AN ABSTRACT OF THE DISSERTATION OF


Title: Exercise, Nutrition, and Homocysteine.

Abstract approved by Melinda M. Manore

Exercise increases B-vitamin (B6, B12, folate) dependent metabolic reactions, especially those related to energy production and the rebuilding and repairing of muscle tissue. These same B-vitamins are also important in maintaining low blood levels of homocysteine (Hcy), a cardiovascular disease (CVD) risk factor. Hcy levels rise when the metabolism of the essential amino acid, methionine increases, a process that requires vitamin B6, B12 and folate. Finally, high intensity exercise can increase methionine metabolism, which can result in elevated Hcy production, particularly if circulating B-vitamins levels are low.

Although exercise is a positive modulator of CVD risk, exercise may increase Hcy production and subsequently increase B-vitamin needs, particularly if the exercise intensity is vigorous. Research examining the effect of exercise on Hcy is limited with no studies carefully controlling for factors that alter Hcy metabolism (age, B-vitamins) to determine if Hcy is higher in active than sedentary individuals. Lack of such knowledge limits our ability to make appropriate dietary recommendations for active individuals. Research on this issue is equivocal due to a variety of study designs and inadequate control of confounding variables.
Furthermore, there is much discussion in the B-vitamin/exercise literature about exercise increasing the need for the B-vitamins, but at the moment these data are limited. Therefore, the primary purpose of this research was to examine the effect of physical activity level (HighPA >420 min/wk; LowPA ≤420 min/wk) on blood Hcy concentrations, independent of B-vitamin status, in non-supplemented young active and sedentary men and women (N=76). A brief introduction (chapter 1) reviews the most recent publications (2005-2007) examining these relationships, research questions, hypotheses, and outcome variables. Then, an extensive review (chapter 2) of Hcy metabolism, blood Hcy as a cardiovascular disease risk factor, and previous publications (1995-2005) on PA and Hcy follows. Finally, chapter 3 describes the study details examining the research questions. Results from this cross-sectional study found no significant differences in blood Hcy concentrations after controlling for plasma B-vitamin levels between HighPA (7.5±1.6 µmol/L) and LowPA (7.7±1.6 µmol/L) groups in young (26±5 y) non-supplemented men (N=38) and women (N=38), unless PA was extremely high (>758 min/wk; >12.5 h/wk).
Exercise, Nutrition, and Homocysteine

by
Lanae Marie Joubert

A DISSERTATION

submitted to
Oregon State University

In partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented May 22, 2007
Commencement June 2008
When I first started this educational expedition, several folks told me a Ph.D. is really a degree in perseverance; indeed it has taken me six years to accomplish. There are many individuals and organizations that assisted me during this learning journey and they deserve special recognition.

Participants
Special thanks are due to the participants whom I respect for taking on all of the challenges of this study. I am grateful for their commitment and precision with keeping 7-day diet and physical activity records and especially thankful for their willingness to give blood for analysis, even though many were squeamish about needles.

Data collection
Special thanks are extended to Terry Deacon, Christa Marney, Kristin Kipp, Sarah Van Vooren for helping with data collection and analysis. Your friendship is just as supportive as your time, energy, and enthusiasm towards this project. I am forever grateful. Also, thank you to Stamatis for organizing the human performance lab schedule and problem solving any equipment errors. I am thankful for Ellen Watrous for her meticulous accounting services, especially in dealing with Good Samartian Health Services billing.
Data Analysis

I am forever indebted to Karin Hardin and Jim Riddlington for showing me the ropes in the lab; juggling chemicals with me and plowing through the HPLC procedures for homocysteine and vitamin B6 analysis. Your expertise and gracious network of friends helped me through what I conceived as the most impossible piece of this entire project. I am also grateful for Dr. Steve Carroll who graciously provided statistical consulting and analysis. His expertise in this area was invaluable.

Graduate Committee

I would also like to express my sincere gratitude and appreciation to my graduate committee: Don Prickel, Therese Waterhous, Christine Snow, Tony Wilcox. All 4 of these individuals have enriched my pursuit of research and education by bringing forward an area of expertise and contributed perpetual support with academics and personal life issues. I'd like to extend this sincere gratitude and appreciation to my major professor, Dr. Melinda Manore. We arrived to OSU nearly the same day and struggled to learn about our new environment together. Our relationship has grown stronger through the years. She has pushed me out of my comfort zone on more than one occasion, encouraging me to be an independent learner and a much improved writer. I especially appreciate her friendship, tireless energy, wisdom, and consistent help.
Financial support

Without financial support, this degree would have only been a dream and not a reality for me. I was fortunate to receive scholarship assistance from the College of Health and Human Sciences at Oregon State University. In addition, this study would not have been possible without the funding that was provided by the Northwest Health Foundation and the American College of Sports Medicine (student grants).

Family

I am indebted to my family and many friends who helped me deal with the challenges and stress of a doctoral program. Their encouragement over the years, especially when I felt like giving up, helped me stay focused and motivated. And finally, a continuing thank you to my husband, Keith, for loving me, believing in me, and encouraging me to be a better person, student, mother, and wife. I am deeply grateful for his patience and understanding throughout my quest for life-long learning.
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER 1: GENERAL INTRODUCTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXERCISE AND NUTRITION</td>
<td>1</td>
</tr>
<tr>
<td>NUTRITION AND HCY</td>
<td>2</td>
</tr>
<tr>
<td>HCY AND EXERCISE</td>
<td>3</td>
</tr>
<tr>
<td>RESEARCH ON HCY, EXERCISE &amp; NUTRITION</td>
<td>4</td>
</tr>
<tr>
<td>RESEARCH QUESTIONS</td>
<td>8</td>
</tr>
<tr>
<td>TABLES</td>
<td>10</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2: MANUSCRIPT 1: REVIEW OF LITERATURE: EXERCISE, NUTRITION, AND HOMOCYSTEINE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>17</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>18</td>
</tr>
<tr>
<td>HOMOCYSTEINE METABOLISM</td>
<td>19</td>
</tr>
<tr>
<td>Role of Nutrition</td>
<td>20</td>
</tr>
<tr>
<td>Role of Physical Activity</td>
<td>21</td>
</tr>
<tr>
<td>HOMOCYSTEINE AND CVD</td>
<td>24</td>
</tr>
<tr>
<td>Homocysteine as a CVD Risk Factor</td>
<td>25</td>
</tr>
<tr>
<td>Mechanism: How does elevated blood homocysteine increase CVD risk?</td>
<td>26</td>
</tr>
<tr>
<td>What is Elevated Blood Homocysteine?</td>
<td>27</td>
</tr>
<tr>
<td>PHYSICAL ACTIVITY AND HOMOCYSTEINE</td>
<td>28</td>
</tr>
<tr>
<td>Epidemiology Research</td>
<td>29</td>
</tr>
<tr>
<td>Cross-Sectional Research</td>
<td>30</td>
</tr>
<tr>
<td>Exercise Interventions</td>
<td>31</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>32</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLES</td>
<td>46</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>50</td>
</tr>
</tbody>
</table>

CHAPTER 3: MANUSCRIPT 2: BLOOD HOMOCYSTEINE LEVELS ARE THE SAME IN NONSUPPLEMENTED YOUNG ACTIVE AND SEDENTARY MEN AND WOMEN WITH DIFFERENT PHYSICAL ACTIVITY LEVELS .............................................................60

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>63</td>
</tr>
<tr>
<td>METHODS</td>
<td>66</td>
</tr>
<tr>
<td>RESULTS</td>
<td>74</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>77</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>84</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLES</td>
<td>85</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>90</td>
</tr>
</tbody>
</table>

APPENDICES ........................................................................96
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGURE 2.1 Vitamins and enzymes involved in homocysteine metabolism. Creatine is formed from guanidinoacetate and s-adenosylmethionine.</td>
<td>44</td>
</tr>
<tr>
<td>FIGURE 2.2 Diagram of relationships between blood homocysteine levels, nutrition, and exercise based on current research literature.</td>
<td>45</td>
</tr>
<tr>
<td>FIGURE 3.1 Mean plasma Hcy concentration comparisons between groups (sex and PA levels) in sub-sample (N=20) ANCOVA.</td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE 1.1 Cross Sectional studies examining relationships between levels of physical activity (PA) and blood homocysteine concentrations [Hcy]</td>
<td>10</td>
</tr>
<tr>
<td>TABLE 1.2 Blood homocysteine concentration [Hcy] response to acute exercise in men</td>
<td>11</td>
</tr>
<tr>
<td>TABLE 1.3 Chronic exercise and blood homocysteine concentration [Hcy] response in sedentary individuals</td>
<td>12</td>
</tr>
<tr>
<td>TABLE 2.1 Cross Sectional studies examining relationships between levels of physical activity and blood homocysteine concentrations</td>
<td>46</td>
</tr>
<tr>
<td>TABLE 2.2 Blood homocysteine response to acute exercise in active individuals</td>
<td>47</td>
</tr>
<tr>
<td>TABLE 2.3 Chronic exercise and homocysteine response in active individuals</td>
<td>48</td>
</tr>
<tr>
<td>TABLE 2.4 Chronic exercise and homocysteine response in sedentary individuals</td>
<td>49</td>
</tr>
<tr>
<td>TABLE 3.1 Characteristics of participants divided by sex and physical activity level</td>
<td>85</td>
</tr>
<tr>
<td>TABLE 3.2 Mean plasma homocysteine (Hcy) and B vitamin levels by sex and physical activity level</td>
<td>86</td>
</tr>
<tr>
<td>TABLE 3.3 Mean dietary intakes of energy, macronutrients, and B-vitamins derived from 7-d weighed food records</td>
<td>87</td>
</tr>
<tr>
<td>TABLE 3.4 Sub-sample comparison of extremely active and sedentary for mean plasma homocysteine (Hcy) and plasma B-vitamin levels</td>
<td>89</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TELEPHONE SCREENING TOOL</td>
<td>97</td>
</tr>
<tr>
<td>HEALTH HISTORY QUESTIONNAIRE</td>
<td>99</td>
</tr>
<tr>
<td>PHYSICAL ACTIVITY QUESTIONNAIRE</td>
<td>104</td>
</tr>
<tr>
<td>EATING ATTITUDES QUESTIONNAIRE</td>
<td>107</td>
</tr>
<tr>
<td>MENSTRUAL HISTORY QUESTIONNAIRE</td>
<td>109</td>
</tr>
<tr>
<td>INSTRUCTIONS FOR RECORDING 7 DAYS PHYSICAL ACTIVITY</td>
<td>111</td>
</tr>
<tr>
<td>7-DAY PHYSICAL ACTIVITY LOG</td>
<td>112</td>
</tr>
<tr>
<td>INSTRUCTIONS FOR RECORDING 7 DAY DIETARY RECORDS</td>
<td>116</td>
</tr>
<tr>
<td>7-DAY FOOD INTAKE RECORD</td>
<td>117</td>
</tr>
<tr>
<td>BLOOD COLLECTION PROCEDURES</td>
<td>118</td>
</tr>
<tr>
<td>HOMOCYSTEINE ASSAY PROCEDURES</td>
<td>120</td>
</tr>
<tr>
<td>PLP ASSAY PROCEDURES</td>
<td>124</td>
</tr>
<tr>
<td>FOLATE/VITAMIN B12 ASSAY PROCEDURES</td>
<td>126</td>
</tr>
</tbody>
</table>
Dedicated to…….

My husband ~

You provide a continuing source of support, encouragement, and love. Never have you tried to discourage me from my dreams. Respectfully, you’ve earned a Ph.T. degree for putting honey through. Thank you for the faith you have in me. I love you more than you know.
It is well known that proper nutrition and regular exercise are powerful lifestyle factors associated with lowering chronic disease risk, including cardiovascular disease (CVD) (17). Blood homocysteine (Hcy), a metabolic product of methionine, is recognized as an emerging independent CVD risk factor (10). Factors known to influence Hcy metabolism include dietary intakes of 3 key essential nutrients, folate, vitamin B6 and vitamin B12 (33), and physical activity (21). The overall goal of this research was to examine the relationships between exercise, nutrition, and homocysteine.

First, regular exercise may reduce the risk of developing CVD by lowering resting heart rate, blood pressure and low density lipoprotein (LDL) cholesterol levels, and by increasing high density lipoprotein (HDL) cholesterol levels (1). Second, proper nutrition may reduce CVD risk by maintaining healthy body weight, blood lipid and glucose levels, and ensuring adequate micronutrients for proper cell metabolism (17). Finally, although Hcy’s link with CVD risk is still not completely understood, elevated levels are thought to contribute to atherosclerosis and thrombosis in several ways: (a) endothelial cell injury and endothelial dysfunction, (b) increased platelet adhesiveness, (c) enhance low density lipoprotein oxidation and deposition in the arterial wall, and (d) direct activation of the coagulation cascade (8). Blood Hcy concentrations are generally higher in males than females and in older than younger populations (20). Independently, Hcy, exercise and nutrition may impact CVD, but they also influence one another.
The interrelationship between these factors is briefly summarized below and addressed in greater depth in Chapter 2.

**EXERCISE AND NUTRITION**

Exercise, especially high intensity exercise, stimulates energy producing metabolic pathways. Energy production and the rebuilding and repairing of muscle tissue rely on key micronutrients, such as B-vitamins (B6, B12, folate) to maintain optimal functioning. Therefore, individuals, such as competitive athletes, who exercise at high intensity levels consistently, may not consume adequate amounts of B-vitamins, especially if energy expenditure is high and dietary intake of energy is low and/or lacks B-vitamin nutrient density. Recently, Rousseau et al (2005) found blood Hcy concentrations were lower in athletes with higher dietary intakes of vitamin B6 and folate and higher energy expenditure (> 4,000 kcal/d) compared to athletes with lower dietary intakes of these same nutrients and energy expenditure (< 3,000 kcal/d). Moreover, nutrient density of folate (µg/kcal) was inversely correlated to blood Hcy concentrations in the athletes (r = -0.33; P = 0.004). They concluded that a high energy intake (> 4,000 kcal/d) provides enough dietary folate (> 500 µg/d) to keep blood Hcy levels low, which is favored by higher levels of physical activity (28). This study was done in France, where dietary recommendations are based on total energy expenditure. Thus, the French folate recommendations increase as energy expenditure increases, whereas the U.S. dietary recommendations are not based on energy expenditure (19). Although it is often assumed that active individuals consume healthier diets, research has not supported this notion. For
example, active healthy elderly women did not have a better nutritional profile than their inactive peers (27). Similarly, a younger group (31-45y) of recreational endurance athletes were deficient in serum levels of folate (15%) and/or vitamin B12 (10%) (14). Therefore, being physically active does not necessarily equate to a healthier nutritional status.

**NUTRITION AND HCY**

Hcy metabolism is dependent on adequate folate, vitamin B6, and vitamin B12 to maintain low blood Hcy levels; concentrations >10 µmol/L in a young population is considered unhealthy (4). Hcy metabolism is discussed in detail in Chapter 2 and shown in Figure 2.1; however, a brief overview is given here. Hcy is a sulfur-containing amino acid metabolized from the dietary essential amino acid, methionine. Metabolized mainly in the liver, Hcy can either gain a methyl group and be converted back into methionine with folate and vitamin B12 as cofactors, or be converted into the amino acid, cysteine in a process using vitamin B6 as a cofactor (31). These 2 pathways may be disrupted by a number of factors, which may increase circulating blood Hcy concentrations: (a) inadequate supply of B-vitamins due to low dietary intake (32), (b) high demand for B-vitamins elsewhere in the body, such as during high intensity exercise (22), (c) high dietary intake of methionine and cysteine (7), such as a diet that is high in animal products, since less Hcy is needed to be remethylated to make de novo methionine, (d) medications that influence B-vitamin metabolism like methotrexate (2), which inhibits folate metabolism for treatment of cancer and autoimmune diseases, and
(e) genetic variants involved in Hcy metabolism (11). The presented research will focus on factors described in a, b, and c.

**HCY AND EXERCISE**

While the nutritional aspects of Hcy metabolism are well studied (31, 33), research on the impact of exercise on blood Hcy concentrations are equivocal (5, 9, 12, 15, 16, 21, 23-26, 28-30, 34, 35). Blood Hcy concentrations may increase during high intensity exercise due to 2 primary factors: (a) low methyl group availability due to high demands for methyl groups to produce exercise dependent substrates, such as creatine for muscle contraction, and (b) higher rates of protein turnover to repair damaged tissue, increasing the rate of methionine metabolism.

**RESEARCH ON HCY, EXERCISE & NUTRITION**

Cross-sectional and intervention studies have examined the relationship of Hcy and exercise, but very few have accounted for the nutritional factors that also impact Hcy metabolism. This research is examined in detail in the published review of literature by Joubert and Manore (2006) in chapter 2 (21). New research since chapter 2 was published will be covered in this section.

**Cross-sectional studies.** Seven cross-sectional studies have been published since the review of literature (Chapter 2) examining the association between level of physical activity and Hcy (see Table 1.1). As shown in Table 1.1, none of these studies accounted for both dietary intakes and blood B-vitamin status, two factors well-known to influence Hcy levels. Another main problem in comparing
this research is the variety of ways physical activity levels are described. Questionnaires probing for amount and/or intensity of physical activity are often used to describe participants’ activeness over the previous year. Some studies (9, 30) have used VO$_2$max data as a more objective measure of physical fitness to describe their participants, yet neglect to account for the amount or intensity of exercise participants currently perform. Since genetic predisposition may account for about 40% of the variation in VO$_2$max (6), a better study design would include both VO$_2$max plus a measure of current plus past physical activity practices in order to reveal a relationship with Hcy. Two studies have examined the relationship between cardiorespiratory fitness levels (estimated VO$_2$max) and blood Hcy concentrations in male and female adolescents (~15 y) and young adults (~30y) (9, 30). Both studies found high fitness levels were associated with low Hcy levels only in their female participants, but not in males. Authors speculate that it was due to high fitness levels in women, low sample size, or hormonal differences between men and women. Two studies using different methods to determine level of PA found higher PA levels were associated with lower Hcy levels. The first study divided a large group of middle-aged women into 2 groups based on estimated energy expenditure (<1,000 kcals or ≥ 1,000 kcals) calculated from a questionnaire. This questionnaire asked participants to estimate the average time per week spent over the past year on different types of recreational activities and then assigned a metabolic equivalent task score to each activity, which calculated PA energy expenditure (24). The second study used a questionnaire with 3 levels of leisure time activity (sedentary,
walking/biking 4 h/wk, and run/swim/tennis 2 h/wk or regular hard exercise or competitive sport activities) in older men and women (63 y) (3).

However, others have not found significant associations between physical activity level and blood Hcy concentrations. Rousseau et al used young (~28 y) male competitive athletes and grouped them in 2 ways; based on estimated energy expenditure (sedentary, 2,200 kcals, 3,100 kcals, 4,000 kcals) calculated from 7-d activity logs and based on the type of sport they practiced (intermittent, aerobic, anaerobic) (28). They found plasma Hcy concentrations were lower in athletes with the highest energy expenditure compared to the sedentary and 2,200 kcal groups. However, once dietary intake of folate was accounted for, the differences between groups was no longer significant (28). Ruiz et al. used both VO$_2$max and an accelerometer to measure current fitness levels and physical activity practices in children (9 y) and adolescents (16 y) and found no association between blood Hcy levels, physical activity, or fitness (29). Finally, Husemoen et al examined a large group of men and women (30-60y) and found no associations between physical activity level and Hcy using questionnaires with 3 physical activity levels (sedentary, moderate activity, and regular exercise or heavy training) (16).

