

CHANGES IN SERUM HOMOCYSTEINE IN RESPONSE TO ORAL CREATINE
SUPPLEMENTATION IN VEGETARIANS

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ABSTRACT

Homocysteine (Hcy) is metabolized through a series of remethylation and transsulphuration processes that require the co-factors vitamins B₆, folate (the nutrient derived from food, and folic acid is supplemental), and B₁₂. Homocysteine metabolism results in the production of creatine (Cr). By way of negative feedback creatine supplements could potentially decrease Hcy production. Low dietary intake of vitamins and creatine, often a consequence of a vegetarian diet may also increase serum Hcy concentrations. It was therefore the purpose of this study to examine the effect of a five day creatine loading period (0.25 g Cr/kg lean body mass (LBM)/day) in female vegetarians (VG) (n=9, age 25 ± 3.6) and non-vegetarians (NV) (n=11, age 24 ± 5.9) on serum levels of Hcy, vitamins B₆ and B₁₂, whole blood folate, and on a number of fitness measures known to be positively affected by creatine supplementation: peak torque and average power during leg extensions; one repetition maximum (1RM) bench and leg press; peak power during repeated Wingate anaerobic tests. Aside from a difference in change scores for leg press, there were no differences between dietary groups for the fitness measures, and therefore the participants were pooled for comparisons between time points. Urinary creatine was significantly greater following the five day loading period (81 ± 113 vs. 647 ± 421 μmol). In contrast, urinary creatinine did not change. A significant increase in plasma vitamin B₆ (pyridoxal 5'-phosphate) was found (72 ± 24 vs. 94 ± 24 nmol/L) (p < 0.05), although there were no between group differences. Serum Hcy, vitamin B₁₂, and whole blood folate levels did not change significantly over time in either group. Participants showed significant increases post-loading in bench press (45 ± 13 vs. 48 ± 12 kg.) and leg press (116 ± 35 vs. 125 ± 40 kg) (p < 0.05). Changes scores for leg press were greater for the vegetarian participants when compared with non-vegetarians (16 ± 16 vs. 3 ± 9 kg). Significant improvements post-loading were also

noted for peak torque (85.0 ± 15.0 vs. 91.8 ± 12.9 Nm) and average power (99.5 ± 14.4 vs. 107.1 ± 14.8 W) ($p < 0.05$). Peak power during repeated Wingate anaerobic tests also significantly improved in response to the intervention (449.2 ± 92.6 vs. 472.4 ± 103.5 W) ($p < 0.05$). This response did not differ however between the two diet groups. These results suggest that a five day creatine loading protocol similarly affects fitness performance measures in both vegetarians and non-vegetarians. There was no significant effect of the supplementation protocol on serum Hcy concentration, suggesting that the supplemented creatine did not decrease the reformation of Hcy, as originally hypothesized.

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DEDICATION

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LIST OF ABBREVIATIONS

ADP – adenosine diphosphate
ANOVA – analysis of variance
ATP – adenosine triphosphate
AGAT - arginine:glycine amidinotransferase
BL – baseline
cal – calorie
CSEP – Canadian Society for Exercise Physiology
Cr – creatine
Cn – creatinine
CBS – cystathionine β -synthase
 $^{\circ}\text{C}$ – degrees Celcius
 $^{\circ}\text{S}$ – degrees per second
d – day
DTT – dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
gly – glycine
g – gram
HME – high meat eater
HPLC – high performance liquid chromatography
Hcy – homocysteine
k - kilo
kg - kilogram
LOV – lacto-ovo vegetarian
LBM – lean body mass

L - litre

LME – low meat eater

MTF – methyl tetrahydrofolate

MTHFR – methylene tetrahydrofolate reductase

MES - 2-(N-morpholino)ethanesulfonic acid

m – milli

mg - milligram

min – minute

M – moles

mol - moles

n – nano

Nm – Newton metre

n.s. – non-significant

NV – non-vegetarian

PAR-Q – Physical Activity Readiness Questionnaire

PARmed-X – Physical Activity Readiness Medical Examination

p - pico

Pl – placebo

PFLC – Professional Fitness and Lifestyle Consultant

RDA – recommended daily allowance

RM – repetition maximum

RT – resistance training

RER – respiratory exchange ratio

SAH – S-adenosyl homocysteine

SAM – S-adenosyl methionine

ser – serine

SE – standard error

THF – tetrahydrofolate

TCr – total muscle creatine

TRIS - tris(hydroxymethyl)aminomethane

UR – urinary

VE – vegan

VG – vegetarian

vs. – versus

VCO₂ – carbon dioxide uptake

VO₂ – oxygen uptake

VO₂max – maximal oxygen uptake

W – watt

wt – weight

Δ – change in

μ - micro

[Cr] – muscle creatine concentration

[PCr] – muscle phosphocreatine concentration

[tCr] – total muscle creatine concentration

5CH₃-THF - 5-methyltetrahydrofolate

5, 10-CH₂-THF - 5, 10-methylene tetrahydrofolate

1. INTRODUCTION

Homocysteine (Hcy) is a sulphur containing amino acid that is created during the production of methionine (Refsum, Ueland, Nygard, & Vollset, 1998). Past research has linked plasma Hcy concentration with a variety of disease states, including cystathionine β -synthase (CBS) deficiencies (Mudd, Levy, & Skovby, 1995), renal (Mudd et al., 1995) and cardiovascular disease (Refsum et al., 1998). Much of the research examining Hcy in disease states has identified altered metabolism of Hcy resulting in elevated Hcy levels as being a key factor in the pathogenetic characteristics of the disease.

Plasma and serum concentrations of Hcy are altered by a number of lifestyle factors, including physical activity, dietary intake and nutritional status, gender and hormones (Chrysohoou et al., 2004). Both gender and hormone status are linked with serum Hcy concentration, with older men generally having elevated concentrations when compared to women of a similar age and to younger populations. Furthermore, postmenopausal women possess greater concentrations when compared to premenopausal or pregnant counterparts, suggesting that increases in female steroid hormones may have a diminishing effect on Hcy concentration (Chrysohoou et al., 2004).

Dietary intake and nutritional status are also closely linked with serum Hcy concentration, because Hcy metabolism is dependent on sufficient intake and blood levels of a number of vitamins, including vitamins B₂, B₆, B₁₂ and folate¹ (Refsum et al., 1998). A significant link between elevated Hcy concentrations in the bloodstream and cardiovascular

disease has been established (Refsum et al., 1998; Selhub, 1999). It is thought that the presence of the amino acid in the bloodstream may damage the vascular system (Nygard et al., 1995; Selhub, 1999; Virtanen et al., 2005). Possible solutions to this problem of elevated Hcy concentrations include vitamin (Bosnaa et al., 2006; Jacques, Selhub, Bostom, Wilson, & Rosenberg, 1999; Lonn et al., 2006; Miller et al., 1992; Nygard, Refsum, Ueland, & Vollset, 1998; Nygard et al., 1995; Refsum et al., 1998) and creatine (Cr) supplementation (Korzun, 2004; Stead, Au, Jacobs, Brosnan, & Brosnan, 2001; Steenge, Verhoef, & Greenhaff, 2001; Taes, 2005; Taes et al., 2004; Taes et al., 2003).

In the metabolism of Hcy, methionine is converted to *S*-adenosyl methionine (SAM), a methyl donor to a number of acceptors. These methylation reactions result in the methylated substrate and *S*-adenosyl homocysteine (SAH). *S*-adenosyl homocysteine is then metabolized to form Hcy. Excess Hcy production leads to its exportation and subsequent increase in the bloodstream (Selhub, 1999).

An important element in the reformation of Hcy is creatine. One methyl acceptor in the transfer from SAM is guanidinoacetate, which results in the production of creatine (Taes et al., 2003). The production of creatine *in vivo* accounts for 70 % of the transmethylation reactions in the body, with the donation of a methyl group by SAM to guanidinoacetate in the liver (Taes, 2005). The role of oral creatine supplementation, a relatively popular sport supplement, in Hcy metabolism has been investigated, with mixed results. Of the few studies looking specifically at the effects of creatine supplementation on Hcy concentration, some have shown that creatine has a lowering effect on Hcy (Korzun, 2004; Stead et al., 2001; Taes et al., 2003) while others have shown no effect (Steenge et al., 2001; Taes, 2005; Taes et al., 2004). These previous researchers have attempted to determine whether oral supplementation with creatine monohydrate reduces

the need for the methylation of guanidinoacetate, thereby decreasing the frequency of Hcy reformation. Due to the contrasting results found in both animal and human studies, further research should be performed to determine the effect of creatine supplementation on Hcy concentration in the bloodstream in humans.

Vegetarians generally have very little natural creatine in their diet, because creatine is found mainly in animal products (Greenhaff, Bodin, Soderlund, & Hultman, 1994). Considering the role of creatine in Hcy metabolism, it is likely that a vegetarian would have higher baseline concentrations of Hcy in her bloodstream, since the need for endogenous production of creatine would be great. By supplementing with creatine monohydrate, this need for endogenous production could decrease, thereby decreasing the amount of Hcy produced.

Creatine monohydrate is commonly used both as an ergogenic aid in high intensity sports and exercise, and to help increase lean tissue mass (Buford et al., 2007). Creatine is required for the formation of phosphocreatine which is required for the rephosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) during exercise and recovery. By increasing the amount of creatine available, stored as phosphocreatine, the amount of energy generated during high intensity bouts of exercise may be increased (Harris, Soderlund, & Hultman, 1992).

Individuals with lower baseline concentrations of total muscle creatine experience greater increases in this measure in response to supplementation (Burke et al., 2003; Syrotuik & Bell, 2004). Vegetarians, who should have a lower baseline concentration of total muscle creatine due to the lack of natural creatine intake in their diet, may experience a significantly greater absorption when supplementing orally. Also, vegetarians may experience greater improvements

in physiological performance in response to oral supplementation, as they possibly would have a greater increase in muscle phosphocreatine available to them during high intensity activity (Burke et al., 2003).

The effects of creatine supplementation on exercise have been investigated thoroughly, though few researchers have studied creatine supplementation and vegetarianism (Burke et al., 2003; Harris et al., 1992; Lukaszuk et al., 2002; Lukaszuk, Robertson, Arch, & Moyna, 2005; MacCormick, Hill, MacNeil, Burke, & Smith-Palmer, 2004; Shomrat, Weinstein, & Katz, 2000), and none have focused on female vegetarians, fitness, and creatine supplementation. A need for information in this area is evident in order to determine whether this population is at risk for elevated concentrations of Hcy in the bloodstream. Also, determining whether supplementation with creatine monohydrate would be beneficial as a treatment to decrease Hcy concentration by decreasing the need for endogenous production of creatine is important.

The current study investigated baseline concentration of Hcy in the bloodstream of vegetarian and non-vegetarian females, as well as the effect of creatine supplementation on this measure. It was hypothesized that vegetarians would have higher concentration of Hcy at the onset of the study, and that creatine supplementation would result in a decrease in Hcy in the bloodstream. As a secondary purpose, measures of muscle strength, power and anaerobic capacity were taken at the onset of the study as well as following the five day creatine loading intervention in order to determine whether supplementation created a response, and if the response differed between vegetarians and non-vegetarians.

2. SCIENTIFIC FRAMEWORK

2.1 Literature Review

2.1.1 Homocysteine

Homocysteine is a non-protein forming sulphur amino acid that is found in the bloodstream (Nygard et al., 1995; Virtanen et al., 2005). Although reported ranges vary significantly (Refsum et al., 1998) normal concentrations of Hcy typically range from five to 15 $\mu\text{mol/L}$.

2.1.1.1 Homocysteine metabolism

The metabolism of Hcy occurs primarily through two pathways; remethylation to methionine and transsulphuration to cystathionine (refer to Figure 1). Methionine is an amino acid that is involved in protein synthesis (Allen, Stabler, Savage, & Lindenbaum, 1993), and is therefore of importance to the body. In the presence of excess methionine, Hcy is directed to the transsulphuration pathway, and is thus catabolized to form cystathionine and then cysteine for disposal (Refsum et al., 1998). The remethylation pathway requires vitamins B₂ (riboflavin), B₁₂ and folate (B₉) in order to proceed, while the transsulphuration pathway requires vitamin B₆ in order to progress optimally (Refsum et al., 1998; Selhub, 1999).

The transsulphuration pathway occurs when methionine supply is plentiful (Refsum et al., 1998). Homocysteine reacts with serine to form cystathionine which is further metabolized to form cysteine (Refsum et al., 1998; Taes et al., 2003). This occurs in the presence of CBS,

which is dependent on vitamin B₆ (Refsum et al., 1998).

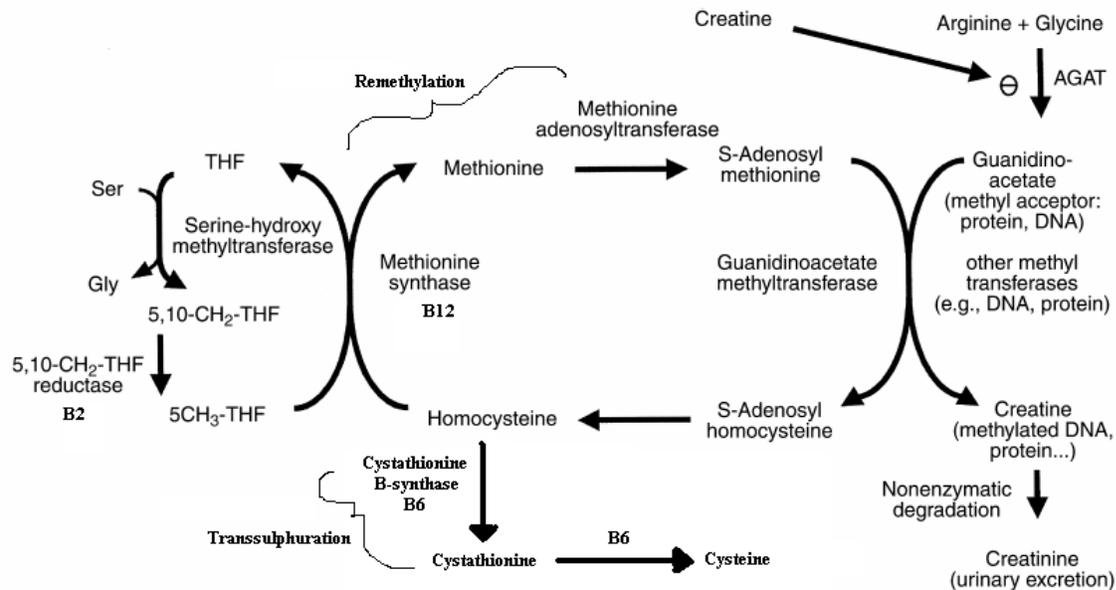


Figure 1. Homocysteine metabolism. Abbreviations: THF, tetrahydrofolate; 5CH₃-THF, 5-methyltetrahydrofolate; 5, 10-CH₂-THF, 5, 10-methylene tetrahydrofolate; Ser, serine; Gly, glycine, AGAT, arginine:glycine amidinotransferase. Modified from Taes et al., 2003.

In the remethylation pathway, Hcy is methylated by 5-methyltetrahydrofolate which forms methionine, using vitamin B₁₂ as a cofactor, and 5-methyltetrahydrofolate as a substrate (Korzun, 2004; Selhub, 1999). 5-methyltetrahydrofolate, upon giving up its methyl group, forms tetrahydrofolate (THF), which then reacts to form 5, 10-methylene tetrahydrofolate. This multi-step folate cycle concludes with the reproduction of 5-methyltetrahydrofolate (Taes et al., 2003) in the presence of methylene tetrahydrofolate reductase (MTHFR), which is dependent on the presence of vitamin B₂ (Refsum et al., 1998). Methionine is then activated to form SAM, which is a methyl donor to a variety of acceptors (Selhub, 1999), including guanidinoacetate, resulting in the formation of creatine (Taes et al., 2003). The use of methyl groups is predominantly

directed towards the formation of creatine (Mudd et al., 1995). SAM is reduced to form SAH, which is then hydrolyzed to regenerate Hcy (Selhub, 1999).

2.1.1.2 Control and regulation of homocysteine metabolism

The determination of the fate of Hcy within the cell is mediated by the dietary concentration of methionine: a) when dietary methionine is high, it is quickly converted to SAM, which inhibits MTHFR activity (multi-step folate cycle) and activates CBS (transsulphuration pathway), causing an increase in the catabolism of Hcy; b) when dietary methionine is low, SAM concentrations are too low to inhibit MTHFR activity or to activate the transsulphuration pathway, which encourages the remethylation pathway via an increase in the availability of substrate for remethylation (Selhub, 1999).

The reactions of the remethylation and transsulphuration pathways are dependent on the availability of various vitamins (B₂, B₆, B₁₂ and folate) (Selhub, 1999). Therefore, the absence or limited amount of any one of the vitamins involved could have a detrimental effect on Hcy metabolism. A well balanced diet that includes each of these vitamins is very important for Hcy metabolism and the resulting protein synthesis.

Decreased availability of vitamin B₂ limits the number of methyl groups available to remethylate Hcy and reform methionine. Without vitamin B₆, the transsulphuration pathway will not proceed optimally and in times of excess methionine availability, Hcy catabolism may be affected. Deficiencies of folate would limit the multi-step folate cycle, and decrease methyl group availability to react with Hcy to form methionine. In the absence of vitamin B₁₂, this remethylation cannot take place, even in the presence of a methyl group and Hcy.

2.1.2 Factors affecting homocysteine concentration

Homocysteine concentration is affected by a number of genetic and lifestyle factors. The accumulation of Hcy in plasma, or hyperhomocysteinemia, typically results from a disruption of Hcy metabolism. This alteration in metabolism precipitates the release of excess Hcy from the cells into the bloodstream to prevent toxicity within the cell (Selhub, 1999). Severe cases of hyperhomocysteinemia are generally associated with genetic defects (Selhub, 1999), while mild to moderate hyperhomocysteinemia (plasma Hcy levels greater than 14 $\mu\text{mol/L}$) has been linked, although not causally, with a number of health problems/diseases.

2.1.2.1 Cystathionine β -synthase deficiency

A deficiency or defect of CBS is the most common disorder that inhibits the transsulphuration pathway (Mudd et al., 1995). In such impairments, Hcy is diverted toward the remethylation pathway, thus increasing the rate of methionine synthesis, resulting in an increase in SAM concentration within the cell (Selhub, 1999). Eventually, SAM concentration will reach levels associated with MTHFR inhibition (Selhub, 1999), which when coupled with the dysfunction of the transsulphuration pathway, causes a buildup of intracellular and then plasma Hcy concentration (Mudd et al., 1995).

This genetic disorder results in a number of clinical abnormalities, such as: vision problems; osteoporosis, generally of the spine and the long bones; problems with reproductive fitness; central nervous system irregularities, such as mental retardation, neurologic and psychiatric abnormalities; and abnormalities of the vascular system, including thromboembolisms (McLean et al., 2004; Mudd et al., 1995). Whether creatine supplementation has an effect on these disorders is unknown.

2.1.2.2 Renal disease/insufficiency

Individuals with renal disease often have impaired excretion of Hcy resulting in mild to moderate hyperhomocysteinemia (Mudd et al., 1995). Bostom and Culleton (1999) reported that 83 % of dialysis patients had fasting Hcy concentrations greater than 13.9 $\mu\text{mol/L}$. This increase in Hcy increases the risk of developing cardiovascular disease in this population. Bostom and Culleton (1999) state that while end stage renal disease patients do have a higher prevalence of cardiovascular disease risk factors such as old age or inactivity, there are also risk factors that are directly related to renal disease, such as elevations of Hcy concentration. Individuals with end stage renal disease have a much greater risk of developing cardiovascular disease, and experience mortality related to cardiovascular disease 10 to 20 times more frequently than the general population (Bostom & Culleton, 1999).

2.1.2.3 Cardiovascular disease

Suggestions have been made stating that increased Hcy concentration in plasma may cause vascular lesions in connective tissue and smooth muscle cells, endothelial cells, and in vessel walls (Refsum et al., 1998). This may affect the blood coagulation system (Selhub, 1999) and could have detrimental effects on vascular tissue (Nygard et al., 1995; Selhub, 1999; Virtanen et al., 2005). It is in this way that the accumulation of Hcy may be linked to cardiovascular disease (Selhub, 1999).

The Hordaland Homocysteine study reveals a number of factors that are associated with increases in Hcy concentration (Nygard et al., 1995). This study included 16,176 individuals randomly selected from the National Population Registry in Western Norway who had not previously been diagnosed with coronary heart disease, cerebrovascular disease, hypertension, or diabetes. In this publication, Nygard and colleagues (1995) reported that increases in plasma

Hcy were associated with several clinical variables that have been linked with cardiovascular disease, such as smoking, cholesterol levels, blood pressure, heart rate, and decreased levels of physical activity in men and women. Early research noted that in individuals with CBS deficiencies who have experienced major atherothrombotic events, receiving treatments designed to decrease Hcy concentration decreased the rate of atherothrombotic events (Mudd et al., 1985; Wilcken & Wilcken, 1997). Hcy lowering treatments also reduce the rate of progression of extracranial carotid artery plaque area (Peterson & Spence, 1998), while treatments designed to increase Hcy concentration (oral L-methionine load) elicit a reduction in flow-mediated brachial artery reactivity (Chambers, McGregor, Jean-Marie, & Kooner, 1997). However, more recent investigations have revealed that decreases in plasma Hcy concentrations do not diminish the occurrence of cardiovascular events in individuals with previous histories of cardiovascular problems or disease (Bonna et al., 2006; Lonn et al., 2006).

Bonna and colleagues (2006) randomly assigned 3,749 men and women who had previously experienced an acute myocardial infarction in the seven previous days to four treatment groups: a) folic acid, vitamin B₁₂, and vitamin B₆; b) folic acid and vitamin B₁₂; c) vitamin B₆; d) and a placebo group. The participants were followed for 40 months, and incidence of a recurrent myocardial infarction, stroke or death due to coronary artery disease was recorded. While Hcy concentration decreased in response to treatment a and b, occurrences of further myocardial events were not decreased. The authors even found a trend towards an increase in myocardial events following the supplementation protocols, and recommend that such therapy should not be recommended following a myocardial event.

Lonn and colleagues (2006) randomly assigned 5,522 men and women with vascular disease or diabetes to two treatment groups: folic acid, vitamin B₆ and vitamin B₁₂; or placebo.

