 ± 2.4	47.4 ± 3.4	NS
Change	3.8 ± 0.6*	1.0 ± 1.1	3.3 ± 0.9*	7.0 ± 1.3*†	0.003
HDL <sub>2</sub> -C					
Baseline	5.1 ± 0.9	7.4 ± 1.5	4.1 ± 1.3	3.9 ± 1.8	NS
Change	0.9 ± 0.4*	0.0 ± 0.6	0.6 ± 0.5	2.1 ± 0.7*	NS
HDL <sub>3</sub> -C					
Baseline	42.5 ± 1.0	42.4 ± 1.8	41.9 ± 1.5	43.1 ± 2.1	NS
Change	3.2 ± 0.5*	0.9 ± 0.9	2.5 ± 0.7*	6.1 ± 1.0*†	0.001

Values are expressed as adjusted means (mg/dL) ± SE.

Sample size: total =65; GG=20; GC=30; CC=15.

\* Indicates significant change within group after training p<0.05.

P = statistical probability for differences among genotype groups.

† = CC significantly different from CG and GG (see Table 9).

Change = Change with exercise training (F-B).

Table 5. NMR measured HDL-C lipids: baseline and change with exercise training

		IL6 Genotype Group			
	Total	GG	GC	CC	P
HDL <sub>5</sub> NMR					
Baseline	5.3 ± 1.0	6.4 ± 1.6	4.0 ± 1.4	5.5 ± 2.0	NS
Change	1.6 ± 0.5*	1.4 ± 0.8	0.0 ± 0.7†	3.4 ± 1.0*	0.03
HDL <sub>4</sub> NMR					
Baseline	10.9 ± 0.9	10.7 ± 1.	9.6 ± 1.3	12.5 ± 2.0	NS
Change	2.0 ± 0.6*	-0.4 ± 1.1	3.1 ± 0.9*	3.2 ± 1.3	0.04
HDL <sub>3</sub> NMR					
Baseline	11.4 ± 1.3	12.8 ± 2.2	12.7 ± 1.8	8.6 ± 2.7	NS
Change	-0.3 ± 0.8	1.2 ± 1.3	-1.8 ± 1.1	-0.3 ± 1.6	NS
HDL <sub>2</sub> NMR					
Baseline	14.4 ± 1.0	13.9 ± 1.7	12.9 ± 1.4	16.4 ± 2.1	NS
Change	-0.0 ± 0.7	-0.9 ± 1.1	1.3 ± 0.9	-0.4 ± 1.4	NS
HDL <sub>3,4,5</sub> NMR					
Baseline	27.5 ± 2.0	29.8 ± 3.3	26.3 ± 2.8	26.5 ± 4.1	NS
Change	3.5 ± 1.2*	1.9 ± 2.0	1.1 ± 1.7	7.5 ± 2.4*	NS
HDL <sub>4,5</sub> NMR					
Baseline	16.2 ± 1.6	17.0 ± 2.7	13.6 ± 2.3	18.0 ± 3.4	NS
Change	3.6 ± 0.8*	1.0 ± 1.3	3.3 ± 1.1*	6.5 ± 1.6*	0.04
HDL <sub>1,2</sub> NMR					
Baseline	15.9 ± 1.0	15.3 ± 1.6	14.2 ± 1.4	18.1 ± 2.0	NS
Change	0.0 ± 0.6	-0.5 ± 1.1	0.9 ± 0.9	-0.4 ± 1.4	NS

Values are expressed as adjusted means (mg /dL) ± SE.

Sample size: total =52; GG=17; GC=24;CC =11.

\* Indicates significant change within group after training p<0.05.

P = statistical probability for the difference among genotype groups.

†= GC significantly different from CC and from GG.

Change = Change with exercise training (F-B).

Table 6. NMR measured LDL-C lipids: baseline and change with exercise training

		IL6 Genotype Group			
	Total	GG	GC	CC	P
IDL					
Baseline	1.9 ± 0.7	3.1 ± 1.2	1.8 ± 1.0	0.7 ± 1.5	NS
Change	-0.9 ± 0.4	-0.1 ± 0.6	-1.2 ± 0.6	-1.7 ± 0.9	NS
LDL <sub>3</sub>					
Baseline	52.7 ± 6.7	48.1 ± 11.2	45.9 ± 9.4	64.2 ± 13.8	NS
Change	10.6 ± 4.1*	-1.8 ± 7.0	13.1 ± 5.8*	20.5 ± 8.6	NS
LDL <sub>2</sub>					
Baseline	36.4 ± 5.4	30.3 ± 9.1	38.3 ± 7.6	40.6 ± 11.2	NS
Change	-3.5 ± 4.3	-7.3 ± 7.3	-0.7 ± 6.1	-2.5 ± 8.9	NS
LDL <sub>1</sub>					
Baseline	42.5 ± 6.6	49.4 ± 11.1	41.5 ± 9.3	36.4 ± 13.7	NS
Change	-7.4 ± 5.1	1.8 ± 8.8	-7.1 ± 7.3	-16.9 ± 10.6	NS

Values are expressed as adjusted means (mg/dL) ± SE.

Sample size: total =52; GG=17; GC=24;CC=11.

\* Indicates significant change within group after training p<0.05.

P = statistical probability for difference among genotype groups.

Change = Change with exercise training (F-B).

Table 7. NMR measured VLDL-TG: baseline and change with exercise training

		IL6 Genotype Group			
	Total	GG	GC	CC	P
VLDL <sub>6</sub> NMR					
Baseline	10.3 ± 4.2	13.5 ± 7.1	11.3 ± 6.0	6.2 ± 8.8	NS
Change	-5.5 ± 1.9	1.3 ± 3.2	-7.9 ± 2.6	-9.9 ± 3.9	NS
VLDL <sub>5</sub> NMR					
Baseline	30.2 ± 4.7	37.6 ± 8.0	30.8 ± 6.7	22.1 ± 9.8	NS
Change	-3.0 ± 4.0	12.3 ± 7.0	-5.2 ± 5.7	-16.0 ± 8.4	NS
VLDL <sub>4</sub> NMR					
Baseline	43.0 ± 3.5	44.2 ± 5.8	45.2 ± 4.9	39.7 ± 7.2	NS
Change	-6.5 ± 2.5*	-7.5 ± 4.3	-3.2 ± 3.6	-8.8 ± 5.2	NS
VLDL <sub>3</sub> NMR					
Baseline	2.6 ± 0.8	2.3 ± 1.3	2.4 ± 1.2	3.2 ± 1.6	NS
Change	1.1 ± 1.1	1.8 ± 2.0	-0.2 ± 1.6	1.7 ± 2.4	NS
VLDL <sub>2</sub> NMR					
Baseline	13.0 ± 1.9	9.8 ± 3.2	17.5 ± 2.7	11.8 ± 3.9	NS
Change	-0.3 ± 1.7	-3.9 ± 3.0	2.2 ± 2.5	2.0 ± 3.6	NS
VLDL <sub>1</sub> NMR					
Baseline	15.1 ± 2.6	14.7 ± 4.3	10.7 ± 3.6	20.0 ± 5.3	NS
Change	-1.2 ± 1.9	0.8 ± 3.2	-1.3 ± 2.7	-3.1 ± 4.0	NS

Values are expressed as adjusted means (mg/dL) ± SE.

Sample size: total =52; GG=17; GC=24;CC=11.

\* Indicates significant change within group after training p<0.05.

P = statistical probability for difference among genotype groups.

Change = Change with exercise training (F-B).

Table 8. NMR measured particle size: baseline and change with exercise training

		IL6 Genotype Group			
	Total	GG	GC	CC	P
VLDL <sub>size</sub>					
Baseline	48.7 ± 1.6	50.0 ± 2.7	50. ± 2.3	45.5 ± 3.3	NS
Change	-2.0 ± 1.3	-1.4 ± 1.9	-1.4 ± 1.9	-5.7 ± 2.8	NS
LDL <sub>size</sub>					
Baseline	20.6 ± 0.1	20.5 ± 0.2	20.5 ± 0.2	20.9 ± 0.2	NS
Change	0.2 ± 0.8*	-0.0 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	NS
HDL <sub>size</sub>					
Baseline	8.7 ± 0.1	8.8 ± 0.1	8.7 ± 0.1	8.7 ± 0.1	NS
Change	0.1 ± 0.0*	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.1*†	0.02
LDL <sub>[particle]</sub>					
Baseline	1570 ± 71	1572 ± 119	1518 ± 100	1620 ± 147	NS
Change	-63 ± 43	-62 ± 73	8 ± 61	-135 ± 89	NS

Values are expressed as adjusted means (nm) ± SE.

Sample size: total =52; GG=17; GC=24;CC=11.

\* Indicates significant change within group after training p<0.05.

P = statistical probability for difference among genotype groups.

† = CC significantly different from CG and GG.

Change = Change with exercise training (F-B).

Table 9. Significant differences in HDL-C and HDL-C subfraction changes with exercise training among genotype groups

		IL-6 Genotype Group			
		GG	GC	CC	P
HDL-C					
	CC v. GC		3.3 ± 0.9*	7.0 ± 1.3*	0.02
	CC v. GG	1.0 ± 1.1		7.0 ± 1.3*	0.001
HDL <sub>3</sub> -C					
	CC v. GC		2.5 ± 0.7*	6.1 ± 1.0*	0.006
	CC v. GG	0.9 ± 0.9		6.1 ± 1.0*	<0.001
HDL <sub>5</sub> NMR					
	GC v. CC		0.0 ± 0.7	3.4 ± 1.0*	0.02
	GC v. GG	1.4 ± 0.8	0.0 ± 0.7		0.04
HDL <sub>4</sub> NMR					
	GG v. CC	-0.4 ± 1.1		3.2 ± 1.3	0.05
	GG v. GC	-0.4 ± 1.1	3.1 ± 0.9*		0.02
HDL <sub>4,5</sub> NMR					
	CC v. GG	1.0 ± 1.3		6.5 ± 1.6*	0.01
HDL <sub>size</sub>					
	CC v. GG	0.1 ± 0.1		0.3 ± 0.1*	0.02
	CC v. GC		0.0 ± 0.0	0.3 ± 0.1*	0.007

Values are expressed as adjusted means (mg/dL or nm) ± SE.

\* Indicates significant change within group after training p<0.05.

P = statistical probability for difference among genotype groups.

Change = Change with exercise training (F-B).