**Intervention trials.** Eight intervention trials have investigated the effects of acute exercise (1 bout) or chronic exercise training (3-5 x wk; 8-24 wks) on blood Hcy concentrations in both sedentary and well-trained men and women. Three
studies examined the effect of acute exercise on blood Hcy levels (Table 1.2). Results showed that marathon running increased blood Hcy levels by 19% in novice male runners (26), while brisk walking on a treadmill at 70-80% of maximal heart rate produced a 7% increase in blood Hcy levels in sedentary men (12). Yet, competitive well-trained men and women had no change in blood Hcy levels during the Hawaii ironman triathlon (~12 h) (23) possibly due to the following factors: weight loss, hemodynamics, or consumption of highly fortified foods during the race. In the five chronic exercise training studies shown in Table 1.3, decreases in blood Hcy levels have most commonly been reported in participants with elevated baseline Hcy levels (>15 µmol/L) (25) or poor baseline fitness levels (34, 35). However, no significant changes in blood Hcy levels were found in response to moderate stair-climbing for 8 weeks in sedentary women (5) or in sedentary men and women after a brisk walking program for 6 weeks (12). Perhaps no significance was found because B-vitamin status was not accounted for or the training wasn’t intense enough to elicit blood Hcy changes.

In summary, the current research examining the relationship between exercise and blood Hcy levels is equivocal due to a variety of study designs and inadequate control of confounding variables. Furthermore, there is much discussion in the B-vitamin/exercise literature about exercise increasing the need for the B-vitamins, but at the moment these data are limited. For example, Hansen et al found dietary vitamin B6 requirements of 1.5 to 2.3 mg/d dietary were necessary to keep blood Hcy levels in young healthy
sedentary women (13). These amounts of dietary vitamin B6 are higher than the current U.S. RDA of 1.3 mg/d (18). An understanding of the interrelationships among exercise, dietary intakes of B-vitamins, and blood Hcy concentrations is necessary in order to make sure our dietary recommendations accommodate increased metabolic demands placed on the body. Therefore, the purpose of this study is to examine whether more physically active individuals, practicing the current physical activity guidelines, put forth by the Institute of Medicine Food and Nutrition Board (IOM) (19), have higher concentrations of blood Hcy than their less active counterparts, while accounting for B-vitamin status.

**RESEARCH QUESTIONS**

**Question 1.** If B-vitamin status is controlled, are blood Hcy levels different in very active individuals compared to less active individuals?

**Hypothesis:** Non-supplementing (no B-vitamin or creatine supplements for ≥ 30d) individuals participating in regular physical activity at the recommended levels (equivalent of walking 4 mph or faster > 7 h/wk) will have higher blood levels of Hcy compared to less active individuals (equivalent of walking 4 mph or faster < 7 h/wk), independent of B vitamin status.

**Outcome Variables:**

- Plasma Hcy (µmol/L)
Question 2. How does B-vitamin status influence blood Hcy levels in very active compared to sedentary individuals?

**Hypothesis:** Highly active individuals with lower levels of all three blood B vitamins will have higher Hcy levels than highly active individuals with higher blood B vitamin levels.

**Outcome Variables:**
- Plasma Hcy (µmol/L)
- Plasma folate (nmol/L), plasma vitamin B12 (nmol/L), Plasma vitamin B6 as pyridoxal 5’ phosphate (PLP) (nmol/L)
- Absolute dietary intakes of folate (µg/d), vitamin B12 (µg/d), vitamin B6 (mg/d)
- Relative dietary intakes of folate (µg/1000kcal/d), vitamin B12 (µg/1000kcal/d), vitamin B6 (mg/1000kcal/d; mg/g/protein/d)
Table 1.1. Cross Sectional studies examining relationships between levels of physical activity (PA) and blood homocysteine concentrations [Hcy].

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample number/sex</th>
<th>Measure of Physical Activeness</th>
<th>[Hcy] &amp; physical activity level</th>
<th>Vitamin Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>(16)</td>
<td>1349 M 1439 W 30-60 y</td>
<td>Questionnaire: 3 activity levels</td>
<td>No association</td>
<td>Not reported</td>
</tr>
<tr>
<td>(29)</td>
<td>163 M 138 F children 9 y 185 M 194 F adolescents 16 y</td>
<td>Accelerometer calculated METS VO$_2$max</td>
<td>No association</td>
<td>Intake (folate, B12)</td>
</tr>
<tr>
<td>(30)</td>
<td>76 M 80 F adolescents</td>
<td>VO$_2$max</td>
<td>High CV fitness of F (very fit) associated w/ low Hcy levels</td>
<td>Blood (folate, B12)</td>
</tr>
<tr>
<td>(9)</td>
<td>49 M 11 F 30 y</td>
<td>VO$_2$max</td>
<td>High CV fitness of F (very fit) associated w/ low Hcy levels</td>
<td>Intake (folate, B6, B12)</td>
</tr>
<tr>
<td>(3)</td>
<td>537 M 571 F 63 y</td>
<td>Questionnaire: 3 activity levels</td>
<td>Low PA levels associated with high Hcy levels</td>
<td>Not reported</td>
</tr>
<tr>
<td>(24)</td>
<td>27,158 F 55 y</td>
<td>Questionnaire: calculated METS</td>
<td>Low PA levels associated with high Hcy levels</td>
<td>Not reported</td>
</tr>
<tr>
<td>(28)</td>
<td>82 M 28 y</td>
<td>Athletes grouped by sport: (aerobic, anaerobic, intermittent)</td>
<td>No association</td>
<td>Intake (folate, B6, B12, methionine)</td>
</tr>
</tbody>
</table>

1 M male; F female
2 Physical activity was measured using questionnaires, accelerometers, and/or estimated VO$_2$max values. Activity levels were determined by reported activity h/wk, category activity levels (i.e. low, moderate, heavy), estimated energy expenditure (kcal or metabolic equivalents), or VO$_2$max values.
3 [Hcy] Blood homocysteine concentrations
4 Dietary intake data was reported using, 24-h dietary recall, food frequency questionnaire, or 7-d food record, respectively.
METS metabolic equivalents
Table 1.2: Blood homocysteine concentration [Hcy] response to acute exercise in men.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample number/sex description</th>
<th>Exercise Intensity &amp; Mode</th>
<th>[Hcy] response to exercise</th>
<th>Vitamin Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>(26)</td>
<td>22 M novice runners</td>
<td>competitive marathon run</td>
<td>19% ↑</td>
<td>Blood (folate, B12)</td>
</tr>
<tr>
<td>(23)</td>
<td>36 M &amp; F well-trained</td>
<td>competitive ironman triathlon</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(12)</td>
<td>13 M &amp; 9 F sedentary</td>
<td>70-80% of MHR treadmill walking</td>
<td>7% ↑</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

1 M male; F female
2 [Hcy] Blood homocysteine concentrations
% percent change in homocysteine values from pre- to post-exercise
↑ increase in homocysteine concentrations from pre- to post-exercise
≠ no change in homocysteine concentrations
MHR maximum heart rate
Table 1.3. Chronic exercise and blood homocysteine concentration ([Hcy]) response in sedentary individuals.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample number/sex&lt;sup&gt;1&lt;/sup&gt; description</th>
<th>Training Program mode, intensity, duration, frequency &amp; intervention length</th>
<th>[Hcy]&lt;sup&gt;2&lt;/sup&gt; response to training</th>
<th>Vitamin Status Blood/Intake&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5)</td>
<td>15 W sedentary</td>
<td>Stairclimbing &lt;i&gt;moderate&lt;/i&gt; 11 min/d 5 x wk for 8 wks</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(34)</td>
<td>49 sedentary</td>
<td>Resistance training &lt;i&gt;moderate&lt;/i&gt; 3 x wk for 24 wks</td>
<td>5% ↓</td>
<td>Not reported</td>
</tr>
<tr>
<td>(35)</td>
<td>16 M 27 W sedentary</td>
<td>Resistance training &lt;i&gt;low verses high&lt;/i&gt; 3 x wk for 24 wks</td>
<td>5% ↓</td>
<td>Intake (folate, B6, B12)</td>
</tr>
<tr>
<td>(25)</td>
<td>365 M 451 F sedentary</td>
<td>Cycle ergometer &lt;i&gt;mod to high&lt;/i&gt;: 30-50 min 3 x wk for 20 wks</td>
<td>15% ↓ or *</td>
<td>Blood (folate, B6, B12)</td>
</tr>
<tr>
<td>(12)</td>
<td>15 M 14 F sedentary</td>
<td>Brisk walking &lt;i&gt;moderate&lt;/i&gt; 6.4-7 km/h 30 min 3 x wk for 6 wks</td>
<td>≠</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

<sup>1</sup> M male; F female

<sup>2</sup> [Hcy] Blood homocystein concentrations

<sup>3</sup> Dietary intake was measured by 3-d diet records

CAD coronary artery disease

≠ no change in homocysteine concentrations

↓ lower [Hcy] at the end of program compared to baseline

↑ higher [Hcy] at the end of program compared to baseline

% percent change in [Hcy] from pre- to post-intervention

* High [Hcy] (>15 µmol/L; n=30) compared to low [Hcy] (<15 µmol/L; n=681)
REFERENCES


Lanae M. Joubert and Melinda M. Manore
Homocysteine is an independent cardiovascular disease (CVD) risk factor modifiable by nutrition and possibly exercise. While individuals participating in regular physical activity can modify CVD risk factors, such as total blood cholesterol levels, the effect physical activity has on blood homocysteine concentrations is unclear. This review examines the influence of nutrition and exercise on blood homocysteine levels, the mechanisms of how physical activity may alter homocysteine levels, the role of homocysteine in CVD, evidence to support homocysteine as an independent risk factor for CVD, mechanisms of how homocysteine increases CVD risk, and cut-off values for homocysteinemia. Research examining the effect of physical activity on blood homocysteine levels is equivocal, which is partially due to a lack of control for confounding variables that impact homocysteine. Duration, intensity, and mode of exercise appear to alter blood homocysteine levels differently, and may be dependent on individual fitness levels.

Key Words: physical activity, cardiovascular disease risk factor, diet, vitamin, intensity, nutrient status

INTRODUCTION
The beneficial effects of exercise for the primary and secondary prevention of cardiovascular disease (CVD) are well established (4). Among patients with established CVD, mortality is lower among those who undertake regular exercise with the level of protection dependent on the type, duration, frequency, and intensity of the activity stimulus (38). In addition, increased physical activity in previously sedentary individuals improves known CVD risk factors, such as blood pressure, total blood cholesterol levels, and blood lipid profiles. However, it is unclear if exercise improves or modifies recently identified CVD risk factors such as circulating levels of C-reactive protein and homocysteine.

Homocysteine was first identified as an important biological compound in 1932 (11) and linked with human disease in 1962 (89) when elevated urinary homocysteine levels were found in children with mental retardation. This condition, called homocysteinuria, was later associated with premature occlusive CVD, even in children. These observations led to research investigating the relationship of elevated homocysteine levels and CVD in a wide variety of populations including middle-aged and elderly men and women with and without traditional risk factors for CVD (6, 16, 62).

Based on the above studies, researchers have identified elevated blood homocysteine as an independent risk factor for CVD further stimulating research examining various factors that impact blood homocysteine levels. We now know
a number of factors influence homocysteine levels, such as age, gender, genetics, medications and lifestyle factors, such as alcohol intake, smoking, nutrition, and physical activity (83).

This review focuses on the impact of two lifestyle factors, nutrition and physical activity on blood homocysteine concentrations. First, homocysteine metabolism is described. Second, the role that nutrition, especially the B-vitamins, has on homocysteine metabolism is reviewed. Third, the mechanisms of how physical activity may alter homocysteine levels are presented. Fourth, the role of homocysteine in CVD is discussed including evidence to support homocysteine as an independent risk factor, mechanisms related to homocysteine and CVD, and a definition of homocysteinemia. Finally, a review of the epidemiological, cross-sectional, and experimental research examining the impact of physical activity on blood homocysteine levels are presented.

HOMOCYSTEINE METABOLISM

Homocysteine is a normal metabolite of the essential amino acid methionine (Figure 2.1). Structurally, it closely resembles methionine and cysteine; all three amino acids contain sulfur. They are metabolically linked to each other as shown in Figure 2.1, methionine is first converted to s-adenosyl-methionine and further processed to s-adenosyl-homocysteine before conversion to homocysteine. Once converted to homocysteine, it has 2 fates: (1) remethylation from homocysteine back into methionine or (2) transsulfuration from homocysteine
into cystathionine with further metabolism to cysteine. Four key enzymes are involved in this complex metabolic pathway, methyl-tetrahydrofolate reductase, methionine synthase, cystathionine-β-synthase, and cystathionine-γ-lyase, which rely on vitamins as cofactors to function properly (Figure 2.1). For more biochemical details of homocysteine metabolism refer to these sources (12, 72).

**Role of Nutrition.** Homocysteine metabolism is driven by several B-complex cofactors. Folate, vitamin B6, and vitamin B12 are used in the remethylation pathway; and vitamin B6 is used in the transsulfuration pathway (Figure 2.1). Deficiencies of folate, vitamin B6 or vitamin B12 can lead to impaired homocysteine metabolism. In addition, the amount of dietary methionine consumed influences homocysteine metabolism. Thus, these factors will either increase or decrease blood homocysteine, depending on dietary levels.

**Dietary Methionine.** When dietary methionine intake is high, the transsulfuration pathway is promoted over remethylation resulting in homocysteine catabolism to cysteine. Conversely, when dietary methionine is low, the remethylation pathway is favored over transsulfuration to elicit de novo methionine synthesis. A methionine-rich meal (e.g. meal high in animal protein) has been shown to cause an acute increase in homocysteine, which may last up to 24 hours in healthy adults (35). Further, methionine-loading tests are often used to study the efficiency of homocysteine metabolism (2). Dietary methionine is found in protein rich foods such as red meat, poultry, and cheese. Individuals consuming diets high in animal protein have reported increased methionine metabolism and higher homocysteine levels than vegetarians (50). However, some vegetarians
and strict vegans may have low dietary intakes of methionine and vitamin B12 resulting in high blood homocysteine levels (44). Thus, individuals consuming lower meat and animal protein diets with adequate intakes of B-vitamins may be able to keep homocysteine concentrations low (24).

**B-Vitamin Status.** The B-vitamins play critical roles in both the remethylation and transulfuration pathways of methionine metabolism (Figure 2.1). The enzyme, methionine synthase, which is involved with the remethylation reaction converting homocysteine to methionine, requires both folate and vitamin B12 as coenzymes. The transsulfuration pathway converts homocysteine to cysteine in a series of vitamin B6 dependent reactions, which requires vitamin B6 as a coenzyme for the enzymes cystathionine-β-synthase and cystathionine-γ-lyase. Therefore, adequate folate, vitamin B12, and vitamin B6 are critical for the enzymes in these pathways to function optimally in order to maintain homocysteine homeostasis. Both the amount of dietary intake and the blood vitamin levels of an individual contribute to their nutritional status. Reduced dietary intake and/or blood levels of these vitamins contribute to an accumulation of blood homocysteine.

Folate insufficiency is the predominant nutritional cause of elevated blood homocysteine in most healthy populations. Research indicates that individuals with diets high in folate or folic acid supplements are able to reduce blood homocysteine concentrations (9, 10, 87), especially in individuals with low folate status and high homocysteine levels (20). Research also shows that folic acid supplements given to individuals with normal folate status can further lower
homocysteine concentrations (46). Vitamin B12 and, to a lesser extent, vitamin B6 supplements are also effective at lowering homocysteine, either individually or in combination with folic acid supplements in individuals who have high blood homocysteine levels (77, 80). Therefore, a major contributing factor to high levels of blood homocysteine is a diet low in these three vitamins.

Based on recent data, not all Americans, especially women, consume enough folate, vitamin B6, or vitamin B12, even with the 1998 mandate of folic acid fortification to cereal grain products (25). According to NHANES data (2001-2002), the percentage of females (19-50y) consuming less than the Estimated Average Requirement (EAR) of folate, vitamin B6, and vitamin B12 were 15%, 22%, and 8%, respectively (61). Additionally, not all Americans, including active individuals, have optimal blood B-vitamin levels (64). Fogelholm et al. (1993) examined vitamin B6 status in 42 physically active college students (18-32 y) before and after 5 weeks of vitamin B-complex supplementation. Prior to supplementation, they reported 43% had poor vitamin B6 status using erythrocyte aspartate aminotransferase activity coefficient as a marker for vitamin B6 status (29). Herrmann et al. (2005) recently examined vitamin B12 and folate status in 72 recreational endurance athletes (31-45 y) (41). They found 10% of the athletes deficient in vitamin B12 and 15% deficient in folate based on serum levels. Thus, being physically active does not necessarily equate to a healthier nutritional status. Active and inactive individuals may have poor nutritional
status, which may influence homocysteine levels independent of the amount, intensity or type of exercise.

**Role of Physical Activity.** In addition to adequate nutrition, there is evidence that physical activity may also alter homocysteine production by increasing protein and/or methyl group turnover. These hypothesized mechanisms are discussed below.

**Protein Turnover.** During exercise, protein turnover could alter homocysteine concentrations by either increasing methionine catabolism, thus lowering homocysteine, or by decreasing B-vitamin availability, which would increase homocysteine.

It is well documented that prolonged high intensity exercise (33, 84) increases protein metabolism and alters blood concentrations of certain amino acids (30). For example, Weiss et al. (1999) reported a 33% reduction in blood methionine levels after a 2.5 h moderate intensity run in college students (88). Reduced methionine availability would promote *de novo* methionine synthesis and, thus, reduce accumulation of homocysteine. In this way, the protein turnover mechanism would lower homocysteine concentrations during high intensity prolonged exercise, as long as folate, vitamins B6 and B12 were adequate.

Conversely, prolonged exercise, where glycogen reserves are reduced, places an increased demand on vitamin B6 dependent reactions. Pyridoxal 5’ phosphate (PLP), the most biologically active form of vitamin B6, is a coenzyme
for transaminases, decarboxylases, and other enzymes used in the metabolic transformations of amino acids and nitrogen containing compounds. PLP is also required for glycogen phosphorylase, the key enzyme in the breakdown of muscle glycogen. In addition, during exercise, gluconeogenesis involves the breakdown of amino acids, with the carbon skeleton used for energy. If exercise increases the demand for vitamin B6 or increases its losses, less vitamin B6 would be available for homocysteine catabolism. In this way, increased protein turnover during prolonged exercise would increase homocysteine concentrations.

**Methyl Group Turnover.** High intensity exercise elicits an increase in methyl group turnover, which could increase homocysteine production. As shown in Figure 1, methionine is first converted to s-adenosyl-methionine, which is the most important methyl group donator in humans. A sufficient supply of methyl groups is important in several biochemical pathways, of which many are exercise related, such as the synthesis of DNA, RNA, carnitine, choline, acetycholine, phosphatidylcholine, epinephrine, adrenalin, methylhistadine, and creatine (60, 72, 82). Creatine synthesis in the liver accounts for nearly 75% of daily homocysteine formation (72), where s-adenosyl-methionine donates its methyl group to guanidinoacetate to form creatine and s-adenosyl-homocysteine (Figure 2.1). High intensity exercise relies on creatine phosphate for muscle contractions, where creatine reacts with the adenosyl-triphosphate (ATP) produced by glycolysis and oxidative phosphorylation, to form ADP and creatine phosphate. During exercise, when muscle ATP is being consumed, the high-energy phosphate group of creatine phosphate is transferred to ADP to restore
ATP. Creatine is then recycled or converted to creatinine, which is excreted in the urine. Thus, high-intensity long-duration physical activity, which increases the demand for creatine, increases homocysteine production compared with less-intense short-duration physical activity. Further, when creatine is taken orally, the endogenous production of creatine decreases, which also decreases the endogenous production of homocysteine. A recent study examined the effects of a 4-week oral creatine supplementation (amount of creatine taken each day was equal to twice their creatinine excretion; 2.1 to 5.1 g/d) in healthy adults ranging in age from 21-58 y (48). The experimental group had a significant reduction in homocysteine production (n=8; -0.9 µmol/L) compared to the control group (n=8; +0.2 µmol/L)(p<0.05), supporting that when endogenous creatine is made, blood homocysteine levels may rise. Thus, an increase in methyl group turnover increases homocysteine production.