Participants were supplemented for an average of five years. The researchers were interested in death from cardiovascular causes, myocardial infarction, and stroke. In spite of a decrease in plasma Hcy concentration (- 2.4 $\mu\text{mol/L}$), the treatment did not decrease the risk of death from cardiovascular causes or myocardial infarction. There were fewer strokes in the supplement group when compared to the active treatment (4.0 vs. 5.3 %), and more participants in the supplement group had unstable angina (9.7 vs. 7.9 %). The authors suggested that these differences were due to either an overestimate of the real effect, or to chance.

Though no causal relationship has been proven, elevated total Hcy pool is a strong predictor of cardiovascular mortality. A graded dose response association was found between plasma Hcy concentration and overall mortality in older adults with a previous history of coronary heart disease (Nygard et al., 1997). This relationship was strongest for plasma Hcy levels greater than 15 $\mu\text{mol/L}$. Also, Boushey, Beresford, Omenn, and Motulsky (1995) reported that a 5 $\mu\text{mol/L}$ increase in total Hcy plasma levels was associated with an increased risk of coronary heart disease of 60 % for men and 80 % for women.

In light of current and past literature, whether otherwise healthy individuals with increased plasma Hcy concentrations would benefit from decreasing these concentrations is unclear. By minimizing plasma Hcy concentration in individuals with high levels initially, there may be a decrease in the risk of developing cardiovascular disease, before a cardiovascular event has occurred. Therefore, determining ways to decrease Hcy in plasma is of great importance.

2.1.2.4 Lifestyle factors affecting homocysteine

Baseline Hcy concentration differs between males and females, and is altered in response to several manageable lifestyle choices such as dietary habits, differences in vitamin status,

physical activity level, medications (e.g. oral contraceptives) and smoking status, to highlight a few key factors.

Smoking: Smoking, on its own, has been reported to cause vascular damage in a number of ways, including by injuring the arterial endothelium and promoting atherogenesis (Powell, 1998). A dose-response relationship between the number of cigarettes smoked and plasma Hcy concentration has been reported (Chrysohoou et al., 2004). While the corresponding increase in Hcy concentration for every 10 cigarettes smoked daily was less for females when compared to males (0.3 vs. 0.7 $\mu\text{mol/L}$), the increases were significant for both genders ($p = 0.01$).

Another study reported that smokers with modestly increased plasma Hcy concentration (greater than 12 $\mu\text{mol/L}$) had a 12-fold increase in risk of cardiovascular disease when compared to non-smokers who had normal plasma Hcy concentration (less than 12 $\mu\text{mol/L}$) (O'Callaghan, Meleady, Fitzgerald, & Graham, 2002). This risk was also found to be greater in men. The authors suggest that due to the aforementioned risk of cardiovascular disease currently associated with smoking and with increased Hcy concentration separately, the interaction of the two conditions would likely produce damage to the vascular system.

In a study examining 16,176 individuals randomly selected from the National Population Registry in Western Norway, Nygard et al. (1995) noted that smokers had higher plasma Hcy concentrations than non-smokers of the same age, and that there was an almost linear increase in Hcy as the number of cigarettes smoked daily increased. They also observed an interaction between smoking and both gender and age with respect to plasma Hcy concentration. Female smokers experienced a greater effect of smoking than males, and the effect of cigarette smoking was greater in the older age groups than in the younger groups (Nygard et al., 1995).

Physical activity levels: Reports conflict on the effect of physical activity on plasma Hcy levels. Nygard and colleagues (1995) reported that there was an inverse relationship between Hcy concentration and physical activity levels, and that Hcy levels were dependent on the intensity of physical activity that the participants were involved in. A more recent investigation of the relationship between physical activity status (sedentary vs. light or moderate vs. vigorous exercise) and plasma Hcy concentration revealed no associations, though specific types of exercise were associated with lower Hcy levels. Chrysohoou et al. (2004) reported that endurance exercise led to lower Hcy levels whereas resistance exercise had no effect in either men or women. Average Hcy concentration for females in endurance training is 8.0 ± 2.1 $\mu\text{mol/L}$, compared with 10.4 ± 1.9 $\mu\text{mol/L}$ for those involved in resistance training, and 12.5 ± 1.8 $\mu\text{mol/L}$ for sedentary females (Chrysohoou et al., 2004). No associations were found between the prevalence of obesity and Hcy concentration (Chrysohoou et al., 2004).

2.1.2.5 Gender and steroid hormones

Males tend to have higher Hcy levels than females (Chrysohoou et al., 2004), which could be associated with an increased risk of developing cardiovascular disease (Nygard et al., 1998; McCarty, 2001). Nygard and colleagues (1995) have reported an average plasma Hcy concentration of 10.84 $\mu\text{mol/L}$ for males and 9.14 $\mu\text{mol/L}$ for females, while other authors have reported average concentrations of 14.5 ± 6 $\mu\text{mol/L}$ for males and 10.8 ± 3.5 $\mu\text{mol/L}$ for females (Chrysohoou et al., 2004). Increases in plasma Hcy concentration in response to age were found in both genders (10.84 , 11.22 , 12.27 $\mu\text{mol/L}$ for men, 9.14 , 9.89 , 11.04 $\mu\text{mol/L}$ for women aged 40-42, 43-64, and 65-67 years) (Nygard et al., 1995).

The difference in Hcy levels is likely due to concentrations of sex steroid hormones such as estradiol and progesterone, which are prevalent in premenopausal females, and have been

found to be slightly but insignificantly negatively correlated with plasma Hcy concentration (Tallova, Tomandl, Bicikova, & Hill, 1999). The lack of significance of the correlation could be due to the small number of participants assessed.

Further evidence in support of a relationship between steroid hormones and plasma Hcy concentration lies in the fluctuation of Hcy levels in response to menstrual cycle phase. Plasma Hcy concentration varies according to menstrual cycle phase in premenopausal females (De Cree et al., 1999; Steegers-Theunissen et al., 1992; Tallova et al., 1999). Homocysteine concentrations increase above normal levels during the follicular phase of the menstrual cycle, when concentrations of estradiol and progesterone are low, and decrease during the luteal phase of the menstrual cycle, when concentrations of steroid hormones are high. Tallova and colleagues (1999) have reported plasma Hcy concentrations of $8.9 \pm 3 \mu\text{mol/L}$ in the follicular phase, and $7.8 \pm 2.8 \mu\text{mol/L}$ in the luteal phase. De Cree and colleagues (1999) found plasma Hcy levels to be $9.44 \pm 1.65 \mu\text{mol/L}$ in the follicular phase, and $8.93 \pm 1.71 \mu\text{mol/L}$ in the luteal phase. In each of these studies, the participants were not taking oral contraceptives, and were premenopausal but not pregnant. These variations indicate that there are cyclical variations of Hcy during the menstrual cycle.

Because of the known changes in Hcy concentration due to different hormone concentrations, similar changes should be expected with oral contraceptives. Steegers-Theunissen and colleagues (1992) studied the effect of sub-50 oral contraceptives on plasma Hcy concentration and vitamin status in premenopausal females. While the authors also found that there were cyclical variations in plasma Hcy concentrations, with increased levels in the follicular phase when compared to the luteal phase, this result was only present in those taking the oral contraceptives. Menstrual cycle phase had no impact on Hcy concentration in the

control group, who were not taking oral contraceptives. Homocysteine levels in the treatment group were 11.0 $\mu\text{mol/L}$ during the early follicular phase and 7.8 $\mu\text{mol/L}$ during the early luteal phase, while in the control group the concentrations were 6.6 $\mu\text{mol/L}$ and 6.4 $\mu\text{mol/L}$ for the respective cycle phases. The authors did however note that the absence of a significant difference between phases in the control group could have been due to the failure of four out of 15 participants to demonstrate a decrease in Hcy. Significant differences between groups in Hcy concentration during the luteal phase were not present.

Menopausal women experience increases in plasma Hcy when compared to premenopausal women (12.0 ± 5.2 vs. 9.9 ± 2.9 $\mu\text{mol/L}$) (Chrysohoou et al., 2004), which corresponds to a decrease in steroid hormones (Refsum et al., 1998).

2.1.2.6 Dietary composition

Folate and vitamin B₁₂ are both involved in the remethylation of Hcy to form methionine, while vitamin B₆ is involved in the transsulphuration of Hcy to form cysteine in the presence of sufficient amounts of methionine (Refsum et al., 1998). Because of the large role that these B vitamins play in Hcy metabolism, exclusion or deficiencies of folate and vitamins B₆, and B₁₂ in the diet can lead to impaired metabolism, which ultimately can result in increased concentration of Hcy in the bloodstream (Selhub, 1999).

2.1.2.7 Folate and vitamin B₆

Folate is a necessary factor in the remethylation pathway, and therefore plays a significant role in resulting plasma Hcy concentrations, as the remethylation of Hcy to form methionine would be severely limited with deficiencies of folate (Refsum et al., 1998; Selhub,

1999). The presence of folate in sufficient quantities in the diet is necessary for the smaller tetrahydrofolate cycle that is the source of the methyl group for Hcy remethylation.

Folate has a significant dose-response relationship with plasma Hcy concentration (Nygard et al., 1998). In this study, baseline data collected from 11,941 participants from the Hordaland Homocysteine Study cohort (Nygard et al., 1995) was used to determine dietary factors that affect total Hcy concentration (Nygard et al., 1998). The authors reported that although folate is readily available in foods such as fruits and vegetables, juices, eggs and meat, large variations observed in plasma Hcy may be due to low folate intake and a lack of folate supplementation (Nygard et al., 1998). Folate is also available in fortified grain products, legumes and beans (Institute of Medicine of the National Academies, 2006). Folate scores were found to be higher in females than males in individuals between the ages of 40 and 42 years (1.8 vs. 1.7 $\mu\text{mol/L}$) while the opposite was true in individuals between the ages of 64 and 67 years (2.4 vs. 2.7 $\mu\text{mol/L}$). Folate status is not likely to be a confounding factor in the current study, due to the age and healthy status of the female participants.

Jacques et al. (1999) investigated the effects of folic acid fortification in individuals participating in the Framingham Heart Study, a study of individuals with heart disease. The authors reported that participants who ate fortified foods experienced a decrease in plasma Hcy concentration (10 %), while those supplementing with both B vitamins and folate or with B vitamins only showed no change. The absence of change in the B vitamin groups could be due to the lower initial Hcy concentrations and higher plasma folate levels in these participants. This could be due to the presence of folic acid in the B vitamins (Jacques et al., 1999). The groups had significant differences in concentration of vitamins B₆ and B₁₂ initially, and so the resulting

change in the folate group cannot be attributed with total certainty to the folate fortification (Jacques et al., 1999).

Cuskelly, Stacpoole, Williamson, Baumgartner, and Gregory (2001), investigated Hcy kinetics while participants were in either a folate or vitamin B₆ deficient state. Homocysteine concentration in the folate deficient group at the end of a six week folate depletion period was 15.9 ± 2.1 $\mu\text{mol/L}$, and in the vitamin B₆ depleted group was 7.7 ± 2.1 $\mu\text{mol/L}$ compared to 7.4 ± 1.7 $\mu\text{mol/L}$ in the control group. There were no significant differences in Hcy concentration between the control and vitamin B₆ groups following the dietary depletion. This indicates that even in a vitamin B₆ depleted state, there is no significant effect on Hcy status. While this vitamin plays a role in Hcy metabolism, changes in B₆ will not impact Hcy in the bloodstream, whereas folate deficiencies result in increases of Hcy past the normal range.

In rats, deficiencies of vitamin B₆ do not seem to have as severe an impact on plasma Hcy concentration as deficiencies of folate (Miller, Nadeau, Smith, & Selhub, 1994). B₆ deficient rats did not have increased plasma Hcy concentrations when compared with controls, while folate deficient rats had significantly higher Hcy levels (Miller et al., 1994). Differences in Hcy concentration between the B₆ deficient and folate deficient groups may be due to the role played by each of the components in Hcy metabolism.

Vitamin B₆ does not play a role in the remethylation of Hcy to form methionine. It is however a factor in the transsulphuration pathway, which occurs in the presence of excess methionine within the cell (Refsum et al., 1998). Vitamin B₆ is available in highly fortified cereals, beef liver and organ meats and fortified soy based meat substitutes (Institute of Medicine of the National Academies, 2006). Vitamin B₆ deficiencies generally cause only a mild

impairment of the transsulphuration pathway (Selhub, 1999). This helps to prevent the precipitation of hyperhomocysteinemia in the presence of a fully active remethylation pathway and residual activity of the transsulphuration pathway when dietary methionine intake is not significant.

Miller and colleagues (1992) explored vitamin B₆ depletion and resulting plasma Hcy concentration in elderly individuals. The participants were first tested following their normal diet, and then following a vitamin B₆ depletion period of less than 20 days. After the depletion period, the participants underwent three successive 21 day repletion periods, whereby vitamin B₆ was returned to their diets in different quantities (Period 1: 15 µg/kg body weight/day; Period 2: 22.5 µg/kg body weight/day; Period 3: 33.75 µg/kg body weight/day). The authors found no significant differences in plasma Hcy concentration in response to the B₆ depletion or in response to the different repletion periods. Miller and colleagues (1992) also studied the B₆ depletion states in different age-grouped (three and 23 month old) rats and compared them to B₆ replete, pair-fed rats, with data collections at six and nine weeks. The authors compared the dietary groups, and different age groups. Plasma Hcy concentration increased in the vitamin B₆ deficient three month old rats at nine weeks, though there were no significant differences at the six week testing point, or at any time point in 23 month old rats. Vitamin B₆ deficiency is therefore not always represented by increases in plasma Hcy concentration.

2.1.2.8 Vitamin B₁₂

Elevation of Hcy concentration in plasma is often due to dietary habits such as vegetarianism, where there are certain factors involved in Hcy metabolism that are lacking in the diet. The disruption at the cellular level is frequently caused by deficiency of nutrients required

in the metabolic pathway of Hcy. These include vitamin B₁₂ and guanidinoacetate (a precursor to creatine), both of which play key roles in Hcy metabolism.

Impairment of remethylation will lead to an extensive increase in plasma Hcy concentration. As a diet moves from omnivorous to a strict vegan diet, decreases in vitamin B₁₂ concentration progress due to decreased intake of vitamin B₁₂ containing foods (Obeid, Geisel, Schorr, Hubner, & Herrmann, 2002). Without the use of fortified foods or supplements, vegetarians have a limited intake of vitamin B₁₂ (Barr & Rideout, 2004). Plasma B₁₂ levels are inversely correlated with plasma Hcy levels, which is likely due to decreases in Hcy metabolism as a result of deficiencies in vitamin B₁₂ intake (Obeid et al., 2002). Vitamin B₁₂ concentrations in omnivorous populations are 403.5 ± 138.9 pmol/L, compared with 207.7 ± 127.1 pmol/L in vegetarians (Hung et al., 2002). Such low values in vegetarians are likely to have a negative impact on cellular Hcy metabolism, subsequently causing increases in plasma Hcy concentrations.

Oral supplementation with vitamin B₁₂ is associated with decreased levels of plasma Hcy, though decreases are of a relatively small magnitude (10-15 %) when compared to individuals with folate deficiencies (25-50 %) (Refsum et al., 1998). Recent attempts at decreasing plasma Hcy concentrations using a combination of folate and vitamins B₆, B₁₂ have found that while supplementation decreased Hcy concentration, the risk of a cardiovascular event increased among patients with baseline plasma Hcy levels greater than 13 μ mol/L (Bonna et al., 2006). Important to note is that the population for this research was chosen based on the occurrence of an acute myocardial infarction within seven days previous to randomization into the intervention groups. Similarly, Lonn and colleagues (2006) based their participant selection on the presence of a history of vascular disease, and did not attempt to determine the effects of supplementation

on a healthy population with increased plasma Hcy concentration. Lonn and colleagues (2006) found that supplementation with different amounts of the same vitamins combined did not prevent the recurrence of cardiovascular events, though plasma Hcy concentration was decreased. Vitamin B₆ was suggested to have adverse effects on vascular modeling and myocardial repair (Bonna et al., 2006). However, since both vitamin B₆ and folate are readily available in a variety of foods, as mentioned previously, supplementation of the diet with this combination of vitamins is often unnecessary. Individuals with normal plasma folate concentrations, but with vitamin B₁₂ deficiencies, such as vegetarians, could benefit from vitamin B₁₂ supplementation. It is possible that a decrease ranging between 10 and 15 % of their initial plasma Hcy concentration would place vegetarians in the normal Hcy range.

Vitamin B₁₂ is mainly found in meats, eggs and milk, and therefore may be absent or available in limited quantities in a lacto-ovo or vegan diet. Folate and vitamins B₂ and B₆ are also found in animal products, but are also prevalent in a number of plant foods and nuts (Obeid et al., 2002). Folate utilization might be compromised in times of depleted vitamin B₁₂ concentration, due to the inability of the cell to produce sufficient amounts of tetrahydrofolate, and this could decrease Hcy metabolism (Obeid et al., 2002). Without proper amounts of nutrients such as those found in animal products, specifically meat products, Hcy metabolism may be limited, resulting in increases in Hcy being released into the bloodstream.

2.1.2.9 Vegetarian diets and homocysteine levels

Vegetarians have higher concentrations of plasma Hcy when compared to non-vegetarians, with the extent of vegetarianism (i.e. lacto-ovo vegetarian vs. vegan) playing a modulating role (Bissoli et al., 2002; Herrmann, Schorr, Purschwitz, Rassoul, & Richter, 2001; Hung et al., 2002; Krajcovicova-Kudlackova, Blazicek, Kopcova, Bederova, & Babinska, 2000;

Obeid et al., 2002). Lacto-ovo vegetarians (does not consume meat, chicken, or fish, but may include dairy products and eggs within their diets (Herrmann et al., 2001)) have increased plasma Hcy levels when compared to non-vegetarians (Bissoli et al., 2002; Herrmann et al., 2001; Hung et al., 2002; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002), and vegans (do not include animal or dairy products in their diets) experience even greater increases in plasma Hcy (Herrmann et al., 2001; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002).

Homocysteine concentrations vary between 10.5 and 17.4 $\mu\text{mol/L}$ in lacto-ovo vegetarians, and between 8.6 and 11.6 $\mu\text{mol/L}$ in non-vegetarians (Bissoli et al., 2002; Herrmann et al., 2001; Hung et al., 2002; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002). Vegans have even higher plasma Hcy concentrations than lacto-ovo vegetarians or non-vegetarians. Plasma Hcy concentrations in vegans range from 12.8 to 26.9 $\mu\text{mol/L}$ (Bissoli et al., 2002; Herrmann et al., 2001; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002). Though it would seem that individuals adhering to a vegan diet would be at a higher risk for increased plasma Hcy concentration, a lacto-ovo vegetarian diet also seems to result in higher than normal concentrations. Due to the association between Hcy and cardiovascular disease, it is of interest to determine ways to decrease plasma Hcy levels.

Elevated concentrations of Hcy are likely due to nutritional deficiencies that are associated with a vegetarian diet, specifically deficiencies in vitamin B₁₂. With regard to plasma folate concentration, lacto-ovo vegetarians and vegans have been found to have similar or greater levels when compared to omnivores (Bissoli et al., 2002; Herrmann et al., 2001; Hung et al., 2002; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002). Therefore, folate deficiencies and therefore limitations with regard to the participation of folate in Hcy metabolism in these

groups were not anticipated. Similarities in this nutritional component are likely due to increased intake of folate-containing plant foods or nuts ingested by vegetarians and vegans.

Table 2.1. Summary of studies that have investigated homocysteine concentration in different dietary groups.

Abbreviations: high meat eater (HME), homocysteine (Hcy), lacto-ovo vegetarian (LOV), low meat eater (LME), non-vegetarians (NV), vegetarians (pooled) (VG), vegan (VE), (male/female).

Reference	Participants	Comparisons	Hcy concentration (μmol/L)	Results
Bissoli et al., 2002	31 VE (19/12); age 45.8 ± 15.8 14 LOV (6/8); age 48.5 ± 14.5 29 NV (19/10); age 43.8 ± 17.3	VG vs. NV VG with vitamin B ₆ + B ₁₂ supplements (n = 9) vs. VG without VE vs. LOV	VEG: 26.9 ± 24.1 LOV: 17.4 ± 11.1 VG: 23.9 ± 21.3 NV: 11.6 ± 4.9	VG > NV VG with supplements < VG without
Herrmann et al., 2001	44 HME (14/30); age 23 29 LME (2/17); age 24 34 LOV (10/24); age 22 7 VE (2/5); age 22	VG, VE, LOV, LME vs. HME	HME: 9.8 (median) LME: 11.8 LOV: 11.0 VE: 15.2 VG: 11.6	LME > HME LOV > HME VE > HME VG > HME
Hung et al., 2001	Healthy females, age 31-45 45 VG; 45 NV	VG vs. NV	VG: 11.20 ± 4.27 NV: 8.64 ± 2.06	VG > NV
Krajcovicova-Kudlackova et al., 2000	32 VE (10/22); age 41.5 ± 1.8 62 LOV (24/38); age 35.1 ± 0.9 59 NV (22/37); age 40.9 ± 0.7	VG vs. NV	VE: 15.79 ± 0.85 LOV: 13.18 ± 0.26 NV: 10.19 ± 0.29	VG > NV
Obeid et al., 2002	Healthy individuals, age 46 ± 15 29 VE (13/16); age 64 LOV (28/36); age 20 LME (8/12); age	VE vs. LME VE vs. LOV LOV vs. LME	VE: 12.8 (median) LOV: 10.5 LME: 8.7	VE > LME VE vs. LOV: p = 0.05

2.1.3 Creatine

Creatine is a compound found in animal products - specifically meats - though in the human body it is also produced endogenously from amino acids in the liver, pancreas and kidneys (Greenhaff et al., 1994; Kraemer & Volek, 1999). In humans, creatine is present primarily in skeletal muscle, with lesser concentrations also found in cardiac and smooth muscle, brain, kidney and spermatozoa (Harris et al., 1992). Creatine, in both the free and phosphorylated form, plays a key role in muscle energy metabolism (Greenhaff et al., 1994). Skeletal muscle force production is dependent on the availability of phosphocreatine. Through the reversible creatine kinase reaction, creatine is phosphorylated to form phosphocreatine (Meyer, Sweeney, & Kushmerick, 1984), providing an energy store at a number of sites, mainly within the cytoplasm, but also in the mitochondrial matrix of muscle cells (Houston, 2001). In the reverse reaction, phosphocreatine donates or transfers a phosphate to ADP to produce ATP and creatine (Meyer et al., 1984). Phosphocreatine therefore plays a central role in the maintenance of ATP homeostasis via the creatine kinase reaction by providing the cell with energy in times of increased ATP hydrolysis (Harris et al., 1992).