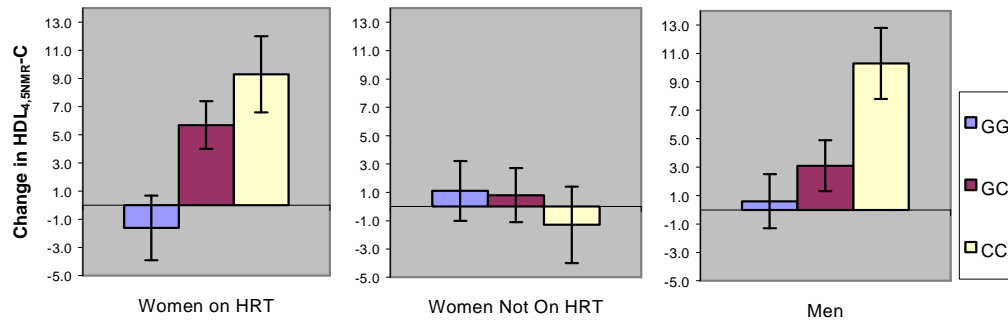


Figure 1. Gender-Hormone Replacement Therapy\*IL6 Genotype Interaction For Exercise Training-Induced Change In HDL<sub>4,5</sub>NMR-C (mg/dL) (p=0.03)

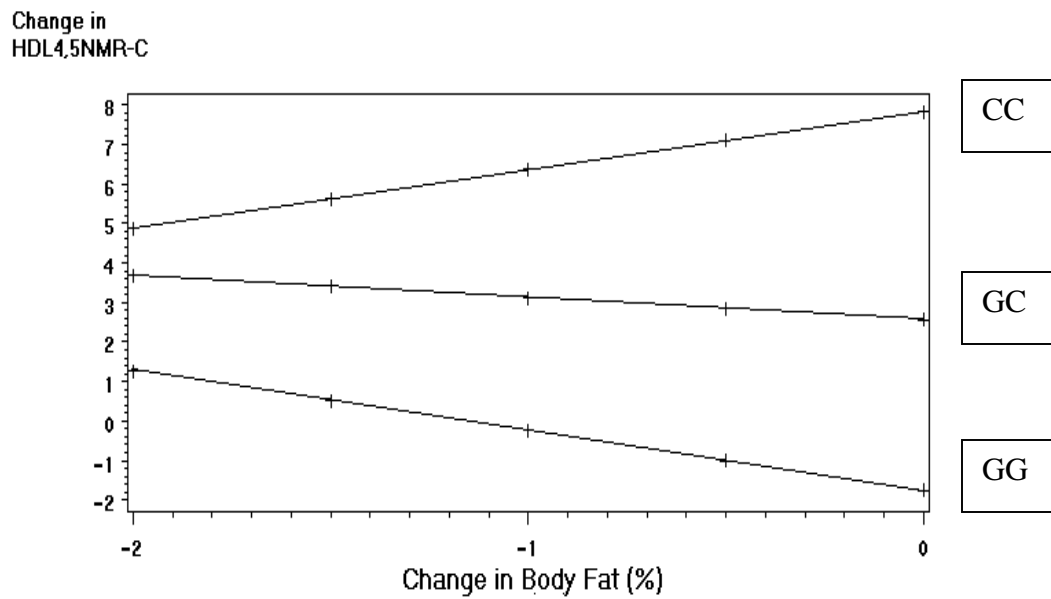


Figure 2. Change In Body Fat\*IL6 Genotype Interaction For Exercise Training-Induced Change In HDL<sub>4,5</sub>NMR-C (mg/dL) (p=0.01)



## DISCUSSION

Plasma lipoprotein lipid levels are independent risk factors for CV disease.<sup>137,140,233</sup> They are also a component of the innate inflammatory response thought to contribute to creating a hostile environment to fight infectious agents and/or provide substrates for energy metabolism, especially during times of starvation.<sup>47,49,54,68,105,106,131,144</sup> Mediating the inflammatory response, the pro-inflammatory cytokine IL-6 has also been independently associated with CV morbidity and mortality, as well as with variation in plasma lipoprotein lipid levels.<sup>9,46,77,126,133,187,188,226</sup> Additionally, both lipids and IL-6 levels are highly variable among individuals, modifiable, and influenced by genetics, environmental factors, and the interaction among genetic and environmental factors.<sup>21,46,53,57,108,174,181,213,232,252</sup> Thus, understanding these genetic and environmental factors is paramount to improving public health through CV disease risk reduction. Contributing to this understanding, we found an association between the IL6 –174G/C promoter polymorphism and exercise training-induced changes in HDL-C, HDL<sub>3</sub>-C, HDL<sub>5NMR</sub>, HDL<sub>4NMR</sub>-C, integrated HDL<sub>4,5NMR</sub>-C, and HDL<sub>size</sub>. These data suggest that IL6 genotype may interact with exercise training to influence changes in HDL-C and HDL-C subfractions.

IL-6 is the primary cytokine regulating the acute phase response.<sup>12,239</sup> It also directly influences other metabolic, endothelial, and coagulation factors.<sup>162,251,252</sup> Of particular interest is the role of cytokines in inducing pro-atherogenic changes in lipoproteins and lipid metabolism.<sup>105,106</sup> These changes include increased TG levels, VLDL-TG, and small dense LDL-C and decreased HDL-C levels.<sup>105,106</sup> Additionally,

enzymes and apolipoproteins associated with HDL-C and its anti-inflammatory, anti-oxidant, and reverse cholesterol transport functions undergo changes with inflammation that may inhibit the normal protective effect of HDL-C against atherosclerosis.<sup>11,105,106</sup> These changes include decreased lecithin:cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), hepatic lipase (HL), and phospholipid transfer protein (PLTP) activity, decreased HDL and apo A-I levels, and increased serum amyloid A (SAA), and apo J.<sup>105,106</sup> While this response is helpful in fighting infection and injury and providing substrates for energy metabolism, chronically elevated levels of IL-6 and subsequent metabolic effects can be detrimental.<sup>47,49,105,106,131,144,252</sup> Recent evidence shows chronic elevation of IL-6 can result from environmental factors (stress, pollution, smoking), metabolic factors (chronic infection, tissue injury, obesity), and genetic factors.<sup>252</sup> Thus, regardless of the cause (host defense or other factors), elevated IL-6 may result in deleterious alterations in lipids and lipid metabolism.

Polymorphisms in the promoter region of the IL6 gene have been associated with alterations in IL6 gene transcription and IL-6 protein levels.<sup>23,51-53,57,98,146,225,232,242</sup> Studied in relation to CV disease, these polymorphisms include the –597G/A, –572 G/C, –373A<sub>n</sub>T<sub>n</sub>, and –174G/C promoter polymorphisms.<sup>23,51-53,57,98,146,225,232,236,242</sup> Of these, the –174G/C polymorphism is the most well-studied and supported as a marker for the most frequently described haplotypes, as well as being associated with IL-6 levels and such health related phenotypes as diabetes, glucose and insulin homeostasis, hypertension, body composition, and lipid profiles.<sup>9,44,46,49,50,93,127,133,138,226,242,243</sup>

After controlling for a number of covariates, we found no significant differences in baseline levels of conventionally measured lipids and lipid subfractions and no differences in NMR measured subfractions among IL6-174G/C genotype groups. However, though not statistically significant, the GG genotype group had the lowest TC and LDL-C levels versus the CC group, higher TG, HDL-C, and HDL<sub>2</sub>-C levels than the GC and CC groups, and lower HDL<sub>3</sub>-C than the CC group. In terms of baseline NMR measured subfractions, we found that the GG group generally had a less atherogenic lipoprotein lipid profile than the CC group. Though not statistically significant, levels of the surrogate marker CRP tended to be higher in carriers of the G allele than the CC group as expected. Although the lipid findings are not statistically significant nor can they be generalized, they could suggest a somewhat less atherogenic baseline lipid profile for the GG group compared to the CC group, a finding opposite of what we hypothesized.

Suggesting a less atherogenic baseline lipid profile for the GG genotype group, the findings in this study differ from other studies that found associations between IL6 genotype and baseline fasting lipid levels.<sup>9,46,133,226</sup> For example, Fernandez Real et al.<sup>46</sup> found an association between carriers of the G allele and increased TG, VLDL-TG, and apo B, decreased HDL<sub>2</sub>-C, and a tendency toward higher IL-6 levels. In epidemiological studies, Mendall et al.<sup>133</sup> found that in men with various CV disease risk factors, higher IL-6 levels were associated with increased plasma TG concentrations. Baggio et al.<sup>9</sup> found that higher IL-6 levels in healthy centenarians were associated with relatively high plasma TG and low HDL-C. Thus, these studies suggest that individuals prone to higher IL-6 production are more

likely to develop higher TG, VLDL-TG, plasma free fatty acids (FFA), and lower HDL<sub>2</sub>-C than those with lower IL-6 levels. With the G allele associated with higher IL-6 levels, carriers of the G allele would appear more susceptible to lipid abnormalities and thus a more pro-atherogenic lipid profile.<sup>46</sup> Using CRP as a surrogate measure of IL6 levels, this investigation found a tendency for the G allele carriers to have non-significantly higher baseline CRP levels compared to the CC homozygotes. Additionally, the G allele carriers tended to have higher, though not significantly different, levels of TG, VLDL<sub>6NMR</sub>, VLDL<sub>5NMR</sub>, and VLDL<sub>4NMR</sub> than the CC homozygotes. Though not statistically significant, the results are of interest as they suggest possible support for the association of higher IL6 levels and somewhat more atherogenic TG related lipid measures in carriers of the G allele as compared to the CC homozygotes. In contrast, while the TG related measures are of interest, the overall baseline lipid profile did appear less atherogenic for the G allele carriers.

Viewed from an evolutionary perspective, the results from this study and others suggesting an association between genotype, higher IL6 levels, and lipid abnormalities are plausible.<sup>47,49,54,131,133,144,252</sup> The common G allele, associated with higher IL-6 levels, would represent an advantageous adaptation to an historic environment of infection, injury, intermittent feeding, and short life expectancy.<sup>44,47,53,113,131,144</sup> However, this advantage is lost in a Westernized society marked by little physical activity, high fat processed foods, and abundant feeding.<sup>47,131,144</sup> Why the baseline profile of the GG group in our study reflected the expected higher TG, VLDL<sub>6NMR</sub>, VLDL<sub>5NMR</sub>, VLDL<sub>4NMR</sub>, and LDL<sub>1NMR</sub>, but not a significantly different and more atherogenic lipid profile overall than the CC

genotype group, is not clear. The lack of expected findings may be due to sample size issues resulting in lack of detection of a genotype effect. It is also plausible that our initially sedentary subjects did not possess levels of IL-6 that were elevated to the point of inducing striking differences in lipid profiles among genotype groups. Additionally, with such a complex interplay among various physiological systems and environmental factors influencing lipid levels, a single gene analysis may not adequately reflect the physiology determining lipid levels and suggests that perhaps this polymorphism is in linkage disequilibrium with others in the IL6 gene that affect protein function rather than levels or with other genes that influence transcription.<sup>241</sup> Another limitation to the interpretation of these results is the lack of direct measures of IL-6 levels for analysis by genotype group and association with lipid levels. It is possible that the genotype groups in this study would not reflect higher IL-6 levels in carriers of the G allele, even though the CRP measures suggest that they would. Thus, additional studies involving additional subjects, direct measures of IL-6, and including investigation of the functional steps potentially influenced by IL-6 (lipid metabolism related enzyme activities) are needed.