**HOMOCYSTEINE AND CVD**

For many individuals, CVD cannot be fully explained by the traditional risk factors such as hypertension and high blood cholesterol levels. For example, 35% of coronary heart disease cases occur in individuals with total cholesterol levels < 200 mg/dL (54). Other risk factors, such as blood homocysteine concentrations, appear to have strong relationships with CVD. This section reviews evidence supporting homocysteine as an independent risk factor for CVD, discusses the proposed mechanisms whereby homocysteine contributes to CVD, and
examines the levels of blood homocysteine concentrations associated with increased CVD risk.

**Homocysteine as a CVD Risk Factor.** Extensive research supporting high blood homocysteine levels as an independent CVD risk factor is summarized in current meta-analyses, review articles, and clinical trials. The first meta-analysis was completed by Boushey et al. (1995), who included 27 studies involving more than 4,000 patients with occlusive vascular disease (cardiovascular, peripheral, and cerebrovascular) and an equal number of controls (8). Results showed that homocysteine was an independent risk factor for atherosclerotic disease in the coronary, cerebral, and peripheral vessels, and that a 5 µmol/L increment increase in total plasma homocysteine levels was associated with a 60% increased risk for coronary heart disease in men and an 80% increased risk for women (8). This study was followed by two more meta-analyses that also supported the relationship between high homocysteine levels and CVD (15, 86). The Homocysteine Studies Collaboration (15), a compilation of prospective and retrospective studies using a total of 5,073 heart disease events and 1,113 stroke events, found that a 25% lower than usual blood homocysteine concentration (~3 µmol/L lower) was associated with an 11% lower heart disease risk (15). In addition, Wald et al. (2002) completed a meta-analysis that used 72 studies in which the prevalence of a mutation in the methyl-tetrahydrofolate reductase gene, which increases homocysteine, was determined in cases and controls (n=16,849) as well as 20 prospective studies with 3,820 participants (86). They concluded from these studies that by lowering blood homocysteine
concentrations by 3 µmol/L from current levels, the risk of ischaemic heart
disease was reduced by 16% and deep vein thrombosis reduced by 25% (86).
Finally, Refsum and Ueland (1998) completed an extensive review examining the
relationship between CVD mortality and blood homocysteine concentrations (66).
They reviewed 80 studies, including more than 10,000 patients of cross-
sectional, case control, nested case control, and cohort populations and found
that blood homocysteine concentration was a prevalent and strong risk factor for
atherosclerotic vascular disease in the coronary, cerebral, and peripheral
vessels, and for arterial and venous thromboembolism. They concluded high
blood homocysteine levels confers a graded increased risk with no threshold, is
independent of and may enhance the effect of the conventional risk factors, and
seems to be a particularly strong predictor of cardiovascular mortality.

Clinical trials have also examined the association between myocardial infarct (MI)
and increased homocysteine levels. Stampfer et al. (1992) completed a clinical
trial using 14,916 male physicians, aged 40 to 84 years, with no prior MI (75).
They measured plasma homocysteine concentrations at baseline and after 5
years. Blood samples from 271 men who subsequently developed MI were
analyzed for homocysteine levels together with paired controls, matched by age
and smoking. They concluded that moderately high levels of plasma
homocysteine were associated with subsequent risk of MI independent of other
coronary risk factors (75). This finding was supported by a more recent clinical
trial by Zylberstein et al. (2004), where 1,368 women were followed for 24 years
These researchers found that blood homocysteine levels in excess of 14.2 µmol/L in middle-aged women was an independent risk factor for future MI, particularly fatal events (93). Collectively, these studies strongly suggest that elevated blood homocysteine levels increase risk of CVD, independent of other CVD risk factors.

**Mechanism: How Does Elevated Blood Homocysteine Increase CVD Risk?**

Homocysteine appears to have a wide variety of adverse effects on vascular physiology that contributes to increased cardiovascular risk. The most commonly suggested mechanisms explaining the link between CVD and homocysteine are endothelial dysfunction and platelet aggregation/thrombosis. These mechanisms are briefly reviewed here.

**Endothelial Dysfunction.** Homocysteine may promote atherosclerosis by inducing endothelial dysfunction, which is characterized as a loss in vasodilation control in blood vessel cells. In cell culture studies, homocysteine inhibits endothelium-dependent anticoagulant reactions (39, 52, 68), induces the expression of procoagulants (31, 68), decreases interactions between endothelial cells and plasminogen activators (36, 37), and impairs the bioavailability of endothelium-derived nitric oxide (74). These findings are supported by animal studies in mini-pigs and rats, which have showed specific structural abnormalities in the large arteries with elevated blood homocysteine levels (13, 59, 69).

Studies in cynomolgus monkeys also showed that high blood homocysteine levels impaired responses to endothelium-dependent vasodilators, such as nitric oxide (53). Nitric oxide allows the smooth muscle of blood vessels to relax or
dilate, which creates greater blood flow when the demand for oxygen is high, such as during exercise. Therefore, an impaired response to nitric oxide would inhibit blood vessel dilation.

Human studies also support a relationship between high blood homocysteine levels and endothelial dysfunction via impaired nitric oxide release (70, 78). Degradation of nitric oxide via abnormal interaction with the free thiol moiety of homocysteine may decrease the bioavailability of nitric oxide. Thiols are proposed to react with nitric oxide to form s-nitrosothiols, which have both potent vasodilatoin and antiplatelet effects. Woo et al. (1997) found endothelial-dependent dilation was significantly lower in subjects with high homocysteine levels when compared to those with low homocysteine levels (p<0.001) (91). In addition, Dinckal et al. (2003) demonstrated the relationship between homocysteine and endothelial-dependent dilation by placing subjects on a 4-week homocysteine lowering diet, which included B-vitamin supplements (26). They found significant reductions in blood homocysteine concentrations and significant improvements in endothelial-dependent dilation in comparison to the placebo group (26).

**Platelet Aggregation and Thrombosis.** Platelet aggregation is a clustering of platelets and/or blood cell fragments, which can lead to the formation of blood clots. Thrombosis is the formation or presence of one or more blood clots that may partially or completely obstruct the flow of blood through the circulatory system. The most convincing research that supports homocysteine’s role in
platelet aggregation and thrombosis is via oxidative stress mechanisms. The oxidative damage caused by hydrogen peroxide during the oxidation of homocysteine may increase platelet activity. Specifically, the SH group of homocysteine is oxidized to a disulfide bond (-S-S-) in a reaction that is coupled to the formation of reactive oxygen species, such as hydrogen peroxide. In turn, these reactive species cause endothelial dysfunction, decrease nitric oxide production, and therefore accelerate atherosclerosis (34). However, direct generation of reactive oxygen species by homocysteine is unlikely to have physiological relevance, but it is probable that oxidative stress is a secondary effect. See the review by Edirisinghe (2004) for this mechanism (28). Animal studies done in mice support a relationship between high blood levels of homocysteine and oxidative stress by activating signal transduction pathways leading to inflammation and apoptosis (90). However, in humans it has been much more difficult to find this relationship. Selhub (1999) argues that human studies provided inconclusive results when looking for thrombogenic abnormalities in patients with high blood levels of homocysteine (72). He suggested the abnormalities observed in human studies are due to inconsistencies in genetic background, dietary habits, and pathology differences among sample populations.

In summary, cell culture, animal and human research all support a relationship between high homocysteine levels and endothelial dysfunction. It appears high blood homocysteine levels are partially responsible for endothelial dysfunction
and high concentrations may attenuate platelet aggregation and thrombosis. Currently, not one unifying hypothesis exists that explains the mechanistic effects of high levels of circulating homocysteine on CVD.

**What is Elevated Blood Homocysteine?** What level of blood homocysteine is associated with a higher risk of CVD? Some researchers argue that the lowest level of blood homocysteine possible is ideal (85); however, not everyone agrees on a single value or range of homocysteine that represents the lowest risk of CVD. The term hyperhomocysteinemia is used to describe an individual with elevated blood homocysteine concentrations, yet there is no standard upper level cut-off value. Hyperhomocysteinemia values vary among published reports, with studies typically using the 95th percentile values as a high cut-off point in their reported control samples.

Stamper et al. (1992) was the first to publish reference ranges based on the 95th percentile cut-off point. They reported a 95th percentile value of 15.8 µmol/L for U.S. white men (75). In South Africa, Ubbink et al. (1995) used men’s responses to vitamin supplementation to develop a mathematical prediction model to calculate the plasma homocysteine concentration that could be expected for individuals treated with a vitamin supplement (81). They predicted that plasma homocysteine concentrations would approach a normal frequency distribution with a 95% reference range of 4.9 – 11.7 µmol/L for adult white men, provided that the vitamin status of the study population is improved (81). Data extracted
from the Third National Health and Nutrition Examination Survey (NHANES 1991-1994) helped to identify reference ranges for serum total homocysteine concentration in U.S. men and women (73). For individuals aged 20-39 y, the 95th percentile for blood homocysteine concentrations were \( \geq 11.4 \mu\text{mol/L} \) for men and \( \geq 10.4 \mu\text{mol/L} \) for women.

Finally, the Nutrition Committee of the American Heart Association suggested a blood homocysteine level <10 \( \mu\text{mol/L} \) is a reasonable therapeutic goal for individuals at increased risk. They determined it was better to have a goal level of blood homocysteine rather than using the definition of "normal" based on population statistical values of the mean \( \pm 2 \) standard deviations (56).

Regardless of the upper cut-off values for blood homocysteine, risk for CVD rises with increasing homocysteine concentrations. In general, blood homocysteine concentrations increase with age and are higher in men than in women. A review by Boushey et al. (1995), found a 60-80% greater risk for CVD for each 5 \( \mu\text{mol/L} \) increment of plasma homocysteine concentration > 10 \( \mu\text{mol/L} \) and suggested this increase in risk is similar to a 0.5 mmol/L (20mg/dL) increase in total blood cholesterol (8). Furthermore, as mentioned earlier, some reports suggest that reducing blood homocysteine concentrations by 3 \( \mu\text{mol/L} \) reduces chronic disease risk by as much as 25% (15, 86). However, recent clinical trials using blood homocysteine lowering therapies (B-vitamin supplementation vs. placebo) in individuals with previous heart attacks or strokes have not been
successful at lowering risk of recurrent events with supplementation (7, 79). These data suggest maintaining the lowest possible blood homocysteine concentration throughout the lifespan may help keep chronic disease risk low.

**PHYSICAL ACTIVITY & HOMOCYTEINE**

Increased physical activity in previously sedentary individuals modifies known risk factors for CVD, including improvements in circulating levels of total serum cholesterol, blood pressure, and cardiorespiratory fitness \( (VO_{2\text{max}}) \) (5). Although the effects of exercise on these traditional risk factors are well documented, few studies have addressed whether physical activity can modify blood homocysteine. This section reviews the current literature that examines the relationship between physical activity and blood homocysteine concentrations in epidemiology, cross-sectional, and intervention/experimental studies.

**Epidemiology Research.** Currently, there has only been one large population-based study that examines the relationship between lifestyle activeness and blood homocysteine concentrations. Nygard et al. (1995) examined the relationship between physical activity and homocysteine in the Hordland Homocysteine Study (Norway) (63). They found self-reported leisure time physical activity was negatively associated with blood homocysteine concentrations \( (p<0.001) \) in both men and women. Participants reported their leisure time physical activity for the year prior to the study in one of 4 categories (sedentary/none, moderate activity, active exercise, heavy training). Those individuals with the highest leisure time physical activity had the lowest levels of
blood homocysteine. The difference in blood homocysteine levels between the sedentary and highly active groups was 0.76 $\mu$mol/L in men and 0.94 $\mu$mol/L in women; however, very few individuals were in the highly active groups (147 men and 51 women; total N = 12,263). Unfortunately, B-vitamin status was not reported for these individuals. However, when the researchers statistically adjusted for higher fruit and vegetable consumption and supplement use, they found significantly lower blood homocysteine levels ($p<0.01$) (63). This study stimulated interest in the relationship between physical activity, diet, and homocysteine levels and prompted many of the research studies discussed in the next section.

**Cross-Sectional Research.** To date, 9 cross-sectional studies have examined the association between physical activity and homocysteine by using self-reported questionnaires, personal standardized interviews, and/or fitness levels determined by measured VO2max to describe individual physical activity levels (Table 2.1). Two of these studies (55, 71) found no significant relationship between the level of blood homocysteine concentrations and physical activity levels. One study by Saw et al. (2001) found higher amounts of physical activity was associated with lower homocysteine levels in Chinese men and women before statistically controlling for folate status, but after multivariate adjustment there was no difference between exercisers and non-exercisers (71). Unfortunately, individuals in this study were considered exercisers if they self-reported participating in at least 0.5 h of physical activity a week, such as brisk walking, bowling, tai chi, chi kung, jogging, tennis, or swimming laps. This
duration of activity may be too low to determine whether blood homocysteine levels are different between exercisers and non-exercisers. Two strengths of this study were that B-vitamin status was evaluated using both dietary intake and blood measurements and hours of television watching was reported to further understand the activeness of their participants (71).

Five cross-sectional studies showed lower blood homocysteine levels in individuals reporting higher levels of physical activity (14, 18, 40, 49, 51), but none of these studies statistically adjusted for B-vitamin status based on blood measures and dietary intake.

Finally, two cross-sectional studies showed a positive relationship between homocysteine and physical activity levels. Rinder et al. (2000) reported higher homocysteine levels in exercisers when compared to non-exercisers. They compared 10 competitive master male athletes (68.5±1.4 y) to inactive controls (64.5±2.3 y) and found blood homocysteine concentrations in the athletes to be significantly higher (10.7±1.3µmol/L) when compared to controls (9.2±1.4µmol/L)(p=0.02) (67). De Bree et al. (2001) also found a similar relationship between physical activity and homocysteine, but only in women (19). They examined a random sample of Dutch men and women (20-65 y) and found no association between physical activity and homocysteine levels in men. However, for women, they found a positive relationship between physical activity and plasma homocysteine levels while adjusting for age, dietary folate intake,
vitamin supplement use, and other lifestyle factors. Thus, as hours of physical activity increased, so did blood homocysteine levels in females (19).

In summary, these 9 cross-sectional studies reported equivocal results with no clear consensus as to whether physical activeness negatively or positively impacts blood homocysteine levels. There are a number of confounders that may have contributed to the mixed observations in these studies: (1) nutritional status parameters were not assessed or accounted for, (2) age ranged widely within each study, (3) individuals self-reported their physical activity levels, which are often overestimated, and (4) level of physical activity and/or fitness level was defined differently among the studies making comparisons between studies difficult.

**Exercise Interventions.** Many experimental studies have examined the impact of physical activity on homocysteine levels (Tables 2.2-2.4) with varying degrees of exercise intensity and length of exercise interventions. Therefore, these studies are divided into 3 categories: (1) acute exercise defined as one episode of physical activity lasting between 10 to 210 minutes, (2) chronic exercise defined as a physical activity program lasting 10 days or more in previously active individuals and (3) in previously sedentary individuals.

**Acute Exercise.** Seven studies have examined the effect of acute exercise on blood homocysteine levels in active individuals. As summarized in Table 2.2, three studies found no effect (21, 22, 92), three studies found exercise to
increase homocysteine levels (42, 47, 88), while one study found acute exercise to decrease blood homocysteine levels (32).

The three studies reporting an increase in homocysteine levels with acute exercise had 60 minutes or longer duration of physical activity, but none of these studies statistically adjusted for B-vitamin status based on both blood measures and dietary intake (42, 47, 88). The three studies finding no effects of acute exercise on blood homocysteine concentrations were all < 60 minutes in duration and used moderate intensity stationary cycling, possibly not rigorous enough to elicit changes (21, 22, 92).

Two factors that may influence the effect of physical activity on blood homocysteine levels are exercise mode and intensity. For example, Herrmann et al. (2003) compared blood homocysteine levels of marathon runners, 100km runners, and mountain bikers before and after their respective races (42). Only marathon runners had significantly higher homocysteine levels after racing when compared to baseline levels (p<0.05). When groups were compared, homocysteine values were significantly different among the groups at 15 minutes post-race (median values: marathon runners (16.1 µmol/L), 100km runners (9.5 µmol/L), and mountain bikers (8.8 µmol/L) (p<0.05). The authors suggest the accumulation of homocysteine is higher in marathon runners because it is a sustained high-intensity, long-duration event, whereas, 100km running is lower in intensity and may have brief occasions of rest similar to mountain biking downhill
To date, no intervention studies have examined the impact of acute exercise on blood homocysteine concentrations in sedentary individuals.

**Chronic Exercise.** Ten studies have examined the effect of chronic exercise, programs lasting from 2 to 25 weeks, on blood homocysteine levels in active (Table 2.3) and sedentary (Table 2.4) individuals.

**Active Individuals.** As shown in Table 2.3, a total of five studies report no consistent blood homocysteine response to exercise training programs in previously active individuals (3, 21, 43, 47, 76). One study showed a 10% increase in blood homocysteine levels in 14 active young (22 y) men training (70-85% max heart rate) for 4 weeks with cycle ergometers (p<0.05) (3). Conversely, König et al. (2003) found 30 days of triathlon training lowered homocysteine levels in well-trained male triathletes (n=39). However, the significantly lower blood homocysteine concentrations were only found in the triathletes (n=9) reporting the highest amount of training volume (>14.9 hrs/wk) when compared to the group with the lowest training volume (<9.1 hrs/wk) (p<0.05) (47).

Herrmann et al. (2003) examined the impact of swimming on blood homocysteine concentrations and found a 10% increase in blood homocysteine levels induced by both high intensity training (20 km/wk) and volume-oriented training (30km/wk), yet these increases were not statistically significant, p=0.070 and p=0.054, respectively (43). De Cree et al (1999) also reported no significant change in blood homocysteine levels with 10 days of high intensity cycle-ergometer training in females (21). Finally, only one study has examined blood
homocysteine response to resistance training (76). There were no differences in blood homocysteine levels found in a small group of women (n=5) participating in a weight-training program for 8 weeks (76). Details about the weight lifting program were not reported. The intensity or frequency of the training program might have impacted the results.

In summary, these studies do not show a consistent effect of chronic exercise on blood homocysteine levels in active individuals. However, only 3 of the 5 studies reported blood B-vitamin levels and none reported B-vitamin intake, thus, adding to the variable results. In addition, it may be that significant effects were not found in 3 of these studies because individuals were described as active prior to the exercise program and therefore, did not perform more exercise than their normal routines.

**Sedentary Individuals.** As shown in Table 2.4, when sedentary individuals participated in exercise training, blood homocysteine levels varied in response. For example, Cooper et al. (2000) and de Jong et al. (2001) found no significant changes in blood homocysteine levels in men who participated in a 6-week walking program or in elderly adults who completed a variety of low-impact exercises for 17 weeks, respectively (17, 23). However, in both of these studies the exercise intensity was very low, and may have not been high enough to elicit a change in blood homocysteine concentrations.
As demonstrated by Duncan et al. (2004), intensity of the exercise may be a factor that modifies blood homocysteine (27). In this study, sedentary individuals were placed into 1 of 4 different exercise programs, high intensity-high frequency, high intensity-low frequency, moderate intensity-high frequency, or moderate intensity-low frequency. Homocysteine levels significantly increased in the high intensity-high frequency and the high intensity-low frequency groups compared to baseline values after 6 months of training (p<0.003), but not in the moderate intensity groups (27). Conversely, others have found that low to moderate intensity exercise decreases homocysteine in individuals with health issues. Ali et al. (1998) reported that a 12-week moderate-intensity aerobic type exercise program decreased blood homocysteine levels in male phase II cardiac rehabilitation patients (1). Randeva et al. (2002) also reported a decrease in blood homocysteine levels in overweight women with polycystic ovary syndrome participating in a walking exercise program for 25 weeks (65).