2.1.3.1 Supplementation protocols

In the past, a number of researchers have set out to determine the best way to maximize the uptake of creatine into muscle via oral supplementation. Originally, supplementation protocols called for the ingestion of 20 grams of creatine a day for a period of five days, divided into four daily servings, in order to “load” muscle cells (Greenhaff et al., 1994; Harris et al., 1992). Greenhaff and colleagues (1994) found that following the creatine loading period, muscle creatine concentration increased by 15 %, and others reported gains of more than 20 % (Harris et al., 1992). More recently, researchers have been supplementing participants with 0.3 grams of

creatine per kilogram of body mass during the loading phase of supplementation (Syrotuik & Bell, 2004). Also, supplementing with 0.25 grams of creatine per kilogram of lean tissue mass has been described as the best way to maximally increase creatine uptake while minimizing excretion of creatine in urine (Burke, Smith-Palmer, Holt, Head, & Chilibeck, 2001). Additionally, research that has evaluated optimal solutions in which to consume creatine has found that carbohydrate solutions are more effective at maximizing whole body creatine retention than ingestion of creatine mixed with water (Green, Simpson, Littlewood, MacDonald, & Greenhaff, 1996).

2.1.3.2 Creatine responders and non-responders

There are responders and non-responders to creatine supplementation (Greenhaff et al., 1994; Harris et al., 1992; Syrotuik & Bell, 2004). In response to the 20 grams per day protocol, Greenhaff and colleagues (1994) classified responders as those who had lower initial concentrations of muscle creatine, and who demonstrated a significant increase in total creatine and an accelerated rate of phosphocreatine resynthesis in the second minute of recovery from intense muscular contraction after supplementation, while non-responders had higher initial concentrations and experienced smaller increases and rate of phosphocreatine resynthesis. Harris and colleagues (1992) also found that increases in total creatine as a result of oral supplementation appeared to be dependent on initial total creatine content, and that those with the lowest initial content showed the greatest increase. It was also noted that 20 to 40 % of the increase in total creatine content was accounted for by an increase in phosphocreatine.

Syrotuik and Bell (2004) defined responders as those whose muscle total creatine concentration (creatine and phosphocreatine) increased by 20 mmol/kg dry weight, and non responders as those who did not demonstrate an increase of more than 10 mmol/kg dry weight.

The authors also described a third group of individuals who fell between responders and non-responders, the quasi responders. Once again, responders were found to have initial total creatine contents that were lower than the mean (111.12 mmol/kg dry weight) and showed the greatest increases when supplemented with creatine monohydrate.

2.1.4 Creatine and exercise performance

Supplementation with creatine monohydrate increases the total body creatine pool (Greenhaff et al., 1994), which therefore can increase the production of phosphocreatine via the creatine kinase reaction (Hultman, Soderlund, Timmons, Cederblad, & Greenhaff, 1996). Creatine supplementation influences muscle metabolism by increasing phosphocreatine levels which allows the resynthesis of ATP during anaerobic exercise (Kraemer & Volek, 1999). Phosphocreatine donates or transfers a phosphate to ADP in the production of ATP to be used in muscle contraction (Meyer et al., 1984). During maximal, short term exercise the store of ATP in skeletal muscle can be depleted within one to two seconds. Therefore a dependency exists for the conversion of ADP to ATP very rapidly, which allows maximal efforts to continue for approximately 30 seconds more (Casey & Greenhaff, 2000).

Suggestions have been made that in order to see an improvement in performance with creatine supplementation, muscle total creatine must increase by at least 20 mmol/kg dry weight (Syrotuik & Bell, 2004). A number of studies have found that oral creatine supplementation has an effect on maximal exercise.

2.1.4.1 Resistance or strength training

When coupled with resistance training, creatine supplementation results in increases in cross sectional area of type I, IIa and IIab muscle fibres (Volek et al., 1997). This increase

translates into improvements in force production when participating in high-intensity, short duration activities, such as resistance or strength training (Volek & Rawson, 2004). Few researchers have focused their efforts on the effects of creatine supplementation on strength in females or vegetarians.

Brenner , Rankin and Sebolt (2000) investigated the effect of six weeks of creatine supplementation (20 g Cr/day for seven days; 2 g Cr/day for five weeks) coupled with resistance training on strength measures in female collegiate lacrosse athletes. Creatine supplementation resulted in an increase in one repetition maximum (1RM) performance in the creatine group when compared to those supplementing with placebo.

Vandenbergh and colleagues (1997) investigated the effects of a 10 week creatine supplementation protocol (4 days of 20 g/day loading, followed by 10 weeks of 2.5 g/day maintenance supplementation) in university aged sedentary females. During the maintenance period, participants began a resistance training program that included one hour of activity three times each week. The participants then continued supplementing (2.5 g/day) for an additional 10 weeks without resistance training. The researchers measured the participants' 1RM for leg press, bench press, leg curl, leg extension, squat, and shoulder press before and after five and ten weeks of training, as well as arm flexion torque before and after four days, five and ten weeks of creatine loading. For the measurement of arm flexion torque, the participants completed five sets of 30 dynamic maximal voluntary contractions separated by two minute rest intervals. At ten weeks, the creatine group had significantly greater performances in 1RM tests for leg press, leg extension, and squat when compared to placebo, and also had non-significant increases in bench press and leg curl performances. No significant differences in arm flexion torque between

groups following the four day loading period were found, though at both five and ten weeks the creatine group had greater scores.

Ferguson and Syrotuik (2006) supplemented resistance trained females with creatine for a period of ten weeks (0.3g Cr/kg body mass for seven days; 0.03 g Cr/kg body mass for nine weeks). During this intervention, the females participated in a resistance training program for four days each week. Increases in strength as assessed by 1RM bench press and incline leg press were found at the end of the ten week period in both the creatine and placebo groups. The authors suggested that the similarities between groups may be due to the presence of non-responders within their subject pool. Burke and colleagues (2003) investigated the effect of creatine supplementation (0.25 g Cr/kg lean tissue mass for seven days; 0.0625 g Cr/kg for seven weeks) on exercise performance in vegetarians and non-vegetarians. The authors assessed the participants' performance on 1RM bench and leg press tests, as measures of strength before and after supplementation coupled with an eight week resistance training program. A significant supplement by time interaction for 1RM bench press was revealed by the statistical analysis, whereby those participants supplementing with creatine experienced a greater increase in bench press when compared to the placebo group.

2.1.4.2 Anaerobic capacity

Supplementation with creatine monohydrate increases total muscle creatine and phosphocreatine concentration, which may allow for improvements in short duration, high intensity activity (Williams & Branch, 1998). Anaerobic capacity is directly related to concentrations of ATP, and by increasing the amount of phosphocreatine available in the muscle, dependence on anaerobic glycolysis is reduced, potentially allowing the muscle to work for

longer periods of time. Very few researchers have focused on the effects of creatine supplementation on anaerobic capacity specifically in females or vegetarians.

Thompson and colleagues (1996) had female competitive swimmers supplement for 56 days (2 g Cr/day), and measured performance on a 100 meter swim. Supplementation had no effect on concentrations of phosphocreatine or ADP in the muscle, and did not improve swim performance when compared to placebo. It is likely that the supplementation protocol used in this situation was too low to cause a difference in muscle metabolites and performance.

Cox, Mujika, Tumilty and Burke (2002) supplemented elite female soccer players with creatine monohydrate for a period of six days (20 g Cr/day), while training with simulated match play. Those supplementing with creatine decreased their sprint times at various points in the simulated match, though the final effect was not statistically different. Creatine supplementation also resulted in decreases during various agility run times throughout the match, though once again the total time did not significantly decrease. The authors suggest that in spite of there being no overall significant differences when looking at sprint times, in a soccer match, having one or two sprints that are improved could help to change the result of the game, and that therefore supplementation with creatine is worthwhile.

2.1.4.3 Aerobic capacity

Because aerobic exercise is characterized by cellular respiration and does not depend primarily on the production of ATP from phosphocreatine and ADP, creatine supplementation is thought to have minimal effect on aerobic performance. However, it is important to recognize that creatine supplementation could potentially affect the phosphocreatine shuttle, which acts as an intracellular system for the transfer of high energy phosphates (Kammermeier, 1986; Meyer et

al., 1984). Specifically, this shuttle allows the transfer of energy between the mitochondrion and cytoplasm of muscle fibres (Kammermeier, 1986). Free creatine in the cytoplasm of muscle fibres moves into the mitochondrion, where it has been found to accept a phosphate from ATP stores, thus regenerating phosphocreatine (Bessman & Carpenter, 1985). The newly formed phosphocreatine then returns to the cytoplasm of the contracting fibre, and allows for the reformation of ATP and the availability of free creatine once again, which returns to the mitochondrion to repeat the cycle. Enhanced levels of creatine, as would occur through supplementation may enable greater mitochondrial ATP availability and subsequently enhance aerobic performance.

It is of note however, that research based on this premise has failed to find a significant effect of creatine supplementation on aerobic performance (Chwalbinska-Moneta, 2003), heart rate response or blood lactate response to aerobic exercise (Vanakoski, Kosunen, Meririnne, & Seppala, 1998), or total work performed and/or average power output during the last 15 minutes of a 28 minute submaximal endurance (Reardon, Ruell, Fiatarone Singh, Thompson, & Rooney, 2006).

2.1.5 Vegetarians and creatine supplementation

Due to the lack of animal products in their diets, vegetarians generally have lower baseline levels of total creatine than individuals adhering to a non-vegetarian diet. Dietary creatine intake accounts for approximately 50 % of the body's daily need for creatine (Syrotuik & Bell, 2004). However, in spite of their creatine depleted diet, biopsied muscle samples from vegetarians had normal total creatine content ($n = 2$; 120.0 and 114.6 mmol/kg dry mass) (Harris et al., 1992). Conversely, a study examining the impact of a lacto-ovo vegetarian diet for 21 days found a significant decrease in total muscle creatine ($n = 12$; mean \pm SE: 126.0 ± 2.4 vs.

113.9 ± 3.9 mmol/kg dry mass) (Lukaszuk et al., 2002). A sample of the participants (n = 6) were then supplemented with creatine monohydrate for a period of five days (0.3 g Cr/kg body mass), and the total creatine stores increased to levels greater than when following an omnivorous diet (n = 7) (148.6 ± 4.5 vs. 141.7 ± 4.5 mmol/kg dry mass), suggesting that there is a potential to increase total muscle creatine stores in both vegetarians and non-vegetarians. A reasonable assumption to make would be that these increases in total muscle creatine stores should allow for improvements in performance on anaerobic fitness and strength measures.

Table 2.2. Summary of studies that have examined the effect of creatine supplementation on fitness in vegetarians.

Abbreviations: baseline (BL), creatine (Cr), muscle creatine concentration ([Cr]), day (d), gram (g), kilogram (kg), lean tissue mass (LTM), non-significant (n.s.), non-vegetarian (NV), muscle phosphocreatine concentration ([PCr]), placebo (Pl), resistance training (RT), standard error (SE), total muscle creatine concentration ([tCr]), urinary (UR), vegetarian (VG), (male/female), change in (Δ).

Reference	Participants	Treatment	Testing	Response
Burke et al., 2003	18 VG 24 NV	Cr: 7d, 0.25 gCr/kgLTM/d; 49d, 0.0635 gCr/kgLTM/d, plus RT. VGCr (n = 10) (5/5); age 31 \pm 2.2 VGPl (n = 8) (3/5); age 34 \pm 4.2 NVCr (n = 12) (7/5); age 33 \pm 2.6 NVPl (n = 12) (5/7); age 32 \pm 2.4	Comparisons of LTM, [tCr], urinary Cr output, 1RM bench and leg press, 50 leg extensions, training volume	Δ LTM: CrVG > CrNV, Cr > Pl [tCr]: NV > VG UR Cr (BL): NV > VG Δ 1RM bench press: Cr > Pl Δ 1RM leg press: n.s. Leg extensions: CrNV > CrVG, Pl Training volume: Cr > Pl
Lukaszuk et al., 2005	26 NV - 12 VG diet; age 24.1 \pm 1.4 - 14 NV diet; age 22.9 \pm 0.6	21d of dietary treatment, 5d Cr/Pl treatment (0.3 gCr/kgLTM/d) VGPl VGCr NVPl NVCr	[tCr] [PCr] [Cr]	Cr > Pl in VG and NV for [tCr], [PCr], and [Cr]
Shomrat et al., 2000	Healthy males 7 VG 17 NV	Cr: 3 x 7g/day for six days NVPl; age 27.4 \pm 1.9 (SE) NVCr; age 26.1 \pm 0.6 VGCr; 29.6 \pm 2.7	Plasma Cr 3 Wingate repetitions	BL Plasma Cr: VG < NV Plasma Cr: \uparrow with Cr Mean Power: \uparrow with Cr Peak Power: \uparrow in NV

2.1.6 Creatine and homocysteine metabolism

Creatine is synthesized endogenously in the human body via the methylation of guanidinoacetate by guanidinoacetate-methyl transferase with SAM donating the methyl group (Taes et al., 2004). This methylation of guanidinoacetate accounts for 70 % of transmethylation reactions in the body (Taes et al., 2004). Creatine supplementation may therefore play a role in the regulation of plasma Hcy levels (Wyss & Schulze, 2002), in that by downregulating the endogenous need for creatine, there would be less need for these transmethylation reactions, thereby decreasing Hcy concentration (Taes et al., 2004).

In the presence of exogenous creatine, such as is supplied by supplementation, the methylation of guanidinoacetate to form creatine may decrease, since the end product of this methylation is creatine. With an excess of creatine, the need for endogenous creatine would be decreased, which would theoretically decrease the prevalence of the reaction. Though guanidinoacetate is only one of the many methyl acceptors that can take part in the remethylation pathway, supplementation with creatine could result in a decrease in methyl donation by SAM. This ultimately would cause a decline in the amount of SAH produced in response to SAM being spared, and subsequently lessen the regeneration of Hcy within the cell (Wyss & Schulze, 2002).

Reports on the effects of creatine supplementation on plasma Hcy levels have been contradictory (Korzun, 2004; Stead et al., 2001; Steenge et al., 2001; Taes, 2005; Taes et al., 2004; Taes et al., 2003). A study comprised of chronic hemodialysis patients who supplemented with creatine reported no decrease in total plasma Hcy concentration (Taes et al., 2004). These participants ingested two grams of creatine each day, an amount that is not the standard for creatine supplementation, and therefore may not have resulted in optimal creatine uptake. In

contrast, Korzun (2004) found that creatine supplementation decreased plasma Hcy concentration in seven out of eight individuals supplementing with creatine. Four of the individuals supplementing showed decreases between 18 % and 27 %. Stead et al. (2001) reported a 27% decrease in plasma Hcy concentration in response to oral creatine supplementation (0.4 % wt/wt creatine) in male rats. Taes and colleagues (2003) reported decreases in plasma Hcy in nephrectomized male rats following creatine supplementation (2 % wt/wt creatine monohydrate), when compared to control fed animals. They also found that plasma Hcy concentration decreased following creatine supplementation when comparing pooled data of nephrectomized and sham operated animals supplementing with creatine to those supplementing with placebo (Taes et al., 2003).

Creatine supplementation and resistance training in non-vegetarians has no significant effect on plasma Hcy levels (Steenge et al., 2001). Including non-vegetarians in the sample may have decreased the possibility of a significant effect, as non-vegetarians' diets would likely already contain optimal doses of creatine. The participants included in this study did not have elevated serum Hcy levels initially, and therefore may not have experienced decreases as might be expected in individuals with elevated concentrations, such as vegetarians sometimes have.

It is apparent from these mixed results that the impact of creatine supplementation on plasma Hcy concentration is dependent on a variety of factors, including baseline Hcy concentration and dietary status. In spite of these mixed results, optimal creatine supplementation techniques in individuals with low dietary intake of creatine and higher Hcy concentration in plasma, as is the case with a vegetarian diet, could decrease plasma Hcy concentration.

Table 2.3. Summary of studies that have examined the effect of creatine supplementation on homocysteine concentration.

Abbreviations: baseline (BL), creatine (Cr), day (d), gram (g), homocysteine (Hcy), milligram (mg), multivitamins (MV), placebo (PI), resistance training (RT), weight (wt), (males/females), change in (Δ)

Reference	Population/Participants	Supplementation	Hcy concentration ($\mu\text{mol/L}$)	Results
Korzun, 2004	Healthy males and females; n = 16	Cr: 28d of 2.1-5.5 g/d MV ($B_6 + B_{12}$) (3/5); age 31 \pm 13 MV + Cr (4/4); age 29 \pm 8	MV BL: 6.2 \pm 1.2 MV Δ : + 0.2 \pm 0.5 MV + Cr BL: 7.1 \pm 1.8 MV + Cr Δ : - 0.9 \pm 0.7	Hcy \downarrow in MV + Cr - 4 participants decreased 18 – 27 %
Stead et al., 2001	Healthy male Sprague Dawley rats	Cr: 14d of 0.4 % wt/wt Control	\downarrow 27 % in Cr fed rats	Hcy \downarrow in response to Cr
Steenge et al., 2001	Healthy women; age 19 – 38	Cr: 5d of 20 g/d, 46d of 3 g/d Cr (n = 7) Cr + RT (n = 8) PI (n = 5)	Cr Δ : - 0.3 \pm 0.5 Cr + RT Δ : - 0.6 \pm 0.5 PI Δ : - 0.6 \pm 0.5	No differences were found with Cr
Taes et al., 2004	Chronic hemodialysis patients, supplementing with 5 mg folic acid, 50 mg pyroxene (B_6), 12 μg B_{12}	Cr: 2 g/d Cr (n = 25) (14/11); age 71 \pm 8 PI (n = 20) (10/10); age 69 \pm 12	BL Hcy (males): 23.3 \pm 3.7; (females): 18.7 \pm 6.1	No differences were found with Cr
Taes et al., 2003	Male Wistar rats with uremia	Cr: 2 % wt/wt Sham operated PI (n = 8) Sham operated Cr (n = 8) Subtotal nephrectomized PI (n = 10) Subtotal nephrectomized Cr (n = 10)	Cr _{post} : 12.4 \pm 2.2 PI _{post} : 14.6 \pm 1.9	\downarrow Hcy in Cr vs. PI Trend towards \downarrow Hcy in sham operated Cr Subtotal nephrectomized PI > sham operated PI, subtotal nephrectomized Cr

2.2 Statement of the problem

While it has been established that males have increased concentrations of Hcy in the bloodstream when compared to females (Chrysohoou et al., 2004), female vegetarians could have elevated levels of serum Hcy when compared with non-vegetarians, due to less exogenous creatine availability and lower vitamin B₁₂ intake (Bissoli et al., 2002). Vegetarians could therefore be at an equal or greater risk for cardiovascular complications. For that reason, it is essential to determine possible mechanisms to decrease the concentration of Hcy in the bloodstream in this population. As such, the primary purpose of this research was to determine the effects of creatine supplementation, taken concurrently with vitamin B₁₂ supplements, on serum Hcy levels in vegetarian and non-vegetarian females.

A secondary purpose was to determine the effects, if any, of oral creatine supplementation on muscle strength, power and anaerobic capacity in female vegetarians and non-vegetarians. The role of creatine in fitness for males has been established and reported in detail. However, few studies have investigated the effect of creatine supplementation on fitness measures in females (Ayoama, Hiruma, & Sasaki, 2003; Cox et al., 2002), and even fewer focusing on those females adhering to a vegetarian diet (Burke et al., 2003).

2.3 Hypotheses

We hypothesized that the plasma Hcy concentration would be higher in vegetarian participants when compared to non-vegetarian participants, and thus hypothesized that the Hcy concentration of vegetarians would decrease in response to supplementation with creatine monohydrate. Also, we hypothesized that the vegetarian group would experience a greater decrease in Hcy due to the greater baseline concentration.

Additionally, we were interested in determining whether physiological performance was affected by the supplementation protocol, and if differences in performance were influenced by diet. We hypothesized that both groups would experience improvements in physiological performance, including anaerobic power, fatigue and strength, in response to the creatine supplementation. We also hypothesized that vegetarian participants would experience a greater improvement, due to lower baseline concentration of creatine.

3. METHODS

3.1 Participant selection and characteristics

3.1.1 Participants and exclusion criteria

Participant selection was based on the availability of volunteers. The objective of recruitment was to engage forty participants in total (twenty for each dietary group) in the study, to achieve 80 % power. Volunteers were recruited from the Saskatoon area based on gender, age, and dietary classification. Recruitment posters were put up in various fitness apparel and grocery stores in Saskatoon, as well as in local yoga studios, and University of Saskatchewan bulletin boards. Participants were also recruited from the University of Saskatchewan Vegetarian Society, through newsletters and announcements, and using an advertisement in the Saskatoon Sun, a weekly newspaper.

Individuals taking medication other than oral contraceptives or vitamins were excluded, whereas those supplementing with vitamins were asked to continue taking them regularly throughout the study period. Participants between 18 and 40 years of age were recruited. Participants were also excluded if they had started or finished taking oral contraceptives within the past year, in order to avoid any hormonal fluctuations (see section 3.1.2 for rationale). All participants recognized themselves as healthy individuals who participated in regular recreational physical activity.

Nygaard and colleagues (1995) stated that plasma Hcy progressively increases with age, and plasma Hcy concentration increases following menopause, which is likely due to a decrease

in the hormones associated with menstruation (Refsum et al., 1998). Therefore, only those with regular menstrual cycles were included in the study. Smokers also tend to have increased plasma Hcy levels (Chrysohoou et al., 2004; Nygard et al., 1995), and therefore were excluded from the study. Participants volunteering to take part in the study met a number of criteria, as described in the following paragraphs.

Participants had not supplemented with creatine for a period of three months prior to the study in order to ensure the absence of any residual effects in plasma and therefore minimal effects on plasma or serum Hcy concentration (Steegers-Theunissen et al., 1992). Participants were asked to maintain their current eating habits, diet, and activity level for the duration of the treatment, so as to limit any confounding factors.

The final participant sample included nine non-smoking female vegetarians (lacto-ovo vegetarians) and eleven non-smoking non vegetarians between the ages of 18 and 40 years. Ethical approval was given by the University of Saskatchewan Biomedical Research Ethics Board (refer to Appendix I). All participants were made aware of the testing procedures and risks associated with the protocols and were asked for their informed written consent to participate in the study (refer to Appendix II). They also were informed that they could withdraw from the study at any time without consequence.