Endurance exercise training is known to influence lipid levels and appears to influence IL-6 levels.<sup>17,19,20,34-38,42,69,70,73,74,84,89,91,97,101,103,114-117,149,153,154,168-172,181,193,207,221,223,234,249</sup> Genetic factors are known to interact with exercise training to influence lipid response, while the interaction of IL6 genotype with exercise training is not known in terms of its influence on IL-6 and lipid levels. Using CRP levels as surrogate indicators of IL-6 levels, no significant changes in CRP levels were found with exercise training, nor were any significant differences found among genotype

groups in terms of change in CRP with exercise training. However, the results did show that while the CC group increased and the GG group decreased, the GC group decreased plasma CRP levels the most. We did find significant differences among genotype groups in exercise training-induced changes in HDL-C, HDL<sub>3</sub>-C, HDL<sub>5NMR</sub>-C, HDL<sub>4NMR</sub>-C, integrated HDL<sub>4,5NMR</sub>-C, and HDL<sub>size</sub> in which the CC group demonstrated the greatest beneficial increases in HDL-C measures. In all cases except HDL<sub>5NMR</sub>-C, the CC group differed significantly from the GG group. For HDL-C, HDL<sub>3</sub>-C, and HDL<sub>size</sub>, the CC group also differed significantly from the GC group. For HDL<sub>5NMR</sub>-C, the CC group differed from the GC group, and the GG group differed from the GC group. With exercise training producing increases in these HDL-C measures for the CC group, the changes can be viewed as more advantageous for the less common allele. This could suggest a more advantageous gene-environment interaction for IL6 CC genotype and exercise training in improving HDL-C levels. With HDL-C being an important molecule in resisting CV disease through its anti-inflammatory, anti-coagulatory, and anti-oxidant properties, such changes in HDL would prove beneficial.

In light of the analysis of the multiple lipoprotein lipid categories and their subfractions, the consistency of direction and variables that were statistically significant provide support for the biological plausibility that the IL6 –174G/C polymorphism may be associated with exercise training-induced changes in HDL C and various HDL-C subfractions. This consistency provides support for the conclusion that the findings were not merely the result of chance due to multiple comparison testing. The significance levels for the significant findings among

genotype groups were  $\leq 0.007$  in four cases,  $\leq 0.02$  in five cases, and  $\leq 0.05$  in two cases, demonstrating that over one-third of the findings would remain statistically significant even with the adoption of an extremely conservative significance level. These findings also occurred entirely in the HDL-C category and were consistent in terms of the groups that differed and the direction in which they changed. While this consistency in significant findings in only the HDL-C outcome variables could be argued as having resulted from the correlation among those variables, the lack of significant findings in the other lipoprotein lipid categories, inter-correlated with each other and the HDL-C measures as well, suggests that, if the results were due to correlation, significant findings should have been found in the other lipoprotein categories as well. Additionally, the study hypotheses were made *a priori* based on specific functional relationships suggested between IL-6 and baseline blood lipids and blood lipid response to exercise training.<sup>5,9,32,46,74,133,226</sup> Thus, the findings are supported both biologically and statistically and demonstrate an association between the IL6-174G/C polymorphism and exercise training induced changes in HDL-C and HDL-C subfractions.

With the argument growing regarding the use of covariates in the statistical models used in gene association studies, this study included the analysis of gene\*covariate interactions by outcome measure.<sup>10,229,230,244</sup> This analysis resulted in two significant interactions (gender-HRT status\*genotype,  $p=0.03$ , and change in body fat\*genotype,  $p=0.01$ ) in one outcome measure (change in HDL<sub>4,5NMR</sub>-C). The findings suggest that for women on HRT, the GG genotype group resulted in a decrease in HDL<sub>4,5NMR</sub>-C while the GC and CC groups increased HDL<sub>4,5NMR</sub>-C. This

suggests a more advantageous response for the C allele carriers. For women not on HRT, genotype appeared less influential. For men, all genotype groups increased HDL<sub>4,5NMR</sub>-C, a favorable response. For the change in body fat percentage\*IL6 genotype interaction, the data suggest that genotype was more influential for the homozygote groups. For example, in the CC group, a greater reduction in body fat percentage resulted in a smaller increase in integrated HDL<sub>4,5NMR</sub>-C. In contrast, the prediction in the GG group is that a greater than 1% reduction in body fat percentage is required for an increase in HDL<sub>4,5NMR</sub>-C. For the heterozygotes, the change in integrated HDL<sub>4,5NMR</sub>-C was essentially the same across the range of changes in body fat percentage. Thus, these analyses demonstrate that interactions between genotype and covariate are of interest.

As little research has been done on the effect of exercise training on IL-6 levels, and no research has been conducted on the interactive effects of IL6 genotype and exercise training on IL-6 levels, any potential mechanisms that could be driving the association between HDL-C changes and IL-6 genotype are not known. While some evidence does suggest that perhaps exercise training can attenuate the inflammatory response and that it may lower IL-6 levels in heart patients and healthy older adults, additional research is needed.<sup>3,13,59,177,235</sup> Following the notion of the “thrifty gene” hypothesis, it is plausible that the less common C allele would reflect a “lower responder” in terms of IL-6 release.<sup>47,49,131,144</sup> As such, exercise induced increases may be lower as well. However, it has been shown that the exercise induced increase in IL-6 comes from contracting skeletal muscle with the majority of the secretion coming from myocytes themselves.<sup>88,224</sup> This IL-6 may be functionally



different from the immune or inflammatory induced release of IL-6 from other cells.<sup>88,170,224,238</sup> Thus, it is not known if the exercise-induced IL-6 secretion from skeletal muscle possesses the same proatherogenic functions of the inflammatory response induced IL-6 secretion. Perhaps the IL-6 secreted in response to different stimuli reflects the operation of different physiological pathways designed to exert specific effects – one primarily immunological and one primarily for energy regulation.<sup>88,170,224,238</sup> Additionally, a recent study showed that IL-6 deficient mice had less endurance capacity and energy expenditure during exercise, suggesting that IL-6 release is essential to normal exercise capacity.<sup>40</sup> This finding, while not in humans, might suggest that individuals with CC genotype, associated with lower IL-6 production, could have lower exercise capacity and perhaps have attenuated release of IL-6 with exercise. Whether this would then affect resting IL-6 levels is unclear. Nevertheless, it is plausible that the less common C allele is associated with a more positive change in HDL should the exercise training interact with the lower responding genotype to produce lower levels of IL-6 and thus produce fewer disadvantageous changes in the lipid profile. Again, if viewed in terms of the “thrifty gene hypothesis”, G allele carriers would be expected to reflect higher IL-6 production and to have a stronger response to stimulatory factors than those with a CC genotype. Hence, with exercise training, perhaps this stronger response is maintained in which G allele carriers continue to have higher levels and to reflect a less beneficial effect of the exercise training in terms of HDL-C change with training.

Thus, while the findings of this study were in contrast to other studies finding an association of the G allele with a more pro-atherogenic baseline lipid profile, the

significant differences among genotype groups in terms of HDL-C changes with exercise training are novel and provide preliminary data of interest for future investigation of the gene\*exercise training interaction in regard to IL-6 genotype. The lack of consistency in findings may lie in the multi-functional nature of the IL-6 molecule itself whereby it may be difficult to discern the effect of the IL-6 secreted in response to different stimuli. It is also plausible that the use of CRP as a surrogate marker may not adequately reflect the exercise training effect on IL-6 levels and calls for additional studies employing the direct measure of IL-6 levels.

## CONCLUSIONS

In summary, the present findings provide further evidence for the association of IL6 genotype with plasma lipoprotein lipid levels, particularly with the change in HDL-C and its subfractions with endurance exercise training. Major strengths of this study include control of dietary composition and baseline physical activity level, a highly standardized prolonged endurance exercise training program, the NMR analyses of plasma lipoprotein lipid subfractions, and the investigation of the interaction of IL6 genotype and endurance exercise training. However, the limitations of this study are the lack of direct measurement of IL-6 levels and the lack of sample size to perform haplotype analyses. While these limitations reduce generalizability, the findings in this study do provide support for additional investigation of the IL-6 promoter polymorphisms and their interaction with endurance exercise training to affect change in plasma lipoprotein lipid profiles. With evidence mounting that IL-6 plays a major role in the initiation and progression of CV disease and in energy regulation, the investigation of potential functional variants in the IL6 gene and their interaction with endurance exercise training may prove useful to clinicians and paramount to public health officials in the fight against CV disease.

## REVIEW OF THE LITERATURE

### Introduction

Cardiovascular (CV) disease is the leading cause of death in the Western world and will soon be the most critical health problem worldwide.<sup>7,137,190,192</sup> Traditionally, the disease has been characterized as a lipid disorder in which excess lipid in the bloodstream accumulates in the arterial walls, resulting in CV disease.<sup>60-62,119-123,135,190,196,197,199,200,203,252</sup> Additionally, the risk of developing CV disease has been assessed based on the evaluation of such conventional risk factors as dyslipidemia, hypertension, age, gender, smoking status, diabetes status, physical inactivity, and obesity.<sup>6,7</sup> However, recent research has focused on novel risk factors that may play a role in the development of CV disease, including factors related to the normal physiological processes involved in coagulation and fibrinolysis, endothelial function, and inflammation.<sup>60-62,118-122,124,125,165,185,185,189,190,192,251</sup> The importance of such research is highlighted by the fact that many individuals presenting with CV disease and coronary events do not have traditional risk factors.<sup>182-184,186,190-192</sup> One major line of this recent CV disease research focuses on the role of inflammation in the development of the disease.<sup>60-62,118-122,124,125,165,185,187,189,190,192,199</sup> This research advances the theory that atherosclerosis is itself an inflammatory condition in which specific molecular and cellular responses occur, including elevation of levels of the inflammatory cytokine interleukin-6 (IL-6), which combined with lipid deposition, endothelial dysfunction, and smooth muscle cell and fibrous material accumulation, result in atherogenesis.<sup>60-62,118-120,122,123,135,183,190,192,198,252</sup> Thus, current research suggests that a chronic inflammatory state, induced by some initiating factor and

characterized by elevated levels of IL-6, may provide the link between the development of the many poor health phenotypes classified as CV disease risk factors and the manifestation of the disease.<sup>60-62,118-120,122,123,183,190,192,198,252</sup> More specifically, although inconclusive, evidence supports an association between circulating levels of the inflammatory cytokine IL-6 and plasma lipoprotein lipid levels, thus providing an additional potential mechanism through which IL-6 may be associated with the development of CV disease.<sup>9,46,56,128,133,143,163,164,176,226,238,254</sup>

Fortunately, CV disease risk can be reduced through such lifestyle modifications as exercise training.<sup>165,233</sup> For example, exercise training is known to improve lipid profiles, although the response to training is highly variable and subject to environmental and genetic influences.<sup>21,35,38,85,109,110,114,115,117,141,181,195</sup> However, the interactive effects of exercise training and IL6 genotype on IL-6 levels and subsequently plasma lipoprotein lipid levels are not known and call for additional research to ultimately discern the potential role of IL-6 in CV disease development.