In summary, all of the exercise intervention programs used diverse exercise programs varying in program length (2 to 25 wk), exercise intensity and mode (brisk walking, cycling, swimming, running, weight training, etc.), and frequency (3 to 7 d/wk), which may be the reason for the varied results. In addition, health status varies among individuals, from those considered healthy to those with compromised health, which influences various metabolic pathways.
CONCLUSIONS

As illustrated in Figure 2.2, there is an interrelationship between nutrition, exercise and homocysteine. Proper intake of vitamins B6, B12, and folate can help to maintain low homocysteine levels and support the increased demands on metabolism during high-intensity and/or prolonged exercise. For example, exercise may increase the need for vitamin B6 since it is involved in many biochemical reactions necessary to fuel working muscles and repair damaged tissue (57, 58).

Based on research conducted over the last 10 years, no consistent relationship exists between physical activity and blood homocysteine concentrations. The impact of physical activity on blood homocysteine concentrations appears to vary based on fitness levels, nutritional status, genetic, or other factors that were either not measured or accounted for in the studies reviewed. The primary variables, excluding dietary factors, which help explain the inconsistencies in blood homocysteine levels, may be the mode, intensity, and duration of exercise. If these variables impact blood homocysteine concentrations, it may be relative to individual fitness levels.

Further studies are needed to help determine the relationship between exercise and blood homocysteine. These studies need to clearly describe the participant’s fitness level prior to the exercise program and detail the intensity, duration, and frequency of the exercises in the program. Furthermore,
researchers need to carefully account for the many factors that influence homocysteine metabolism, such as dietary intake and blood vitamin levels of folate, vitamin B6, and vitamin B12, in order to reduce variability between individuals and studies.
Figure 2.1. Vitamins and enzymes involved in homocysteine metabolism. Creatine is formed from guanidinoacetate and s-adenosyl-methionine.

Footnotes
Adapted with permission from reference (45)
ATP-adenosine triphosphate
Pi –inorganic phosphate
Ppi –inorganic pyrophosphate
TH$_4$ -tetrahydrofolate
Figure 2.2. Diagram of relationships between blood homocysteine levels, nutrition, and exercise based on current research literature.

Blood Homocysteine Levels

Nutrition
- Folate ↓
- Vitamin B6 ↓
- Vitamin B12 ↓

Exercise
- *Intensity ↑↓≠
- *Duration ↑↓≠
- **Mode walk, bike, swim, run, weight train

Footnotes
- ↓ decreases blood homocysteine concentration
- ↑ increases blood homocysteine concentration
- ≠ no change in blood homocysteine concentration
- * impact of intensity and duration of exercise on blood homocysteine may be relative to individual fitness level
- ** impact of mode on blood homocysteine may depend on size of muscle mass involved and/or type of muscle contraction/loading
Table 2.1. Cross Sectional studies examining relationships between levels of physical activity and blood homocysteine concentrations.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample</th>
<th>Measure of Physical Activeness</th>
<th>[Hcy]$^3$ &amp; physical activity level</th>
<th>Vitamin Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>(55)</td>
<td>204 F 380 M</td>
<td>Interview &amp; questionnaire: activity levels</td>
<td>No relationship</td>
<td>Blood (folate, B6, B12)</td>
</tr>
<tr>
<td>(67)</td>
<td>20 M</td>
<td>VO$_{2\text{max}}$ &amp; questionnaire: h/wk</td>
<td>↑</td>
<td>Not reported</td>
</tr>
<tr>
<td>(19)</td>
<td>1319 F 1297 M</td>
<td>questionnaire: h/wk</td>
<td>↑ females only</td>
<td>Intake (folate)</td>
</tr>
<tr>
<td>(71)</td>
<td>270 F 216 M</td>
<td>Interview &amp; questionnaire: h/wk</td>
<td>No relationship</td>
<td>Blood &amp; Intake (folate, B6, B12)</td>
</tr>
<tr>
<td>(14)</td>
<td>1154 F 1128 M</td>
<td>questionnaire: activity levels</td>
<td>↓ endurance exercisers only*</td>
<td>Intake</td>
</tr>
<tr>
<td>(18)</td>
<td>192 F 231 M</td>
<td>interview: h/wk</td>
<td>↓</td>
<td>Not reported</td>
</tr>
<tr>
<td>(49)</td>
<td>90 F 87 M</td>
<td>questionnaire: activity levels</td>
<td>↓</td>
<td>Intake (folate, B6, B12)</td>
</tr>
<tr>
<td>(40)</td>
<td>191 F 196 M</td>
<td>interview: activity levels</td>
<td>↓</td>
<td>Not reported</td>
</tr>
<tr>
<td>(51)</td>
<td>730 F 714 M</td>
<td>VO$_{2\text{max}}$: fitness levels</td>
<td>↓</td>
<td>Blood (folate &amp; B12)</td>
</tr>
</tbody>
</table>

1 M male; F female  
2 Physical activity was measured with questionnaires, interviews, and/or estimated VO$_{2\text{max}}$ values. Activity levels were determined by activity h/wk, category activity levels, such as low, moderate, and heavy, or VO$_{2\text{max}}$ values.  
3 [Hcy] Blood homocysteine concentrations  
4 All dietary intake data was reported using Food Frequency Questionnaires, which measured average intake per week over the previous year for consumption of particular foods or food groups. Approximate frequency of consumption was then quantified in terms of the number of times a month a food was consumed.  
↓ Lower homocysteine concentrations in the group most physically active  
↑ Higher homocysteine concentrations in the group most physically active compared with resistance exercise or a sedentary lifestyle
Table 2.2. Blood homocysteine response to acute exercise in active individuals.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample¹</th>
<th>Exercise Intervention minutes (mode)</th>
<th>[Hcy]² response to exercise</th>
<th>Vitamin Status Blood/Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>(92)</td>
<td>20 M</td>
<td>30 (cycling) 70% max heart rate</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(21)</td>
<td>15 F</td>
<td>~10 (cycling) VO₂max test</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(88)</td>
<td>13 M</td>
<td>150 (running) ~70% max heart rate</td>
<td>↑</td>
<td>Not reported</td>
</tr>
<tr>
<td>(22)</td>
<td>7 M</td>
<td>60 (cycling) sustained 60% VO₂max</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(47)</td>
<td>39 M</td>
<td>~67 (triathlon) competitive race</td>
<td>↑</td>
<td>Blood (folate &amp; B12)</td>
</tr>
<tr>
<td>(42)</td>
<td>100 B</td>
<td>220-264 (running) 328-390 (mtn. biking) 630-715 (100k running) competitive race</td>
<td>↑ ≠ ≠</td>
<td>Blood* (folate &amp; B12)</td>
</tr>
<tr>
<td>(32)</td>
<td>12 M</td>
<td>~12 (cycling) VO₂max test</td>
<td>↓</td>
<td>Intake (folate, B6, B12)</td>
</tr>
</tbody>
</table>

¹ M male; F female
² [Hcy] Blood homocysteine concentrations
↓ lower homocysteine concentrations at the end of exercise compared to baseline
↑ higher homocysteine concentrations at the end of exercise compared to baseline
≠ no change in homocysteine concentrations
* blood vitamins only measured/statistically controlled for in individuals with >12 µmol [Hcy] (n=23)
Table 2.3. Chronic exercise and homocysteine response in active individuals.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample</th>
<th>Training Intervention</th>
<th>[Hcy](^2) response to training</th>
<th>Vitamin Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>(21)</td>
<td>15 F</td>
<td>Cycling ~60 min. <em>high</em>: 2 x 5 day periods ~2 wks apart</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(3)</td>
<td>14 M</td>
<td>Cycling 20-30 min. <em>high</em>: 3 x wk 4 wks</td>
<td>↑</td>
<td>folate &amp; B12</td>
</tr>
<tr>
<td>(76)</td>
<td>5 F</td>
<td>Resistance training <em>unknown</em>: 3 x wk 8 wks</td>
<td>≠</td>
<td>Not reported</td>
</tr>
<tr>
<td>(47)</td>
<td>39 M</td>
<td>Triathlon training <em>unknown</em>: 9.1 – 14.9 h/wk 4 wks</td>
<td>↓*</td>
<td>folate &amp; B12</td>
</tr>
<tr>
<td>(43)</td>
<td>19 M/F</td>
<td>Swimming + dryland 20 or 30 km/wk <em>mod or high</em>: 6 x wk 3 wks</td>
<td>≠</td>
<td>folate, B6 &amp; B12</td>
</tr>
</tbody>
</table>

1 M male; F female
2 [Hcy] Blood homocysteine concentrations
≠ no change in homocysteine concentrations
↓ lower homocysteine concentrations at the end of program compared to baseline
↑ higher homocysteine concentrations at the end of program compared to baseline
* only subgroup had significant difference: triathletes training 14.9 h/wk had a decrease from 12.7 to 11.7 µm/L as compared with levels of 12.5 to 12.86 µm/L in the triathletes training 9.1 h/wk (p<0.05).
Table 2.4. Chronic exercise and homocysteine response in sedentary individuals.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample</th>
<th>Training Intervention</th>
<th>[Hcy]\textsuperscript{2} response to training</th>
<th>Vitamin Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mode, duration, intensity frequency, intervention length</td>
<td></td>
<td>Blood/Intake</td>
</tr>
<tr>
<td>(1)</td>
<td>11 M</td>
<td>Variety of exercises 60 min moderate: 3 x wk for 12 wks</td>
<td>↓</td>
<td>Blood (folate)</td>
</tr>
<tr>
<td>(17)</td>
<td>6 M</td>
<td>Walking 30 min low: 5 x wk for 6 wks</td>
<td>≠</td>
<td>Blood (folate &amp; B12)</td>
</tr>
<tr>
<td>(23)</td>
<td>30 M/F</td>
<td>Variety of exercises 45 min low: 2 x wk for 17 wks</td>
<td>≠</td>
<td>Blood (folate, B6, B12)</td>
</tr>
<tr>
<td>(65)</td>
<td>12 F</td>
<td>Walking 24 min low: 3 x wk for 25 wks</td>
<td>↓</td>
<td>Blood (folate &amp; B12)</td>
</tr>
<tr>
<td>(27)</td>
<td>324 M/F</td>
<td>Walking 30 min mod or high: 3-4 or 5-7 x wk for 25 wks</td>
<td>≠ML ≠MH ↑HH ↑HL</td>
<td>Intake (folate, B6, B12)</td>
</tr>
</tbody>
</table>

\textsuperscript{1} M male; F female

\textsuperscript{2} [Hcy] Blood homocysteine concentrations

↓ lower homocysteine concentrations at the end of program compared to baseline

≠ no change in homocysteine concentrations

↑ higher homocysteine concentrations at the end of program compared to baseline

ML moderate intensity, low frequency

MH moderate intensity, high frequency

HH high intensity, high frequency

HL high intensity, low frequency
REFERENCES


CHAPTER 3 – MANUSCRIPT 2: BLOOD HOMOCYSTEINE LEVELS ARE THE SAME IN NONSUPPLEMENTED YOUNG ACTIVE AND SEDENTARY MEN AND WOMEN WITH DIFFERENT PHYSICAL ACTIVITY LEVELS

Lanae M. Joubert and Melinda M. Manore

For submission to:
Medicine & Science in Sports & Exercise
Lippincott Williams & Wilkins
16522 Hunters Green Parkway
Hagerstown, MD 21740
ABSTRACT

Exercise increases methionine metabolism, which also increases homocysteine (Hcy), an amino acid intermediate of this metabolic pathway. High levels of Hcy increase cardiovascular disease (CVD) risk. Folate, vitamins B6 and B12 are known to reduce Hcy levels; increased intakes through diet, supplementation and fortified foods can effectively reduce Hcy. Regular exercise improves many CVD risk factors, yet the research exploring the relationship between exercise and Hcy is equivocal. **PURPOSE:** To determine if blood Hcy values (µmol/L), independent of plasma B-vitamin concentrations (plasma folate, vitamin B6 [pyridoxal 5’ phosphate-PLP], B12 levels), are higher in active (HighPA; >420 min/wk) than less active (LowPA; ≤420 min/wk) males (M) and females (F). **METHODS:** Subjects (M=38; F=38) were healthy, young (26±5y), used no B-vitamin supplements in last 30-d, and reported a consistent physical activity (PA) level for the last 5y. PA groups were based on moderate + high intensity PA (min/wk) using 7-d PA records. Dietary intakes of folate, vitamin B6 and B12 were assessed using 7-d weighed food records. The differences of Hcy between PA and sex groups were examined using ANCOVA with plasma B-vitamin levels as covariates. **RESULTS:** Mean PA was 220 min/wk for LowPA (n=36; VO₂max=42.8±8.8 ml/kg/min) and 652 min/wk for HighPA (n=40; VO₂max=54.2±9.7 ml/kg/min). There were no significant differences in plasma Hcy values between PA levels (LowPA=7.5±1.6; HighPA=7.7±1.6 µmol/L, P=0.36) or sex (M=7.8±1.7; F=7.4±1.1 µmol/L, P=0.13). Plasma folate was the only significant covariate (p<0.001) and accounted for 19% of variance, yet no
participants were deficient in plasma folate (<3.4 nmol/L). To examine whether those with the highest level of PA had higher blood Hcy levels than the lowest level of PA, a secondary analysis of the data were done (N=20). Plasma Hcy levels were significantly higher in those with the most PA (ExHighPA; range 758-1085 min/wk; n=11) and highest fitness levels (>90th% VO2max) compared to those with the least PA (ExLowPA; range 9-130 min/wk; n=9) and lowest fitness levels (<70th% VO2max); 8.6±1.8 vs. 6.7±1.5 µmol/L; P=0.007, respectfully.

CONCLUSION: Plasma Hcy concentrations, independent of plasma B-vitamin levels, were not different between physical activity levels in non-supplementing young adults, unless PA of moderate and high intensities was extremely high (≥758 min/wk).

Key Words: diet, nutrient status, exercise, fitness, CVD risk factor
INTRODUCTION

Homocysteine (Hcy) is an amino acid intermediate metabolite in the methionine pathway. Circulating levels of blood Hcy increase when methionine catabolism is elevated. Methionine is considered the most important methyl group source in humans (51) since it donates its methyl groups for many functional molecules, such as neurotransmitters, DNA, RNA, and creatine. Exercise increases methionine catabolism (18, 58) and induces a higher turnover for many of these methyl containing molecules, particularly creatine during high intensity exercise (39). Thus, it is possible that high intensity exercise-induced methionine turnover could increase circulating levels of Hcy.

Elevated blood concentrations of Hcy are associated with an increased risk of cardiovascular disease (CVD) (7), while lowering Hcy is associated with reduced incidence of heart attack and strokes (57). Identifying modifiable factors that influence Hcy is important in order to reduce CVD risk. Nutritional factors, such as low dietary intake of folate, vitamin B6 and vitamin B12, are known to be major contributors of elevated blood Hcy (54). Less is known about the relationship of exercise and blood Hcy levels.

The Institute of Medicine’s (IOM) Food and Nutrition Board set physical activity (PA) recommendations for weight maintenance at 1-hour per day based on studies of how much energy is expended on average each day by individuals who maintain healthy weight (27). These guidelines suggest exercise can be
cumulative throughout the day with daily PA to reach the recommended 60 minutes. Examples include activities of daily living, such as housecleaning, and more moderate intensity aerobic activities, such as walking at 4 miles per hour, or higher intensities, jogging 30 minutes at a pace > 4 miles per hour. These moderate and high intensity aerobic activities can help to develop or maintain a high level of cardiorespiratory fitness, which is associated with reduced CVD risk (1).

B-vitamin (B6, B12, folate) dependent metabolic reactions in the body, such as energy production and the rebuilding and repairing of muscle tissue, are in high demand during moderate and high intensity exercise (37). Individuals who regularly exercise at these levels may have less available B-vitamins for methionine metabolism, induce methionine turnover and increase creatine production and therefore, may have higher blood Hcy concentrations in comparison to their less active counterparts, particularly if dietary intakes of folate, vitamin B12 and B6 are inadequate.

In general, males have higher levels of blood Hcy than females (29), perhaps due to higher amounts of fat free mass (FFM) in males and protective estradiol influences in females. Blood Hcy concentrations commonly increase with age, with levels in post-menopausal females converging with those of males at older ages (43), supporting the notion that estrogen may protect younger females from higher blood Hcy levels.
Researchers have examined the impact of exercise on blood Hcy without much consensus. Some cross-sectional studies have found low blood Hcy concentrations associated with higher levels of physical activity or fitness (3, 8, 9, 11, 23, 32, 34, 40, 46, 48), yet others report the opposite relationship (12, 45), or no relationship at all (26, 35, 47, 50). Although a few studies have shown that one bout of vigorous exercise (acute) may increase blood Hcy levels (20, 24, 30, 31, 44, 58), others have found no significant changes (13, 14, 38, 59).

Furthermore, evidence of long-term (chronic) exercise training, which includes moderate and high intensity levels, is equivocal (5, 20, 30, 42, 55, 56). Research on blood Hcy and exercise do not permit general conclusions primarily because of poorly described fitness and/or physical activity levels, and limited statistical power with low sample sizes, no control for B-vitamin status, and use of diverse study designs.

The purpose of this study was to determine if, independent of B-vitamin status, young (19-35 y), non-supplementing individuals who regularly participate in moderate and high intensity PA (>5 y; >420 min/wk) have higher plasma Hcy levels than less active (<420 min/wk) individuals. We hypothesized plasma Hcy levels are higher, independent of blood B-vitamin levels, in non-supplementing (no B-vitamin supplements for ≥ 30d) very active individuals participating in consistent moderate and high intensity exercise, as per current recommendations (27), than less active counterparts.
METHODS

Participants. Volunteers were recruited using flyers posted on campus and e-mail sent to local athletic clubs. Participants were first screened via telephone interview and then invited to participate in the study if they met the inclusion criteria: (1) participated in > 7 h/wk of moderate and high intensity PA plus were recreational or competitive athletes (HighPA) or, (2) participated in ≤ 7 h/wk (LowPA) for at least 5 y. This 7 hrs/wk criterion was based on the Institutes of Medicine's (IOM) PA recommendation to maintain healthy weight and reduce risks for chronic disease (27). Eighty young volunteers (19-35y; 50% male) were invited to participate in this cross-sectional investigation during 2005-2006. Four participants did not follow appropriate 7-d recording procedures and were excluded from all analyses, thus 76 participants were included in demographic and blood analyses. For the analysis of dietary intake, an additional 12 participants were eliminated due to under-reporting or abnormal dietary patterns. Participants were considered under-reporters if their total energy intake was 200 kcal/d less than that calculated by estimated resting metabolic rate [based on Cunningham’s equation (10)] multiplied by Goldberg’s factor of 1.3 (21). Therefore, 64 participants were included in the dietary analysis portion of the study.