3.1.2 Menstrual status and oral contraceptives

Oral contraceptives are associated with increased levels of plasma Hcy during the follicular phase of the menstrual cycle, while there are no differences in the luteal phase (Steegers-Theunissen et al., 1992). This indicates that participants taking oral contraceptives may have heightened baseline serum Hcy concentration. All participants were taking oral contraceptives for one year or longer, or not at all, in order to standardize testing protocols, as

measurements were carried out at particular times within each participant's menstrual cycle. Including this as a control allowed us to eliminate hormone fluctuations as a potential variable influencing Hcy concentration, body mass or composition. Because there were no changes in contraceptive status, the magnitude of Hcy response to creatine supplementation should not have been different than those participants who do not take oral contraceptives. Participants began the treatment in the early follicular phase in order to ensure the greatest baseline mean Hcy concentration so that a response to creatine supplementation would be more obvious.

3.2 Experimental design

At the onset of the study, participants were given an appointment listing detailing each of the upcoming five laboratory visits (refer to Appendix III). The first appointment fell on day two of the participants' menstrual cycles. During this meeting, participants were given vitamin B₁₂ supplements to take daily for the duration of the study, and completed a maximal effort aerobic fitness test. Participants had their body mass, percentage body fat measurements, and total work performed during leg extensions measured during appointment two. Body mass and percentage body fat measurements were carried out in order to determine the appropriate amount of creatine for supplementation. Participants were also given three day food diaries, urine collection containers and instructions to complete before the next meeting. Appointment two occurred on approximately day 24 of the menstrual cycle. Day two of the following cycle marked the third appointment, during which participants were given creatine monohydrate to consume for the next five days. Blood samples were taken, followed by strength and anaerobic fitness tests. During this appointment, participants also handed in their urine samples collected during the prior 24 hours. Participants supplemented with creatine for five days, then returned for follow up blood work, mass and body fat measurements, and measurement of work performed during

leg extensions. At this time, they were given new urine collection containers, and were instructed to collect for the next 24 hours. The final meeting took place on approximately day twelve (11.5 ± 2.4) of the menstrual cycle, and included follow up measurements of strength and anaerobic fitness. A visual representation of the participant timeline is given in Figure 2.

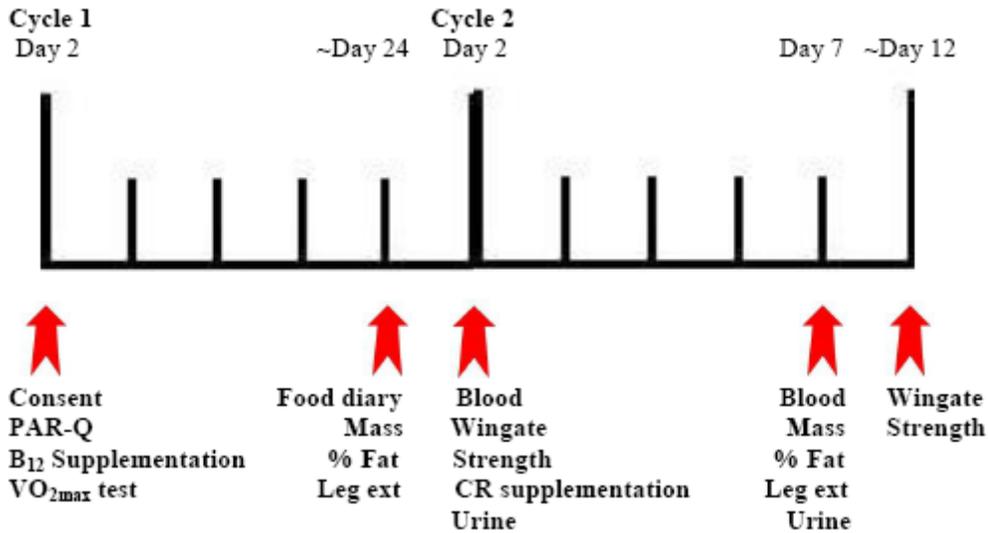


Figure 2. Graphical representation of participant timeline.

3.2.1 Dietary intake

Participants completed a three day food diary, including all products ingested prior to the study intervention (refer to Appendix IV). Participants were asked to complete the diaries as accurately as possible, and to include two week days and one weekend day. Diaries were analyzed using NutriBase 50-Node Network Edition, v. 6.21 (CyberSoft, Inc., Phoenix, AZ), which allows for the quantification of vitamin B₂, B₆, B₁₂ and folate ingested in the diet, as well as the identification of products fortified with folate. Energy, protein, carbohydrate, fat and cholesterol intake were also included in the analysis. Creatine intake was calculated from the food diaries based on the creatine content of foods (milk: 0.1 g/kg; beef: 4.5 g/kg; pork: 5 g/kg; salmon: 4.5 g/kg) (Balsom, Soderlund & Ekblom, 1994).

3.2.2 Supplementation

3.2.2.1 Vitamin B₁₂

Participants were supplied with unopened bottles of vitamin B₁₂ (cyanocobalamin) tablets (25 µg, Jamieson Natural Sources, Toronto, ON) and were asked to begin taking the supplements on day two of their menstrual cycle preceding the supplementation period. This helped to ensure that time did not play a role in any effect of the vitamin on serum Hcy concentration, and to ensure that each participant was satisfying the dietary requirements in terms of vitamin B₁₂ intake. This concentration was used in order to surpass the recommended dietary allowance for this population of vitamin B₁₂ (2.4 µg) (Institute of Medicine of the National Academies, 2006), thereby ensuring that each participant was meeting the minimum daily requirements, but also because most of the greater concentrations contain additives, such as calcium, which were not tested within this study, and could be considered confounding factors. The dose chosen was the smallest possible dose of the vitamin available commercially. The participants were instructed to ingest one vitamin supplement each day with a meal, as per the supplement instructions.

3.2.2.2 Creatine

Participants were supplied with pre-measured daily amounts of creatine monohydrate (Dymatize, Peak Performance Products, Toronto, ON), based on their lean body mass (LBM) as determined by air displacement plethysmography using a BodPod (Life Measurement, Inc., Concord, CA) one week prior to the supplementation period. The sum of four skinfolds (triceps, biceps, subscapular and iliac crest) (Durnin & Womersley, 1974) were used to determine LBM for the remaining participants, because of technical problems with the BodPod. All supplements were mixed with a carbohydrate containing powdered juice mix, and mixed with 250 mL of water by the participant. This protocol of ingesting creatine with carbohydrate maximizes the

amount of creatine retained by the body (Green et al., 1996). A five day creatine loading period was used. Participants ingested 0.25 grams of creatine per kilogram of LBM (Burke et al., 2003), separated into four equal portions and taken at four hour intervals each day (Hultman et al., 1996). Seven vegetarian and six non-vegetarian participants were measured using the BodPod prior to the intervention, as well as following. The remaining participants had either a mix of methods, or were measured using solely skinfolds, and were therefore not included in the statistical analysis of percentage body fat.

3.2.3 Body mass and percentage fat

Body mass and percentage fat were determined at the onset of the study as well as following the intervention in order to determine the daily amount of creatine supplement to be ingested by each participant, as well as to provide body composition data. Initially, these measures were carried out using a BodPod Gold Standard Body Composition Tracking System, which determines percentage body fat and LBM using densitometry. Following technical difficulties with the BodPod, participants' body mass was taken using a Model 1938 Toledo DigiTOL scale (Toledo Scale Corporation, Spartanburg, SC), and percentage body fat was determined using the Durnin-Womersley method (Durnin & Womersley, 1974). This method uses the sum of four skinfolds (biceps, triceps, subscapular and iliac crest sites), which is converted to a percentage body fat using normative data from the 1988 Campbell's Survey on Well-Being in Canada, conducted by the Canadian Fitness and Lifestyle Research Institute (unpublished data, Canadian Society of Exercise Physiology [CSEP], 1996). This method was chosen because it had been validated in both males and females between the ages of 16 and 72 years.

3.2.4 Physical fitness assessments

All fitness testing was preceded by a Physical Activity Readiness Questionnaire (PAR-Q) (refer to Appendix V), and for one participant who had a history of asthma, a Physical Activity Medical Examination (PARmed-X) (refer to Appendix VI). Participants underwent pre and post treatment testing for muscular strength, anaerobic power, and total work performed during repeated isokinetic movements. Aerobic fitness was tested prior to supplementation with vitamin B₁₂, whereas the other fitness tests were performed during the week prior to and following creatine supplementation.

3.2.4.1 Strength

Strength testing was carried out using 1RM assessments for bench and leg press. These tests were completed according to the “PFLC Resource Manual” (CSEP, 1996). Participants were allowed three to five warm up repetitions at approximately 50 % of their estimated 1RM. They were then asked to lift the weights as quickly and with as much force as possible, while maintaining proper form. The weight was progressively increased until the maximum weight was reached. Two minutes of rest was allowed between each attempt in order to allow for ATP and phosphocreatine stores to restore. Participants were allowed up to six attempts to determine their 1RM value (Chrusch, Chilibeck, Chad, Davison, & Burke, 2001).

For the determination of 1RM bench press, a Hammer Strength barbell was used (Go Mango, Calgary, AB). Each participant was instructed to lie on her back on the bench and grip the bar with her thumbs shoulder width apart with a closed overhand grip. At the initiation of the attempt, the bar was even with the top of the chest, and the participant was asked to push the bar to full extension while keeping the buttocks on the bench and without arching the back.

For the determination of 1RM leg press, the seat of a TechnoGym leg press machine

(TechnoGym USA Corp, Seattle, WA) was positioned so that the participant had a 90 ° starting angle at the knee with the foot flat on the pedals and the buttocks on the seat and the participant was then instructed to fully extend their legs without locking the knees.

3.2.4.2 Anaerobic power

Anaerobic power was assessed with repetitions of the Wingate anaerobic test (Bar-Or, 1987). This is a 30 second maximal performance test on a cycle ergometer. The participant warmed up for five minutes on a stationary bicycle in order to attain a heart rate of approximately 150 beats per minute (bpm). Following the warm up period, participants pedaled as hard as they could for a period of 30 seconds in a seated position against a resistance set at 0.075 kiloponds per kilogram of body weight (Bulbulian, Jeong, & Murphy, 1996). The resistance was applied when the participant was pedaling at maximal effort. Three repetitions of the Wingate test were performed (Trial 1, 2, 3), separated by two minutes to allow time for phosphocreatine stores to be replenished. Peak and average power outputs were derived from the Wingate tests. Comparisons were made between each of the test repetitions, as well as between time points during the study. Anaerobic capacity was determined using a Monark cycle ergometer (Monark, Sweden).

3.2.4.3 Total work performed

The total work performed during 50 isokinetic knee extensions was used as a measure of muscular endurance (Burke et al., 2003). An isokinetic dynamometer (Biodex System 3, Biofit Medical, London, ON) was used, with an angular velocity of 180 °/second, and a range of motion of 90 to 170 degrees of flexion at the knee of the dominant leg (Burke et al., 2003). Participants were stabilized in a sitting position with 85 degrees of flexion at the hip, and their back supported. Stabilizing straps were placed across the chest, over the lap, and across the thigh of

the test leg. Participants warmed up by completing ten repetitions of extension of the lower leg, followed by five minutes of rest before the test. For the test the participants were asked to extend their lower leg as quickly and with as much force as possible for 50 repetitions, giving their maximal effort.

3.2.4.4 Aerobic power

Aerobic power was tested using a SensorMedic Vmax 29 series metabolic cart (Sensor Medics, Yorba Linda, CA) in accordance with the method of Thoden (1991) as slightly modified by the Canadian Society for Exercise Physiology (CSEP, 1996). Participants were familiarized with the equipment at a walking pace (~5.0 km/hour) for two minutes, after which the speed of the treadmill (TechnoGym USA Corp, Seattle, WA), was increased to a jogging speed for three minutes to allow for a warm up before the test. Participants finished the warm up period at a speed that they felt they could run at for 20 minutes if the treadmill were to remain at a 0 % grade. The chosen speed was maintained and the grade increased by 2 % at the end of each two minute stage until the participant could no longer continue. Expired gases were collected continuously throughout the test, and were analyzed for volume and percentage of oxygen and carbon dioxide. Twenty second averages were calculated from the breath by breath measurements. Heart rate was measured, as well as ventilation volume, oxygen uptake (VO_2), carbon dioxide output (VCO_2) and the respiratory exchange ratio (RER) were calculated. Maximal oxygen uptake ($\text{VO}_{2\text{max}}$) values were used to compare between groups. $\text{VO}_{2\text{max}}$ was determined by a peak and plateau or decrease in breath by breath twenty second averages. Other criteria used to determine that $\text{VO}_{2\text{max}}$ was achieved was a heart rate close to (generally within five beats per minute) or above the age predicted maximum, and a RER of 1.1 or greater (CSEP, 1996).

3.2.5 Urine collection and analyses

3.2.5.1 Urine collection

Twenty-four hour urine samples were collected by the participants on day one and day seven of their second menstrual cycle (the intervention cycle). These days represent the day before the initiation of, and the day following the cessation of creatine supplementation. Along with a urine collection container (VWR International, Mississauga, ON), participants were given instructions on how to collect the 24 hour samples. Twenty-four hour samples were required in order to avoid fluctuations due to time of day and water intake (Smith-Palmer, 2002). Participants were instructed to begin the collection with the second urination of the collection day, and to collect all subsequent urinations into the jug. The importance of including each urination during the 24 hour collection period was stressed with each participant. Each urination up to and including the first urination of the following day (day two of the cycle) was collected in the jug. Participants were given instructions to keep the urine collection jug securely closed in order to prevent leakage and contamination of the sample, and to keep the sample out of direct sunlight.

3.2.5.2 Urine analyses

An aliquot of urine was removed from the collection container after the sample was mixed and the volume recorded. The aliquots were frozen at -80 °C until further analyses could be completed. Samples were diluted 100 fold prior to analysis (Burke et al., 2001), and were analyzed for the presence of creatine and creatinine. The analyses were conducted using high performance liquid chromatography (HPLC), using a potassium phosphate and TBAHS (2.0 g : 0.8 g) adjusted to pH 5.0 with aqueous potassium hydroxide (Burke et al., 2001). Analyses were carried out using a Waters 600 controller, a Waters 486 Tunable Absorbance Detector, and a

Waters 715 Ultra WISP sample processor. Twenty microlitres (μL) of supernatant was injected into a 250 x 3.0 mm Varian column for each trial, and concentrations were determined from their peak heights in comparison to a standard curve. The standard curve was derived using creatine and creatinine standards of concentrations between one and 500 μmol and was created with Millennium 32 Software. A standard was run after every five samples (Burke et al., 2001). Reproducibility for this method has been reported to be $\pm 1.0\%$ for creatine and $\pm 1.4\%$ for creatinine (MacNeil et al., 2005).

3.2.6 Blood collection and analyses

3.2.6.1 Blood collection

All blood collections were performed according to the recommendations of Refsum and colleagues (1998). Blood was drawn in the morning in an overnight fasted state; with the participant sitting with a standardized posture so as not to affect albumin concentration (a determinant of protein bound Hcy). Hematocrit values were measured using a Thermo IEC Micro-MB microcentrifuge (Fisher Scientific, Nepean, ON) prior to each blood collection in order to ensure that any concentration changes were not a result of changes in blood volume. Blood samples were collected from an antecubital vein of each participant into chilled vacutainer tubes (VWR International, Inc., Edmonton, AB) using venipuncture techniques (Garza & Becan-McBride, 2002).

Collections were made on the morning of the initiation of the creatine loading phase, prior to the ingestion of the first supplement, in order to determine baseline concentrations of the hematological components of interest. Blood was again collected on the morning of day seven of the menstrual cycle, or the morning following the cessation of the creatine loading period (following the same procedures). Three tubes containing approximately 6 mL of whole blood

each were removed during each collection.

3.2.6.2 Serum homocysteine

Serum Hcy concentrations were analyzed using a commercially available Immulite 2500 Competitive Immunoassay kit (Diagnostic Products Corporation, Los Angeles, CA) at The Provincial Laboratory (Regina, Saskatchewan). Samples were collected in Serum Plus Blood Collection vacutainer tubes, and centrifuged following the clotting period, in order to separate the red cells and serum. The samples were centrifuged for 15 minutes at 5,710 times gravity using an Allegra 6R RFG centrifuge at approximately 4 °C (VWR International Inc, Edmonton, AB) following clotting. The serum was removed and frozen for later analysis. Serum samples have been reported to remain stable when kept frozen at -20 °C for six months for Hcy analyses in the Immulite 2500 Competitive Immunoassay kit (refer to Appendix VII).

The immunoassay kit uses chemiluminescence to detect the amount of a particular component in a sample, which in this case is the amount of Hcy in human serum. The assay kit involves two 30 minute incubation cycles. The first cycle involves pretreating the serum with SAH hydrolase and dithiothreitol (DTT) solution. The second cycle involves moving the sample to a second reaction tube, containing a SAH-coated polystyrene bead and an alkaline phosphate-labeled antibody specific for SAH. Finally, the sample is washed to remove any unbound enzyme conjugate. Diagnostic Products Corporation reports a coefficient of variation range between 3.4 and 7.4 % within run, and a total range between 4.1 and 10.4 %.

3.2.6.3 Serum B₁₂

Analyses of serum B₁₂ concentrations were carried out using an Architect B₁₂ Assay (Abbott Laboratories, Abbott Park, IL, refer to Appendix VII) at the Provincial Laboratory. Samples were collected in Serum Plus Blood Collection vacutainer tubes, and centrifuged

following the clotting period, in order to separate the red cells and serum. Blood samples to be used for vitamin B₁₂ analysis were kept in a dimly lit area for the process of centrifugation. This has been found to prevent loss or degradation of the substance, as vitamin B₁₂ has been reported to be unstable in the light (Briddon, 2003). Following removal, serum samples were stored at -80 °C for up to 78 days before they were batch analyzed.

The samples were first incubated with sodium hydroxide with potassium cyanide, alpha monothioglycerol and EDTA, and cobinamide dicyanide in borate buffer with protein stabilizers. Following incubation, microparticles (porcine coated microparticles in borate buffer) and assay diluent (borate buffer with EDTA) were mixed in; the sample was again incubated, and then washed. A B₁₂ acridinium-labeled conjugate in MES buffer was added, and the sample was again mixed, incubated and washed. Pre trigger (hydrogen peroxide) and trigger (sodium hydroxide) solutions were added, and the chemiluminescent emission was measured to determine the quantity of vitamin B₁₂ in the sample. The within run coefficient of variation reported ranged between 3.7 and 8.4 %, while the total coefficient of variation ranged from 5.3 to 9.5 %.

3.2.6.4 Plasma B₆

Samples for B₆ (measured as pyridoxal 5'-phosphate) analysis were collected in EDTA vacutainer tubes, and immediately centrifuged for 15 minutes. Following centrifugation, the plasma was removed and frozen at -80 °C until further analysis at the Provincial Laboratory.

Analysis of plasma B₆ concentrations were carried out using Hewlett Packard 1100 series system high performance liquid chromatography (HPLC) with a degasser and fitted with a diode array detector. Samples were analyzed according to procedures outlined by Deitrick, Katholi, Huddleston, Hardiek and Burrus (2001). Briefly, protein free plasma samples were prepared by protein precipitation using perchloric acid. The samples were then injected into the HPLC

system with two mobile phases (A and B). The Mobile phase A was a 0.1 mol/L potassium dihydrogen phosphate buffer, and mobile phase B was a 30 % acetonitrile and water buffer. For quantification, the peak area from each sample was used as compared with a standard calibration curve for vitamin B₆. The coefficient of variation for this method was reported to fall between 1.10 and 2.36 % (Deitrick et al., 2001).

3.2.6.5 Whole blood folate

Whole blood samples used for the determination of folate concentration were collected in EDTA tubes, and kept refrigerated for less than seven days until analysis, as recommended in the immunoassay kit. Samples were analyzed at the Provincial Laboratory using an Abbott Competitive Immunoassay Kit on an Architect i2000 instrument (refer to Appendix VII). The assay determines folate concentration using Chemiluminescent Microparticle Immunoassay technology.

The whole blood sample was first mixed with DTT in acetic acid buffer with EDTA, followed by incubation. Next, the sample was mixed and incubated with potassium hydroxide, followed by the addition of microparticles (anti-folate binding protein coupled to microparticles with affinity bound with folate binding protein in TRIS buffer) and assay specific diluents (borate buffer). The samples were mixed, incubated and washed. Conjugate (Pteric acid-acridinium labeled conjugate in MES buffer) was added and the samples were mixed, incubated and washed again. Pre-trigger (hydrogen peroxide) and trigger (sodium hydroxide) solutions were added, and the chemiluminescent emission was measured to determine the quantity of folate in the sample. Abbott Laboratories reported a within run coefficient of variation ranging from 1.4 to 6.2 %, and a total coefficient of variation between 2.7 and 9.5 %.

3.2.7 Statistical analyses

Following calculations of descriptive statistics for each of the dependent variables, outliers were removed for all measures except for dietary intake. Outliers were determined by distinguishing which dependent variables were skewed (skewness/standard error $> \pm 2.5$), which violates the assumption of normality within the population. The dependent variables were also plotted in box plots using SPSS 16.0 (SPSS Inc. Headquarters, Chicago, IL). Data that was identified as an outlier in the box plot by the statistical program, that was also from a skewed population was removed before further analysis.

Independent samples t tests were used to make comparisons between groups for aerobic fitness ($VO_2\max$), daily amount of creatine ingested, and nutritional intake reported in the food diaries. The average dietary intakes of vitamin B₂, B₆, B₁₂ and folate were compared between groups to ensure that there were no significant differences in dietary components that could have an effect on the resulting plasma or serum concentrations of vitamin B₁₂ or Hcy. Comparisons of energy, protein, carbohydrate, fat, cholesterol and creatine intake between groups were also carried out. Oral contraceptive use was compared between groups using Fisher's exact test, due to the small sample sizes. Two factor analysis of variance (2 x 2 factorial ANOVA) with a between groups factor (vegetarian vs. non-vegetarian) and a repeated measures factor (pre vs. post-supplementation) was used to analyze mass and percent fat, bench and leg press strength, peak torque and average power during leg extensions, hematocrit, plasma concentrations of vitamin B₆, serum concentrations of vitamin B₁₂ and Hcy, whole blood measures of folate, and urinary creatine and creatinine. Comparisons between vegetarians and non-vegetarians for change score on leg press were made using a one factor ANOVA. Comparisons of peak and average power were made using 2 (group) x 2 (time) x 3 (set) factorial ANOVAs, with repeated

measures on the last two factors, followed by a Bonferroni post hoc test. A p value of 0.05 was used to determine significance.

4. RESULTS

4.1 Participant characteristics

Twenty healthy females between the ages of 18 and 40 volunteered to participate in the current study. All claimed to be recreationally active, that they were non smokers, had regular menstrual cycles, and were healthy individuals, free from disease. Each participant also indicated that they did not take medications for reasons other than birth control.