### **The Inflammatory Response, Lipid Metabolism, and CV Disease**

Historically, the human physiological response to environmental threats, including infection, injury, and starvation, has involved the induction of the inflammatory response and concomitant alterations in metabolism.<sup>11,45,47,49,54,65,68,76,105,106,128,131,133,144,252</sup> These physiological changes were designed to protect the body from invading organisms, facilitate repair of damaged tissue, and to help prevent further injury and included increased levels of inflammatory cytokines (interleukin-1, tumor necrosis factor- $\alpha$ , IL-6), increased

levels of positive acute-phase proteins (CRP, serum amyloid A), decreased levels of negative acute-phase proteins (albumin, transferrin), and pro-atherogenic alterations in lipid metabolism.<sup>105,106</sup> While these responses are protective and promote human survival in the short-term, the prolonged presence of these metabolic changes could be detrimental to human health.<sup>47,49,54,144,252</sup> As the major regulator of this response, IL-6 may prove to be the link between many CV disease risk factors and development of the disease itself.<sup>252</sup>

As part of the inflammatory response, lipid metabolism and concentrations of plasma lipoproteins and lipids undergo pro-atherogenic changes.<sup>76,105,106</sup> These potentially proatherogenic changes include increased TG, VLDL-TG, and small dense LDL-C levels and decreased HDL-C levels.<sup>76,105,106</sup> Additionally, enzymes and apolipoproteins associated with HDL-C and its anti-inflammatory, anti-oxidant, and reverse cholesterol transport functions undergo changes with inflammation that may inhibit the normal protective effect of HDL-C against atherosclerosis.<sup>11,76,105,106</sup> These changes include decreased LCAT, CETP, HL and PLTP activity, decreased HDL and apo A-I levels, and increased SAA and apo J.<sup>76,105,106</sup> These changes and their effects are summarized in the following table.<sup>106</sup>

**POTENTIAL CHANGES IN LIPIDS AND  
LIPID METABOLISM DURING INFECTION AND INFLAMMATION**

<b>HDL</b>	<b>Change</b>	<b>Effect</b>
HDL and apo A-I	↓	Impaired apolipoprotein-mediated cell cholesterol removal
LCAT	↓	Impaired cholesterol removal from cells by diffusion
CETP	↓	Impaired cholesterol transfer to triglyceride-rich lipoprotein
HL	↓	Reduced pre-beta HDL generation
PLTP	↓	Reduced pre-beta HDL generation; decreased HDL phospholipid content and impaired cholesterol removal by increasing cholesterol flux from HDL into cells
SAA	↑	Decreased availability of cholesterol in HDL to be metabolized by hepatocytes; increased cholesterol uptake by macrophages
sPLA2	↑	Decreased HDL phospholipid content and impaired cholesterol removal by increased cholesterol flux from HDL into cells
PAF-AH activity	↑	Increased lysophosphatidycholine
PON	↓	Decreased anti-oxidant function of HDL (-LDL oxidation)
transferrin	↓	Impaired anti-oxidant function of HDL (- LDL oxidation)
apo J	↑	Increased smooth muscle cell differentiation in arterial wall
<b>LDL</b>	<b>Change</b>	<b>Effect</b>
Small dense LDL	↑	Increased susceptibility to oxidation; increased penetration of LDL into endothelium; increased interaction with arterial wall proteoglycans and LDL retention in arterial wall
PAF-AH activity	↑	Increased lysophosphatidycholine
sPLA2	↑	Released polyunsaturated fatty acids from phospholipids that can be oxidized
Sphingolipid content	↑	Facilitated LDL aggregation and uptake by macrophages
Ceruloplasmin	↑	Increased oxidation of LDL
<b>VLDL</b>	<b>Change</b>	<b>Effect</b>
VLDL levels	↑	Increased availability of lipid substrates for macrophage uptake
LPL and HL	↓	Decreased triglyceride-rich lipoprotein clearance
Sphingolipid content	↑	Decreased triglyceride-rich lipoprotein clearance
Tissue apo E expression	↓	Decreased lipoprotein clearance

Khovidhunkit, W., Memon, R.A., Feingold, K.R., and C. Grunfeld. Infection and inflammation-induced proatherogenic changes of lipoproteins. *The Journal of Infectious Diseases*. 181(Suppl 3):S462-72, 2000.

As the major regulator of the inflammatory response, IL-6 may play a role in initiating some of these alterations in lipids and lipid metabolism.<sup>27,80,82,87,162,239</sup>

Systemically, IL-6 is the major mediator of the hepatic acute phase response and stimulates the hepatic secretion of the acute phase reactants C-reactive protein (promotes endothelial dysfunction such as secretion of vascular adhesion molecules and selectins and reduces nitric oxide and prostacyclin (PGI<sub>2</sub>)), serum amyloid A (reduces HDL), and fibrinogen (promotes coagulation).<sup>16,49,131,194,226,237,251,252</sup> Locally, IL-6 induces the expression of vascular adhesion molecules (ie, vascular cell adhesion molecule-1 (VACM-1), intercellular adhesion molecule-1 (ICAM-1)) and selectins, the reduction of nitric oxide secretion and PGI<sub>2</sub>, and the expression of monocyte recruitment factors and macrophage colony stimulating factors, all of which result in the process of inflammation, coagulation, and tissue healing.<sup>82,135,162,239,250,252</sup> It also inhibits adipose lipoprotein lipase, which is responsible for the breakdown of TG in TG-rich lipoproteins, and stimulates lipolysis, increases in FFA levels, and increased secretion of TG by the liver, all of which can lead to increased TG, LDL-C, and FFA in the blood and reduced HDL-C.<sup>66-68,143,252</sup> Thus, IL-6 is designed to promote survival when functioning appropriately.<sup>27,68</sup> However, when IL-6 levels become dysregulated and chronically elevated, these metabolic alterations may also become chronic and deleterious to survival.<sup>47,54,250,252</sup> In particular, with a potential role in mediating some of the pro-atherogenic changes in lipoproteins and lipid metabolism, either directly or indirectly through other factors which it stimulates, chronically elevated IL-6 may play a role in determining pro-atherogenic lipid profiles. With gene sequence variation being one possible mechanism for inducing elevated IL-6



levels, the potentially functional –174G/C promoter polymorphism may be associated with lipid profiles.

In Paleolithic times, individuals who exhibited a strong response to infection, inflammation, and/or starvation, were conferred a survival advantage.<sup>47,131,144</sup> Life expectancy was short, life was characterized by physical activity and intermittent feedings, and infection and injury were common.<sup>47,131,144</sup> Individuals possessing genetic variation conferring them “high responder status” would survive and carry that variation forward.<sup>47,131,144</sup> Thus, humans evolved during a hunter – gatherer lifestyle in which they face severe environmental threats, while they now live in a time of abundant food and physically inactive lifestyles.<sup>47,144</sup> This “maladaptation” of lifestyle to a once advantageous genome may be contributing to the spread of many poor health conditions.<sup>47,144</sup> In light of the known effects of inflammation on lipids and lipid metabolism, a genetic predisposition for mounting a greater defense against infection and injury (greater inflammatory response) might result in some individuals exhibiting a more atherogenic lipoprotein lipid profile as a result of possessing a “high responder genotype”.<sup>47,105,106,144</sup> With IL-6 being the major regulator of the inflammatory response, IL6 genotype may play a role in determining who is “advantaged” or “disadvantaged” in terms of plasma lipoprotein lipid levels.

### **IL6 Gene**

IL6 is a single copy gene located on the short arm of chromosome 7 (7p21) and is comprised of 5 exons and 4 introns.<sup>53,231,232</sup> Gene transcription is tightly regulated by the NFIL6, NFkB, Fos/Jun, CRBP, and glucocorticoid receptor transcription factors which have binding sites in the –180 to –123 base pair region of

the promoter.<sup>232</sup> In HeLa cells, this region has been shown to be crucial for virus, second messenger, and cytokine induction of transcription with promoter activation occurring upon the synergistic action of the NFIL6 (-158 to -145 base pair) and NFkB (-73 to -64 base pair) transcription factors.<sup>232</sup> Repression of IL6 expression by steroid hormones (estrogen and glucocorticoids) occurs by binding of the hormone receptor ligand complexes directly to the transcription factors which prevents their binding to the IL6 DNA.<sup>232</sup> Thus, the coordinated action of many factors, binding at distinct promoter sites, regulates IL6 transcription.<sup>232</sup>

Several allelic variants have been identified in the 5' flanking region that may influence IL6 transcription.<sup>23,51-53,57,98,146,225,232,242</sup> These polymorphisms include the -597G/A, -572 G/C, -174G/C, and -373A<sub>n</sub>T<sub>n</sub> polymorphisms.<sup>23,51-53,57,98,146,225,232,242</sup> However, with previous work supporting strong allelic associations among these 5' flanking region polymorphisms and giving estimates for 94% of the haplotype combinations, the literature suggests that the -174G/C promoter polymorphism can serve as a marker for the most frequent haplotypes (see Methods on haplotypes).<sup>23,51-53,57,98,146,225,232,236,242</sup> Additionally, studies have shown that the -174G/C polymorphism has plausible biological significance in terms of influencing IL6 gene transcription and expression and consequently circulating IL6 levels.<sup>51-53,232,241</sup> Positioned close to the glucocorticoid receptor binding site, the C allele of this polymorphism creates a novel binding site for the NF-1 transcription factor, a structurally related family of transcription factors active in many cell types, which could repress expression.<sup>53</sup> Supporting this, Fishman et al used 5' region constructs and showed that the IL-6 -174 C allele was associated with lower basal and

stimulated expression than the G allele in HeLa cells.<sup>53</sup> Additional studies have also provided support for a potential functional role for the IL6 –174 G/C polymorphism in influencing IL6 gene transcription and thus regulation of IL-6 levels, as well as suggesting that the C allele results in decreased promoter activity and thus lower circulating levels of IL-6.<sup>51-53,232,241</sup> However, while evidence supports the investigation of the hypothesis that the G allele is associated with higher IL-6 levels and potentially detrimental health phenotypes, other investigations using constructs of the 5' flanking region have found evidence of an opposite effect.<sup>23,25,96</sup> For example, Jones et al.<sup>96</sup> found the C allele associated with higher IL-6 levels in a study of patients with aneurismal disease. Burzotta et al.<sup>25</sup> also found higher IL-6 levels associated with the C allele. The inconsistency of findings among the in vitro studies may be due to the use of different cell lines and culture conditions, use of basal state or stimulated cells, nature of the stimulation if used, and presence of other regulatory elements in sequences that may induce or repress expression.<sup>23</sup> Findings in in vivo studies may differ due to differing sample populations. Thus, while no concrete conclusions can be drawn, these studies support further research into the potential effect of the IL6 promoter polymorphisms on gene transcription and expression and ultimately on circulating IL-6 levels and their association with health phenotypes.