All participants were non-supplement users and agreed to not use any form of B-vitamin supplements, such as multivitamin/mineral pills, or any form of creatine supplements at least 30 days prior to the blood test. In addition, they were asked
to refrain from consuming sport type products with a daily value of %50 or higher for folate, vitamin B12, or vitamin B6, such as Powerbars® and/or Lunabars® for at least 30-d prior to the scheduled blood test. Exclusion criteria were as follows: not within young age range (19-35y), history or current status of menstrual dysfunction or disordered eating (assessed by questionnaire), cigarette smoking within past year, body mass index (BMI) >28 kg·m$^2$, currently taking medications known to alter blood Hcy, currently injured, or had a health related problem such as kidney disease, diabetes, or heart disease. This study was approved by the Internal Review Board of Oregon State University and written informed consent was obtained from all participants.

**Study Procedures.** Participants visited the research laboratory on 2 different days (~ 4 h total). The first visit included 1) completing questionnaires, 2) learning how to keep detailed weighed food and PA logs, 3) assessment of height, weight, and body composition, 4) and a treadmill VO$_2$ max fitness test. Participants were assigned specific dates to record concurrent food and activity records. These dates were to represent a typical week (i.e. no special events like weddings, holidays). Females were instructed to complete their 7-d records and schedule their blood draw during the follicular phase of their menstrual cycle. The second visit transpired on the morning of the 8$^{th}$ day, after completion of the 7-d recording period, a fasting blood draw was done and activity and diet records were reviewed.

**Questionnaires.** Individuals completed 3 detailed questionnaires: (1) health history to screen for injuries and/or contraindicated health status, (2) previous
year leisure and occupational PA (33) to help interpret the 7-d PA record, and (3) eating attitudes test (EAT-26) (19) to screen for disordered eating behaviors. Additionally, all females completed a menstrual history questionnaire to screen for abnormal menstrual cycles (missed more than 6 menstrual periods within the past 12 months or not menstruated within the past 3 months) since menstrual cycle may influence Hcy levels (13). No participants had disordered eating or abnormal menstrual cycles.

7-d weighed dietary intake records. Participants attended a 30-min instruction course on how to keep accurate food and PA records. Calibrated food scales were issued to each participant to weigh the amounts of food items. Consistent detailed instruction was provided by the same trained research assistant for all participants. Food models were used to show examples of how to estimate amounts of food if the scale was unavailable (i.e. restaurant dining). Participants were asked to describe in detail all foods and beverages including the type of food, how it was prepared, manufacturer if possible, and the amount consumed on the provided record forms (see Appendix) and personal recording dates were assigned. They were instructed to maintain normal dietary and PA patterns during the 7-d of recording. Energy intake and dietary composition was assessed using Food Processor (ESHA version 8.3; Salem, OR). Recipes were analyzed when provided and manufacturer food labels were turned in with records to help select closest foods to Food Processor’s database.

7-d physical activity records. Each participant recorded time spent in resting, sitting, standing, moderate, and high intensity activities over 7 consecutive 24
hour periods (see Appendix). Intensity of activity was described to each participant as follows: resting (laying down; sleeping), very light (all sitting activities; studying, driving, computer work), light (all standing activities; standing around, teaching, walking below 4mph pace, housework), moderate (i.e. fast walk > 4mph pace, jogging, swimming, biking), and heavy (exercise at this intensity could not be sustained for more than 3 minutes, i.e. sprinting). Each minute of the day (1440 minutes) needed to be accounted for and individuals were instructed to keep records with them at all times during the recording period. Examples of completed food and PA records were issued to each participant to reduce recording errors. Participants were encouraged to contact researchers if they had questions during their 7-d recording period. Time spent in each category of intensity for each day was tallied and totals were then recorded as minutes spent in each intensity category for each of the 7 days. Total minutes for all 7 days, recorded in both moderate and heavy intensity categories were used to assign PA groups (HighPA > 420 min/wk; LowPA ≤ 420 min/wk), based on current PA recommendations (27).

Anthropometrics. Height in meters was measured with no shoes using a stadiometer, a digital scale weighed subjects only wearing swim suits. BMI was calculated from weight (kg) divided by height (m^2). Body composition was measured using the Bod Pod (Life Measurements Instruments; Concord, CA). Body density was calculated as body weight divided by the corrected body volume and Siri’s two-compartment formula was used to calculate percent body
fat from body density (53). From percent body fat and body weight, total fat mass (kg) and total fat-free mass (FFM) (kg) were calculated.

**VO₂max testing.** Participants performed a continuous incremental running treadmill (Trackmaster, model TMX22; JAS Manufacturing Co., Inc., Newton, KS) test to exhaustion to determine the cardiorespiratory response to maximal exercise (VO₂max ml/kg/min) based on a modified protocol (25). Respiratory rate was measured using indirect calorimetry (TrueOne 2400; Parvo Medics, Salt Lake City, UT). A heart rate monitor (Polar USA, NY) measured heart rate (bpm) and was worn during the entire fitness testing procedure. Average VO₂ and heart rate data were recorded every 30 seconds. After a brief warm-up and stretching period, individuals were connected to the indirect calorimetry system with an air hose connected to a mouthpiece and head support. The test started at a speed (mph) that elicited a heart rate of ~130 bpm consistently for 2 minutes and incline of 3% grade. Rate of Perceived Exertion (RPE: Borg Scale 6-20) (6) was displayed for each individual to describe their level of exhaustion every 2 minutes. An increase in speed (0.5 mph) each minute continued until the subject felt they were at a “somewhat hard” intensity level (14-15 on the RPE scale). The speed remained constant from this point forward and the incline increased 1% in grade every minute until the subject reached exhaustion. Verbal encouragement was provided throughout the test. Respiratory exchange ratio (>1.1), RPE, and maximum heart rate (95% of age predicted max) were used to provide objective evidence of leveling of VO₂ and maximal effort endpoint for VO₂max testing.
Blood biochemical analyses. Participants were instructed to refrain from vigorous exercise 12-h and fast at least 8-h before arrival at the laboratory for the second visit. Blood was collected during the first 10-d of the menstrual cycle for all female participants. Fasting venous blood was collected in EDTA at the end of the 7-d food and activity recording period (blood taken on day 8). Blood samples were sent to a local laboratory (Good Samaritan Health Services; Corvallis, OR) for lipid, hematocrit, and chemistry analysis. All other blood analysis was performed in the Nutrition Science Laboratory of the Department of Nutrition and Exercise Sciences at Oregon State University. Blood was immediately centrifuged at 2000xg for 15 min at 10°C. Plasma was removed and aliquoted (0.6mL) into 2mL cryo-vials and stored in the freezer (-80°C) until day of analysis.

Homocysteine. Fasting plasma Hcy concentrations were measured using a modification of the reversed-phase HPLC with fluorescence detection according to the method described by Durand et al. (1996) (16) (see Appendix). Each sample was reduced by tri-n-butyl phosphine, followed by precipitation of plasma proteins with perchloric acid. Precipitates were then combined with 7-fluoro-2,1,3-benzodiazole-4 sulfonamide (SBF) and incubated in a heat block at 60°C for 1-h, then filtered and injected into an integrated HPLC system (Waters Separations Module; Waltham, MA 02454) and separated on a reverse-phase C18 column (Alltech ODS-2 5µ 150x4.6mm; Deerfield, IL 60015). Plasma Hcy was detected by fluorescence (Waters Fluorescence Detector; Waltham, MA
02454) at 385nm excitation and 515nm emission. The intra and inter-assay coefficients of variation (CV) were 1.3% and 1.8%, respectively.

**B-vitamins.** For plasma levels of vitamin B6, fasting plasma pyridoxal-5’-phosphate (PLP) was measured using a modification of the reversed-phase HPLC with fluorescence detection according to the method described by Rybak and Pfeiffer (2004) (49) (see Appendix). Metaphosphoric acid was used to precipitate plasma proteins from each sample. The supernatant was then filtered and injected into an integrated HPLC system (Waters Separations Module; Milford, MA 01757) and separated on a reverse-phase C18 column (ThermoElectron BDS Hypersil150x3mm; Waltham, MA 02454). Sodium chlorite was introduced into the HPLC post-column effluent flow using an automated post-column pump (Waters Reagent Manager; Waltham, MA 02454). The combined flow was passed through a heated (75 °C) coiled tubing (300 µL stainless steel) for improved detection. PLP was then detected by fluorescence (Waters Fluorescence Detector; Waltham, MA 02454) at 300nm excitation and 400 nm emission. The intra and interassay coefficients of variation were 1.3% and 6.7%, respectively. For plasma levels of folate and vitamin B12, fasting plasma folate and vitamin B12 were measured using RIA kit (SimulTRAC-SNB folate/B12; MP Biomedicals, Solon, OH 44139). Radioactivity of I\(^{125}\) and Co\(^{57}\) was counted simultaneously in the pellets using an auto-gamma counter (Cobra II 5002, Packard Instrument Co.). Interassay CV for this method was <12%. Red blood cell folate levels were analyzed using fasting red blood cell folate in participants reporting the lowest plasma folate levels (n=9; range:17.63-23.57 nmol/L) to
eliminate the possibility of folate tissue deficiencies (deficiency considered $<3.4\text{nmol/L}$) (41). Whole blood was collected in EDTA tubes. Well-suspended blood (100 $\mu$L) was added to 2 mL of freshly prepared 0.2% ascorbic acid solution (w/v) and inverted several times for mixing. The whole blood hemolysate was stored in the freezer (-80°C) until day of analysis. Hematocrit values were determined and recorded on day of blood draw by local laboratory (Good Samaritan Health Services; Corvallis, OR). Red blood cell folate (nmol/L) was measured using RIA kit (SimulTRAC-SNB folate/B12; MP Biomedicals, Solon, OH 44139). Radioactivity of $^{125}$I was counted in the pellets using an auto-gamma counter (Cobra II 5002, Packard Instrument Co.). No participants had deficient levels. Red blood cell folate values (mean = 1123±180 nmol/L; $N$=9) were well above red blood cell folate deficiency criteria ($<272$ nmol/L) (41).

**Statistical Analysis.** Power analysis indicated sample sizes of 17 and 9 per group was needed to provide 90 and 80% power respectively, to detect a Hcy concentration difference of 3 $\mu$mol/L. The assumptions of normality for sampling distributions, linearity, homogeneity of variance, homogeneity of regression, and reliability of covariates were all satisfactory. PA group (LowPA, HighPA) comparisons for demographic data were conducted for each biological sex (M, F) by independent samples $t$-tests. Analysis of covariance was performed on plasma Hcy concentrations with biological sex and PA groups as independent variables. Plasma folate, PLP, and B12 were included in the full model as covariates to account for their impact on Hcy levels. Only significant covariates
(p<0.05) were included in the final model. For sub-sample ANCOVA analysis with the same B-vitamins as covariates, participants were divided into 2 groups based on extreme upper and lower PA percentile criteria. The description of these groups is given below:

**Extremely High Physical Activity (ExHighPA)** (N=9; 4 M, 5 F) This group’s criteria was a Tukey’s Hinge >75th percentile for moderate and high intensity PA (min/wk) plus age and sex appropriate VO2max (ml/kg/min) >90th percentile values for maximal aerobic power (2).

**Extremely Low Physical Activity (ExLowPA)** (N=11; 6 M, 5 F) This group’s criteria was a Tukey’s Hinge <25th percentile for moderate and high intensity PA (min/wk) plus age and sex appropriate VO2max (ml/kg/min) <70th percentile values for maximal aerobic power (2).

Results are expressed as mean ± SD; statistical significance was determined at P<0.05. All analyses were performed by SPSS statistical software package (SPSS, Inc. Chicago, IL; version 15.0).

**RESULTS**

Characteristics of participants (N=76) are shown in Table 3.1. Mean age for all participants was 26±4 y. Both males and females in the HighPA group were significantly taller and older compared to the LowPA group (P<0.05). Males in the HighPA group had more FFM (kg) than the LowPA group (P<0.05). By design, combined weekly min of moderate and high intensity PA were significantly different between HighPA and LowPA groups (P<0.01). On
average, the LowPA group \((N=36)\) completed 219±130 min/wk \((\text{VO}_2\text{max} 42.8±8.8 \text{ ml/kg/min})\), while the HighPA group \((N=40)\) completed 652±191 min/wk \((\text{VO}_2\text{max} 54.2±9.7 \text{ ml/kg/min})\). Data in Table 3.1 is expressed by biological sex and PA groups.

**PA & Hcy.** The primary objective of this research was to determine if, independent of blood B-vitamin levels, blood Hcy concentrations were different in healthy young non-supplementing adults compared to their less active counterparts. Overall, accounting for blood B-vitamin levels, blood Hcy levels \((\mu\text{mol/L})\) were not significantly different between HighPA \((7.7±1.6; N=40)\) and LowPA groups \((7.5±1.6; N=36)\) \((P=0.36)\), regardless of biological sex. Although males \((N=38)\) had higher blood Hcy values than females \((N=38)\), they were not significantly different \((7.9±1.7 \text{ vs. } 7.4±1.4 \mu\text{mol/L}, \text{respectively})\) \((P=0.13)\).

**Blood B-vitamins & Hcy.** Since Hcy metabolism is supported by B-vitamin cofactors, we used plasma folate, vitamin B12, and vitamin B6 (PLP) concentrations as covariates in our analysis. Plasma folate was the only statistically significant B-vitamin covariate \((P<0.01)\) and explained 19% of the variance in plasma Hcy in our final model. Table 3.2 displays plasma values for Hcy and each B-vitamin by groups. No participants had plasma folate levels of <3.4 nmol/L, which is the cut-off value for folate deficiency \((41)\). Mean plasma vitamin B12 and PLP levels were in the normal range; however, 13% of the participants had low PLP values \(<30 \text{ nmol/L})(17)\). The following individuals had low plasma PLP values \((\text{nmol/L})\) expressed as mean±SD by
group: 2 males in LowPA had 26.1±2.4; 2 males in HighPA had 28.4±0.9; 4 females in LowPA had 24.3±5.1; 2 females in HighPA 28.3±0.9 nmol/L; and 1 female in LowPA was considered deficient (<20 nmol/L) (28) with a PLP value of 17.9 nmol/L. No participants had low plasma vitamin B12 (<120 pmol/L) (41).

Dietary intakes. Table 3.3 shows energy and dietary intake data for participants with completed 7-d weighed dietary intake records (N=64). As one would expect, energy intake was significantly different between HighPA and LowPA groups (P<0.05), with the HighPA male group consuming ~400 more kcal/d than the LowPA male group and the HighPA female group consuming ~240 more kcal/d than the LowPA female group. Although mean dietary intakes of both vitamin B6 and folate were above the recommended dietary allowance (RDA), not all participants consumed recommended levels. One male in the LowPA group did not meet the RDA for dietary vitamin B6 (RDA=1.3mg/d), and 13 participants (22%) did not meet the RDA for dietary folate (RDA=400 µg/d) (9 females in LowPA, 1 female in HighPA, 2 males in HighPA, 1 male in LowPA). All individuals met the RDA for vitamin B12 (2.4µg/d). Further, one female in the LowPA group was below the estimated average requirement (EAR) for folate (320 µg/d), one LowPA male was below the EAR for vitamin B6 (1.1 mg/d), but all were above the EAR for vitamin B12 (2.0 µg/d).

Extreme PA & Hcy. If PA increases blood Hcy concentrations, it is most likely to occur in those who are consistently highly active. Thus, we compared the most
active and most fit (ExHighPA; \(N=11\)) participants to the least active and least fit participants (ExLowPA; \(N=9\)) based on percentile criteria for both fitness level (\(\text{VO}_2\text{max}\)) and minutes of PA (min/wk) (see footnotes of Table 3.4). Participants in ExHighPA (PA range: 750-1085 min/wk) participated in competitive triathlons, cycling, running, or rowing events consistently for at least 5 y, while ExLowPA were sedentary (PA range: 9-130 min/wk). As shown in Figure 3.1, there were significant differences in Hcy (\(\mu\text{mol/L}\)) between ExLowPA (\(N=9\)) and ExHighPA groups (\(N=11\)) (\(P=0.007\)), and between males (\(N=10\)) and females (\(N=10\)) (\(P=0.014\)). Three participants had Hcy values >10 \(\mu\text{mol/L}\) (all in ExHighPA male group). Plasma B12 was the only significant covariate (\(P=0.042\)). All sub-sample participants had normal plasma B12 (>120 pmol/L) \((41)\) and plasma folate (>3.5 nmol/L) \((41)\) values, while plasma PLP was low in 1 male and 3 females (<30 nmol/L) \((17)\). Of the sub-sample, 14 of the 20 participants had complete 7-d dietary records. Even though plasma folate levels were adequate in this sub-sample, 3 males and 3 females consumed less than the RDA for dietary folate (400 \(\mu\text{g/d}\)) and 1 male ate less than the RDA for vitamin B6 (1.3 mg/d). All participants consumed the RDA for dietary vitamin B12 (2.4 \(\mu\text{g/d}\)).

**DISCUSSION**

The primary objective of this study was to determine if, independent of plasma B-vitamin levels, plasma Hcy concentrations were higher in active vs. less active individuals. We did not find a significant difference in plasma Hcy concentrations between PA groups as defined by > 420 min/wk or ≤ 420 min/wk of moderate
and high intensity activity in young participants. However, in a secondary analysis, individuals who participate in extremely high levels of PA (≥758 min/wk) had significantly higher plasma Hcy values compared to sedentary individuals (≤130 min/wk). Furthermore, we do not know if the differences in plasma Hcy concentrations remained statistically significant when B-vitamins were used as covariates. Even though there were statistical significance in plasma Hcy levels between ExLowPA and ExHighPA, mean levels were still within a healthy range (<10 µmol/L). Furthermore, the mean difference (1.75 µmol/L) between ExHighPA and ExLowPA is not physiologically relevant. For example, a meta-analysis by Boushey et al. found a 5 µmol/L increment of plasma Hcy concentration greater than 10 µmol/L was necessary to increase risk for CVD by 60-80% (7) in mostly middle-aged populations. Further, a meta-analysis by Wald et al. suggested lowering homocysteine concentrations by 3 µmol/L would reduce the risk of ischaemic heart disease by 11-20%, deep vein thrombosis by 8-38%, and stroke by 15-33% (57). Further research is needed to explore whether or not keeping blood Hcy levels low over a lifespan keeps risk for CVD low.

To our knowledge, this is the first study in young men and women examining the relationship between PA levels and plasma Hcy concentrations while also accounting for blood and dietary intakes of all 3 B-vitamins that influence Hcy concentrations. Factors known to influence blood Hcy were controlled for in this study. We used young men and women within a small age range. We measured all females during the same phase of the menstrual cycle,
screened for menstrual dysfunction, disordered eating behaviors, and under-reporters of energy intake. Both detailed 7-d weighed food records to measure dietary intakes of B-vitamin as well as other dietary factors that influence Hcy. Finally, fitness levels and specific PA intensity levels of the participants were well described.

**PA & Hcy.** Considering the hypothesis that high creatine synthesis and protein turnover increases the blood Hcy levels, it's possible only highly intense or exhaustive exercise, relying on anaerobic or protein-derived energy sources, produces significant increases in blood Hcy concentrations. We only found significant differences in plasma Hcy levels between individuals who participated in high levels of PA and were physically fit (ExHighPA ≥ 758 min/wk; 57.2 ml/kg/min) compared to those that were sedentary and unfit (ExLowPA ≤ 130 min/wk; 35.1 ml/kg/min) when both PA and VO_{2}max were combined to define the groups. When we only used PA (min/wk) or VO_{2}max alone to define these groups there were no differences in plasma Hcy concentrations. This suggests fit individuals who chronically exercise have higher plasma Hcy levels than their unfit sedentary counterparts. This was supported in the present study, since both males and females in the ExHighPA groups had significantly higher blood Hcy levels than the ExLow PA groups. These results are supported by other cross-sectional studies that found higher blood Hcy values in the groups most physically active (12, 45). Conversely, other cross-sectional research have found the opposite (3, 8, 9, 11, 23, 32, 34, 40, 46, 48) or no relationship (26, 35, 47, 50). Discrepancies in
the research may be because there was no account for the combination of both the participants’ amount of consistent PA and fitness levels, or due to different modes and intensities of exercise, which were not well described in these cross-sectional studies. As reported by Hermann et al (2003), marathon runners had higher post-race blood Hcy levels than 100km runners or mountain bike competitors, which they explain by different load profiles of the three disciplines (24).