4.1.1 Age, height and oral contraceptive use

Age ($p = 0.557$), oral contraceptive use ($p = 0.197$) and height ($p = 0.607$) were not different between vegetarian and non-vegetarian participants (Table 4.1).

4.1.2 Baseline aerobic fitness

Baseline aerobic fitness levels were not statistically different between vegetarian and non-vegetarian participants ($p = 0.167$, Table 4.1).

4.1.3 Dietary intake

Analyses of dietary intake (Table 4.2) revealed no significant differences between groups for protein ($p = 0.14$), carbohydrate ($p = 0.075$) or fat ($p = 0.107$) intake. No significant differences between groups for energy intake ($p = 0.59$), vitamin B₂ ($p = 0.304$), vitamin B₆ ($p = 0.82$), vitamin B₁₂ ($p = 0.245$), total folate ($p = 0.602$) iron ($p = 0.87$), or methionine ($p = 0.15$) were found. Dietary intake of cholesterol was also found to differ ($p = 0.025$) between groups. There was a significant difference in creatine intake between groups ($p = 0.001$).

Table 4.1. Participant characteristics for vegetarian (VG) and non-vegetarian (NV) groups.

Measure	Vegetarian (n = 9)	Non-Vegetarian (n = 11)
Age (years)	25.4 ± 3.6	24.1 ± 5.9
Height (cm)	164.8 ± 5.7	163.3 ± 6.8
Oral contraceptive use (% using)	22.2	54.5
Aerobic fitness (ml·O ₂ kg ⁻¹ ·min ⁻¹)	43.1 ± 5.4	47.2 ± 7.1
Creatine supplement (g d ⁻¹)	11.2 ± 1.0	11.57 ± 1.2

Baseline measures were analyzed using independent sample t tests ($p < 0.05$). $n_{VG} = 9$, $n_{NV} = 11$. Values are means ± standard deviation.

4.1.4 Creatine supplementation

Groups were not different for the daily amount of measured creatine supplementation given ($p = 0.512$, Table 4.1).

4.1.5 Body composition

Body mass did not differ significantly between dietary groups at either time point ($p = 0.170$, Table 4.3). When data were pooled however, there was a significant time effect of creatine supplementation on body mass ($p = 0.017$).

Vegetarians had higher percentage body fat at the onset of the study when compared to non-vegetarians ($p = 0.001$, Table 4.3). There was no significant effect of creatine supplementation on body fat in either group ($p = 0.512$). Body composition measures using BodPod were completed for seven vegetarians and six non-vegetarians.

Table 4.2. Dietary intake from foods for vegetarian (VG) and non-vegetarian (NV) groups.

Measure	Vegetarian	Non-Vegetarian
Energy (kcal)	2233 ± 435	2106 ± 566
Vitamin B ₂ (mg)	1.4 ± 0.9	3.5 ± 6.0
Vitamin B ₆ (mg)	1.2 ± 0.8	1.3 ± 0.5
Total folate (µg)	228 ± 176	261 ± 104 ^a
Vitamin B ₁₂ (µg)	2.4 ± 2.8	4.0 ± 1.8 ^a
Iron (mg)	16.4 ± 5.8	15.9 ± 7.8
Methionine (g)	0.5 ± 0.3	0.7 ± 0.5
Protein (g)	73.0 ± 12.4	86.1 ± 22.7
Carbohydrate (g)	320 ± 80	253 ± 77
Fat (g)	65 ± 23	86 ± 33
Cholesterol (mg)	88 ± 72	279 ± 173 ^a
Creatine (g)	0.02 ± 0.03	0.50 ± 0.33 ^a

Independent sample t tests were used to compare between vegetarians and non-vegetarians for nutritional intake. ^a: Significant effect of diet ($p < 0.05$). $n_{VG} = 9$, $n_{NV} = 11$. Values are means ± standard deviation.

4.2 Fitness results

4.2.1 Leg extensions

A significant main effect of time on absolute peak torque was observed ($p = 0.002$, Table 4.4), indicating an increase in peak torque for pooled participants. The response to creatine was not significantly different between groups ($p = 0.339$). There was also a significant main effect of time on absolute average power ($p = 0.002$), whereby the pooled participants increased over

time (Table 4.4). However, with respect to this measure, vegetarians and non-vegetarians did not respond differently to the supplement ($p = 0.667$).

Table 4.3. Body mass and percentage fat for vegetarians (VG), non-vegetarians (NV), and participants pooled by time point.

Measure	Pre (VG)	Post (VG)	Pre (NV)	Post (NV)	Pre (pooled)	Post (pooled)
Body Mass (kg)	63.5 ± 6.3	64.0 ± 6.6	60.0 ± 4.8	60.3 ± 4.9	61.6 ± 5.7	62.0 ± 5.9 ^a
Body Fat (%) (n_{VG} = 7, n_{NV} = 6)	28.9 ± 2.5	28.1 ± 3.0	20.2 ± 3.7 ^b	22.5 ± 4.7 ^c	24.9 ± 5.4	25.5 ± 4.7

Comparisons of body mass and fat were made using two by two factorial ANOVAs. For each analysis, time (pre and post) and dietary group (VG and NV) were used as factors. ^a: Significant effect of time ($p < 0.05$); ^b: Significant effect of diet at onset of study ($p < 0.05$); ^c: Significant effect of diet during post measures ($p < 0.05$). $n_{VG} = 9$, $n_{NV} = 11$ unless otherwise indicated. Pooled values include vegetarian and non-vegetarian data. Values are means ± standard deviation.

4.2.2 Strength

Strength assessed by bench press was significantly affected over time ($p = 0.001$, Table 4.5), as indicated by an increase in one repetition maximum in response to creatine supplementation. Performance on one repetition maximum bench press did not differ between vegetarians or non-vegetarians ($p = 0.105$).

A significant effect of time on strength assessed by one repetition maximum of leg press was also found ($p = 0.003$, Table 4.5). There was a significant dietary condition by time interaction whereby vegetarians increased more than non-vegetarians ($p = 0.035$), though the difference between groups post-supplementation was not significant ($p = 0.374$). Comparisons of change scores from the leg press measures revealed that there was a significant effect of diet

on strength assessed by leg press ($p = 0.035$), whereby vegetarians experienced an increase of 16 ± 16 vs. 3 ± 9 kg.

Table 4.4. Peak torque and average power during 50 repetitions of leg extension on a BioDex dynamometer for vegetarians (VG), non-vegetarians (NV), and participants pooled by time point.

Measure	Pre (VG)	Post (VG)	Pre (NV)	Post (NV)	Pre (pooled)	Post (pooled)
Peak Torque (Nm)	80.3 ± 11.3	90.1 ± 8.1	89.0 ± 17.1	93.1 ± 16.1	85.1 ± 15.0	91.8 ± 12.9^a
Average Power (W) ($n_{VG} = 9$, $n_{NV} = 10$)	100.7 ± 17.6	108.9 ± 15.3	98.4 ± 11.8	105.5 ± 14.9	99.5 ± 14.4	107.1 ± 14.8^a

Two by two factorial ANOVAs were used to compare pre and post measures (for VG and NV) of peak torque and average power during fifty repetitions of a leg extension using a Biodex isokinetic dynamometer. ^a: Significant effect of time ($p < 0.05$). $n_{VG} = 9$, $n_{NV} = 11$ unless otherwise indicated. Pooled values include vegetarian and non-vegetarian data. Values are means \pm standard deviations.

4.2.3 Repeated Wingates

The response to creatine supplementation as reflected by peak power during repetitions of Wingate tests was not different between vegetarians and non-vegetarians ($p = 0.793$, Table 4.6). When the groups and trials were pooled, peak power was found to increase following the intervention ($p = 0.025$; 449 ± 93 vs. 472 ± 104 W), though there was no change in average power in response to creatine supplementation ($p = 0.755$; 345 ± 73 vs. 348 ± 81 W). A significant effect of trial on peak power was found when groups and time points were pooled ($p < 0.001$). Post hoc analyses using a Bonferroni adjustment revealed decreases in peak power between trial 1 and 2 ($p < 0.001$; 506 ± 95 vs. 447 ± 98 W), and decreases between trial 1 and 3

($p < 0.001$; 506 ± 95 vs. 430 ± 88 W), but no significant differences between trial 2 and 3 ($p = 0.231$). No significant interactions were found between trial and diet ($p = 0.654$), time and trial ($p = 0.554$) or time, trial and diet ($p = 0.714$).

A significant main effect of trial on average power during Wingate tests, when groups and time points were pooled was found ($p < 0.001$). Post hoc analyses using a Bonferroni adjustment revealed decreases in average power between trial 1 and 2 ($p < 0.001$; 393 ± 69 vs. 336 ± 72 W), between trial 1 and 3 ($p < 0.001$; 393 ± 69 vs. 311 ± 67 W), and between trial 2 and 3 ($p < 0.001$). However, there were no differences between vegetarians and non-vegetarians in response to the intervention ($p = 0.839$), and there was not a significant effect of time ($p = 0.755$). Finally, there were no significant interactions between time and diet ($p = 0.449$), trial and diet ($p = 0.062$), time and trial ($p = 0.775$) or time, trial and diet ($p = 0.108$).

Table 4.5. One repetition maximum for bench and leg press for vegetarians (VG), non-vegetarians (NV), and participants pooled by time point.

Measure	Pre (VG)	Post (VG)	Pre (NV)	Post (NV)	Pre (pooled)	Post (pooled)
1RM Bench Press (kg)	45 ± 7	43 ± 8	49 ± 15	52 ± 14	45 ± 13	48 ± 12^a
1RM Leg Press (kg)	121 ± 43	137 ± 48	112 ± 29	115 ± 31	116 ± 35	125 ± 40^a

Strength assessed by a one repetition maximum (1RM) bench press and leg press was compared using two by two factorial ANOVAs. Factors included were time (pre and post) and dietary group (VG vs. NV). ^a: Significant effect of time ($p < 0.05$). $n_{VG} = 9$, $n_{NV} = 11$ unless otherwise indicated. Pooled values include vegetarian and non-vegetarian data. Values are means \pm standard deviations.

Table 4.6. Peak and average power during three repetitions of a Wingate anaerobic test for vegetarians (VG), non-vegetarians (NV), and participants pooled by time point.

Measure	Pre-test: T1	Pre-test: T2	Pre-test: T3	Post-test: T1	Post-test: T2	Post-test: T3
Peak Power (W): VG	485 ± 77	436 ± 81	419 ± 106	514 ± 85	453 ± 117	434 ± 106
Peak Power (W): NV	503 ± 101	434 ± 97	426 ± 80	527 ± 115.	464 ± 105	439 ± 74
Peak Power (W): pooled	490 ± 90	435 ± 88	423 ± 90	521 ± 100	459 ± 108	437 ± 87
Average Power (W): VG	376 ± 58	339 ± 64	319 ± 71	384 ± 81	334 ± 90	305 ± 76
Average Power (W): NV	404 ± 70	329 ± 72	303 ± 64	402 ± 73	343 ± 71	315 ± 67
Average Power (W): pooled	391 ± 65	334 ± 67	310 ± 66	394 ± 76	339 ± 78	311 ± 69

Peak and average power measures were compared between dietary groups, over time (pre and post) and by repetition (trials 1, 2, 3) using two by two by three factorial ANOVAs ($p < 0.05$). $n_{VG} = 9$, $n_{NV} = 11$ unless otherwise indicated. Pooled values include vegetarian and non-vegetarian data. Values are means \pm standard deviations.

4.3 Blood measures

4.3.1 Hematocrit

A significant interaction between time and diet was found ($p = 0.047$, Table 4.7).

Independent samples t tests revealed that vegetarian participants had lower hematocrit values prior to the intervention ($p < 0.001$) but not following ($p = 0.49$).

4.3.2 Serum homocysteine

Creatine did not have a significant effect on serum Hcy concentration across time ($p = 0.21$, Table 4.7) or by dietary classification ($p = 0.28$).

4.3.3 Vitamins in blood

Plasma concentrations of vitamin B₆ were significantly affected over time ($p = 0.001$, Table 4.7). The pooled population experienced an increase in vitamin B₆ concentration. However, vegetarians and non-vegetarians did not respond differently to creatine supplementation ($p = 0.869$).

No significant differences were found in response to creatine supplementation for whole blood folate ($p = 0.448$, Table 4.7) or serum concentrations of vitamin B₁₂ ($p = 0.301$, Table 4.7). Vegetarians and non-vegetarians did not respond differently to the intervention for whole blood folate ($p = 0.303$) or vitamin B₁₂ ($p = 0.489$).

4.4 Urinary measures

4.4.1 Urinary creatine and creatinine

A main effect of time for urinary creatine concentration was evident ($p < 0.001$, Table 4.8), whereby the grouped participants experienced an increase in response to creatine supplementation. Baseline concentrations of urinary creatine were not different for vegetarians and non-vegetarians. Also, the response to creatine supplementation was not significantly different between groups ($p = 0.357$). No differences were found in urinary creatinine concentration in response to creatine supplementation ($p = 0.628$, Table 4.8). Finally, the urinary creatinine response was not different for vegetarians and non-vegetarians ($p = 0.890$).

Table 4.7. Blood measures for vegetarians (VG), non-vegetarians (NV), and participants pooled by time point.

Measure	Pre (VG)	Post (VG)	Pre (NV)	Post (NV)	Pre (pooled)	Post (pooled)
Hematocrit (%)	35.4 ± 2.6	38.9 ± 4.2	39.6 ± 1.2 ^a	40.0 ± 2.4	37.7 ± 2.8	39.5 ± 3.3 ^b
Serum Hcy (µmol/L)	6.3 ± 1.7	6.9 ± 1.7	5.7 ± 1.9	5.9 ± 1.7	5.9 ± 1.8	6.3 ± 1.7
Plasma B₆ (nmol/L)	78.8 ± 23.0	89.9 ± 25.8	67.0 ± 26.2	98.5 ± 23.3	72.3 ± 24.9	94.7 ± 24.2 ^b
WB folate (nmol/L)	1480.9 ± 279.6	1388.7 ± 391.3	1341.3 ± 279.0	1306.6 ± 225.7	1404.1 ± 281.0	1343.5 ± 305.0
Serum B₁₂ (pmol/L) (n _{VG} = 8, n _{NV} = 11)	294.3 ± 261.9	217.0 ± 142.4	322.0 ± 171.0	276.7 ± 144.5	310.3 ± 207.6	251.6 ± 142.8

Blood measure comparisons were made between dietary groups (VG and NV) and over time (pre and post) using two by two factorial ANOVAs. ^a: Significant effect of diet ($p < 0.05$); ^b: Significant effect of time ($p < 0.05$). WB = whole blood. n_{VG} = 9, n_{NV} = 11 unless otherwise indicated. Pooled values include vegetarian and non-vegetarian data. Values are means ± standard deviations.

Table 4.8. Creatine (Cr) and creatinine (Cn) measures in urine for vegetarians (VG), non-vegetarians (NV), and participants pooled by time point.

Measure	Pre (VG)	Post (VG)	Pre (NV)	Post (NV)	Pre (pooled)	Post (pooled)
Urine Cr (µmol) (n _{VG} = 8, n _{NV} = 11)	76.6 ± 84.9	537.4 ± 255.8	84.8 ± 136.6	736.9 ± 513.8	81.1 ± 113.5	647.1 ± 420.5 ^a
Urine Cn (µmol)	10308 ± 4290	10479 ± 2893	10256 ± 5185	11063 ± 5688	10279 ± 4680	10800 ± 4543

Two by two factorial ANOVAs (between dietary groups and over time) were used to compare urinary creatine and urinary creatinine outputs. ^a: Significant effect of time ($p < 0.05$). n_{VG} = 9, n_{NV} = 11 unless otherwise indicated. Pooled values include vegetarian and non-vegetarian data. Values are means ± standard deviations.

5.0 DISCUSSION

Homocysteine has been linked with cardiovascular disease when found in high concentration in the bloodstream, though no causative role has been distinguished (Selhub, 1999). Homocysteine metabolism involves the methylation of a number of compounds, including the formation of creatine from guanidinoacetate (Taes et al., 2003). Following the methyl donation, Hcy is reformed. The purpose of this research was to determine whether five days of oral supplementation with the popular sports supplement creatine monohydrate (0.25 g Cr/kg LBM per day) elicits a change in serum concentration of Hcy and/or changes in selected fitness measures in vegetarian and non-vegetarian females. Individuals following a lacto-ovo vegetarian or vegan diet have increased concentrations of Hcy in the bloodstream when compared to non-vegetarian counterparts (Obeid et al., 2002). While this is due to decreased dietary intake of natural sources of vitamin B₁₂ (Barr & Rideout, 2004), it is possible that the lack of dietary creatine may be a contributing factor. However, in the current study, there were no differences in urinary creatine output between groups prior to or following the creatine intervention. Because the groups were at similar concentrations prior to supplementation, it was unlikely that differences in response to creatine would be present.

While increases in urinary creatine output were noted which would be indicative of a positive response to the loading protocol per se, serum Hcy concentration was not affected by creatine supplementation. There was no effect of diet on urinary creatine at baseline or following the supplementation period. When pooled, participants experienced a performance

improvement in strength assessed by a 1RM bench press and leg press, improved their peak torque and average power during fifty repetitions of the leg extension, and improved their peak power during three repetitions of the Wingate anaerobic test, in response to the creatine supplementation protocol.

Dietary intake was analyzed in order to identify any differences between groups that may have an effect on serum concentration of Hcy. Given the importance of the vitamins B₂, B₆, B₁₂ and folate in the metabolism of Hcy, either in the formation of methionine, or in the disposal of Hcy through the transsulphuration pathway (Selhub, 1999) it was important to discern any baseline differences in these micronutrients. Deficiencies of vitamin B₁₂ and folate increase Hcy concentration in the bloodstream. In the current study, no differences in dietary intake of these vitamins were noted prior to the supplementation period.

Although the mean values for both groups satisfied the recommended daily allowance (RDA) intake for vitamin B₂ (1.1 mg per day) (Institute of Medicine: Food and Nutrition Board, 1998) only five of the nine vegetarian participants met the RDA, whereas all but one of the non-vegetarian participants met or surpassed this recommendation. Three participants in total (one vegetarian, two non-vegetarians) met the RDA of 400 µg per day of folate. The mean values conform to previous findings that vegetarians consumed the same amount of vitamin B₂ and folate when compared to non-vegetarians over a seven day period (Millet, Guillard, Fuchs, & Klepping, 1989). Eleven participants (two vegetarians, nine non-vegetarians) consumed enough vitamin B₁₂ daily to satisfy the RDA values (2.4 µg per day). It is important to note however, that each participant was supplementing with 25 µg of vitamin B₁₂, to ensure that deficiencies would not be a factor affecting serum concentrations of Hcy. While the mean intake reported for the vegetarian participants satisfies the RDA recommendation, non-vegetarians reported

ingesting 1.5 times the RDA for vitamin B₁₂. Also, RDA values may not reflect the true requirement of an individual for the particular nutrient of interest (Institute of Medicine of the National Academies, 2006). Though an individual may not satisfy the RDA values, they may still be including sufficient quantities in their diet for their own requirements.

In the case of folate and vitamin B₁₂, fortified or enriched foods were not reported as such, and therefore were not included in the dietary analysis. Food diaries (refer to Appendix IV) are often used as a source of information about nutritional intake, though reports of the reliability of self reporting reveal that energy intake and portion sizes are generally underreported (Howat et al., 1994). Dietary intake was used in the current study to determine whether there were differences between vegetarians and non-vegetarians for nutrients of interest, and to use as a benchmark for participants' dietary intake throughout the study. Participants were given instructions on the completion of food diaries, and were encouraged to include as much information as possible. However, participants did not include information about fortification. Fortified foods may not have been specified in their records, since instructions to include information in the food diaries about fortification in products were not given. This weakness would be a factor in both groups, though the greatest impact with regard to vitamin B₁₂ might be noted in the vegetarian group.

With vegetarian products, fortified foods were likely included in their diets, since most popular processed protein options for vegetarians include supplemental vitamin B₁₂. Many vegetarian burgers, textured proteins, soy milks, vegetable and sunflower margarines and breakfast cereals are fortified with vitamin B₁₂. Also, vitamin B₂, folate and vitamin B₁₂ are available naturally in a variety of foods. Folate can be found in high quantities in beans, spinach, romaine lettuce, orange and pineapple juice, many fruits and berries, nuts, and vegetables.

Vitamins B₂ and B₁₂ are found naturally in meat, dairy products and eggs. Vitamin B₂ can also be found in green leafy vegetables, enriched and whole grains and cereals, nuts and fruits. While the NutriBase software includes folate fortification for vegetarian products, it may not accurately assess quantities of vitamins B₂ or B₁₂ fortification (CyberSoft, Inc., 2006). Nutritional information was derived from the USDA Nutrient Database for Standard Reference and Brand Name foods, and the Canadian Nutrient Files.

Both vegetarians and non-vegetarians had normal concentrations of whole blood folate and serum vitamin B₁₂. Normal values for whole blood folate are greater than 317 nmol/L (Bailey, 1990), and for serum concentrations of vitamin B₁₂ are greater than 150 pmol/L (Millet et al., 1989). Values in the current study meet these criteria.

Also, there was no effect of creatine on whole blood folate or serum B₁₂ concentration. While creatine, folate and B₁₂ are all involved in Hcy metabolism, they are involved in different metabolic pathways. Creatine is created as a result of the methylation of guanidinoacetate, whereas folate and B₁₂ are involved as cofactors in the methylation of Hcy to form methionine (Korzun, 2004; Selhub, 1999).

No differences were found in energy intake, vitamin B₆, iron or methionine. Interestingly, mean values for iron intake were slightly, but not significantly, higher for vegetarian participants. Each group reported ingesting slightly less than the 18 mg RDA for iron (Institute of Medicine: Food and Nutrition Board, 1998). Though the general belief is that iron is found in the highest concentration in meat and seafood, beans, tofu, a variety of seeds, and enriched breakfast cereals each have greater amounts of iron per serving size than meats. Nieman (1999) states that iron intake in vegetarians is generally above the recommended

amount, though nonheme iron from plant food sources is not as readily absorbed as heme iron from animal products, resulting in decreased concentrations in the bloodstream. While vegetarians reported consuming a number of sources of nonheme iron, their reported intake remained slightly below the RDA.

Vegetarian participants in the current study made an effort to include these nonheme iron concentrated foods and other nutrients and vitamins in their diet, which therefore resulted in similar intakes when compared to non-vegetarians. This agrees with conclusions made by Millet and colleagues (1989), that vegetarians may be more diet-conscious than non-vegetarians.