### **IL-6 Protein Structure, Secretion, and Function**

As the final IL6 gene product, IL-6 is an 184 amino acid inflammatory cytokine that has a very short biphasic half-life (initial 3 minute and then second 55 minute clearance), marked diurnal variability (low in the morning and high at bedtime), and large within and between individual variation (30% and 44%,

respectively).<sup>12,26,30,33,132</sup> It is secreted from multiple cell types, including monocytes, macrophages, lymphocytes, fibroblasts, mast cells, astrocytes, microglia, osteoblasts, intestinal epithelial cells, endothelial cells, vascular smooth muscle cells and adipocytes, as well as contracting skeletal muscle.<sup>87,139,139,162,171,172,223,231,252</sup> IL-6 is a unique, pleiotropic cytokine in that it has autocrine, paracrine, and endocrine functions.<sup>80,81,83,139,162,252</sup> It exerts its wide range of actions by binding first to a low-affinity membrane bound receptor (IL6-R or gp80) that is not a signal transducer.<sup>80,81,87,252</sup> This IL-6 receptor complex then binds to the high affinity gp130 which is a signal transducer and, once the IL-6-gp80 complex binds, begins the signaling cascade and produces action.<sup>80,81,87,252</sup> The IL-6-R is active in both membrane and soluble forms which allows cells that do not contain the membrane bound form of the gp80 receptor to be targets of IL-6 by binding the IL-6 – soluble receptor complex and producing action through gp130.<sup>252</sup> Its normal physiological functions include roles in neural development, immunity and inflammation, and reproduction.<sup>80,81,87,139,162,252</sup> However, research has shown that a multitude of factors, including the acute phase response mediated release of IL-6, aging, obesity, and genetic variation, may result in dysregulation and elevation of circulating IL-6 levels, potentially leading to many pathophysiologic outcomes.<sup>15,23,44,46,50,53,92,94,112,113,243,250,252</sup> Thus, while IL-6 is required for many normal physiological functions, its positive, healing role may turn pathological by nature of its widespread and redundant actions, health outcomes (including conditions such as a chronic inflammatory state, insulin resistance, hypertension, endothelial

dysfunction, and dyslipidemia), and potential role in the development of atherosclerosis.<sup>15,23,44,46,50,53,54,92,94,112,113,162,243,250,252</sup>

### **IL6 –174G/C Promoter Polymorphism, IL-6 Levels, and Health Phenotypes**

As discussed, the IL6 –174G/C promoter polymorphism has been shown to have biological significance in terms of IL-6 levels and various outcome phenotypes, including plasma lipid levels.<sup>15,23,44,46,50,53,92,94,112,113,243,250,252</sup> For example, Fernandez-Real et al.<sup>44,46</sup>, Fishman et al.<sup>53</sup>, and Kubaszec et al.<sup>113</sup> found an association between the C allele and decreased levels of circulating IL-6. In contrast, Brull et al.<sup>23</sup> found an association between the C allele and increased levels of IL-6. In terms of health outcomes, Fernandez-Real et al.<sup>46</sup> found an association between carriers of the G allele and lipid abnormalities (increased TG, VLDL-TG, and apo B and decreased HDL<sub>2</sub>-C), and, in a second study, found an association between the C allele and increased insulin sensitivity. However, Kubaszec et al.<sup>113</sup> found an association between the C allele and decreased insulin sensitivity. Humphries et al.<sup>92</sup> found that in healthy men levels of the IL-6 surrogate C-reactive protein (CRP) were higher, though not significantly different, in C allele carriers compared to those with the GG genotype and that the C allele was associated with significantly higher systolic blood pressure (SBP) than in the G allele homozygotes. Berthier et al.<sup>15</sup> found an association between the polymorphism and indices of obesity such that the –174G allele was more commonly observed in lean subjects, and Brull et al.<sup>23</sup> found an association between the CC genotype and endothelial function in which subjects with the CC genotype showed a trend toward greater flow mediated dilatation. These

studies show that associations have been found between the –174G/C polymorphism and levels of IL-6 and various health outcomes, but the results are inconsistent with respect to the deleterious allele. Thus, based on the known effect of inflammation in inducing pro-atherogenic changes in lipoproteins and lipid metabolism, the association of IL-6 with many poor health phenotypes, and the findings associating IL-6 levels with increased TG and VLDL and lower HDL-C, the further investigation of the potential association between IL6 genotype and plasma lipoprotein lipid levels is warranted.

### **Exercise Training and Plasma Lipoprotein Lipid Response**

Plasma lipoprotein lipid levels are an important independent risk factor for CV disease that can be modified through exercise training.<sup>21,35,85,109,114-117,141,165,166,195</sup> For example, Durstine et al.<sup>35</sup> summarized current cross-sectional and longitudinal studies and established that 15-20 miles of brisk walking or jogging per week (expenditure of 1200-2200 kcals per week) was associated with increases in HDL-C of 2 to 8 mg/dL. Kraus et al.<sup>109</sup> recently found that a relatively high amount of regular exercise significantly improved overall lipoprotein profiles, even without clinically significant weight loss. Leon et al.<sup>114</sup> also found that 20 weeks of endurance exercise training significantly increased HDL-C levels on average by 1.4 mg/dL. However, the response of plasma lipoprotein lipids to even standardized exercise training is highly variable among individuals.<sup>35,64,109,114,116,246</sup> For example, while Williams et al.<sup>246</sup> found average increases in HDL-C of 4.2 mg/dL in 46 men with a one year exercise training program, they also reported that individual changes

in HDL-C ranged from a 10 mg/dL decrease to a 20 mg/dL increase in HDL-C. Strong evidence suggests that this variability in plasma lipid response to exercise training may be due to genetic factors.<sup>8,90,91,114,116,181</sup> For example, Rice et al.<sup>181</sup> recently studied 115 African American and 99 white families in a 20 week exercise training intervention and found that heritable factors accounted for 25-38% of the variance in lipid response to exercise training. For change in HDL<sub>2</sub>-C in whites, they found heritability in response to exercise training as high as 60%. In another study, Leon et al.<sup>114</sup> found that genetic contributors accounted for 26-29% of the variance in HDL-C changes with training in 675 sedentary, healthy, white and African American men and women aged 17-65 years of age. Additionally, a number of recent studies have begun to investigate the potential associations between exercise training-induced changes in plasma lipoprotein lipids and common polymorphic variations at specific genetic loci.<sup>14,17,70-73,148,149,217,228,248,249,249</sup> For example, in preliminary data from the Gene Exercise Research Study, endothelial lipase (LIPG) genotype had a significant effect on baseline lipoprotein lipid levels and also showed significant associations with changes with exercise training.<sup>74</sup> Thus, this evidence suggests that plasma lipoprotein lipid levels and their response to exercise training may be regulated by the interaction of lipid metabolism-related genes, their variants, and environmental factors.<sup>14,17,20,70-73,148,149,174,181,207,209,217,228,248,249,249</sup>

## **Exercise Training and IL-6**

Exercise has been shown to result in increased secretion of IL-6 from both skeletal muscle and adipose tissue.<sup>40,88,89,103,104,168-172,210,219,223,224,238</sup> For example, IL-

6 levels have been shown to increase 100-fold following a marathon.<sup>43,172</sup> However, neither the mechanisms resulting in the increased secretion nor the implications of the increased secretion are fully understood.<sup>40,43,88,89,168-172,224,238</sup> Furthermore, the influence of the type, intensity, and duration of exercise, while known to increase secretion, is not fully understood. Additionally, the role of genetic variation in influencing the response to exercise has not been studied, nor have the effects of a regular prolonged exercise-training program been studied in healthy older adults. However, researchers have begun to investigate this area and to formulate hypotheses regarding exercise, IL-6, and the role of excess secretion and the mediation of exercise-induced metabolic changes.<sup>40,43,88,89,168-172,224,238</sup>

With acute exercise, IL-6 secretion increases in both skeletal muscle and adipose tissue.<sup>40,43,88,89,168-172,224,238</sup> However, the mechanism behind and action of the increased IL-6 differs between the two tissues. In skeletal muscle, the mechanism for increased secretion appears to be the muscle contraction itself and a state of low muscle glycogen.<sup>168-172</sup> The resulting increase in IL-6 produces local action on the skeletal muscle, plus endocrine action as the IL-6 moves through the circulation and acts on the liver, adipose tissue, and arterial walls.<sup>170</sup> In the liver, the IL-6 stimulates glycogenolysis, resulting in the release of glucose that can be used by working muscles.<sup>170</sup> In adipose tissue, the IL-6 inhibits lipoprotein lipase and stimulates lipolysis, resulting in the release of free fatty acids.<sup>129,169,171,172</sup> In the artery, the IL-6 is thought to inhibit the TNF-alpha induced inflammatory effects of insulin resistance and atherogenesis (an interesting finding as IL-6 is thought to play its own role in the development of such inflammatory response conditions).<sup>168-172</sup> In adipose tissue,



circulating IL-6 from the contracting muscle acts to stimulate lipolysis while, after exercise, sympathetic nervous system stimulation is thought to result in increased IL-6 secretion with a local effect of increased lipolysis and FFA release.<sup>129</sup> Thus, these proposed mechanisms suggest that IL-6 may play a role in mediating exercise-induced metabolic changes. Current research as summarized by Pedersen et al.<sup>168-172</sup> suggests that IL-6 may serve to maintain glucose availability to working muscles during exercise, as well as mediate the mobilization of free fatty acids. This work suggests that IL-6 is an additional factor involved in the maintenance of glucose levels during exercise.<sup>168-172</sup>

Thus, in summary, acute exercise has been shown to augment IL6 transcription rates, mRNA, and release.<sup>168,219,220,223</sup> The effect of exercise on changes in IL6 gene expression and IL-6 release is influenced by the mode, intensity and duration of the exercise, as well as muscular glycogen status.<sup>154,168,169,171</sup> The primary source of the increase in IL-6 with exercise is thought to be the contracting skeletal muscle with the release stimulated by the muscle contraction itself, low intramuscular glycogen levels, or changes in energy turnover.<sup>40;43,88,89,103,104,168-172,224,238</sup> Other organs, including adipose tissue, also release IL-6 during exercise.<sup>97,219,223,224</sup> Thus, acting as an energy sensor and signaling molecule between the contracting muscle and other organs, the IL-6 protein appears to play a role in energy regulation during exercise as it functions to maintain glucose availability and mediate the mobilization of free fatty acids.<sup>224</sup>

In terms of exercise training, few data exist on the IL-6 response in healthy older adults. However, some evidence suggests that endurance exercise training may

attenuate the IL-6 response to exercise.<sup>3,13,59,177,235</sup> Additionally, we know of no investigations looking at the interactive effects of endurance exercise training and IL6 genotype on IL-6 levels and subsequent effects on plasma lipoprotein-lipids in healthy older adults.

### **Exercise Training, IL-6, And Plasma Lipoprotein Lipid Response**

While the effect of exercise training is unclear but potentially positive for IL-6 and known to be favorable for plasma lipoprotein-lipids, the interactive effect of IL6 genetic variation and endurance exercise training has not been investigated.