Participants in the present study performed a variety of moderate and high intensity exercises. Collectively, only 30% of the HighPA participants (mean PA=11 h/wk; N=40) completed 15 minutes or more of high intensity exercise daily, which was attributed to interval/sprint training as well as resistance weight training. Yet, approximately 75% of the sub-sample ExHighPA participants (mean PA=15 h/wk; N=11) completed more than 15 min of high intensity exercise daily, including interval training for swimming, cycling, running, rowing and strength weight lifting. Duncan et al (2004) reported blood Hcy levels in sedentary participants increased significantly in response to 25 weeks of high intensity walking (65-75% heart rate reserve), but not with moderate intensity walk training (45-55% heart rate reserve) (15). Moreover, Rousseau et al (2005) reported significant differences in Hcy values between intermittent sport athletes (10.6±2.6 µmol/L), competing in soccer, water-polo, and rugby, compared with athletes in aerobic type sports (9.2±2.0 µmol/L).
such as running and swimming (46), suggesting mode and/or intensity of exercise may influence Hcy.

Acute exercise has been shown to significantly increase blood Hcy levels and these levels remain high several hours post-exercise (20, 24, 31, 44, 58). In the present study, we controlled for the effect of acute exercise by asking participants to refrain from any moderate to high intensity exercise at least 12-h prior to the blood draw.

**Blood B-vitamins & Hcy.** In the total group (N=76), plasma folate was the only significant covariate involved in the modulation of plasma Hcy concentrations. However, in the sub-sample analysis (N=20), only plasma B12 was a significant modulator of plasma Hcy. Unfortunately, several diet records in this sub-sample were not complete enough to examine this observation in more detail. It’s possible the sub-sample group (N=20) ate more meat and thus, had higher dietary vitamin B12 intakes than the group as a whole (N=76).

**Dietary intakes.** Sehlub et al. suggests nutrient deficiencies of vitamin B6, folate, and vitamin B12 contribute to two-thirds of all cases of hyperhomocysteinemia (52). In our study, 22% of the participants consumed less than the RDA for the B-vitamins. In general, these participants consumed less energy or selected less nutrient-dense foods (such as potato chips and soda), and ate higher amounts of processed foods. Even though energy intake was higher in our very active participants, they did not
necessarily consume higher amounts of B vitamins (see Table 3.3 for B-vitamin/1000kcal). Other dietary factors can also affect plasma Hcy levels. High dietary intake of methionine could lead to high Hcy concentrations if adequate dietary folate intake is not consumed, yet mean intakes between initial groups (N=76) for methionine and folate were similar. Moreover, plasma Hcy concentrations are reported to be higher in alcoholics (4) and heavy coffee drinkers (22). We had no known alcoholics in our study. We monitored coffee consumption and found that ~ 55% of our participants consumed coffee during the 7-d diet record session, but did not control for this in the analyses.

Limitations/strengths. This investigation had a few inherent limitations. Due to the cross-sectional design of this study, causality cannot be established. However, the increased levels of Hcy in our ExHighPA sub-sample warrants further research in order to determine if people who exercise ≥758 min/wk have higher B-vitamin needs to keep blood Hcy levels as low as possible. In addition, the majority of our participants were healthy young white college students. Results may be different in older adults who have subsequent CVD risk factors. The reliability of self-reported PA records has been disputed. Without the use of heart rate monitors, pedometers, or some other objective measure of exercise intensity during the 7-d recording period, we had to rely on personal interpretation of PA intensity levels. We minimized error and exaggeration by providing a detailed description of appropriate PA categories (i.e. moderate = walking faster than 4 mph; high = breathless
sprinting that can’t carry on for more than 1-2 minutes). Participants completed VO₂max fitness testing as an objective measure of fitness levels between groups and to help support their reported activity. Finally, it is well recognized that the methylene tetrahydrafolate reductase (MTHFR) genotype is a major determinant of plasma Hcy. Unfortunately, measurement of the MTHFR polymorphism was beyond the financial resources for the project.

Conversely, this research had several strengths. This study accounted for several discrepancies in previous research. Since blood Hcy concentrations increase with age, we used a young and discrete age range (19-35 y) (29). Menstrual influences were accounted for by drawing blood during the follicular phase in all female participants, which has been shown to influence blood Hcy levels in previous research (13). We used both males and females, where many of the previous studies have used only male participants. All 3 key B-vitamins involved in Hcy metabolism were measured accounted for where previous studies have mainly only focused on blood folate levels. This research contributes to the small amount of existing dietary intake data for highly active individuals, especially B-vitamin intake.

CONCLUSION

The aim of this study was to determine if plasma Hcy concentrations are higher in active vs. less active individuals while controlling for B-vitamin status. To our knowledge, this is the first study to examine the relationship
between exercise and Hcy while controlling for B-vitamin intake and blood levels.

Overall, there were no significant differences in plasma Hcy concentrations between PA groups as defined by > 420 min/wk or ≤ 420 min/wk of moderate and high intensity activity in young non-supplementing men and women, unless PA of moderate and high intensities was extremely high (>758 min/wk). These data suggests high amounts of strenuous exercise may increase Hcy metabolism and thus, plasma levels. It’s possible that a higher dietary intake of B-vitamins may be necessary to keep Hcy levels low in extremely active young individuals, particularly vitamins B12 and folate, to reduce CVD risk later in life.

ACKNOWLEDGEMENTS

This work was supported in part by Northwest Health Foundation and American College of Sports Medicine student grants.
FIGURE 3.1. Mean plasma Hcy concentration comparisons between groups (sex and PA levels) in sub-sample (N=20) ANCOVA.

† (P=0.014) statistical significance between males (n=10) and females (n=10) for plasma Hcy concentrations
‡ (P=0.007) statistical significance between extremely high physical activity (n=11) and extremely low physical activity (n=9) for plasma Hcy concentrations

PA = physical activity in moderate + high intensity for 7 days
ExLowPA = extremely low levels of moderate + high intensity physical activity (min/wk) based on Tukey’s Hinge criteria of ≤ 25th percentile plus VO₂max (ml/kg/min) <70th percentile based on ACSM criteria
ExHighPA = extremely high levels of moderate + high intensity physical activity (min/wk) based on Tukey’s Hinge criteria of ≥ 75th percentile plus VO₂max (ml/kg/min) >90th percentile based on ACSM criteria
<table>
<thead>
<tr>
<th>Variables</th>
<th>Males (N=38)</th>
<th>Females (N=38)</th>
<th>Females (N=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LowPA (N=15)</td>
<td>HighPA (N=23)</td>
<td>LowPA (N=21)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>29 (4)</td>
<td>25 (5)</td>
<td>27 (5)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178.4 (6.6)</td>
<td>185.0 (7.2)</td>
<td>164.1 (6.0)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.9 (12.0)</td>
<td>83.8 (10.2)</td>
<td>61.8 (9.6)</td>
</tr>
<tr>
<td>BMI (kg·m(^2))</td>
<td>24.0 (2.5)</td>
<td>24.4 (2.4)</td>
<td>22.8 (2.7)</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>17.0 (6.9)</td>
<td>14.9 (6.6)</td>
<td>27.1 (7.3)</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>63.4 (7.7)</td>
<td>71.0 (6.5)</td>
<td>44.8 (5.8)</td>
</tr>
<tr>
<td>VO(_2)max (ml·kg·min(^{-1}))</td>
<td>48.6 (8.8)</td>
<td>58.8 (7.7)</td>
<td>38.7 (6.2)</td>
</tr>
<tr>
<td>PA-mod/high (min/wk)</td>
<td>230 (149)</td>
<td>621 (151)</td>
<td>210 (118)</td>
</tr>
</tbody>
</table>

Values are means (SD)

a (P<0.05) within sex statistical significance compared with LowPA
b (P<0.001) within sex statistical significance compared with LowPA
PA = physical activity in moderate + high intensity for 7 days
LowPA = low physical activity ≤ 420 min/wk
HighPA = high physical activity > 420 min/wk
BMI = body mass index (kg·m\(^2\))
Body Fat (%) = derived from Bod Pod analysis
FFM = Fat free mass calculated [total body weight (kg)] - [body fat (%) x total body weight (kg)]
TABLE 3.2. Mean plasma homocysteine (Hcy) and B vitamin levels by sex and physical activity level.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Males (N=38)</th>
<th></th>
<th>Females (N=38)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LowPA (N=15)</td>
<td>HighPA (N=23)</td>
<td>LowPA (N=21)</td>
<td>HighPA (N=17)</td>
</tr>
<tr>
<td>Plasma Hcy (umol/L)</td>
<td>7.8 (1.5)</td>
<td>7.9 (1.8)</td>
<td>7.4 (1.6)</td>
<td>7.4 (1.1)</td>
</tr>
<tr>
<td>Plasma PLP (nmol/L)</td>
<td>58.2 (23.5)</td>
<td>63.8 (24.6)</td>
<td>48.1 (18.6)</td>
<td>49.6 (19.7)</td>
</tr>
<tr>
<td>Plasma Folate</td>
<td>32.24 (7.45)</td>
<td>36.94 (12.96)</td>
<td>32.63 (8.99)</td>
<td>35.20 (6.83)</td>
</tr>
<tr>
<td>Plasma B12</td>
<td>466 (187)</td>
<td>504 (110)</td>
<td>395 (162)</td>
<td>357 (159)</td>
</tr>
</tbody>
</table>

Values are means (SD)
No statistical significance between LowPA & HighPA for males or females
PA = physical activity in moderate + high intensity for 7 days
LowPA = low physical activity < 420 min/wk
HighPA = high physical activity > 420 min/wk
Hcy = homocysteine
PLP = pyridoxal 5’ phosphate, the active form of vitamin B6
Normal plasma values: homocysteine (<10 umol/L)(36), PLP (>30 nmol/L)(17), folate (>3.4 nmol/L)(41), vitamin B12 (>120 pmol/L)(41)
TABLE 3.3. Mean dietary intakes of energy, macronutrients, and B vitamins derived from 7-d weighed food records.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Males (N=31)</th>
<th>Females (N=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LowPA (N=10)</td>
<td>HighPA (N=21)</td>
</tr>
<tr>
<td></td>
<td>LowPA (N=17)</td>
<td>HighPA (N=16)</td>
</tr>
<tr>
<td>Total Energy (kcal/d)</td>
<td>2938 (349)</td>
<td>3372 (850)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Energy/TBW (kcal/kg/d)</td>
<td>38.4 (7.7)</td>
<td>40.9 (11.1)</td>
</tr>
<tr>
<td>Total Energy/FFM (kcal/kg/d)</td>
<td>46.6 (6.9)</td>
<td>47.8 (12.4)</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>120 (16)</td>
<td>137 (33)</td>
</tr>
<tr>
<td>Protein/TBW (g/kg/d)</td>
<td>1.6 (0.3)</td>
<td>1.7 (0.4)</td>
</tr>
<tr>
<td>Protein/FFM (g/kg/d)</td>
<td>1.9 (0.3)</td>
<td>2.1 (0.6)</td>
</tr>
<tr>
<td>Methionine (g/d)</td>
<td>1.86 (0.40)</td>
<td>2.28 (0.78)</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td>392 (50)</td>
<td>458 (112)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carbohydrate/TBW (g/kg/d)</td>
<td>5.1 (1.0)</td>
<td>5.6 (1.6)</td>
</tr>
<tr>
<td>Carbohydrate/FFM (g/kg/d)</td>
<td>6.2 (1.1)</td>
<td>6.5 (1.7)</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>103 (23)</td>
<td>112 (51)</td>
</tr>
<tr>
<td>Fat/TBW (g/kg/d)</td>
<td>1.3 (0.4)</td>
<td>1.4 (0.6)</td>
</tr>
<tr>
<td>Fat/FFM (g/kg/d)</td>
<td>1.6 (0.4)</td>
<td>1.6 (0.7)</td>
</tr>
<tr>
<td>Vit B6 (mg/d)</td>
<td>2.8 (1.2)</td>
<td>3.1 (0.7)</td>
</tr>
<tr>
<td>Vit B6/Energy (mg/1000kcal/d)</td>
<td>0.937 (0.396)</td>
<td>0.930 (0.220)</td>
</tr>
<tr>
<td>Vit B6/Protein (mg/g/d)</td>
<td>0.023 (0.009)</td>
<td>0.023 (0.005)</td>
</tr>
<tr>
<td>Folate (µg/d)</td>
<td>568 (148)</td>
<td>661 (165)</td>
</tr>
<tr>
<td>Folate/Energy (µg/1000kcal/d)</td>
<td>194 (46)</td>
<td>200 (46)</td>
</tr>
<tr>
<td>Vit B12 (µg/d)</td>
<td>7.1 (5.3)</td>
<td>7.5 (3.0)</td>
</tr>
<tr>
<td>Vit B12/Energy (µg/1000kcal/d)</td>
<td>2.43 (1.83)</td>
<td>2.32 (1.13)</td>
</tr>
</tbody>
</table>
TABLE 3.3. Mean dietary intakes of energy, macronutrients, and B vitamins derived from 7-d weighed food records. (Continued)

Values are means (SD)

\(^a(P=0.05)\) within sex statistical significance compared with LowPA

\(^b(P<0.05)\) within sex statistical significance compared with LowPA

PA = physical activity in moderate + high intensity for 7 days

LowPA = low physical activity \( \leq 420 \text{ min/wk} \)

HighPA = high physical activity \( > 420 \text{ min/wk} \)

TBW = total body weight

FFM = fat free mass

Recommended dietary allowances for men and women 19-50 y: protein (0.8g/kg body weight/d), folate (400 µg/d), vitamin B6 (1.3 mg/d), vitamin B12 (2.4 µg/d)

Estimated average requirements: folate (320 µg/d), vitamin B6 (1.1 mg/d), vitamin B12 (2.0 µg/d)

Participants were considered under-reporters if their calculated estimated energy expenditure multiplied by a factor of 1.3 \((10, 21)\) was within 200 kcal of their reported mean dietary energy intake, thus \(N=64\).
TABLE 3.4. Sub-sample comparison of extremely active and sedentary for mean plasma homocysteine (Hcy) and plasma B-vitamin levels.

<table>
<thead>
<tr>
<th>Variables</th>
<th>ExLowPA (N=9)</th>
<th>ExHighPA (N=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>44%</td>
<td>55%</td>
</tr>
<tr>
<td>Plasma Hcy (umol/L)</td>
<td>6.9 (3.9-9.1)</td>
<td>8.6 (6.1-12.3)*</td>
</tr>
<tr>
<td>Plasma PLP (nmol/L)</td>
<td>41.6 (17.9-73.1)</td>
<td>63.4 (27.8-100.3)</td>
</tr>
<tr>
<td>Plasma Folate (nmol/L)</td>
<td>32.8 (17.6-41.9)</td>
<td>33.6 (23.0-44.4)</td>
</tr>
<tr>
<td>Plasma B12 (pmol/L)</td>
<td>389 (161-545)</td>
<td>474 (180-582)</td>
</tr>
<tr>
<td>VO(_2)max (ml·kg·min(^{-1}))</td>
<td>35.1 (29.1-44.3)</td>
<td>57.2 (48.5-68.7)**</td>
</tr>
<tr>
<td>PA moderate + high (min/wk)</td>
<td>53 (9-130)</td>
<td>900 (758-1085)**</td>
</tr>
</tbody>
</table>

Mean (range) values are displayed  
*(P=0.007) ANCOVA statistical significance between ExHighPA and ExLowPA  
**(P<0.001) Independent t-test statistical significance between ExLowPA and ExHighPA

PA = physical activity in moderate + high intensity for 7 days  
ExHighPA = Extremely high physical activity group  
ExLowPA = Extremely low physical activity group  
PLP = pyridoxal 5' phosphate, the active form of vitamin B6  
Normal plasma values: Hcy (<10 umol/L)(36), PLP (>30 nmol/L)(17), folate (>3.4 nmol/L)(41), vitamin B12 (>118 pmol/L)(41)
REFERENCES


APPENDICES
TELEPHONE SCRIPT (for screening purposes):

Thanks for responding to our flyer. I need to learn more about you so I can determine if you fit all our criteria for this study. This interview is anonymous and confidential. If you fit the criteria, you can ask questions and then we’ll try and schedule you to participate.

Are you in a place you feel comfortable to answer some personal questions? This interview will take about 5 minutes.

Have you been within 5 pounds of your current weight for the past year?
Yes
No

Have you taken any supplements (containing creatine or B vitamins within last 30 days)?
Yes
No

If yes, would you be willing to stop supplementing for 30 days to participate in this study?
Yes
No

Are you on any special type of diet? Do you eat meal replacements or sports bars or beverages that have been fortified?
Yes
No

Power bar, protein shakes, Odwalla, Propel, clif bar, balance bar, promax bars, luna bars, etc.

Do you have any health or medical problems, such as diabetes, kidney or heart problems?
Yes
No

Have you smoked cigarettes within the past 2 months?
Yes
No

Do you drink alcoholic beverages?
Occassionally
How much do you drink when you do drink alcohol?
Often
Rarely

Describe your coffee consumption:
Is your menstrual cycle regular? Do you use oral contraceptives
Yes Yes
No No

Would you consider yourself an active individual?
Yes
No

Have you competed in any particular sport in the past year? Describe these events:
Yes
No

How much training or programmed physical activity do you do on average per week?
>6 hours each week
3-6 hours each week
1-3 hours each week
no training

Describe the type of exercise or training:

How long have you been doing this for?

Describe the type of weight training or resistance exercise (amount of weight, sets, reps):

How many minutes a day on average do you _________?