Hematocrit values are often used as an indicator of iron status. The vegetarians in the current study had significantly lower hematocrit values when compared with non-vegetarians, suggesting that they were not absorbing enough nonheme iron. Iron is fundamental in both oxygen delivery and utilization during aerobic exercise (Lukaski, 2004). However, diet, along with differences in hematocrit values, did not result in significant differences in aerobic fitness, as measured by the VO_2 max test. Differences in hematocrit across time are likely due to differences in hydration status.

Vegetarian participants satisfied the Acceptable Macronutrient Distribution Ranges for protein (13 ± 2 vs. 10 – 35 %), carbohydrate (59 ± 7 vs. 45 – 65 %) and fat (25 ± 6 vs. 20 – 35 %) intake (Institute of Medicine of the National Academies, 2006). Non-vegetarians complied with these guidelines for protein (16 ± 3 %) and carbohydrate (47 ± 8 %) intake, though the mean value for fat was slightly greater than the recommended range (36 ± 8 %). When protein intake relative to body weight (g/kg) was compared between vegetarians and non-vegetarians, no differences were found. Both groups satisfied the RDA values (0.80 g/kg/day). Significant

differences were found between groups for cholesterol intake. Non-vegetarian participants had greater cholesterol intake when compared to vegetarians, which is likely a result of the high amounts present in foods of animal origin (Institute of Medicine of the National Academies, 2006).

Dietary intake of creatine via animal products in the diet was significantly different between groups. Non-vegetarians, who ingest meat and fish, were found to have significantly higher dietary creatine intake when compared to vegetarians. This was expected, since vegetarians have only milk and cranberries available to them, each of which have very small creatine content (milk: 0.1 g/kg; cranberries, 0.02 g/kg). No intake of cranberries was listed in the food diaries. While non-vegetarians consumed greater amounts of creatine in the diet, their intake remained lower than expected (Bemben & Lamont, 2005). Bemben and Lamont (2005) report that a normal mixed diet includes approximately 1 gram of creatine per day, which is twice the average intake in the current work. The non-vegetarian females in this study did not ingest animal tissues with each meal, nor did some of them ingest animal tissues every day, thereby limiting the intake of creatine in the diet. This low dietary intake likely had an effect on urinary creatine output, decreasing the difference between vegetarians and non-vegetarians and resulting in non-significant baseline concentration.

In the current study, twenty healthy females supplemented with creatine (0.25 g Cr/kg LBM) for a period of five days. The rationale for supplementing with creatine was to examine the serum Hcy response to creatine supplementation. It was thought that Hcy production would be decreased due to a diminished need for endogenous production of creatine (Taes et al., 2003). The healthy females involved in the current project did not experience a change in serum Hcy concentration in response to a five day loading phase of creatine supplementation. In fact,

though there was no trend ($p = 0.214$), the mean values increased slightly from the onset of the intervention to the conclusion. If creatine supplementation has a Hcy lowering effect, such a response could occur only in those with elevated Hcy concentration at the onset of supplementation. Homocysteine concentration in the vegetarian participants was lower than expected, since previous research has established that vegetarians have higher Hcy concentration in the bloodstream than non-vegetarian counterparts (Bissoli et al., 2002; Herrmann et al., 2001; Hung et al., 2002; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002). In fact, Hcy concentration found in the current study was lower in both vegetarians and non-vegetarians when compared to these previous studies, which suggests not only that the participants were very healthy, but that they had ample vitamin B₁₂ and folate available for Hcy metabolism. Therefore, further decreases in Hcy concentration in the bloodstream would be unlikely to occur.

The fact that these healthy females did not experience decreases in Hcy concentration falls in line with previous research examining healthy (Steenge et al., 2001) and unhealthy individuals (Taes et al., 2004). Steenge et al. (2001) had healthy female participants load with creatine for five days (20 g Cr/day), then ingest three grams each day for an additional eight weeks. While there was a trend towards a decrease in Hcy concentration, statistical significance was not achieved. Homocysteine concentration prior to the intervention was not particularly elevated (~9.5 $\mu\text{mol/L}$), though they were greater than the values found in the current work.

Taes et al. (2004) investigated the Hcy response to creatine supplementation for four weeks (2 g Cr/day) in hemodialysis patients. Participants were already being treated with vitamins B₆, B₁₂ and folate for their condition. The authors found that their supplementation protocol did not further reduce plasma concentrations of Hcy.

However, Korzun (2004) reported a statistically significant decrease in plasma Hcy concentration in healthy volunteers following four weeks of multivitamin and creatine supplementation (twice their urinary creatinine excretion). Four of the eight participants supplementing with creatine experienced decreases of 18 to 27 percent of their baseline Hcy concentration.

The participants in the current study may not have experienced a decrease in their Hcy concentration because the supplementation period was not long enough (five days vs. four weeks). The participants in Korzun's (2004) study were taking a single dose of creatine that was slightly greater than each of the four doses taken by the vegetarians and non-vegetarians in the current study. Baseline Hcy concentrations were comparable, though concentrations were slightly less in the current study, which may have been a factor (6 ± 2 vs. 7.1 ± 1.8 $\mu\text{mol/L}$). Korzun (2004) reported a decrease of 0.9 ± 0.7 $\mu\text{mol/L}$. This post-intervention concentration remains greater than the starting point for the vegetarians and non-vegetarians in the present study.

While Korzun (2004) appears to be the sole researcher to have found a statistically significant decrease in plasma Hcy concentration in humans, animal research has produced similar results. Taes and colleagues (2003) reported a decrease in plasma Hcy concentration in an animal model of uremia. However, other attempts at decreasing the concentration in humans have failed (Taes et al., 2004). Due to these conflicting results, it is difficult to say whether plasma or serum Hcy concentrations are altered by the ingestion of creatine monohydrate. In the present study, healthy females did not respond to creatine supplementation with a decrease in serum Hcy concentration.

The amount of supplemented creatine may not have been large enough to bring about a change. Participants supplemented with 11.42 ± 1.10 g of creatine a day, or 0.25 grams per kilogram of LBM (Burke et al., 2003). Burke et al. (2003) found that this supplementation protocol resulted in the least urinary creatine output during a loading phase. This indicates that participants had available to them the maximum amount of creatine with which to suppress endogenous production of creatine and therefore the reformation of Hcy. A supplementation protocol of 20 grams of creatine each day is also widely accepted (Greenhaff et al., 1994; Harris et al., 1992), and may have been more effective in having an effect on serum Hcy concentration.

There was, however, an impact of creatine supplementation on plasma concentrations of vitamin B₆. Though there was not a significant effect of diet, there was a trend towards a time by diet interaction. This would indicate that the dietary groups changed differently. The mean increase in vegetarians was approximately 10 nmol/L, whereas non-vegetarians increased by approximately 30nmol/L. When groups were pooled, concentrations increased following the intervention. This was unexpected, since creatine is not known to have an effect on vitamin B₆. While previous research has reported that increases in estrogen are associated with a decrease in plasma concentrations of vitamin B₆ (Rose, 1978), it is unlikely that this was a factor in the findings of the current study since plasma concentrations of B₆ actually increased as the participants moved into the follicular phase and therefore experienced increases in estradiol concentration.

The lack of Hcy response was not a result of a deficiency of creatine uptake. As mentioned, urinary creatine output increased in response to the supplementation protocol. Urinary creatine output is used as an indicator of the amount of creatine retained in the body (Burke et al., 2001). Improvements on strength and anaerobic test performances corresponding

with creatine supplementation are likely due to increases in muscle creatine stores. Although muscle total creatine was not measured in the current study, urinary creatine measures taken from 24 hour urine samples did show a tenfold increase in vegetarians and an eightfold increase in non-vegetarians.

Burke et al. (2001) reported an increase in urinary creatine output of approximately twenty-four times the baseline output following supplementation. This approximates 50 % creatine retention. The authors had twenty male varsity athletes supplement with 0.1 grams of creatine per kilogram of LBM for a period of seven days. The athletes had a mean body mass of 91 ± 21 kg, approximately 30 kg greater than those involved in the current study. The lower levels of urinary creatine output observed in the current study may have been a result of a greater uptake and retention rate. This may be due either to lower intake of creatine in food sources, or lower baseline concentration in muscle. Burke et al. (2001) suggest that their participants had maximal concentration of creatine in their muscles at the onset of the study, thereby prohibiting further uptake with supplementation, which may also account for the differences in urinary creatine output with the current study.

In the current study, differences in baseline urinary creatine output were expected, but not found. Burke et al. (2003) looked at urinary creatine output and muscle total creatine content in vegetarian and non-vegetarian participants before and after eight weeks of creatine supplementation (7 day loading, 7 week maintenance dose) and resistance training (8 week program). Participants collected 24 hour urine samples prior to supplementation, on the fifth day of supplementation, and following the eight week supplementation period. Urinalysis revealed that baseline concentration of urinary creatine was greater for non-vegetarian participants, as was

expected, due to the nature of a vegetarian diet, lacking animal meats and therefore excluding most sources of creatine (Burke et al., 2003).

Urinary creatinine concentration in the current study was not different across groups or time. This lack of difference may have occurred because there were no significant differences in body mass observed between groups at either time point, and these concentrations are based on lean tissue mass (Wang, Gallagher, Nelson, Matthews, & Heymsfield, 1996), which was not statistically different between groups or over time. Also, no changes with supplementation were expected, since the changes in body mass, while significant, were minimal. This is in accordance with previous research (Burke et al., 2003).

The secondary purpose of the current study was to determine the effect of creatine supplementation on measures of muscle strength, power and anaerobic capacity in female vegetarians and non-vegetarians. No significant effects of diet were found for any of the fitness measures, excepting a group by time interaction for strength as assessed by leg press. Therefore the data from vegetarians and non-vegetarians were pooled.

Previous research looking at differences in body composition measures and dietary intake in vegetarians and non-vegetarians reported significantly greater body mass for non-vegetarians participants (7 kg greater for female participants) (Millet et al., 1989). In the current study, while there were body mass increases in response to the intervention, and differences in percentage body fat between groups at both time points, vegetarians and non-vegetarians did not differ in mass at either time point, and the magnitude of the increase in body mass in response to creatine supplementation was not significantly different between groups. Also, neither group experienced a change in percentage body fat over time. Therefore, it is unlikely that these

increases in body mass were due to a significant increase in skeletal muscle mass, as has been previously reported (Vandenberghe et al., 1997).

A reasonable conclusion for this increase in body mass in the absence of a change in percentage body fat is that while there may have been a positive change in fat free mass, it was coupled with an increase in fat mass as well. As such, percentage of total mass represented by fat remains the same. There are three possible causes for this increase in body mass: increase in fat mass due to juice mix, increase in fluid retention, and increase in protein synthesis.

Weight gain may have been due to the carbohydrate containing juice mix combined with the creatine monohydrate, adding approximately 400 to 500 calories daily. Because the participants were instructed to maintain their dietary habits and physical activity regime prior to the supplementation period, these added calories would likely cause an increase of approximately 2000 to 2500 calories consumed over the five day loading period. The participants may have decreased their caloric intake to account for the additional intake, though they were instructed to maintain their previous eating habits throughout the intervention. Approximately 0.32 kilograms of fat mass could have been accumulated in the course of the creatine loading phase. Obviously this small an increase in fat would not be enough to cause a significant difference in percentage of body fat, but it does account for most of the mass gained on average in vegetarians and non-vegetarians (0.44 kg and 0.37 kg).

Previous work suggests that creatine supplementation may cause fluid retention by skeletal muscle, which would be responsible for weight gain (Balsom, Harridge, Soderlund, Sjodin, & Ekblom, 1995), or that creatine may cause an increase in protein synthesis by stimulating the rate of myosin heavy chain synthesis (Ingwall, Weiner, Morales, Davis, &

Stockdale, 1974). Vandenberghe and colleagues (1997) reported an increase in body mass corresponding with an increase in fat free mass following creatine supplementation in sedentary females. Body mass and composition were not measured directly after the four day creatine loading phase (20 g Cr/day), but following five and ten weeks of maintenance supplementation (5.0 g Cr/day) coupled with resistance training. They reported that those in the creatine group experienced a greater increase in fat free mass and a trend towards greater body mass and decrease in percentage body fat when compared with a placebo group. The authors suggest that creatine supplementation allowed for this increase in fat free mass, and enhanced the benefits of resistance training by improving both maximal muscle strength and maximal intermittent arm power output (Vandenberghe et al., 1997).

In a study investigating the effects of creatine supplementation (7 days of 0.25 g Cr/kg lean tissue mass/day followed by 7 weeks of 0.0625 g Cr/kg lean tissue mass/day) in vegetarians and non-vegetarians participating in an eight week high intensity, heavy load resistance training program, Burke et al. (2003) found that vegetarian participants experienced greater lean tissue increases when compared to non-vegetarians (2.4 vs. 1.9 kg). Also, when pooled according to supplement and compared to participants supplementing with a placebo, the creatine group (vegetarians and non-vegetarians) experienced greater increases in lean tissue mass.

In the current study, we succeeded in causing an ergogenic response with creatine supplementation. Creatine monohydrate has been studied in depth for its positive effect on exercise performance. Creatine supplementation increases muscle creatine content (Greenhaff et al., 1994). These increases in muscle creatine content may allow for quicker resynthesis of phosphocreatine which subsequently allows a longer period of ADP re-phosphorylation (Greenhaff et al., 1994). In the current study, healthy females took part in a five day creatine

loading phase (0.25 g Cr/kg LBM) in order to elevate the concentration of creatine in both the bloodstream and in muscle. Though muscle total creatine was not measured, urinary creatine output was analyzed, which is closely correlated to uptake of creatine by muscle.

Previous research investigating uptake of creatine into muscle and phosphocreatine resynthesis in response to a five day creatine loading period reported a mean creatine increase of 15 % in muscle, and by the second minute of recovery the rate of phosphocreatine resynthesis was increased by 42 %, though these differences were not significant when comparing time points (Greenhaff et al., 1994). Muscle biopsies were carried out after three maximal contractions and electrical stimulation of the knee extensors.

Smith, Montain, Matott, Zientara, Jolesz and Fielding (1999) investigated the effect of creatine supplementation (0.3g Cr/kg/day) and exhaustive exercise on ATP cost. Baseline concentrations of phosphocreatine, resynthesis following single leg extensions completed to exhaustion, and hydrolysis were determined. Following creatine supplementation (0.3g Cr/kg/day), resting muscle phosphocreatine increased by 15 %, and phosphocreatine hydrolysis during exercise increased by 13 %. There was no effect of creatine supplementation on phosphocreatine resynthesis immediately following exercise. However, ATP cost was not affected by creatine supplementation, suggesting that the availability of phosphocreatine may not cause differences in ATP cost of contraction.

Similar muscular endurance tests were carried out by Burke and colleagues (2003) with a group of vegetarian and non-vegetarian participants. Participants supplemented with 0.25 g Cr/kg of lean tissue mass per day for a seven day loading phase, followed by seven weeks of maintenance supplementation (0.0625 g Cr/kg of lean tissue mass per day) and resistance

training. Participants completed 50 repetitions of leg extensions and curls at 180 °/s on an isokinetic dynamometer before and after the supplementation period began. The authors reported a significant group by supplement by time interaction, where vegetarians supplementing with creatine experienced an improvement in performance that was statistically greater than all other groups. The authors attribute this improvement to corresponding increases in total muscle creatine and phosphocreatine (Burke et al., 2003).

Vandenbergh and colleagues (1997) had their female participants perform five bouts of 30 repetitions of bicep curls on an isokinetic dynamometer, separated by two minute rest intervals before and after loading, mid and post maintenance supplementation. The authors used the mean torque for the five bouts to represent performance. While a creatine loading phase was insufficient in creating a statistically significant impact on performance, significant differences between groups were found following five and ten weeks of maintenance supplementation. The authors suggest that creatine supplementation allowed the participants to continue optimal training for a prolonged period of time when compared to the placebo group. The participants in the current study were not introduced to a resistance training protocol for the duration of the five day intervention, though were encouraged to continue their regular physical activity regimen. During this regimen, the supplemented creatine may have allowed them to prolong their activities, which could therefore have an impact on the post intervention performance tests.

Contrasting research has shown no effect of creatine supplementation on performance on an isokinetic dynamometer. A study that compared the effects of creatine supplementation on knee extensor strength using an isokinetic dynamometer in men and women found no significant effect of creatine supplementation following a four day creatine loading period (20g Cr/day) (Tarnopolsky & MacLennan, 2000). Participants performed three sets of 10 repetitions of

maximal leg extensions with one minute of rest between sets, at an angular velocity of 30 or 180 °/s. No response to creatine supplementation was reported at either velocity.

There are some major differences between the current study and that of Tarnopolsky and MacLennan (2000). In the current study, participants performed one set of fifty repetitions before and after the supplementation protocol. Thus the availability of creatine in muscle could have allowed them to exercise with higher torque outputs for a longer period of time, whereas Tarnopolsky and MacLennan (2000) were more interested in strength during shorter sets of leg extensions. Average power increased in the current study, suggesting that participants experienced less fatigue when compared to pre testing.

These results are substantiated by Chrusch and colleagues (2001), who conducted muscular endurance and fatigue tests using an isokinetic dynamometer in older men supplementing with 0.3 g Cr/kg of body weight per day for five days, which was followed up with maintenance supplementation (0.07 g Cr/kg body weight per day) for a total supplementation period of 12 weeks. The authors also implemented a whole body resistance training program. Participants were asked to complete three sets of ten repetitions of leg extensions and curls at 60 °/s. Average power was measured. The authors reported that creatine supplementation improved average power during leg extensions and curls. The authors suggest that the lack of improvement in strength and endurance may be due to a lack of statistical power, or insufficient time to overcome a learning curve.

A learning effect too could have played a role in the current study, since most of the participants had a very limited experience with an isokinetic dynamometer, despite all having experience with the leg extension exercise itself. Although participants were instructed to warm

up with the equipment prior to the test, by the second testing session, they might have been more comfortable and familiar with the leg extensions on the dynamometer. However, due to the high level of significance for both peak torque and average power ($p = 0.002$), we can conclude that while there may have been a learning effect, a statistically significant response to creatine supplementation exists as well.

When pooled, the participants in the current study experienced a significant increase in strength assessed using a 1RM bench press and leg press test, in response to creatine supplementation. There were no differences in response due to dietary group for strength as assessed by bench press. For leg press, there was a significant group by time interaction, and a significant effect of diet on change scores. Each group experienced an increase in strength during this measure, though the vegetarians improved their mean maximal lift by 16 kg, whereas non-vegetarians increased by only 3 kg. However, neither pre nor post measures were significantly different. While there may have been a slight learning effect on the results, most of the participants involved had previous experience with both bench and leg press exercises. Creatine supplementation likely fueled the improvements in both leg and bench press following the creatine loading phase.

Increases in strength generally accompany creatine supplementation due to increases in muscle creatine content, causing a decrease in fatigue rates during exercise, generally allowing the individual to maintain physical activity for longer periods of time (Hoffman et al., 2006). Due to such a short period of time between pre and post testing, it is unlikely that strength gains were due to an increase in training volume allowed by creatine supplementation. Alternatively, previous work suggests that creatine supplementation increases intramuscular concentration of phosphocreatine, thereby delaying fatigue during medium to high intensity exercise (Cady,

Jones, Lynn, & Newham, 1989) and allowing for work at a higher training load (Chrusch et al., 2001). This is a more probable explanation for the gains in strength achieved in the current study.

Many studies report that strength increases when creatine supplementation is combined with a resistance training program (Burke et al., 2003; Chrusch et al., 2001; Hoffman et al., 2006; Vandenberghe et al., 1997). Participants in the current study likely would have experienced a greater increase in strength if maintenance supplementation and a resistance training program had been included in the study. However, we are able to compare the changes in the current study to the differences between creatine and placebo groups in the aforementioned studies.

Burke and colleagues (2003) investigated the effects of creatine supplementation (7 day loading, 7 week maintenance dose) and resistance training (8 week program) on strength in vegetarian and non-vegetarian participants. They found that when pooled by diet and compared by supplement, those supplementing with creatine experienced a greater increase in their 1RM bench press, but not leg press. When bench press differences were compared to those of the current study, it is apparent that the resistance training protocol was very effective, as the participants of Burke et al. (2003) experienced an increase of approximately 16 kg on their bench press when supplementing with creatine, compared to our increase of 6 kg. The participants supplementing with placebo increased their bench press by approximately 9 kg due to the resistance training protocol. Taking this training program into account, the changes in 1RM bench press are comparable to the current work. There were no differences reported between vegetarians and non-vegetarians (Burke et al., 2003). The authors attribute the improvement in performance to the ability to increase their training volumes more quickly than those

supplementing with placebo, since their creatine group had a greater training volume than the placebo group.

Chrusch and colleagues (2001) looked at the effects of creatine loading (5 days of 0.3 g Cr/kg body weight) followed by maintenance supplementation (0.07 g Cr/kg body weight) combined with resistance training (12 weeks, 3 times a week) on strength in older males. The authors reported an increase in 1RM leg press. Following six weeks of supplementation, the creatine group increased their 1RM leg press by approximately 35 kg, whereas the placebo group improved by only 20 kg. Following the 12 weeks of supplementation and resistance training, this score was increased by another 20 kg for the creatine group, and 15 kg for the placebo group. The difference between groups following the intervention was 20 kg, which is very comparable to the increase in the current study. There were no differences between groups (creatine vs. placebo) for bench press measures. The authors suggest that bench press did not increase because the resistance training program had less upper body exercises than lower body. An alternate possibility is that the ability of older adults to respond to bench press training may be less than lower body training (Chrusch et al., 2001).

Hoffman and colleagues (2006) looked at the effects of creatine supplementation and a four day per week, split routine resistance training program (10.5 g Cr/day for 10 weeks) on a number of fitness measures in males (Hoffman et al., 2006). These measures included strength assessed by 1RM tests for bench press and the squat. The researchers found that both creatine and control groups experienced an improvement in each of the strength measures (Hoffman et al., 2006). Those supplementing with creatine experienced a greater increase over time than the control group for both bench press and squat, though both groups experienced an increase due to the resistance training program. The creatine group increased their mean 1RM bench press by

approximately 15 kilograms, and their squat by approximately 24 kilograms, compared with five and seven kilograms for the placebo group. When the differences are compared to the current work (10 and 17 kg), we find that Hoffman et al. (2006) had slightly greater changes in bench press, and slightly lower changes for squat than leg press in the current study.