Speculatively, exercise training may have a beneficial effect on inflammatory pathways by improving endothelial function, increasing insulin sensitivity and heightening the possible anti-inflammatory effects of insulin, and reducing CRP levels.<sup>224,235</sup> Whether or not these potential benefits are mediated through or reflect alterations in IL-6 will require direct functional investigation in future studies.

However, with much evidence supporting positive changes in plasma lipoprotein lipids with exercise training and some evidence supporting the attenuation of exercise training on IL-6 levels, it is plausible that these genetic and environmental factors may interact in a positive manner to improve lipid profiles. To date, no research has been conducted on this topic, or, even more simply, solely on the interaction of exercise training and IL6 genotype on IL-6 levels. Consequently, in light of the growing public health crisis related to the spread of CV disease and the potential primary role of IL-6 in the development and progression of that disease, the investigation of the interaction between exercise training, IL6 genetic variation, and

the impact on plasma lipoprotein lipid response to training is of great interest and importance.

### **CRP As A Surrogate Marker For IL-6 Levels**

In 2002, the American Heart Association (AHA) and the Centers for Disease Control (CDC) convened a workshop to formulate a consensus regarding the use of assays of markers of inflammation in clinical practice. The result was an evidence-based scientific paper presenting the major findings and conclusions of the workshop.<sup>166</sup> The paper presents evidence for inflammation as a mechanism in atherosclerosis, provides support for the association of inflammatory markers with CV disease, and supports the use of CRP as the inflammatory marker of choice.

While a number of studies have investigated the potential association between the IL-6 –174G/C polymorphism and IL-6 levels, the results have varied widely.<sup>23,46,53,96,180,241</sup> However, the discrepancies in results may be due to random error in the small sample sizes used in these studies that may limit intra-individual replicability of IL-6 measures, the lack of timed blood sampling in light of the marked diurnal variability of plasma IL-6, and differences in sample population (healthy v. existing CV disease).<sup>99,215,216,240</sup> These studies highlight the difficulty in using direct measures of IL-6 due to its diurnal variability and need for precise, consistent collection procedures. Thus, as IL-6 is the major regulator of the acute phase response and the expression of the CRP gene, and CRP is stable and highly reproducible, studies have begun to investigate the association between IL6 gene variants, and specifically the –174G/C polymorphism, and plasma CRP levels.<sup>23,51,96,102,130,132,145,235,241</sup> While the first few studies found no association, which

may be due to limited sensitivity in the CRP assay and the use of subjects with existing CV disease, Vickers et al.<sup>160,241</sup> recently found an association between the –174G/C polymorphism and plasma CRP levels using a family model and determined that baseline plasma CRP level is highly heritable (0.39) which supported the findings of Pankow et al (CRP heritability of 0.35-0.4). Vickers et al.<sup>241</sup> concluded that their results strongly justified the use of typing of the IL-6 –174G/C polymorphism together with measures of plasma CRP. Ferrari et al.<sup>51</sup> also found an association between the –174G/C polymorphism and CRP levels. Additionally, Bataille et al.<sup>12</sup> conducted experiments in which they found that human CRP production in vivo was dependent on IL6 production, thus concluding that CRP levels are a direct indicator of IL-6 levels in humans. Thus, at the present time, the use of CRP as a surrogate marker for IL-6 is supported by the literature and is currently the most meaningful, viable method of assessing IL-6 response among the subjects in this study. This will provide an opportunity for replication of the approach using CRP as a measure of IL-6 and will set the stage for future research directly measuring IL-6.

The subjects for the proposed study are comprised of individuals who completed the study over the past five years, as well as those who are currently finishing. Thus, the use of samples from previous cohorts for measuring IL-6 levels will not meet rigorous standardization procedures for sample collection, processing, and analysis. For accurate comparison, samples should be collected at a consistent time and be processed and stored according to assay procedures. Additionally, samples should be analyzed within a year to two years, the same kit should be used to analyze baseline and final samples from the same subject, and repeated freeze-thaw cycles of samples

should be avoided. However, unlike IL-6, CRP is highly stable and measurable over time and is accepted as a surrogate marker for IL-6.

## **APPENDIX A – Limitations of the Study**

### **Limitations**

1. Subjects self-reported many factors related to health and lifestyle such as physical activity habits, dietary habits, medication regimens, and medical conditions. It is possible that inaccurate self-reports may adversely affect the results of the study.
2. While each subject does serve as their own control, there is no separate control group in this study. Thus, it is possible that seasonal variation or subject aging may influence the results.
3. The NMR lipid analyses were conducted on single plasma samples, therefore the results could be affected by the known daily variation in plasma lipoprotein lipid levels.
4. Plasma IL-6 levels were not measured directly in this study, and CRP levels were used as a surrogate measure. The use of CRP measures in place of direct measures of IL-6 is based on the following:
  - 1) the AHA/CDC Scientific Statement recommendations regarding markers of inflammation and cardiovascular disease and the application to clinical and public health practice<sup>166</sup>;
  - 2) the precedent found in the literature for the use of CRP as a measure of IL-6 and the nature of the IL-6 molecule<sup>12,145</sup>; and
  - 3) the difficulties in assuring consistency in sample collection, processing, and analysis procedures for subjects having already completed the GERS study.

## **Delimitations**

1. Approximately 65 subjects recruited from the University of Maryland – College Park campus and surrounding areas were evaluated for plasma lipoprotein-lipid levels and response to endurance exercise training.
2. Due to the low frequency of the less common C allele in African Americans (0.08) as compared to Caucasians (0.37-0.44) and the relatively small projected sample size and genotype/allele frequency for African Americans (N=30; GG=24, GC=4, CC=1; G=55, C=5), the analyses included only Caucasians as the inclusion of African Americans would result in merely adding to the common allele pool without contributing to analysis of the rare allele.<sup>28,78,94,98,150</sup>
3. Subjects were healthy males and females, 50-75 years of age, sedentary as determined by questionnaire and personal interview, non-diabetic as determined from a 2-hour glucose tolerance test, free from cardiovascular disease as determined by a graded exercise test (GXT), and not taking medications known to affect lipid metabolism. Therefore, the results most specifically apply to populations with similar characteristics.
4. All female subjects enrolled in the study were at least 2 years postmenopausal and remained either on or not on hormone replacement therapy (HRT) throughout the duration of the study. Since HRT may affect lipid metabolism and IL-6 action, female subjects maintained their current HRT status throughout the study. While statistical control can be applied, it is possible that HRT status may affect genotype and/or training effects on the outcomes.

5. Participants were asked to follow the American Heart Association's Dietary Guidelines for the General Population with <30% of calories from fat and cholesterol intake of <300 mg/d for the duration of the study.<sup>111</sup>
6. In addition to the -174G/C promoter polymorphism, three additional polymorphisms that have been identified in the 5' flanking region of the IL6 gene were not assessed in this study (-597G/A, -572 G/C, -373A<sub>n</sub>T<sub>n</sub>).<sup>23,51-53,57,98,146,225,232,236,242</sup> However, previous work supports strong allelic associations among these 5' flanking region polymorphisms, gives estimates for 94% of the haplotypes at these loci, and suggests that the -174G/C promoter polymorphism serves as a marker for the most frequent haplotypes.<sup>23,51-53,57,98,146,225,232,236,242</sup> Therefore, knowing the -174G/C genotype allows for the estimation of 94% of the haplotype combinations for these three loci (GGG 54%; AGC 40%), and, with only 65 subjects, typing of additional loci would only identify 4 additional subjects beyond the 61 identified by the -174G/C site. Thus, as this study consists of only a modest sample size and the genotyping and analysis of these additional SNPs would provide no additional information above that found from analyzing the -174 G/C site alone, the present investigation focuses only on the -174G/C locus.
7. Lipids were analyzed using conventional methods at the Baltimore Veterans Administration Medical Center (VAMC) using a CDC standardized Hitachi 717 autoanalyzer<sup>4,205</sup>, as well as by using nuclear magnetic resonance (NMR) techniques (LipoScience, Raleigh, NC) previously standardized and validated against conventional methods of separation and analysis.<sup>157-159,190,212</sup> Body



composition was analyzed at the Baltimore VAMC using dual-energy x-ray absorptiometry (DPX-L; Lunar Corp, Madison, WI).<sup>142</sup>

8. All exercise training sessions were conducted at the Wellness Research Laboratory at the University of Maryland – College Park.

## APPENDIX B – Definition of Terms

*Cytokine.* Secreted by many cells types, these low-molecular weight, hormone-like proteins regulate immune response intensity and duration, as well as mediate cell-to-cell communication.

*Interleukin.* A group of multifunctional cytokines synthesized by lymphocytes, monocytes, macrophages, and certain other cells. They have a known amino acid structure.

### *Lipoprotein Nuclear Magnetic Spectroscopy Measured Subfractions.*

Traditionally, lipoprotein analysis has recognized VLDL, LDL, and HDL subclasses of increasing density and decreasing size (VLDL – large, intermediate, small; LDL – (IDL1 and 2), I, II a, II b, III a, III b, IV a, IV b; HDL – HDL2b, HDL2a, HDL3a, HDL3b, HDL3c). Nuclear magnetic resonance spectroscopy analysis also distinguishes between these subfractions, but designates them as follows: VLDL - V6, V5, V4, V3, V2, V1; LDL – IDL, L3, L2, L1; HDL – H5, H4, H3, H2, H1; respectively. The approximate range in nanometers for these particles is as follows:

$$\text{VLDL (V6-V1)} = 150 \pm 70, 70 \pm 10, 50 \pm 10, 38 \pm 3, 33 \pm 2, 29 \pm 2$$

$$\text{LDL (IDL, L3-L1)} = 25 \pm 2, 22 \pm 0.7, 20.5 \pm 0.7, 19 \pm 0.7$$

$$\text{HDL (H5-H1)} = 11.5 \pm 1.5, 9.4 \pm 0.6, 8.5 \pm 0.3, 8.0 \pm 0.2, 7.5 \pm 0.2$$

The NMR analysis takes advantage of the fact that different sizes of lipoprotein particles have specific proton NMR spectroscopic signatures.<sup>149,155-159,212</sup>

*Sedentary.* Sedentary individuals are those who are not physically active. In the proposed study, sedentary is defined as performing aerobic exercise for no more than 20 minutes two or more times a week over the past 6 months.

*Triglyceride.* Triglycerides are a type of fat in the blood that are comprised of three fatty acid molecules esterified to a glycerol molecule.

## **APPENDIX C – Human Subjects Approval and Consent Form**

**Title:** Interleukin-6 (IL6) Genotype, Plasma Lipoprotein Lipids, and Their Response To Exercise Training

**Applicant:** Amy Halverstadt

**Advisor:** Dr. James Hagberg

### **1. Abstract**

A small body of literature suggests an association between the interleukin-6 (IL6) G-174C gene polymorphism and plasma lipoprotein-lipid levels such that the G allele may be associated with a more pro-atherogenic lipid profile than the C allele. However, no research exists regarding the potential IL6 gene x physical activity interaction. Thus, the purpose of this proposed study is to assess the association between the IL6 G-174C gene polymorphism, plasma lipid profiles, and their response to exercise training. It will be of particular interest to determine whether individuals carrying the G allele demonstrate a pro-atherogenic lipid profile versus non-carriers and to compare their lipid changes with exercise training. In a more general sense, the proposed study will contribute to understanding the great inter-individual variability in plasma lipoprotein lipid levels and their response to exercise training and may aid in the design of individualized treatment and prevention strategies targeted at improving plasma lipid profiles. The study will employ a standardized exercise training intervention in healthy 50-75 year olds, conduct baseline and final testing that includes NMR measurement of plasma lipoprotein lipids, genotype subjects at the identified IL6 gene locus, and assess the potential

association of the IL6 –174G/C polymorphism with plasma lipoprotein lipid levels and their responses to exercise training.