How long have you been doing this for? Describe any other exercise you do:

How old are you? _____ (19-35) Height: ________ Weight ________

Gender:
Male
Female
HEALTH HISTORY QUESTIONNAIRE

1. Age: _______  2. Date of Birth: ___________ (mm/dd/yr)

3. Gender:
   - Male
   - Female

4. Predominant place of residence: ___________________________

5. Height: ___________ (feet’ & inches”)  6. Present Weight ________ (pounds)

7. Most ever weighed ______ (pounds) & year ______

8. Length of time you have maintained current weight ______________

9. Are you currently employed:
   - Yes
   - No

10. If yes, describe your job duties: ____________________________

11. Race:
   - White, European American, Non-Hispanic
   - Asian or Asian American
   - Black, African American, Non-Hispanic
   - Middle Eastern or Middle-Eastern American
   - North African or North African-American
   - Pacific Islander
   - Hispanic or Latino American
   - American Indian or Alaskan Native
   - Please use your own description if none of the choices above apply to you:

12. Are you currently on a special diet:
   - Yes
   - No

13. If yes, was it prescribed by a doctor, dietitian, or nurse:
   - Yes
   - No
   - Please describe: ____________________________

14. If yes, for how long have you been on this diet: __________
15. If dieting, is your dieting associated with any commercial weight loss programs:
   □ Yes  If yes, please specify what program: ______________________________
   □ No

16. Please list foods which you refuse to eat, can not eat, or prefer not to eat:

17. Please list any food allergies or food intolerances, such as lactose intolerance:

18. Do you currently take vitamins/minerals or herbal supplements:
   □ Yes daily
   □ Yes frequently (3 to 6 times/wk)
   □ Often (1 or 2 times/wk)
   □ Occasionally (< 1 time/wk)
   □ Never

19. List the name brand  20. How many per day  21. For how long have you taken it

22. Do you take any other nutritional supplements (fiber, supplement drinks, etc.)
   □ Yes
   □ No

23. List the name brand  24. How many per day  25. For how long have you taken it

26. Do you currently smoke:
   □ Yes
   □ No

27. If yes, what age did you start _____  30. What age did you quit_____?

28. If you do not currently smoke, did you ever smoke:
   □ Yes
   □ No
   □ Only a few times

29. If only a few times, was it within the past 2 months:
   □ Yes
   □ No
30. Do you drink beverages containing alcohol:
   □ Yes
   □ No

31. If yes, how many times do you drink per week _______

32. If yes, check the types of drinks and list how many you consume each time:
   □ Beer _______ fluid ounces (12 fluid ounces in one can)
   □ Wine _______ fluid ounces (5 fluid ounces in one glass)
   □ Liquor _______ fluid ounces (1.5 fluid ounces in one shot)
   □ Other _______

33. Do you drink beverages containing caffeine
   □ Yes
   □ No

34. If yes, check the types of drinks and list how much you consume each time:
   □ Coffee _______ cups per day
   □ Tea _______ cups per day
   □ Soda _______ fluid ounces (12 fluid ounces in one can) per day

35. Check any condition for which you have been medically diagnosed. Please indicate
   AGE at diagnosis:
   □ AIDS _______  □ Pancreatitis _______
   □ Diabetes _______  □ Ulcerative colitis _______
   □ Hypoglycemia _______  □ Recurring gastritis _______
   □ Hypothyroidism _______  □ Allergies/hayfever _______
   □ Hyperthyroidism _______  □ Spastic colon/diverticulitis _______
   □ Goiter _______  □ Rheumatoid arthritis _______
   □ Osteoporosis _______  □ Systemic lupus erythematosus _______
   □ Hepatitis _______  □ Asthma _______
   □ Cirrhosis _______  □ Insomnia requiring medication _______
   □ Kidney stones _______  □ Emphysema _______
   □ Nephritis _______  □ Heart problems _______
   □ High blood pressure _______  □ Tuberculosis _______
   □ Angina _______  □ Hereditary condition _______
   □ Ulcer _______  □ Premenstrual syndrome _______
   □ Hypoadrenalism (Addison's disease) _______
   □ Mental depression requiring regular medication _______
   □ Chronic headache or other pain (specify) _______
   □ Cancer (specify type) _______
   □ Chronic infection (specify) _______
   □ Other condition (specify) _______

Comments:
36. Are you currently suffering from any cold, flu, or allergy symptoms:
   □ Yes   If yes, please specify __________________________
   □ No

37. Please specify any type of surgery you have had, the year of surgery and your age at the time:
   Operation                                Age and Year

38. Do you currently have any muscular injury(s)?
   □ Yes
   □ No

39. If yes, please explain the type of injury(s) and the length of time it has persisted

40. Have you had any muscular injury(s) in the past?
   □ Yes
   □ No

41. If yes, please report how long ago you had the injury(s):

42. Do you currently have any bone or joint injury(s):
   □ Yes
   □ No

43. If yes, please report how long ago you had the injury(s):

44. Do you currently have any soft tissue injury(s):
   □ Yes
   □ No

45. If yes, please report how long ago you had the injury(s):

46. If you’ve recently (within the past 6 months) suffered any other type of injury(s) please list them here:

47. What is the one primary sport or physical activity you participate in: __________________
48. Please list any other sports or physical activity you participate in regularly:

49. Please complete the following about your exercise program (if applicable):

**Aerobic Exercise** (e.g., running, swimming, biking, etc.):

<table>
<thead>
<tr>
<th>Type of exercise</th>
<th>Minutes/Session</th>
<th>Intensity/Pace</th>
<th>Times/Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>a.</td>
<td>a.</td>
<td>a.</td>
</tr>
<tr>
<td>b.</td>
<td>b.</td>
<td>b.</td>
<td>b.</td>
</tr>
<tr>
<td>c.</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
</tr>
<tr>
<td>d.</td>
<td>d.</td>
<td>d.</td>
<td>d.</td>
</tr>
<tr>
<td>e.</td>
<td>e.</td>
<td>e.</td>
<td>e.</td>
</tr>
</tbody>
</table>

**Resistance Exercise**

<table>
<thead>
<tr>
<th>Type of Training</th>
<th>Minutes/session</th>
<th>Times/Week</th>
</tr>
</thead>
</table>

50. How long have you participated in an aerobic exercise program? ____________

51. How long have you participated in a resistance exercise program? ____________

52. Check any of which you take on a regular basis. Please indicate how often and for how long:

- Sleeping tablets
- Aspirin
- Cold medicines
- Barbiturates
- Tranquilizers
- Diuretics
- Blood pressure tablets
- Antibiotics
- Thyroid hormones
- Oral contraceptives
- Insulin
- Oral hypoglycemics
- Corticosteroids
- Estrogens
- Isoniazid
- Pain medications
- Muscle relaxants
- Theophylline
- Antiarrhythmics
- Ulcer medications
- Antacids
- Antidepressants
- Seizure medications
- Methotrexate
- Other medications (please specify) ________________

Thank you for your honest answers. Please recheck your answers for completeness.
PHYSICAL ACTIVITY QUESTIONNAIRE

1. Please circle all activities listed below that you have done more than 10 times in the past year:

- Jogging (outdoor, treadmill) 1
- Swimming (laps, snorkeling) 2
- Bicycling (indoor, outdoor) 3
- Softball/Baseball 4
- Volleyball 5
- Bowling 6
- Basketball 7
- Skating (roller, ice, blading) 8
- Martial Arts (karate, judo) 9
- Tai Chi 10
- Calisthenics/toning exercises 11
- Wood chopping 12
- Water/oral hauling 13
- Football/soccer 14
- Racquetball/Handball/Squash 15
- Horseback riding 16
- Hunting 17
- Fishing 18
- Aerobic Dance/Step Aerobics 19
- Water Aerobics 20
- Dancing (Square, Line, Ballroom) 21
- Gardening or Yard work 22
- Bedminton 23
- Strength/Weight training 24
- Rock climbing 25
- Scuba diving 26
- Stair Master 27
- Fencing 28
- Hiking 29
- Tennis 30
- Golf 31
- Canoeing/Rowing/Kayaking 32
- Water skiing 33
- Jumping rope 34
- Snow skiing (X-country/Nordic trk) 35
- Snow skiing (downhill) 36
- Snow shoeing 37
- Yoga 38
- Other 39
- Walking for exercise (outdoor, indoor at mall or fitness center, treadmill) 40

For each activity that you circled above, list in the “Activity” box below. Check the months you did each activity over the past year (12 months) and then estimate the average amount of time spent in that activity (please let research assistant know if you need more space to record your activities).

<table>
<thead>
<tr>
<th>Activity</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>Average # of Times per month</th>
<th>Average # of Minutes each time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. In general, how many HOURS per DAY do you usually spend watching television? ________ hrs

3. Over this past year, have you spent more than one week confined to a bed or chair as a result of an injury, illness, or surgery? (please circle yes or no) Yes No
If yes, how many weeks over this past year were you confined to a bed or chair? _____ weeks

4. Do you have difficulty doing any of the following activities? (please circle yes or no)
   a. Getting in or out of a bed or chair? Yes No
   b. Walking across a small room without resting? Yes No
   c. Walking for 10 minutes without resting? Yes No

5. Did you ever compete in an individual or team sport (not including any time spent in sports performed during school physical education classes)? (please circle yes or no) Yes No

   If yes, how many total years did you participate in competitive sports? _______ years

6. Have you had a job for more than one month over this past year? (please circle yes or no) Yes No

   If yes, from last ____ to this ____
   month     month

List all JOBS that the individual held over the past year for more than one month. Account for all 12 months of the past year. If unemployed/disabled/retired/homemaker/student during all or part of the past year, list as such and probe for job activities of a normal 8 hour day, 5 day week.

<table>
<thead>
<tr>
<th>Job Name</th>
<th>*Job Code</th>
<th>Walk or ride to/from work</th>
<th>Average Job Schedule</th>
<th>Hours spent sitting at work</th>
<th>Check the category that best describes Job Activities* when not sitting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A     B     C</td>
</tr>
</tbody>
</table>

*See next page for Job Codes and Job Activities

Out of the total # of “Hrs/Day” you reported working at a particular “job”, how much of this time was usually spent sitting? Enter this # in “Hrs Sitting” column, then place a check “v” in the category which best describes their job activities when you were not sitting.
Job Activities:

**Category A**
(includes all sitting activities)
- Sitting
- Standing still w/out heavy lifting
- Light cleaning: ironing, cooking, washing, dusting
- Driving a bus, taxi, tractor
- Jewelry making/weaving
- General office work
- Occasional/short distance walking

**Category B**
(includes carrying light loads most indoor activities)
- Heavy cleaning: mopping, continuous walking, sweeping, scrubbing, vacuuming
- Gardening: planting, weeding
- Painting/Plastering
- Plumbing/Welding
- Electrical work
- Sheep herding

**Category C**
(heavy industrial work, outdoor construction, farming)
- Carrying moderate - heavy loads
- Heavy construction
- Farming: hoeing, digging, mowing, raking
- Digging ditches, shoveling
- Chopping (ax), sawing wood
- Tree/pole climbing
- Water/coal/wood hauling

Job Codes:

- **Not employed outside of the home:**
  1. Student
  2. Home Maker
  3. Retired
  4. Disabled
  5. Unemployed

- **Employed (or volunteer):**
  6. Armed Services
  7. Office worker
  8. Non-office worker
**EAT-35 QUESTIONNAIRE**

This questionnaire is strictly confidential. We are interested in some of your behaviors and beliefs about food. Most of the questions are directly related to food or eating patterns, although other types of questions have been included. For the following statements, please **circle** the response that best applies to you.

Please answer each question carefully with the following criteria in mind:

<table>
<thead>
<tr>
<th>Statement</th>
<th>Always</th>
<th>Very often</th>
<th>Often</th>
<th>Sometimes</th>
<th>Rarely</th>
<th>Never</th>
</tr>
</thead>
<tbody>
<tr>
<td>I am terrified about being overweight.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I avoid eating when I am hungry.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I find myself preoccupied with food.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think that my stomach is too big.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I have gone on eating binges when I have felt like I couldn’t stop.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I cut my food into small pieces.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think that my thighs are too large.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I am aware of the calorie content of foods that I eat.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I particularly avoid foods with high carbohydrate content.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I feel that others would prefer if I ate more.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think that my stomach is just the right size.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I feel satisfied with the shape of my body.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I vomit after I have eaten.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I feel extremely guilty after eating.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I like the shape of my buttocks.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I am preoccupied with a desire to be thinner.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think about burning calories when I exercise.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Other people think I am too thin.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I am preoccupied with the thought of having fat on my body.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I take longer than others to eat meals.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think my hips are too big.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I avoid food with sugar in them.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I eat diet foods.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think that my thighs are just the right size.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I feel that food controls my life.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I display self-control around food.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I feel that others pressure me to eat.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>I think my buttocks are too large.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
I give too much time and thought to food. 1 2 3 4 5 6
I feel uncomfortable after eating sweets. 1 2 3 4 5 6
I engage in diet behavior. 1 2 3 4 5 6
I think that my hips are just the right size. 1 2 3 4 5 6
I like my stomach to be empty. 1 2 3 4 5 6
I enjoy trying new rich foods. 1 2 3 4 5 6
I have the impulse to vomit after meals. 1 2 3 4 5 6
MENSTRUAL HISTORY QUESTIONNAIRE

1. How old were you when you started menstruating: _______ yrs old

2. What is the average number of days of flow during your period: ______ days

3. What is the average number of days between each period: ______ days

4. Do your menstrual cycles occur regularly (every 28-34 days):
   Yes
   No

5. How many menstrual periods have you had within the previous 6 months:
   I've had _____ periods within the past 6 months.

6. How many menstrual periods have you had within the previous 12 months:
   I've had _____ periods within the past 12 months.

7. Are you currently using oral contraceptives (birth control pills):
   Yes
   No

8. If yes, how long have you been using oral contraceptives (birth control pills):
   For several years
   About one year
   Between 6 to 11 months
   Less than 6 months
   Less than 3 months

9. Have you ever been pregnant:
   Yes
   No

10. If yes, how many times:
    I've been pregnant _____ number of times.

11. What was the date of your last birth:
    My last child was born on ______________.
        month/ day/ year

12. Amount of weight gain with your last child:
    I gained ____ pounds with my last child.

13. Amount of weight lost after 1 year of your last child’s birth:
    I lost _____ pounds after my last child’s birth.

Continue only if you have missed more than 6 menstrual periods within the past 12 months or if you have not menstruated within the past 3 months.
14. Are there any changes that you made in your lifestyle that occurred just prior to your menstrual periods stopping (weight loss, increased stress, increased physical activity, competition, etc.):
   Yes
   No

If yes, please be as specific and detailed as possible:

15. Have you noticed an increase in injuries since you stopped menstruating:
   Yes
   No

If yes, please describe:

16. Have your menstrual periods restarted:
   Yes
   No

17. If yes, what changes in lifestyle or physical activity did you make just prior to your menstrual period starting again. Please be as specific and detailed as possible:
INSTRUCTIONS FOR RECORDING 7 DAYS OF PHYSICAL ACTIVITY

1. Please maintain your normal activity level -- do not change your normal intensity (how difficult) or duration (how long) of activities.

2. Be as prompt as possible when recording your activities. Try to record all physical activities on your activity log as soon as you have completed them in minutes. Also, be as specific and accurate as possible when recording intensity and length of time the activity was performed.

3. Please record all activity for the same 24-hour periods starting at 5am each day and continuing until 5am the next day. Estimate as closely as possible the length of time sleeping as well as length of time for each activity.

Example:
Sunday 5am - Monday 5am = day 1
Monday 5am - Tuesday 5am = day 2
Tuesday 5am - Wednesday 5am = day 3
Wednesday 5am - Thursday 5am = day 4
Thursday 5am - Friday 5am = day 5
Friday 5am - Saturday 5am = day 6
Saturday 5am - *Sunday 5am = day 7

Day 8 is the day to have your blood drawn
(For this example, blood would be drawn on *Sunday between 7am-9am)

4. Please monitor your heart rate (pulse), training pace, and exact distance during any programmed physical activity or workouts.

5. Please complete the 7-day activity log during the same 7 days you complete the dietary records.

Example of how to record in log:

<table>
<thead>
<tr>
<th>Clock Time</th>
<th>Total Minutes</th>
<th>Activity Description</th>
<th>Intensity of Activity (record minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:00am - 7:15am</td>
<td>135</td>
<td>sleeping</td>
<td>135</td>
</tr>
<tr>
<td>7:16am - 8:30am</td>
<td>74</td>
<td>Eat, shower, dress</td>
<td>72 2</td>
</tr>
<tr>
<td>8:31am - 8:54am</td>
<td>23</td>
<td>Bike to school</td>
<td>4 13 3 3</td>
</tr>
<tr>
<td>8:55am - 10:59pm</td>
<td>848</td>
<td>walk to class &amp; sit</td>
<td>793 50 5</td>
</tr>
<tr>
<td>11:00pm - 5:00am</td>
<td>360</td>
<td>sleeping</td>
<td>360</td>
</tr>
<tr>
<td>TOTAL =</td>
<td>1440 minutes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total the minutes for each level of intensity:
495 869 65 8 3
## 7-DAY PHYSICAL ACTIVITY LOG

<table>
<thead>
<tr>
<th>Clock Time</th>
<th>Total Minutes</th>
<th>Activity Description</th>
<th>Describe workout pace (min/mile)</th>
<th>Intensity of Activity (allocate minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:00 - 5:15 AM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:15 – 5:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:30 - 5:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:45 - 6:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:00 - 6:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:15 – 6:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:30 - 6:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:45 - 7:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:00 - 7:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:15 - 7:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:30 – 7:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:45 – 8:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:00 - 8:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:15 – 8:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:30 – 8:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:45 – 9:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:00 – 9:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:15 - 9:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:30 – 9:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:45 – 10:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:00 – 10:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:15 - 10:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:30 – 10:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:45 - 11:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock Time</td>
<td>Total Minutes</td>
<td>Activity Description</td>
<td>Intensity of Activity (allocate minutes)</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>----------------------</td>
<td>----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>11:00 – 11:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:15 - 11:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:30 – 11:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:45 - 12:00 PM NOON – 12:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:15 – 12:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:30 – 12:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:45 – 1:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:00 – 1:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:15 – 1:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:30 – 1:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:45 – 2:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:00 – 2:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:15 – 2:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:30 – 2:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:45 – 3:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:00 – 3:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:15 – 3:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:30 – 3:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:45 – 4:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:00 - 4:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:15 - 4:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:30 - 4:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:45 - 5:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:00 – 5:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock Time</td>
<td>Total Minutes</td>
<td>Activity Description</td>
<td>Describe workout pace (min/mile)</td>
<td>Intensity of Activity (allocate minutes)</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>----------------------</td>
<td>----------------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>5:15 - 5:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:30 - 5:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:45 – 6:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:00 – 6:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:15 – 6:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:30 - 6:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:45 - 7:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:00 - 7:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:15 - 7:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:30 – 7:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:45 – 8:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:00 - 8:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:15 – 8:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:30 – 8:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8:45 – 9:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:00 – 9:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:15 - 9:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:30 – 9:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:45 – 10:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:00 – 10:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:15 - 10:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:30 – 10:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:45 - 11:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:00 – 11:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:15 - 11:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock Time</td>
<td>Total Minutes</td>
<td>Activity Description</td>
<td>Describe workout pace (min/mile)</td>
<td>Intensity of Activity (allocate minutes)</td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>----------------------</td>
<td>----------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>11:30 – 11:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:45 - 12:00 AM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midnite - 12:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:15 – 12:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:30 – 12:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:45 – 1:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:00 – 1:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:15 – 1:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:30 – 1:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:45 – 2:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:00 – 2:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:15 – 2:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:30 – 2:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:45 – 3:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:00 – 3:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:15 – 3:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:30 – 3:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:45 – 4:00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:00 - 4:15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:15 - 4:30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:30 - 4:45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:45 - 5:00 AM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TOTAL = 1440 minutes
Total the minutes for each level of intensity:
INSTRUCTIONS FOR RECORDING 7-DAY DIETARY RECORDS

Please record each food and beverage item you consume on a separate line. Be sure to include all snacks.

Record each item after weighing in exact amounts:
- liquids in cups or fluid ounces
- vegetables and fruits in cups, grams, or ounces
- beans, grains, and pasta in cups dry or cups cooked
- bread in slices, indicate what kind of bread (brand name and type)
- meats, fish, poultry and cheeses in ounces
- nuts in cups, ounces, or grams
- chips or other snack type foods in cups, ounces, or grams
- Spread (butter, cream cheese, margarine, etc.) in tsp or Tbs

Please specify if food is consumed raw. Also indicate if it was prepared from fresh, frozen, or canned products.

Indicate how the foods were prepared, such as fried, baked, boiled, etc.

If a food has a mixture of ingredients (sandwich or casserole), list the major ingredients separately in their proportions or amounts.

Use brand names whenever possible, or mention comparable brand.

For fruits and vegetables, please indicate if the skin was removed.

Indicate if dairy products are whole, 2%, or skim.

Be sure to include sauces, gravies, milk/sugar in coffee, etc.

Check food labels for weights, etc. Candy bars, cheeses, cookies, juices are all labeled with their weights -----Write this information down!

Provide any other information you feel might be helpful, such as food labels and/or recipes.

Record EVERYTHING edible that goes in your mouth.