It is interesting to note that the strength test performances in the current study compare very closely to these previous studies that involved supplementing with creatine for many weeks, whereas our protocol had only a five day creatine loading period. It is possible that increases in strength due to supplementation are achievable in only a short period of time, though it is likely that there was also a learning effect involved in the current study. Further research should be carried out to establish whether this is the case.

Due to a decrease in the available stores of ATP and phosphocreatine, performance on subsequent repetitions of Wingate anaerobic tests decreased, with reductions in peak power from trial 1 to 2 and 1 to 3, and in average power from trials 1 to 2, 1 to 3 and 2 to 3. It was expected that creatine supplementation would improve performance and therefore decrease the magnitude of power drop on trials 2 and 3, due to increases in phosphocreatine available in muscle for the production of ATP (Greenhaff et al., 1994).

There were improvements in peak power following the supplementation period, although the pooled participants did not experience an improvement in average power following the intervention. The increase in peak power with time, and the decrease in performance with repetitions fall in line with previous research using repeated bouts of anaerobic activity (Mujika, Padiall, Ibanez, Izquierdo, & Gorostiaga, 2000; Preen et al., 2001; Shomrat et al., 2000; Tarnopolsky & MacLennan, 2000). However, contrasting evidence also exists, stating that

creatine does not cause such an improvement in performance (Finn et al., 2001; Hoffman, Stout, Falvo, Kang, & Ratamess, 2005; Odland, MacDougall, Tarnopolsky, Elorriaga, & Borgmann, 1997). These contrasting reports may be due to differences in protocol, but may also be due to the characteristics of the participants at baseline.

Results for the Wingate anaerobic tests were similar to work performed by Shomrat et al. (2000). Comparisons of the effects of creatine supplementation on maximal exercise performance in vegetarians were made, using three repetitions of 20 second Wingate anaerobic tests. The authors found that the intervention affected both vegetarians and non-vegetarians similarly (Shomrat et al., 2000). Vegetarians had significantly lower baseline concentration of plasma creatine, and both vegetarians and non-vegetarians experienced similar increases with supplementation. Non-vegetarians experienced a significant increase in peak power output following supplementation, while the vegetarians did not differ significantly.

Generally, research investigating creatine and anaerobic power has examined the response in non-vegetarians. Since there were no differences between vegetarians and non-vegetarians in the current work, it is possible to make comparisons between the present study and these previous studies. The response to supplementation in the current study falls in line with previous work involving repetitions of anaerobic activity (Preen et al., 2001; Tarnopolsky and MacLennan, 2000).

Tarnopolsky and MacLennan (2000) studied the effects of creatine supplementation (4 days of 20 g Cr/day) on exercise performance in males and females during two repetitions of 30 second Wingate anaerobic tests, separated by a period of four minutes. The resistance set for the tests was seven percent of the participant's mass. Peak power was significantly higher in the

creatine group, while there were no changes in average power. While this investigation allowed a longer period of time between anaerobic test repetitions, a significant increase with creatine supplementation was found in both this and the current research, suggesting that an adequate amount of phosphocreatine resynthesis had also occurred in the two minute break given. It is possible that with a longer break between tests, the drop in peak power from one trial to the next may not have been as great.

A group of Australian researchers (Preen et al., 2001) studied the effects of creatine supplementation on an 80 minute exercise trial including 10 sets of either five or six by six second maximal bike sprints, with varying recover times (24, 54, or 84 seconds between sprints). The supplementation protocol included five days of creatine loading (20 g/day). The creatine group had a greater increase in total work performed and peak power following set five and eight when compared to the placebo group, who did not experience any significant differences with time. In the current study, creatine supplementation resulted in an increase in peak power when the trials were pooled. The creatine group also had increases in total muscle creatine and phosphocreatine concentration following supplementation (Preen et al., 2001). They suggest that the increase in peak power and work performed may be due to a greater energy store allowed by the increases in muscle phosphocreatine concentration following the creatine intervention.

5.4 Limitations

There were a few limitations associated with this study.

With respect to food diaries, specific instruction to include information about folate or vitamin B₁₂ fortification was not given. This may have resulted in an underestimation of the amount of these vitamins actually being included in the diet. Analysis of dietary intake did not

reveal differences in intake of either folate or vitamin B₁₂. Though non-vegetarians reported a mean intake of approximately 1.5 times more vitamin B₁₂ in their food records, the difference was not significant.

Oral contraceptive use in this study was not used as an exclusion criterion. Those using (or not using) oral contraceptives for a period of at least one year were allowed to participate in the study. Oral contraceptive use was evenly distributed between the vegetarian and non-vegetarian groups ($p = 0.197$), which allows us to conclude that there was no effect on Hcy concentration or physical fitness measures over the course of the intervention.

A familiarization test was not used in the current research. All individuals stated that they were familiar with bench and leg press, as well as leg extensions. Few of the participants had completed repetitions of the Wingate anaerobic test in the past, which may have had an effect on the results for this measure. It is possible that participants who were not familiar with the test did not give as great an effort on the post intervention measure, which would have a negative impact on the results. In future work, a familiarization opportunity for all fitness measures should be included, to decrease the possibility of non-maximal efforts being recorded, and to limit the effect of learning that may take place without familiarization.

Scheduling and time limitations made it impossible to complete all post measures on all participants immediately upon cessation of the loading protocol. As such, for some participants there were several days between the end of the loading period and post fitness measures. Since participants were not supplemented with creatine during the time between the five day loading period and their final fitness test day it is possible that this may have resulted in a decrease in creatine availability. This may have resulted in a lesser magnitude of improvement in strength as

assessed by 1RM bench and leg press, and performance during repetitions of the Wingate anaerobic test. Finally, there is a possibility for type II error to occur, due to the small sample sizes available to us, resulting in less than the 80 % power calculations we were originally aiming for (20 vegetarians, 20 non-vegetarians). With a greater sample size, a significant interaction between trial and diet for average power measured during the Wingate anaerobic tests may have been elicited, as a trend was revealed with the current sample sizes ($p = 0.062$). The possibility of type I error exists as well due to the number of statistical tests used in determining the location of statistical significance.

5.5 Conclusions

The current research project is the first to focus on the Hcy response to creatine supplementation in female vegetarians and non-vegetarians. Whether due to the length of the supplementation protocol or the low baseline levels found in the participants, there was no effect of Cr supplementation on Hcy concentration. While Korzun (2004) reports statistically significant decreases in Hcy in humans following creatine supplementation, the current study, and those of others (Taes et al., 2004) failed to find an observable impact. Due to these conflicting results, it remains difficult to say whether creatine supplementation would result in a decrease in Hcy concentration in the bloodstream.

Baseline concentrations of Hcy were quite low in both vegetarian and non-vegetarian participants when compared to other healthy individuals (Bissoli et al., 2002; Herrmann et al., 2001; Hung et al., 2002; Krajcovicova-Kudlackova et al., 2000; Obeid et al., 2002). These low concentrations may also have limited the potential for an effect of creatine supplementation on serum Hcy concentration.

While vegetarians have been previously reported to have increased Hcy concentrations when compared to non-vegetarians (Bissoli et al., 2002; Herrmann et al., 2001; Hung et al., 2002; Krajcovicova-Kudlackova et al., 2000), which may put them at increased risk of developing cardiovascular disease, there are a number of other lifestyle factors that may counteract this risk. These include, but are not limited to healthy physical activity levels, non-smoking habits, and dietary constituent consumption. The current vegetarians had significantly lower cholesterol intake when compared to non-vegetarian participants, and satisfy the Acceptable Macronutrient Distribution Range, which estimates that 20-35 percent of energy should be derived from fat (Institute of Medicine of the National Academies, 2006). Consequently, while it is of importance to determine the usefulness of creatine supplementation on elevated Hcy concentrations, this may have limited direct application to vegetarians. In instances when vegetarians do have elevated Hcy levels, finding treatments may not be as critical as they generally have few other risk factors. In future research, baseline testing of plasma or serum Hcy could be performed to determine whether concentrations are elevated prior to an intervention, to increase the potential for change.

Creatine supplementation had a positive effect on fitness performance during 1RM bench press and leg press tests, as well as during repeated Wingate anaerobic tests and fifty repetitions of a leg extension. Vegetarian participants responded very similarly to non-vegetarians for all fitness measures except strength as assessed by leg press. In this case, there was a significant interaction between diet and time, whereby vegetarians experienced a greater improvement when compared to non-vegetarians, when change scores were considered.

REFERENCES

- Allen, R.H., Stabler, S.P., Savage, D.G., & Lindenbaum, J. (1993). Metabolic abnormalities in cobalamin (vitamin B₁₂) and folate deficiency. *FASEB Journal*, 7, 1344-1353.
- Ayoama, R., Hiruma, E., & Sasaki, H. (2003). Effects of creatine loading on muscular strength and endurance of female softball players. *Journal of Sports Medicine and Physical Fitness*, 43, 481-487.
- Bailey, L.B. (1990). Folate status assessment. *The Journal of Nutrition*, 120, 1508.
- Balsom, P.D., Harridge, S.D.R., Soderlund, K., Sjodin, B., & Ekblom, B. (1995). Creatine supplementation per se does not enhance endurance exercise performance. *Acta Physiologica Scandinavica*, 149, 521-523.
- Balsom, P.D., Soderlund, K., & Ekblom, B. (1994). Creatine in humans with special reference to creatine supplementation. *Sports Medicine*, 18, 268-280.
- Bar-Or, O. (1987). The Wingate anaerobic test: an update on methodology, reliability and validity. *Sports Medicine*, 4, 381-394.
- Barr, S.I., & Rideout, C.A. (2004). Nutritional considerations for vegetarian athletes. *Nutrition*, 20, 696-703.
- Bemben, M.G., & Lamont, H.S. (2005). Creatine supplementation and exercise performance. *Sports Medicine*, 35, 107-125.
- Bessman, S.P., & Carpenter, C.L. (1985). The creatine-creatine phosphate energy shuttle. *Annual Review of Biochemistry*, 54, 831-862.
- Bissoli, L., Di Francesco, V., Ballarin, A., Mandragona, R., Trespidi, R., Brocco, G., et al. (2002). Effect of vegetarian diet on homocysteine levels. *Annals of Nutrition & Metabolism*, 46, 73-79.
- Bonaa, K.H., Njolstad, I., Ueland, P.M., Schirmer, H., Tverdal, A., Steigen, T., et al. (2006). Homocysteine lowering and cardiovascular events after acute myocardial infarction. *The New England Journal of Medicine*, 354, 1578-1588.
- Bostom, A.G., & Culleton, B.F. (1999). Hyperhomocysteinemia in chronic renal disease. *Journal of the American Society of Nephrology*, 10, 891-900.
- Boushey, C.J., Beresford, S.A., Omenn, G.S., & Motulsky, A.G. (1995). A quantitative assessment of plasma Hcy as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *The Journal of the American Medical Association*, 274, 1049-1057.

- Brenner, M., Rankin, J.W., Sebolt, D. (2000). The effect of creatine supplementation during resistance training in women. *Journal of Strength and Conditioning Research*, 14, 207-213.
- Bridson, A. (2003). Homocysteine in the context of cobalamin metabolism and deficiency states. *Amino Acids*, 24, 1-12.
- Buford, T.W., Kreider, R.B., Stout, J.R., Greenwood, M., Campbell, B., Spano, M., et al. (2007). International Society of Sports Nutrition position stand: creatine supplementation and exercise. *Journal of the International Society of Sports Nutrition*, 4, 6.
- Bulbulian, R., Jeong, J. W., & Murphy, M. (1996). Comparison of anaerobic components of the Wingate and Critical Power tests in males and females. *Medicine & Science in Sports & Exercise*, 28, 1336-1341.
- Burke, D.G., Chilibeck, P.D., Parise, G., Candow, D.G., Mahoney, D., & Tarnopolsky, M. (2003). Effect of Creatine and Weight Training on Muscle Creatine and Performance in Vegetarians. *Medicine & Science in Sports & Exercise*, 35, 1946-1955.
- Burke, D.G., Smith-Palmer, T., Holt, L.E., Head, B., Chilibeck, P.D. (2001). The effect of 7 days of creatine supplementation on 24-hour urinary creatine excretion. *Journal of Strength and Conditioning Research*, 15, 59-62.
- Cady, E.B., Jones, D.A., Lynn, J., & Newham, D.J. (1989). Changes in force and intracellular metabolites during fatigue of human skeletal muscle. *Journal of Physiology*, 418, 311-325.
- Canadian Society for Exercise Physiology. (1996). Professional Fitness & Lifestyle Consultant Resource Manual. Ottawa, ON: Author.
- Casey, A., & Greenhaff, P.L. (2000). Does dietary creatine supplementation play a role in skeletal muscle metabolism and performance? *American Journal of Clinical Nutrition*, 72, 607S-617S.
- Chambers, J.C., McGregor, A., Jean-Marie, J., & Kooner, J.S. (1997). Acute hyperhomocysteinaemia and endothelial dysfunction [Letter]. *Lancet*, 351, 36-37.
- Chrusch, M.J., Chilibeck, P.D., Chad, K.E., Davison, S. & Burke, D.G. (2001). Creatine supplementation combined with resistance training in older men. *Medicine & Science in Sports & Exercise*, 33, 2111-2117.
- Chrysohoou, C., Panagiotakos, D.B., Pitsavos, C., Zeimbekis, A., Zampelas, A., Papademetriou, L., et al. (2004). The associations between smoking, physical activity, dietary habits and plasma homocysteine levels in cardiovascular disease-free people: the 'ATTICA' study. *Vascular Medicine*, 9, 117-123.

Chwalbinska-Moneta, J. (2003). Effect of creatine supplementation on aerobic performance and anaerobic capacity in elite rowers in the course of resistance training. *International Journal of Sport Nutrition and Exercise Metabolism*, 13, 173-183.

Cox, G., Mujika, I., Tumilty, D., & Burke, L. (2002). Acute creatine supplementation and performance during a field test simulating match play in elite female soccer players. *International Journal of Sport Nutrition and Exercise Metabolism*, 12, 33-46.

Cuskelly, G.J., Stacpoole, P.W., Williamson, J., Baumgartner, T.G., & Gregory, J.F. 3rd (2001). Deficiencies of folate and vitamin B(6) exert distinct effects on homocysteine, serine, and methionine kinetics. *American Journal of Physiology, Endocrinology and Metabolism*, 281, E1182-1190.

CyberSoft, Inc. (2006). *NutriBase 6 User's Manual*. Phoenix, AZ.

De Cree, C., Malinow, M.R., van Kranenburg, G.P., Geurten, P.G., Longford, N.T., & Keizer, H.A. (1999). Influence of exercise and menstrual cycle phase on plasma homocyst(e)ine levels in young women – a prospective study. *Scandinavian Journal of Medicine & Science in Sports*, 9, 272-278.

Deitrick, C.L., Katholi, R.E., Huddleston, D.J., Hardiek, K., & Burrus, L. (2001). Clinical adaptation of a high-performance liquid chromatographic method for the assay of pyridoxal 5'-phosphate in human plasma. *Journal of Chromatography B*, 751, 383-387.

Durnin, J.V., & Womersley, J. (1974). Body fat assessed from total body density and its estimation from skinfold thickness: measurements of 481 men and women aged from 16 to 72 years. *British Journal of Nutrition*, 32, 77-97.

Ferguson, T.B., & Syrotuik, D.G. (2006). Effects of creatine monohydrate supplementation on body composition and strength indices in experienced resistance trained women. *Journal of Strength and Conditioning Research*, 20, 939-946.

Finn, J.P., Ebert, T.R., Withers, R.T., Carey, M.F., Mackay, M., Phillips, J.W., & Febbraio, M.A. (2001). Effect of creatine supplementation on metabolism and performance in humans during intermittent sprint cycling. *European Journal of applied Physiology*, 84, 238-243.

Garza, D., & Becan-McBride, K. (2002). *Phlebotomy Handbook: Blood Collection Essentials* (6th Edition). New Jersey: Prentice Hall.

Green, A.L., Simpson, E.J., Littlewood, J.J., MacDonald I.A., & Greenhaff, P.L. (1996). Carbohydrate ingestion augments creatine retention during creatine feeding in humans. *Acta Physiologica Scandinavica*, 158, 195-202.

- Greenhaff, P.L., Bodin, K., Soderlund, K., & Hultman, E. (1994). Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *American Journal of Physiology*, 266, E725-E730.
- Harris, R.C., Soderlund, K., & Hultman, E. (1992). Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clinical Science*, 83, 367-374.
- Herrmann, W., Schorr, H., Purschwitz, K., Rassoul, F., & Richter, V. (2001). Total homocysteine, vitamin B₁₂, and total antioxidant status in vegetarians. *Clinical Chemistry*, 47, 1094.
- Hoffman, J., Ratamess, N., Kang, J., Mangine, G., Faigenbaum, A., & Stout, J. (2006). Effect of creatine and β -alanine supplementation on performance and endocrine responses in strength/power athletes. *International Journal of Sport Nutrition and Exercise Metabolism*, 16, 430-446.
- Hoffman, J.R., Stout, J.R., Falvo, J., Kang, J., & Ratamess, N.A. (2005). Effect of low-dose, short-duration creatine supplementation on anaerobic exercise performance. *Journal of Strength and Conditioning Research*, 19, 260-264.
- Houston, M.E. (2001). *Biochemistry Primer for Exercise Science* (2nd Ed.). Champaign, IL: Human Kinetics.
- Howat, P.M., Mohan, R., Champagne, C., Monlezun, C., Wozniak, P., & Bray, G.A. (1994). Validity and reliability of reported dietary intake data. *Journal of the American Dietetic Association*, 94, 169-173.
- Hultman, E., Soderlund, K., Timmons, J.A., Cederblad, G., & Greenhaff P.L. (1996). Muscle creatine loading in men. *Journal of Applied Physiology*, 81, 232-237.
- Hung, C.J., Huang, P.C., Lu, S.C., Li, Y.H., Huang, H.B., Lin, B.F., et al. (2002). Plasma homocysteine levels in Taiwanese vegetarians are higher than those of omnivores. *Journal of Nutrition*, 132, 152-158.
- Institute of Medicine: Food and Nutrition Board (1998). *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B₆, folate, vitamin B₁₂, pantothenic acid, biotin, and choline*. Washington, DC: National Academy Press.
- Institute of Medicine of the National Academies (2006). *DRI: Dietary reference intakes: The essential guide to nutrient requirements* (J.J. Otten, J. Pizzi Hellwig & L.D. Meyers, Eds.). Washington DC: The National Academies Press.
- Jacques, P.F., Selhub, J., Bostom, A.G., Wilson, P.W.F., & Rosenberg, I.H. (1999). The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *The New England Journal of Medicine*, 340, 1449-1454.

- Korzun, W. J. (2004). Oral creatine supplements lower plasma homocysteine concentrations in humans. *Clinical Laboratory Science, 17*, 102-106.
- Kraemer, W.J., & Volek, J.S. (1999). Creatine supplementation: its role in human performance. *Clinics in Sports Medicine, 18*, 651-666.
- Krajcovicova-Kudlackova, M., Blazicek, P., Kopcova, J., Bederova, A., & Babinska, K. (2000). Homocysteine levels in vegetarians versus omnivores. *Annals of Nutrition & Metabolism, 44*, 135-138.
- Lonn, E., Yusuf, S., Arnold, M.J., Sheridan, P., Pogue, J., Micks, M., et al. (2006). Homocysteine lowering with folic acid and B vitamins in vascular disease. *The New England Journal of Medicine, 354*, 1567-1577.
- Lukaski, H. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition, 20*, 632-644.
- Lukaszuk, J.M., Robertson, R.J., Arch, J.E., Moore, G.E., Yaw, K.M., Kelley, D.E., et al. (2002). Effect of creatine supplementation and a lacto-ovo vegetarian diet on muscle creatine concentration. *International Journal of Sport Nutrition and Exercise Metabolism, 12*, 336-348.
- Lukaszuk, J.M., Robertson, R.J., Arch, J.E., & Moyna, N.M. (2005). Effect of a defined lacto-ovo-vegetarian diet and oral creatine monohydrate supplementation on plasma creatine concentration. *Journal of Strength and Conditioning Research, 19*, 735-740.
- MacCormick, V.M., Hill, L.M., MacNeil, L., Burke, D.G., & Smith-Palmer, T. (2004). Elevation of creatine in red blood cells in vegetarians and nonvegetarians after creatine supplementation. *Canadian Journal of Applied Physiology, 29*, 704-713.
- MacNeil, L., Hill, L., MacDonald, D., Keefe, L., Cormier, J.F., Burke, D. G., & Smith-Palmer, T. (2005). Analysis of creatine, creatinine, creatine-d₃ and creatinine-d₃ in urine, plasma, and red blood cells by HPLC and GC-MS to follow the fate of ingested creatine-d₃. *Journal of Chromatography B, 827*, 210-215.
- McCarty, M. F. (2001). Supplemental creatine may decrease serum homocysteine and abolish the homocysteine 'gender gap' by suppressing endogenous creatine synthesis. *Medical Hypotheses, 56*, 5-7.
- McLean, R.R., Jacques, P.F., Selhub, J., Tucker, K.L., Samelson E.J., Broe, K.E., et al. (2004). Homocysteine as a predictive factor for hip fracture in older persons. *New England Journal of Medicine, 350*, 2042-2049.
- Meyer, R.A., Sweeney, H.L., Kushmerick, M.J. (1984). A simple analysis of the "phosphocreatine shuttle". *American Journal of Physiology, 246*, C365-C377.