## **2. Subject Selection**

### **a) Who will be the subjects? How and from where will they be obtained?**

The subjects in this study will have taken part in the Gene Exercise Research Study “APO E genotype and HDL changes with exercise training” study (IRB approval number 00494) at the University of Maryland. These individuals are men and women from the population in the vicinity of the University of Maryland (Washington, D.C. metropolitan area) who responded to media advertisements (newspaper and magazine advertisements, radio public service announcements, flyers mailed to citizens in the metropolitan area).

### **b) Are the subjects being selected for any specific characteristics?**

Subjects in the Gene Exercise Research Study are screened via telephone and with the use of laboratory assessments to meet eligibility requirements. Subjects must be sedentary (aerobic exercise  $\leq 2$  times/week,  $< 20$  minutes/session, sedentary job), 50-75 years of age, nondiabetic, normotensive or blood pressure controlled with non-lipid and non-glucose altering medication, no history of chronic obstructive lung disease, a body mass index (BMI)  $< 37 \text{ kg/m}^2$ , and no physical or orthopedic conditions that would preclude vigorous exercise. In addition, women must be postmenopausal (absence of menses for  $\geq 2$  years) and agree to maintain their hormone replacement status during the study, either receiving or not receiving hormone replacement therapy.

**c) State why the selection is made on the basis given in (2).**

These criteria are necessary to ensure subjects have no known conditions limiting participation in any of the Gene Exercise Research Study testing procedures and to help control for extraneous variables, which could affect plasma lipoprotein lipid levels. In addition, individuals aged 50-75 were chosen as the subject population since they represent a high risk group for cardiovascular disease (CVD) and preventive measures could make an impact on their health.

**3. Procedures**

Data from subjects in the Gene Exercise Research Study will be used to separate the subjects into groups based on their IL6 –174G/C genotypes. Then, the subjects' plasma lipoprotein lipid levels and responses to exercise training will be compared among genotype groups. Possible confounding factors, including gender, body composition, and age, will be used as covariates in the statistical analyses to control for their effects.

*Orientation Visit:* Telephone screened subjects interested in participating in the Gene Exercise Research Study come to the laboratory and have their medical history forms reviewed to determine if they meet inclusion criteria. They then have the Gene Exercise Research Study explained to them. Any questions are answered and those interested in participating provide their written informed consent. The subjects are then scheduled for Screening Visit 1.

*Screening Visit 1:* Following an overnight fast, subjects have a blood sample drawn for standard blood chemistry tests. Subjects are excluded if they have plasma triglycerides > 400 mg/dL, a hematocrit < 35, evidence of renal or liver disease, or

fasting glucose > 126 mg/dL. The fasting blood sample is immediately evaluated using a One-Touch Glucometer. If the fasting blood glucose is > 126 mg/dL, the subject does not continue with the remaining Screening Visit 1 procedures. If clinical laboratory analysis confirms a fasting glucose > 126 mg/dL, the subject is excluded from the study. If the laboratory analysis does not confirm a fasting glucose > 126 mg/dL, then the subject is rescheduled for a second Screening Visit 1 appointment.

Subjects also undergo a 2-hour OGTT, where they drink a 75-gram glucose solution and have a blood sample drawn 2 hours later. If the blood glucose level 2 hours after the glucose challenge is > 200 mg/dL, the subject is excluded from the study. To be included in the study, subjects must also have at least one National Cholesterol Education Program lipid abnormality (cholesterol > 200 mg/dL, HDL < 35 mg/dL, triglyceride > 200 but < 400 mg/dL, or LDL > 130 mg/dL). Height and weight are also measured to calculate BMI to ensure subjects meet the BMI < 37 kg/m<sup>2</sup> criteria.

During Screening Visit 1, DNA is also extracted from the fasting blood sample for genotyping. DNA is isolated from peripheral monocytes and genotyped in the University of Pittsburgh Laboratory of Dr. Robert Ferrell. Genotyping of the IL6 -174G/C gene involves the use of DNA that was frozen for future studies associated with the goals of the Gene Exercise Research Study.

*Screening Visit 2:* Those individuals continuing to qualify for the study undergo a second screening visit consisting of a general physical examination and a maximal graded treadmill exercise test, using the Bruce protocol, to screen for CVD. Blood pressure, electrocardiogram (ECG), and heart rate are recorded before the test,

at the end of every stage in the test, and every other minute for 6 minutes during active recovery from the exercise test. A subject is excluded from the study if his or her blood pressure response to exercise is abnormal, the test is terminated due to cardiovascular symptoms, or the subject has ST segment depression greater than 2 mV.

*Dietary Stabilization:* After screening into the study, subjects attend dietary lectures on the principles of an American Heart Association low-fat diet, twice a week for 6 weeks. Subjects maintain the diet throughout the duration of the study, with periodic dietary recalls and food frequency checks to ensure adherence. In addition, subjects are instructed to maintain caloric intake throughout the study, since weight loss can influence many health and fitness variables. Subjects are weighed weekly and those losing more weight than expected are counseled to increase their caloric intake.

*Baseline Testing:* After dietary stabilization, the subjects undergo baseline testing, which includes body composition scans, a 3-hour OGTT, and plasma lipoprotein lipid measures. The 3-hour OGTT takes place following a 12-hour overnight fast. A catheter is placed in an antecubital vein, and blood sampling occurs before and every 30 minutes after the ingestion of a 75-gram glucose solution, for 3 hours. Body composition is measured via dual energy x-ray absorptiometry at the Baltimore VA Medical Center, using standard procedures.

To analyze plasma lipoprotein lipid levels, subjects have fasting samples drawn on two separate occasions, with the values averaged. If these measures differ by > 10%, a third separate measurement is included in the average. Plasma TG and



cholesterol levels are analyzed using a CDC standardized Hitachi 717 autoanalyzer. HDL-C is measured after precipitation with dextran sulfate, and LDL-C is calculated using the Friedewald equation. HDL<sub>2</sub>-C and HDL<sub>3</sub>-C are separated using a second high-molecular weight dextran sulfate precipitation with HDL<sub>3</sub>-C measured and HDL<sub>2</sub>-C calculated. Nuclear magnetic resonance (NMR) techniques (LipoScience, Raleigh, NC) are used to determine plasma lipoprotein lipids, using techniques previously standardized and validated against conventional methods of separation and analysis. NMR measures are made on a single blood sample that is frozen at –80° C and analyzed at the same time as final test samples.

This sub-study of the Gene Exercise Research Study will be conducted in the Department of Kinesiology at the University of Maryland, College Park. Gene Exercise Research Study procedures take place cooperatively at the University of Pittsburgh, the University of Maryland at Baltimore, and the University of Maryland, College Park.

#### **4. Risks and Benefits**

There are potential risks associated with participation in the Gene Exercise Research Study and, consequently, this sub-study. There are risks involved with graded treadmill exercise testing of approximately 1 nonfatal event in 10,000 maximal treadmill tests and 1 fatal cardiac event in 70,000 maximal treadmill tests. To minimize these risks, American Heart Association CPR and AED certified personnel administer the tests. In addition, a physician experienced in arrhythmia detection and exercise testing will be present at every treadmill test. Subjects will be screened with a physical exam and resting ECGs in the supine, seated, and standing

positions prior to starting the graded exercise test. An emergency cart with medications and an AED will be present at all tests.

There is a potential risk of bruising and infection with the blood drawing procedures. Using aseptic techniques and having trained laboratory technicians draw the blood samples will minimize these risks.

There is a risk of malignancies due to x-ray exposure during the body composition scans. Exposing the subject to 0.02 mRem/scan, which is equal to 30 minutes of natural background radiation exposure, will minimize these risks.

There are no risks associated with the genetic testing since the participants are not provided with information regarding their genetic test results. The laboratories that perform the genetic tests are not clinically certified, thus the genetic results cannot be used for diagnostic purposes.

The OGTT involves risks due to blood drawing, the potential for a hypoglycemic reaction at the end of the test, and the possibility of an upset stomach after ingestion of the glucose solution. To minimize these risks, the blood drawing techniques described above will be employed, subjects will receive juice and a snack after the test to prevent a hypoglycemic response, and subjects will be monitored closely throughout the test so that a drink and snack can be provided if symptoms of hypoglycemia appear.

Each subject in the Gene Exercise Study receives his/her dietary assessments, test results (except genetic tests), and interpretations of the results. As part of the Gene Exercise Study, the subjects also receive six months of aerobic exercise training. Overall, the results of this study will help explain if genetic polymorphisms

affect the variability in HDL-C, its subfractions, and responses to exercise training in 50-75 year old men and women. With the prevalence of cardiovascular disease in society today, identifying factors that influence plasma lipoprotein lipid metabolism and composition is beneficial to helping understand mechanisms of disease and possible treatment strategies.

## **5. Confidentiality**

Gene Exercise Research Study subject data is entered into a database with access granted only to qualified study personnel. All blood samples for genetic analysis are numbered and sent to the University of Pittsburgh and University of Maryland at Baltimore for analysis, so the samples there are unrelated to any specific research subjects. All samples sent to LipoScience in Raleigh, NC for NMR measured lipid analyses are also numbered such that the samples are unrelated to any specific research subject. Only the Gene Exercise Research Study Primary Investigator and Study Coordinator know the subject names corresponding to the coded numbers. Presentations of study results will not reference subject names and all data from subjects will be grouped together for reporting purposes.

## **6. Information and Consent Forms**

Subject study results (except genetic tests) are provided to the subject in written form with explanations attached. Also, it is indicated that the results are not for diagnostic use, as they are not generated from clinically approved laboratories. A subject's written consent is obtained following an orientation meeting, where the study specifications and consent form are explained in detail by the investigators.

## **7. Conflict of Interest**

Investigators do not have a real or potential conflict of interest.

## **8. HIPAA Compliance**

The researchers working on this project are not employed by the University Health Center and thus are not subject to specific HIPAA requirements regarding the creation, use, disclosure, or access of protected health information. Subject personal identification information will remain confidential by the methods described in this and the original IRB applications. The attached informed consent details the procedures for the handling of confidential information.

## CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

Project Title: APO E genotype and HDL Changes with Exercise Training

I state that I am over 18 years of age and wish to participate in a program of research being conducted by Dr. James Hagberg in the Department of Kinesiology, University of Maryland.