MOST IMPORTANTLY, eat as you normally would -- please don't change your usual eating habits or modify your portion size.
7-DAY FOOD/BEVERAGE INTAKE RECORD

Please measure and weigh all food and beverages you eat throughout the day and write them down as you eat them. Remember to give as many details as possible, keep the food label if you think it will help describe the food better than you are able to. Providing us with recipes for homemade foods is helpful for us, too. Please list any vitamin or mineral supplements or any other supplements taken on bottom of this form and attach these labels if possible. It's best to be as descriptive as possible!

<table>
<thead>
<tr>
<th>Time</th>
<th>Food or Beverage item</th>
<th>Brand/source (manufacturer)</th>
<th>Type of preparation (bake, boil, fry, etc.)</th>
<th>Amount/weight (cups, oz, grams, tsp, TBS, fluid ounces)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

List any vitamin/mineral pills or other supplements here:

Add any additional comments here:
**BLOOD COLLECTION PROCEDURES**

**Ask each subject:**
Name
Subject number
Have you had anything to drink this morning?
Have you had anything to eat this morning?
When was the last time you ate or drank something?
When was the last time you exercised?
How many hours have you slept?
How are you feeling this morning?
How did you get here this morning? Walk? Bike? Run?

**Inform each subject:**
We are taking about 4 tablespoons of your blood to be analyzed for cholesterol, some B vitamins, and homocysteine. Your results will be mailed to you at the completion of this study, which may be as long as a year from now. If we detect anything that causes concern we will inform you immediately.

**Draw whole blood:**
5 mL in purple tube (EDTA) for hospital
5 mL in red/yellow tube (SERUM SEPARATOR) for hospital
8 mL in DNA tube for polymorphisms
2 x 10 mL in purple tubes (EDTA) for storage

**Preparation of blood samples:**
Place DNA tube for polymorphism analysis in -20 C freezer for 2 days before placing in -80 C freezer
Place all purple tubes on ice immediately.

**Prep Ascorbic Acid:**
Prepare ascorbic acid solution (0.02g ascorbic acid in 10 mL H2O for each subject)
Pipette 2 ml of ascorbic acid into each 4mL storage tube labeled red cell folate.

**Prep Red Cell Folate for storage:**
Use 1 purple top whole blood tube for red cell folate procedures as follows:
4 storage tubes should be prepared with 2 mL of freshly prepared 0.2% ascorbic acid solution (w/v). To each of the 4 storage tubes labeled red cell folate add 100 uL of well-suspended whole blood (this is a 1:21 dilution). Mix by inversion
several times; avoid foaming. Place in appropriate labeled box and store in -20 freezer for a few days then move to -80 freezer.

Spin 2 x 10 mL whole blood tubes (purple) to get plasma for storage and spin 10mL red/yellow tube to separate serum for hospital (15 minutes). Place tubes for hospital (5 mL purple; 10 mL red/yellow) in ziplock bag with paper towel. Attach billing slip for hospital from inside subject’s folder. Place in cooler for transport.

**Aliquot Plasma as follows:** (Use 12 x 1.8 mL plastic tubes for -80 freezer storage with screw on lids for each subject)
0.6 mL plasma into 2 separate tubes for B-6 assay
0.6 mL plasma into 2 separate tubes for plasma folate/B12 assay
0.6 mL plasma in 2 tubes for HoloTC assay
0.6 mL plasma into 1 tube for HCY assay
0.6 mL plasma in as many tubes that is left over labeled EXTRA
Place in appropriate labeled box and store in -20 freezer for a few days then move to -80 freezer.

<table>
<thead>
<tr>
<th>For Analyzing:</th>
<th>Type of Tube</th>
<th>Size of Tube</th>
<th>Number of Tubes</th>
<th>Type of Blood Needed</th>
<th>Amount Needed (duplicate)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B6</td>
<td>EDTA (purple)</td>
<td>10 mL</td>
<td>2</td>
<td>plasma</td>
<td>1.2 mL</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>EDTA (purple)</td>
<td>10 mL</td>
<td>2</td>
<td>plasma</td>
<td>0.6 mL</td>
</tr>
<tr>
<td>RIA kit whole blood for Red cell folate</td>
<td>EDTA (purple)</td>
<td>10 mL</td>
<td>2</td>
<td>whole blood</td>
<td>0.4 mL</td>
</tr>
<tr>
<td>RIA kit for plasma folate and Vitamin B12</td>
<td>EDTA (purple)</td>
<td>10 mL</td>
<td>2</td>
<td>plasma</td>
<td>1.2 mL</td>
</tr>
<tr>
<td>RIA kit plasma HoloTC</td>
<td>EDTA (purple)</td>
<td>10 mL</td>
<td>2</td>
<td>plasma</td>
<td>1.2 mL</td>
</tr>
<tr>
<td>Polymorphism</td>
<td>Special tube (blue)</td>
<td>8 ml</td>
<td>1</td>
<td>whole blood</td>
<td>~5 mL</td>
</tr>
<tr>
<td>Extra</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>plasma</td>
<td>~3-5 mL</td>
</tr>
<tr>
<td>Good Sam:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBC</td>
<td>EDTA (purple)</td>
<td>5mL</td>
<td>1</td>
<td>whole blood</td>
<td>~15 mL</td>
</tr>
<tr>
<td>ChemScreen</td>
<td>Ser. Sep. (red/yellow)</td>
<td>10mL</td>
<td>1</td>
<td>whole blood</td>
<td>~15 mL</td>
</tr>
</tbody>
</table>
HOMOCYTEINE PROCEDURES [updated 4-25-06 by Lanae Joubert]

These procedures are adapted from Christine Hansen’s work who used modifications from Durand P, Fortin LJ, Lussier-Cacan S, Davington J, and Blache D. Hyperhomocysteinemia induced by folic acid deficiency and methionine load – applications of a modified HPLC method. Clinica Chemica Acta 1996; 252: 83-93. Pay close attention to the actual chemicals we used here since formula weights are different with “hydrated” chemicals.

**Mobile Phase** (0.1 mol/L sodium acetate/acetic acid, pH 4.0; 2% methanol V:V)
6.8 g sodium acetate trihydrate → 500 mL pure water
Separately, mix 11.45 mL glacial acetic acid → 2000 mL water
Add enough acid mixture to bring sodium acetate mixture to pH 4.0 (~ 2300mL acid mixture)
Add methanol to a concentration of 20 mL/L [final volume (L) of acetic acid + acetate x 20] (2.8L x 20 = 56 mL methanol)

**Borate Buffer** (0.125 M with 4 mmol/L EDTA, pH 9.5)
49.96 g Potassium tetraborate tetrahydrate + 1.647 g EDTA dihydrate
Mix potassium hydroxide pellets with water to make basic solution
Add enough potassium hydroxide to bring pH to 9.5
Bring up to volume of 1000 mL with pure water

**NaCl mixture** (0.9% NaCl with 4 mmol/L EDTA)
9 g NaCl + 1.647 g EDTA dihydrate
Bring up to volume of 1000 mL

**Perchloric Acid mixture** (0.6 mol/L with 1 mmol/L EDTA)
25.075 mL perchloric acid (60%) + 0.103 g EDTA dihydrate
Bring up to volume of 250 mL with pure water
Keep refrigerated

**NaOH mixture** (1.55 mol/L)
12.4 g NaOH up to 200 mL pure water

**Freezer Homocysteine Calibration Standard** (1 mmol/L)
0.0169 g DL homocysteine + 20 mL NaCl mixture
Place in sonicator until small bits are dissolved (may take~10 min)
Bring up to volume of 25 mL with NaCl mixture (5 mmol/L)

Take 20 mL of above solution (5 mmol/L) + 80 mL NaCl mixture
This is 100 mL total volume (1 mmol/L)

Aliquot & Freeze: Place 1.60 mL (1mmol/L) in 2.0mL conical plastic snap-cap vials
Retrieve from freezer (thaw for 10 min):
1 x 2.0 mL snap-cap vial of 1 mmol/L HCY standard (invert ~ 5 times)
1 x 2.0 mL cryo-vial of pooled plasma for standards (vortex briefly)
1 cryo-vial of sample plasma to run in duplicate (only need 120 uL per
snap-cap; vortex briefly)
*Allow to be in room temperature for only 10 minutes - set timer!

Label Vials: 2.0 mL snap-cap microcentrifuge vials as follows:
Standards: 0 stnd, 5 stnd, 20 stnd
Samples: in duplicate (01A, 01B; 02A, 02B; 03A, 03B, etc.)
Controls: Pool 1, Pool 2

Make-up just before using:

Tri-n-butyl phosphine (10% in dimethylformamide V:V)
For 4 vials + 2: For 7 vials + 2:
15 uL TBP + 135 uL dimethylformamide 22.5 uL TBP + 202.5 uL
dimethylformamide

For 10 vials + 2: For 16 vials + 2:
30 uL TBP + 270 uL dimethylformamide 45 uL TBP + 405 uL
dimethylformamide

Incubation Solution (SBD-F stored in fridge)
[(175 uL borate buffer) x (number of samples + 1)] + [(0.05 mg SBD-F) x (number
of samples + 1)] + [(10 uL NaOH mixture) x (number of samples + 1)] add NaOH
last!

For 13 vials +1: (5 samples in duplicate, 3 standards, and a little extra):
2450 uL borate buffer + 0.7 mg SBD-F (small vial in fridge) + 140 uL NaOH
mixture
Vortex well to dissolve SBD-F.

For 7 vials +1: (2 samples in duplicate, 3 standards, and a little extra):
1400 uL borate buffer + 0.4 mg SBD-F (small vial in fridge) + 80 uL NaOH
mixture
Vortex well to dissolve SBD-F.

For 29 vials +1: (13 samples in duplicate, 3 standards, and a little extra):
5250 uL borate buffer + 1.5 mg SBD-F (small vial in fridge) + 300 uL NaOH
mixture
Vortex well to dissolve SBD-F.
**Internal Standard** (N-acetyl-L-cysteine **12.5 mmol/L**) chemical stored in fridge
Make this to use in Homocysteine calibration standards:
0.051 g N-acetyl-L-cysteine → 25 mL NaCl mixture (**12.5 mmol/L**)  

**Diluted Internal Standard** (N-acetyl-L-cysteine **1.25 mmol/L**)  
Make this to use in all sample vials and 0 stnd:  
Dilute to 1.25 mmol/L:  
0.5 mL **12.5 mmol/L** + 4.5 mL NaCl mixture (**1.25 mmol/L**)  

**Homocysteine (HCY) Calibration Standard** (DL-Homocysteine)  
Get HCY aliquots from freezer (1 mmol/L) and thaw for 10 min.  
Use 1 mL of thawed 1 mmol/L HCY standard + 9 mL NaCl mixture (100 umol/L)  

5 umol/L calibration standard:  
0.25 mL 12.5 mmol/L internal standard + 0.5 mL 100 umol/L HCY standard +  
1.75 mL NaCl mixture  

20 umol/L calibration standard:  
0.5 mL 12.5 mmol/L internal standard + 4.0 mL 100 umol/L HCY standard + 0.25 mL NaCl mixture  

**Prepare empty labeled snap-cap vials:**  
Add 30 uL 1.25 mmol/L internal standard to each sample vial  
Add 30 uL 1.25 mmol/L internal standard to the 0 stnd vial  
Add 30 uL of 5 umol/L calibration standard to its vial  
Add 30 uL of 20 umol/L calibration standard to its vial  
Add 120 uL sample plasma to each sample vial  
Add 120 uL pooled plasma to each standard vial (0 stnd, 5 stnd, 20 stnd)  
Add 25 uL 10% TBP in dimethylformamide to all vials while vortexing.  
Incubate in ice-water bath for 30 minutes.  
Add 150 uL perchloric acid mixture (stored in fridge) to each tube while vortexing.  
Let stand at room temperature for 10 minutes.  
Centrifuge in microcentrifuge for 10 minutes (prepare heat block to 60° C).  

**Label second set of Vials:** 2.0 mL snap-cap microcentrifuge vials as follows:  
Standards: 0 stnd, 5 stnd, 20 stnd
Samples: in duplicate (01A, 01B; 02A, 02B; 03A, 03B, etc.)
Controls: Pool 1, Pool 2
Add 185 uL incubation solution to each vial.

Add 50 uL supernatant from the first set of vials to the second set of vials. Vortex.

Incubate at 60° C in heat block for 60 minutes.

Cool in ice bath. Turn on Fluorometer!

**Label microautosampler vials** (plastic vials with inserts as one unit):
Label 0.2 mL microautosampler vials as follows:
Standards: 0 stnd, 5 stnd, 20 stnd
Samples: in duplicate (01A, 01B; 02A, 02B; 03A, 03B, etc.)
Controls: Pool 1, Pool 2

Use hypodermic needle screwed onto BD 3mL syringe to draw sample into syringe
Take off needle and screw on filter (4mm nylon syringe filter)
Filter into microautosampler vials (push plunger slowly)
Crimp tops with blue caps
Place in HPLC carousel

**HPLC Procedure:**
Column: Alltech Assoc., Inc. [fax PO to 1-302-292-8520; www.timtec.net $330 in 2006] Allsphere ODS-2 (4.6 x 150mm; 5µ) [part # 778545]
Flow rate: 1.0 mL/min
Fluorescence: excitation at 385 nm and emission at 515 nm
Injection volume: 15 uL
Run time: 11 min.
Check needle injection length [3mm]

Empowers software:
ID: System
Password: Manager
Advanced button: PRO (this indicates the Pro interface)
HCY Trial 2695-2475
Browse Project
Methods tab
Choose date from previous run – open
Save as - new date
Update vial names
Save/Close

Run Samples
File: new sample set method: based on existing sample set method
Green icon to run samples

Shutdown procedure:
Turn off fluorometer
Run H2O 10 minutes and then run methanol 10 minutes
VITAMIN B6 (PLP & 4-PA) PROCEDURES
[updated 6-14-06 by Lanae Joubert]


Mobil Phases
Solvent A: 50mM Sodium Phosphate (pH 3.1) containing 0.2% Acetonitrile
900 mL H₂O + 2.9 mL phosphoric acid
Add NaOH (5M) to pH 3.1 while mixing
Add 2 mL acetonitrile
Bring up to final volume of 1 L with H₂O

Solvent B: Methanol

Solvent C: Water

Protein Precipitator
5% (w/v) Metaphosphoric Acid 4°C
To make 10 mL:
0.5 g metaphosphoric acid + 9.5 mL H₂O

Lipid Extractor
150 uL per sample dichloromethane
[We did not use for our fasting samples. This step requires interesting extraction]

Post Column Reagent
Sodium Chlorite Solution (0.25 g/L; 2.76 mM)
0.125g Sodium Chlorite → 500 mL H₂O

Freezer Standards: Freeze in 1.5 mL aliquots at -80°C
PLP standard (1mM)
0.2652 g PLP →1L H₂O

4-PA standard(1mM)
0.183g 4-PA→1L H₂O

Serial Dilution of Standards:
Add 1 mL each of [1mM] PLP and 4-PA up to 100 mL H₂O [this makes 10 uM]
Add 0.5 mL of 10uM solution up to 50 mL H₂O [this makes 100 nM]

Calibrators (6)
Mix aqueous calibrators ranging in concentration from 0– 100 nmol/L [100nM]

<table>
<thead>
<tr>
<th></th>
<th>0 std</th>
<th>12 std</th>
<th>25 std</th>
<th>50 std</th>
<th>75 std</th>
<th>100 std</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP H₂O</td>
<td>4 mL</td>
<td>3.5 mL</td>
<td>3 mL</td>
<td>2 mL</td>
<td>1 mL</td>
<td>0 mL</td>
</tr>
<tr>
<td>4 mL</td>
<td>100nM</td>
<td>0.5 mL</td>
<td>1 mL</td>
<td>2 mL</td>
<td>3 mL</td>
<td>4 mL</td>
</tr>
</tbody>
</table>
**Sample Preparation**

Label 2mL snap cap vials:
- Standards: Calibrators 0-100
- Sample plasma: in duplicate (#1A, 1B, 2A, 2B, etc)
- Control plasma: in duplicate (Pool 1, Pool 2)

Place 150 uL of sample plasma into sample vials
Place 150 uL of control plasma into pool vials
Place 150 uL of standards into calibrator vials

Add 150 uL metaphosphoric acid to all vials (including blank) while vortexing

Vortex (2000 rpm) for 10 seconds

Centrifuge (8,000rpm) for 12 minutes

Label autosampler vials:
- Standards: Calibrators 0-100
- Sample plasma: in duplicate (#1A, 1B, 2A, 2B, etc)
- Control plasma: in duplicate (Pool 1, Pool 2)

Extract top aqueous (top) layer from each vial and filter with syringe & filter into autosampler vials

**HPLC Procedure:**

- Column Temperature: 35°C
- Flow rate: 0.7 mL/min
- Fluorescence: excitation at 325 nm and emission at 425 nm
- Injection volume: 10 uL [Check needle injection length [3mm]]
- Run time: 16 min.

Post-Column pump:
- Sodium chlorite (.25g/L) at 0.1 mL/min
- Heat block 75°C
- Make sure postcolumn pump is on and set at 0.1mL/min

Empowers software:
- ID: System  Password: Manager
- Advanced button: PRO (this indicates the Pro interface)
- Run Samples:
  - Project to acquire data: PLP_May_2006
  - Chromatographic systems: PLPsetup
  - File: New sample set method
  - Based on existing sample set method:
    - PLP sample set method (or previous date w/similar setup)
  - Make sure separations module (2695) and fluorescence detector (2475) are turned on
  - Make sure heat block is actual 75°C
  - Click green icon to run samples

Shutdown procedure:
- Purge postcolumn pump with methanol (store in methanol)
- Run H20 (C) 10 minutes and then run methanol (B) 10 minutes & TURN OFF ALL MACHINES
PLASAMA FOLATE/VITAMIN B12 PROCEDURES
MPBiomedicals SimulTRAC-SNB RIA Kit-2004

Collect sample and control plasma from freezer
Collect reagent kit from fridge

Turn lights off (yellow lamp for diffused light ok)

Assay Procedures:
Add 200 µL std A to tubes 3, 4, 5, 6
Add 200 µL std B to tubes 7, 8
Add 200 µL std C to tubes 9, 10
Add 200 µL std D to tubes 11, 12
Add 200 µL std E to tubes 13, 14
Add 200 µL std F to tubes 15, 16
Add 200 µL control plasma (pool plasma) to *1, *2 tubes
Add 200 µL sample plasma to appropriate sample tubes
Add 200 µL DTT/TRACER (see reagent prep in box above) solution to all tubes & vortex
Incubate (18-25° C) for 15 minutes at room temperature
Add 100 µL REAG EXT to all tubes except tubes 1, 2 & vortex
Incubate (18-25° C) for 10 minutes at room temperature
Mix thoroughly REAG BLANK. Add 1 mL REAG BLANK to tubes 3, 4
Mix thoroughly BINDER (shake vigorously). Set Timer! Add 1 mL BINDER to tubes 5-16, control plasma, and sample plasma tubes & vortex.
Incubate (18-25° C) for 60 minutes at room temperature
Place reagent kit back in fridge
Centrifuge all tubes except tubes 1, 2 in cold (4°C) for 10 minutes (2000xg)
Remove supernatant (dump into liquid waste); remove excess liquid w/ Q-tip

Gamma Counter Procedures (Cobra II; 5002)
Place tubes in cassettes – protocol 58 first cassette, stop cassette at end F3 (protocols); Enter protocol #58; F2 (SC commands); F5 (count); F1 x 3

Reference Values: Count for tubes 1, 2 = 10,000 – 25,000 for Co\textsuperscript{5}; Count for tubes 1, 2 = 15,000 – 35,000 for I\textsuperscript{125}

Reagent Preparation (20mL):
Add DTT to TRACER (1:1 ratio) (use within 30 days of mixing)