The purpose of this study is to determine the role that genetics may play in determining how my blood cholesterol levels change with exercise training.

I already completed a telephone interview that determined that I am not physically active, am 50 – 75 years of age, not a diabetic or have controlled diabetes, not taking cholesterol-lowering medications, have normal blood pressure or high blood pressure controlled on medications not affecting my cholesterol levels, have no evidence of lung disease, have an appropriate weight for my height, and have no other medical problems that would keep me from exercising vigorously. Furthermore, if I am a woman, I must be postmenopausal, defined as no menstrual cycles for at least the last 2 years. I understand that if I am a woman and change my hormone replacement therapy regimen during the study, my participation in the study will be terminated. I also understand that if I have a prior history of ulcers or bleeding disorders, I will be excluded from one test that is part of this study. I also understand that I must have somewhat abnormal levels of cholesterol to enter the study.

I understand that I will complete one orientation and two screening visits. The orientation session will present all aspects of the study and my written informed consent will be provided after all of my questions have been answered. For my first Screening Visit, I will report to the laboratory in the morning after an overnight fast and a blood sample will be drawn for blood chemistries and blood cholesterol levels. I understand that I may be excluded from the study if this initial blood sample shows elevated levels of glucose in my blood. I understand that a part of this blood sample will be used to obtain my DNA. A blood sample will also be drawn 2 hours after I drink a sugar solution. I understand that a total of 3 tablespoons of blood will be drawn during this visit. I understand that I will be excluded from the study at this point if I have low cholesterol levels, high triglyceride levels, a low red blood cell count, evidence of kidney or liver disease, or evidence of diabetes. I understand that if I remain qualified to this point, on my second Screening Visit I will undergo a treadmill exercise test to determine if I have heart disease. A physical examination will precede the exercise test. I will then complete a test on an exercise treadmill where the treadmill speed and grade will increase every 3 minutes until I cannot continue or symptoms of heart disease develop. Blood pressure, heart rate, and ECG will be recorded before, during, and after the test. I understand that I will be excluded from the study at this point if I have evidence of heart disease.

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I understand that if I meet all of these requirements to enter the study, I will undergo 6 – 8 weeks of instruction in the principles of an American Heart Association low-fat diet and must follow this diet for the remainder of this study. After this I will undergo Baseline Testing that includes the following tests that will be completed in 5 testing sessions. I will have blood drawn on 2 or 3 occasions from a vein in my arm in the morning after an overnight fast to measure my cholesterol levels and to assess my immune system. I understand that a maximum of 2 tablespoons of blood will be drawn during these visits. I understand that I will also undergo a second exercise test on a treadmill to measure my cardiovascular fitness. This test will start at 70% of the highest heart rate achieved on my first exercise test and the treadmill grade will increase by 2% every 2 minutes. Blood pressure, heart rate, and ECG will be monitored before, during, and after the test. The test will be stopped when I can no longer continue. During this test I will have a nose clip on my nose and I will breathe through a mouthpiece so that the air that I breathe out can be analyzed. I also understand that my dietary habits will be measured by having me record for 7 days all of the food items that I eat. I understand that on another morning after an overnight fast I will have blood samples drawn before and for 3 hours after I drink a glucose solution to assess my risk of developing diabetes; I will also have additional blood drawn prior to this test that will be frozen for future studies that relate directly to the goals of the present study. I understand that 5 tablespoons of blood will be drawn during this visit. I understand that on another occasion after an overnight fast, I will have blood samples drawn from a line (catheter) in my arm before and for 4 hours after drinking approximately 1 – 2 cups of a high-fat liquid meal. The high-fat meal is made of heavy whipping cream with small amounts of chocolate, sugar, and powdered milk and tastes similar to a rich chocolate shake. I understand that 10 tablespoons of blood will be drawn during this test and will be used to measure how my body absorbs and uses fat from a meal and how my blood clotting, and substances that affect hunger are affected by a fat meal. Before and after I drink the high-fat meal, I understand that I will breath through a mouthpiece wile my nose is closed-off with a nose clip and the air that I breathe out will be collected and used to determine how much fat I use for energy while sitting at rest. I understand that these tests will be done at the University of Maryland College Park.

I understand that in the morning after an overnight fast I will have blood samples drawn to assess my cholesterol levels and my blood clotting system. I will then have a substance that temporarily stops blood from clotting injected into my arm vein. Blood samples are drawn 10 min later for measurement of chemicals that affect blood cholesterol levels. I understand that if I have a prior history of ulcers or bleeding disorders I will not undergo this test. I understand that I will remain in the laboratory for 2 – 3 hours after this test with pressure on the site where blood samples were drawn to make sure that all bleeding is stopped. I understand that 4 tablespoons of blood will be drawn during this visit. I understand that how much fat and muscle I have will be measured using x-rays while I lay quietly on a table for 15 to 30 minutes. I understand that the amount of fat I have around my waist will be measured with a

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CAT scan while I lie quietly on a table. I also understand that these last 3 tests will be done at the VA Medical Center in Baltimore.

I understand that during this Baseline Testing a total of 21 tablespoons of blood will be drawn, which is about two-thirds of the amount of blood given when donating blood.

I understand that after completing this testing, for 6 months I will complete 3 exercise sessions each week supervised by study personnel. I understand that I will be instructed on appropriate warmup and stretching exercises to perform prior to each exercise training session. I will be taught to measure my heart rate and to use heart rate monitors to control how hard I am exercising. The first training sessions will consist of 20 minutes of light exercise. The amount of exercise and how hard I exercise will increase gradually until I am completing 40 minutes of moderate intensity exercise every session. Exercise modes include walk/jogging, stairstepping, and cycle, cross-country ski, and rowing ergometry. I will be asked to add a 45-60 minute walk to my exercise program on weekends after the first 10 weeks of the exercise program. I understand that some of the supervised exercise sessions may be done outside of the exercise facility, but still under the direct supervision of study personnel. I understand that if I lose more weight than expected from exercise, I will be counseled by a dietitian against restricting how much food I eat. I will also be asked to complete food records during the exercise training program and if major dietary changes have occurred, I will also be counseled by a dietitian to resume my original dietary habits.

I understand that after completing 6 months of exercise training, I will have everything reevaluated that was measured before I began the exercise program. I understand that during this testing a maximum of 21 tablespoons of blood will be drawn, which is about two-thirds of the amount of blood given when donating blood.

I understand that if I qualify for this study that my DNA will be isolated from my blood and analyzed at a number of sites for differences in DNA that may affect how my cholesterol levels change with exercise training. I understand that some of my DNA will also be frozen for future studies. However, these studies can only analyze my DNA at sites that might affect how my cholesterol levels, glucose and insulin levels, bone density, body composition, immunology (disease-fighting), and cardiovascular and blood clotting systems change with exercise training.

All information collected in this study is confidential, and my name will not be identified at any time. I understand that my DNA (genetic material) will be sent to collaborating genetics laboratories that are part of this study and that a sample of my DNA will be kept in the University of Maryland Department of Kinesiology laboratories. I understand that my coded blood samples will also be sent to other collaborating laboratories to measure compounds in my blood that relate to blood cholesterol levels and cardiovascular disease risk. I understand, however, that all my blood samples sent to collaborating laboratories will be identified only by a numeric code. I understand that only investigators at the University of Maryland College Park will know whose name is associated with each coded number. I further understand

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that the list of names and codes will be retained at the University of Maryland College Park for up to 25 years.

I understand the following risks are associated with my participation in this study. (1) The risk of maximal exercise testing is approximately 1 nonfatal event in 10,000 tests and 1 fatal cardiac event in 70,000 tests. Risks will be minimized by having the test administered by a physician and personnel trained in such tests and emergency procedures. I will be screened with a resting ECG and a physical examination prior to this test. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all testing sessions. (2) There is minimal risk of bruising and infection associated with the blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The risk if the body composition testing is the exposure to X-rays. The amount of X-ray exposure for this test is the same as that occurring during 30 minutes of any activity outside in the sun. (4) The risk associated with the test requiring the injection into an arm vein of a substance that temporarily stops blood clotting is bleeding. This risk will be minimized by excluding persons with bleeding disorders, peptic ulcers, or other blood disorders from the study. The risk is further minimized by placing a pressure bandage on the intravenous access site after the blood sampling and observing the subject for 2-3 hours after the injection. (5) The risk associated with the CAT scan to measure abdominal fat is the exposure to X-rays. The X-ray exposure is less than the maximum radiation dose individuals are permitted to be exposed to each year in their occupation. (6) The risks associated with the blood clotting and immune system studies are those related to blood drawing as listed above. (7) The risks associated with the oral glucose tolerance test and the high-fat meal test are those associated with blood drawing, the possibility of having low blood sugar levels at the end of the test, and the possibility of having an upset stomach, primarily a stomach ache, after drinking the glucose and /or high fat meals. The risk of low blood sugar levels at the end of the test will be minimized by providing you with a drink and small snack. (8) The risk of exercise training is the possibility of a heart attack or other cardiovascular event. A large physical activity center reported that 1 nonfatal cardiovascular event occurred in 1.7 million walk/jogging miles. These risks will be minimized because I will undergo a cardiovascular evaluation before beginning exercise training. Exercise sessions will be supervised by experienced personnel trained in emergency procedures. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all supervised exercise training sessions. Two study personnel will supervise the outside exercise sessions done at the University of Maryland, College Park though no emergency equipment will be directly available during these sessions. (9) There are no risks associated with the genetic testing because no results of these tests will be given to the participants. This has to be the case because the genetic results are not from clinically-approved laboratories.

I understand that this study is not designed to help me personally, but may help the investigators to determine who exercise might benefit the most. I understand that I will be provided with my study results and they can be sent to my physician if I

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request this in writing. I understand that these results are not to be used for clinical diagnostic purposes and that I will not receive the results of my genetic testing. I understand that I am free to ask questions or to withdraw from participation at any time without penalty. I understand that I will be paid \$50 for completing Baseline Testing after the dietary stabilization period. I also understand that I will be paid another \$50 for completing 3 months of exercise training and another \$100, for a total of \$200, for completing 6 months of exercise training and all final testing, if I complete at least 90% of my exercise training and testing sessions. I understand that if my participation in the study has to be terminated because I change my hormone replacement therapy regimen, I will only be paid for the portion of the study that I have already completed, that is, which of the stages above that I have completed.

In the event of a physical injury resulting from participation in the study, I understand that immediate medical attention is available at the Washington Adventist Hospital or the Baltimore VA Medical Center. However, I understand that the University of Maryland does not provide any medical or hospitalization coverage for participants in this research study nor will the University of Maryland provide any compensation for any injury sustained as a result of participation in this research except as required by law.

Principal Investigator: James Hagberg, PhD, Department of Kinesiology, HLHP Building, University of Maryland, College Park, MD 20742-2611, telephone 301-405-2487.

\_\_\_\_\_  
Subject's signature

\_\_\_\_\_  
Date

\_\_\_\_\_  
Witness

\_\_\_\_\_  
Date

\_\_\_\_\_  
Investigator

\_\_\_\_\_  
Date

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