- Miller, J.W., Nadeau, M.R., Smith, D., & Selhub, J. (1994). Vitamin B-6 deficiency vs folate deficiency: comparison of responses to methionine in rats. *American Journal of Clinical Nutrition*, 59, 1033-1039.
- Miller, J.W., Ribaya-Mercado, J.D., Russell, R.M., Shepard, D.C., Morrow, F.D., Cochary, E.F., et al. (1992). Effect of vitamin B-6 deficiency on fasting plasma homocysteine concentrations. *American Journal of Clinical Nutrition*, 55, 1154-1160.
- Millet, P., Guillard, J.C., Fuchs, F., & Klepping, J. (1989). Nutrient intake and vitamin status of healthy French vegetarians and non-vegetarians. *American Journal of Clinical Nutrition*, 50, 718-727.
- Mudd, S.H., Levy, H.L., & Skovby, F. (1995). Disorders of transsulfuration. In C.R. Scriver, A.L. Beaudet, W.S. Sly, & D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease* (pp. 1279-1327). New York: McGraw-Hill, Inc. Health Professions Division.
- Mudd, S.H., Skovby, F., Levy, H.L., Pettigrew, K.D., Wilcken, B., Pyeritz, R.E., et al. (1985). The natural history of homocystinuria due to cystathionine beta-synthase deficiency. *American Journal of Human Genetics*, 37, 1-31.
- Mujika, I., Padiall, S., Ibanez, J., Izquierdo, M., & Gorostiaga, E. (2000). Creatine supplementation and sprint performance in soccer players. *Medicine and Science in Sports and Exercise*, 32, 518-525.
- Nieman, D.C. (1999). Physical fitness and vegetarian diets: is there a relation? *American Journal of Clinical Nutrition*, 70, 570S-575S.
- Nygaard, O., Nordrehaug, J.E., Refsum, H., Ueland, P.M., Farstad, M., & Vollset, S.E. (1997). Plasma homocysteine levels and mortality in patients with coronary artery disease. *The New England Journal of Medicine*, 337, 230-236.
- Nygaard, O., Refsum, H., Ueland, P.M., Vollset, S.E. (1998). Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. *The American Journal of Clinical Nutrition*, 67, 263-270.
- Nygaard, O., Vollset, S.E., Refsum, H., Stensvold, I., Tverdal, A., Nordrehaug, J.E., et al. (1995). Total plasma homocysteine and cardiovascular risk profile: the Hordaland Homocysteine Study. *Journal of the American Medical Association*, 274, 1526-1533.
- Obeid, R., Geisel, J., Schorr, H., Hubner, U., & Herrmann, W. (2002). The impact of vegetarianism on some haematological parameters. *European Journal of Haematology*, 69, 275-279.

- O'Callaghan, P., Meleady, R., Fitzgerald, T., & Graham, I. (2002). Smoking and plasma homocysteine. *European Heart Journal*, *23*, 1580-1586.
- Odland, L. M., MacDougall, J. D., Tarnopolsky, M. A., Elorriaga, A., & Borgmann, A. (1997). Effect of oral creatine supplementation on muscle [PCr] and short-term maximum power output. *Medicine and Science in Sports & Exercise*, *29*, 216-219.
- Peterson, J.C., & Spence, J.D. (1998). Vitamins and progression of atherosclerosis in hyperhomocyst(e)inaemia [Letter]. *Lancet*, *351*, 263.
- Powell, J.T. (1998). Vascular damage from smoking: disease mechanisms at the arterial wall. *Vascular Medicine*, *3*, 21-28.
- Preen, D., Dawson, B., Goodman, C., Lawrence, S., Beilby, J., & Ching, S. (2001). Effect of creatine loading on long-term sprint exercise performance and metabolism. *Medicine and Science in Sports and Exercise*, *33*, 814-821.
- Reardon, T.F., Ruell, P.A., Fiatarone Singh, M.A., Thompson, C.H., & Rooney, K.B. (2006). Creatine supplementation does not enhance submaximal aerobic training adaptations in healthy young men and women. *European Journal of Applied Physiology*, *98*, 234-241.
- Refsum, H., Ueland, P.M., Nygard, O., & Vollset, S.E. (1998). Homocysteine and cardiovascular disease. *Annual Review of Medicine*, *49*, 31-62.
- Rose, D.P. (1978). The interactions between vitamin B₆ and hormones. *Vitamins and Hormones*, *36*, 53-99.
- Selhub, J. (1999). Homocysteine metabolism. *Annual Review of Nutrition*, *19*, 217-246.
- Shomrat, A., Weinstein, Y., & Katz, A. (2000). Effect of creatine feeding on maximal exercise performance in vegetarians. *European Journal of Applied Physiology*, *82*, 321-325.
- Smith, S.A., Montain, S.J., Matott, R.P., Zientara, G.P., Jolesz, F.A., & Fielding, R.A. (1999). Effects of creatine supplementation on the energy cost of muscle contraction: a ³¹P-MRS study. *Journal of Applied Physiology*, *87*, 116-123.
- Smith-Palmer, T. (2002). Separation methods applicable to urinary creatine and creatinine. *Journal of Chromatography B*, *781*, 93-106.
- Stead, L.M., Au, K.P., Jacobs, R.L., Brosnan, M.E., & Brosnan, J.T. (2001). Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. *American Journal of Physiology: Endocrinology and Metabolism*, *281*, E1095-E1100.

- Steegers-Theunissen, R.P.M., Boers, G.H.J., Steegers, E.A.P., Trijbels, F.J.M., Thomas, C.M.G., & Eskes, T.K.A.B. (1992). Effects of SUB-50 oral contraceptives on homocysteine metabolism: a preliminary study. *Contraception*, *45*, 129-139.
- Steenge, G.R., Verhoef, P., & Greenhaff, P.L. (2001). The effect of creatine and resistance training on plasma homocysteine concentration in healthy volunteers. *Archives of Internal Medicine*, *161*, 1455-1456.
- Syrotuik, D.G., & Bell, G.J. (2004). Acute creatine monohydrate supplementation: A descriptive physiological profile of responders vs. nonresponders. *Journal of Strength and Conditioning Research*, *18*, 610-617.
- Taes, Y.E.C. (2005). Analytical and biochemical aspects associated with supraphysiological creatine intake. *Clinica Chimica Acta*, *351*, 217-219
- Taes, Y.E.C., Delanghe, J.R., De Bacquer, D., Langlois, M., Stevens, L., Geerolf, I., et al. (2004). Creatine supplementation does not decrease total plasma homocysteine in chronic hemodialysis patients. *Kidney International*, *66*, 2422-2428.
- Taes, Y.E.C., Delanghe, J.R., De Vriese, A.S., Rombaut, R., Van Camp, J., & Lameire, N.H. (2003). Creatine supplementation decreases homocysteine in an animal model of uremia. *Kidney International*, *64*, 1331-1337.
- Tallova, J., Tomandl, J., Bicikova, M., & Hill, M. (1999). Changes of plasma total homocysteine levels during the menstrual cycle. *European Journal of Clinical Investigation*, *29*, 1041-1044.
- Tarnopolsky, M.A., & MacLennan, D.P. (2000). Creatine monohydrate supplementation enhances high-intensity exercise performance in males and females. *International Journal of Sport Nutrition and Exercise Metabolism*, *10*, 452-463.
- Thoden, J.S. (1991). Testing aerobic power. In J.D. MacDougall, H.A. Wenger, & H.J. Green (Eds.), *Physiological Testing of the High-Performance Athlete* (pp. 107-173). Illinois: Human Kinetics Books.
- Thompson, C.H., Kemp, G.J., Sanderson, A.L., Dixon, R.M., Styles, P., Taylor, D.J., & Radda, G.K. (1996). Effect of creatine on aerobic and anaerobic metabolism in skeletal muscle in swimmers. *British Journal of Sports Medicine*, *30*, 222-225.
- Vanakoski, J., Kosunen, V., Meririnne, E., & Seppala, T. (1998). Creatine and caffeine in anaerobic and aerobic exercise: effects on physical performances and pharmacokinetic considerations. *International Journal of Pharmacology and Therapeutics*, *36*, 258-262.
- Vandenbergh, K., Goris, M., Van Hecke, P., Van Leemputte, M., Vangerven, L., & Hespel, P. (1997). Long-term creatine intake is beneficial to muscle performance during resistance training. *Journal of Applied Physiology*, *83*, 2055-2063.

- Virtanen, J.K., Voutilainen, S., Alfthan, G., Korhonen, M.J., Rissanen, T.H., Mursu, J., et al. (2005). Homocysteine as a risk factor for CVD mortality in men with other CVD risk factors: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. *Journal of Internal Medicine*, 257, 255-262.
- Volek, J.S., Kraemer, W.J., Bush, J.A., Boetes, M., Incledon, T., Clark, K.L., & Lynch, J.M. (1997). Creatine supplementation enhances muscular performance during high-intensity resistance exercise. *Journal of the American Dietetic Association*, 97, 765-770.
- Volek, J.S., & Rawson, E.S. (2004). Scientific basis and practical aspects of creatine supplementation for athletes. *Nutrition*, 20, 609-614.
- Wang, Z., Gallagher, D., Nelson, M.N., Matthews, D.E., & Heymsfield, S.B. (1996). Total-body skeletal muscle mass: evaluation of 24-h urinary creatinine excretion by computerized axial tomography. *American Journal of Clinical Nutrition*, 63, 863-869.
- Wilcken, D.E., & Wilcken, B. (1997). The natural history of vascular disease in homocystinuria and the effects of treatment. *Journal of Inherited Metabolic Disorders*, 20, 295-300.
- Williams, M.H., & Branch, J.D. (1998). Creatine supplementation and exercise performance: An update. *Journal of the American College of Nutrition*, 17, 216-234.
- Wyss, M., & Schulze, A. (2002). Health implications of creatine: Can oral creatine supplementation protect against neurological and atherosclerotic disease? *Neuroscience*, 112, 243-260.

APPENDIX I: Certificate of Research Ethics Board approval



University of Saskatchewan

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR Carol Rodgers

DEPARTMENT Kinesiology

Bio# 06:108

INSTITUTION (S) WHERE RESEARCH WILL BE CARRIED OUT College of Kinesiology

105 Gymnasium Place

Saskatoon SK S7N 5C2

SUB-INVESTIGATOR(S)

Philip D. Chilibeck

Gordon A. Zello

STUDENT RESEARCHER(S) Vanessa MacCormick SPONSORING AGENCIES

SPORT MEDICINE AND SCIENCE COUNCIL OF SASKATCHEWAN

TITLE:

Changes in Plasma Homocysteine in Response to Oral Creatine Supplementation in Vegetarians

ORIGINAL APPROVAL DATE

CURRENT EXPIRY DATE

APPROVAL OF

13-Jul-2006

OI-Jul-2007

Revised Researcher's Summary Form (03-Jul-2006)

Revised Research Participant Information and Consent Form (03-Jul-2006)

Recruitment Advertisement/Poster

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board has reviewed the above-named research project at a full-board meeting (any research classified as minimal risk is reviewed through the expedited review process). The proposal was found to be acceptable on ethical grounds. The principal investigator has the responsibility for any other

administrative or regulatory approvals that may pertain to this research project, and for ensuring that the authorized research is carried out according to governing law. This Approval is valid for the above time period provided there is no change in experimental protocol or in the consent process.

ONGOING REVIEW REQUIREMENTS/REB ATTESTATION

In order to receive annual renewal, a status report must be submitted to the Chair for Committee consideration within one month of the current expiry date each year the study remains open, and upon study completion. Please refer to the following website for further instructions: "<http://www.usask.ca/research/ethics.shtml>". In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This approval and the views of this REB have been documented in writing.

APPROVED.

Michel Desautels, Ph.D., Chair

University of Saskatchewan

Biomedical Research Ethics Board

APPENDIX II: Consent form

Research Participant Information and Consent Form

Title: Changes in serum homocysteine and fitness in response to oral creatine supplementation in vegetarians and non-vegetarians concurrently supplementing with vitamin B₁₂.

Investigators: Carol Rodgers, Ph.D., Dean of the College of Kinesiology, University of Saskatchewan. Phone: 306-966-1061; Email: carol.rodgers@usask.ca.

Vanessa MacCormick, B.Sc., M.Sc. candidate, Student researcher (supervised by Dr. Rodgers) College of Kinesiology, University of Saskatchewan. Phone: 306-966-1123, Email: vanessa.maccormick@usask.ca.

Gordon Zello, Ph.D., College of Pharmacy and Nutrition, University of Saskatchewan. Phone: 306-966-5825, Email: gordon.zello@usask.ca.

Philip D. Chilibeck, Ph.D., College of Kinesiology, University of Saskatchewan. Phone: 966-1072, Email: phil.chilibeck@usask.ca.

Sponsorship: This research is being sponsored by the Saskatchewan Academy of Sports Medicine (SASM).

You are being invited to participate in a research study because we want to determine the effects of creatine supplementation on blood homocysteine levels, and exercise performance in vegetarians. Before you decide whether to participate, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study and the possible benefits, risks and discomforts.

Homocysteine is a compound which is a risk factor for cardiovascular disease and is elevated in vegetarians. Creatine is a compound that is naturally produced in the body, but is also found in meat products. Supplementation with creatine has been shown to lower homocysteine levels in some studies. Because vegetarians do not consume meat, their creatine levels are lower than non-vegetarians and supplementation with creatine may have a greater effect. By providing additional creatine through supplementation to the body, the necessity for the body's own metabolic pathway to produce creatine is lessened.

If you wish to participate, you will be asked to sign this form. Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. If you do decide to take part in this study, you are free to withdraw at any time without giving any reasons for your decision and your refusal to participate will not affect your relationship with any of the researchers. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide.

Purpose: The purpose of the proposed research is to determine whether creatine monohydrate has an effect on serum homocysteine levels and fitness measures in females who adhere to a vegetarian diet vs. a non-vegetarian diet, and who are supplementing with vitamin B₁₂.

Possible benefits of this study: It is possible that supplementation with vitamin B₁₂ or creatine monohydrate will reduce your serum homocysteine concentrations. By participating in this study you will also have the opportunity to gain information about your body composition and fitness levels. Also, it is possible that with creatine supplementation your strength and anaerobic fitness will improve. These benefits, however, are not guaranteed.

Procedures: If you agree to participate in the study, the following things will happen:

Initially, you will be asked to complete a physical activity readiness questionnaire (PAR-Q) that will let the researchers know whether participation in fitness testing is an acceptable thing for you, based on your current health. Once this questionnaire is completed and approved, then we can carry on with the remainder of the study.

Each group, the vegetarian group and non-vegetarian group, will supplement with creatine monohydrate for a five day loading period, beginning on day two of their menstrual cycle. Day two is defined as the second day of menses, or your period. You will be “loading” with 0.25 grams of supplement per kilogram of lean body mass each day. This loading period will allow your body to absorb the maximal amount of creatine in a short time. These daily supplements will be divided into four equal servings and will be mixed with a carbohydrate containing juice mix that you will mix with water, and drink. This should be done every four hours until your four servings are finished for that day. You will receive the supplements in addition to your regular diet.

You will begin taking vitamin B₁₂ four weeks prior to the initiation of the supplementation period, in order to ensure that Hcy metabolism is not limited by vitamin B₁₂ intake. If this metabolism were limited, there could be an increase of Hcy in the blood. Vitamin B₁₂ supplementation will consist of 24µg tablets to be taken once a day with a meal for the duration of the study.

Before the supplementation begins you will be asked to complete a three day food diary. By completing the food diary, the researchers can determine your average intake of nutrients. You will be asked to continue with your normal diet and physical activity or exercise levels for the whole study.

Body composition (i.e. lean tissue, fat mass) will be assessed at the beginning and end of the study by air displacement plethysmography using a BodPod. A BodPod calculates the amount of lean tissue and fat mass from the amount of air displaced by your body in a sealed chamber, or the Pod. You will be required to change into a swimsuit and will be provided with a swim cap for this test. The BodPod involves sitting quietly in a measuring chamber for two or three 50 second measurements. The BodPod measures the amount of air that a person displaces while sitting in a chamber, and determines whole body density from the person's mass and volume. Body fat and lean mass can then be calculated. From this we can determine your fat and lean mass. The results of this will be given to you at the end of the study.

Before we begin the supplementation, we need to do some fitness testing and take some blood samples for analysis:

One of the fitness tests that we will do is a VO_2max test, which tests your aerobic fitness, or your body's capability to deliver oxygen to your muscles while you're exercising on a treadmill. Following test instructions and a warm up, you will be asked to run at a constant speed until you cannot maintain either the speed or your running form. The incline of the treadmill will increase each two minutes of the test. During the test you will breathe into a mouthpiece so that the gas composition of your breath can be determined. This measure will be done prior to the supplementation period. Though the test itself will take only approximately 10 to 15 minutes, please allow an hour to become familiar with the procedure and to prepare for the test.

The second fitness test that we will do is a strength test, where we will need to determine the greatest amount of weight you can lift on a bench press and a leg press. After the process has been explained fully, and you have warmed up, a weight will be chosen that is approximately 20% less than the predicted maximal amount that you can lift. Two minutes of rest will be given between trials, until your maximal weight is determined. For the bench press, you will lie on an exercise bench on your back and grip the bar with your thumbs at shoulder width apart, your buttocks in contact with the bench, and the bar starting position even with the top of the chest. You will be asked to push the bar to full extension of your arms. For the leg press test, from a sitting position on a leg press machine with your knees bent to a 90 degree position and a flat foot on the pedals, the pedals are to be pushed out to full knee extension.

The third fitness test we will do is called a repeated Wingate test. This test measures your anaerobic fitness, or the ability of your body to work at a very high intensity on a stationary bicycle for 30 seconds. Following a warm up and explanation of the test, you will be asked to pedal as hard as possible immediately after the "GO" command is given, and to give an all out effort for the 30 seconds of exercise, while remaining seated on the stationary bicycle. At the beginning of the 30 second test, a resistance chosen based on your weight will be applied, while pedaling at 80 revolutions per minute (rpm). Following the test you will be allowed 2 minutes to rest while seated on the bicycle, and then the test will be repeated (for a total of three tests).

From your performance we can determine changes in mean and peak power, which gives information about your anaerobic capacity.

Another exercise test that will be performed requires you to do 50 knee extensions and flexions with maximal effort on a device that measures your force output. The purpose of this test is to measure total work performed by the knee extensors and flexors. An isokinetic dynamometer will be set at a constant speed, and you will be asked to move your lower leg from a bent to straight position, or vice versa, while in a seated position. After a warm up and familiarization set of 10 motions, you will be asked to complete 50 repetitions of knee extensions or flexions.

In order to determine how much of the supplement you are retaining, we will need to analyze 24 hour urine samples. These samples must be collected before and after the supplementation period, so that we can compare the different concentrations of creatine in your urine. You will be supplied with a urine collection container, and will be asked to collect your urine on the particular day beginning with the second urination of the day. Once the whole 24 hour sample has been collected, you can drop it off with the researchers.

Finally, a trained individual will collect 3 tubes of blood from your arm for analysis, and take a finger prick blood sample. Blood collections, the strength and total work tests and Wingate tests will be done twice, prior to and following the creatine loading period. The strength and Wingate tests will take approximately 30 minutes to conduct, and will be done on the same day as the blood collections. The test of work performed will be conducted at least 48 hours after the other fitness measures in order to give your muscles time to heal.

In summary, you will be required to visit the University of Saskatchewan a total of five times. Initially we will meet in order to go over the study protocols and answer any questions that you have, run the aerobic VO_2 max test, and to give you your vitamin B₁₂ supplements. The second meeting will be to conduct the body composition, and strength and Wingate tests, and will take approximately one hour. The third meeting will consist of the isokinetic dynamometer test of work performed, as well as the blood collections and the distribution of supplement. This meeting will take between 45 minutes and one hour. Blood collections will occur again during the fourth meeting, as will the body composition, strength and Wingate tests, and the final meeting will consist of the post measure of the isokinetic dynamometer test of work performed.

Risks and discomforts: Creatine supplementation has been shown to be associated with minimal side effects, especially with the low dose given in this study. There have been anecdotal reports of increased muscle cramping or muscle pulls during creatine supplementation, but when this is compared to subjects receiving placebo, there is no differences in rates of occurrence of muscle cramping or pulls. Creatine supplementation has been shown, on two occasions, to worsen kidney function in individuals who already had kidney disease. If you have any problems with kidney function you should not participate in this study.

With blood sampling there is always a risk of bruising or local infection, though with proper techniques and an experienced person performing the blood collections, these risks should be minimal.

The exercise tests may cause you some discomfort because they will be performed to exhaustion. Injuries that may result include muscle pulls, strains or cramps, but this will be minimized by having you perform a proper warm up and stretching before the exercise tests. There is a possibility that you will feel muscle or joint soreness in the days following your exercise testing.

There may be unknown or unforeseen risks during the study or after the study is completed.

Costs and Reimbursements: There will be no reimbursements for time commitments or pocket expenses such as transportation.

Research-Related Injury: There will be no cost to you for participation in this study. You will not be charged for any research procedures. In the event you become ill or injured as a result of participating in this study, necessary medical treatment will be made available at no additional cost to you. By signing this document you do not waive any of your legal rights.

Alternatives to this study: You do not have to participate in this study to have your fitness or body composition assessed. You can schedule a fitness assessment in the College of Kinesiology, University of Saskatchewan.

Confidentiality: While absolute confidentiality cannot be guaranteed, every effort to make certain that your participation in the study, and information gathered during the study period will be kept confidential will be made. Your identity will be protected, and all data and information regarding the study will be kept together, locked in a cabinet in the College of Kinesiology for a period of twenty-five years, in the care of the principal investigator, Dr. Carol Rodgers. All reporting of data in presentation or publication format will be done in aggregate form and will not refer directly to individual data.

Voluntary Withdrawal: Your participation in this research is entirely voluntary. You may withdraw from this study at any time. If you decide to enter the study and to withdraw at any time in the future, there will be no penalty or loss of benefits to which you are otherwise entitled.

If you are a student at the University of Saskatchewan, refusal to participate or withdrawal from the study will not affect your academic standing or relationship with University instructors.

If you choose to enter the study and then decide to withdraw at a later time, all data collected about you during your enrolment in the study will be retained for analysis.

If you have questions concerning the study you can contact Dr. Carol Rodgers (principal investigator) at 306-966-1061 or Vanessa MacCormick (student researcher) at 306-966-1123.

If you have questions about your rights as a research subject, you can contact the Chair of the Biomedical Research Ethics Board, c/o Ethics Office, University of Saskatchewan, at (306) 966-4053.

We will advise you of any new information that will have a bearing on your decision to continue in the study.

By signing below, I acknowledge that I have read or have had this read to me and that I understand the research subject information, the study procedures, and the consent form. I understand that my participation in this study is voluntary, and that we can withdraw from the study at any time without penalty. I have received a copy of the consent form for my own purposes.

Participant's signature: _____ Date: _____.

Researcher's signature: _____ Date: _____.

APPENDIX III: Appointment listing given to participants

APPOINTMENT LISTINGS

..... APPOINTMENT 1 (Day 2 of menstrual cycle)

(Consent form and PAR-Q should have been completed already)

B₁₂ supplementation begins

VO₂max test

Familiarization with testing procedures (Wingate test, strength tests, BioDex)

PRELIMINARY INSTRUCTIONS FOR FITNESS TESTING:

Please bring: Running shoes

Clothes to exercise in

Please avoid: Eating or drinking for two hours prior to the test

Caffeinated beverages for two hours prior to the test

Alcoholic beverages for six hours prior to the test

Smoking for two hours prior to the test

Strenuous physical activity for six hours prior to the test

..... APPOINTMENT 2

BodPod

Repeated Wingates

Given food diary

Given urine collection container

Please bring: Running shoes

Clothes to exercise in

Swimsuit (to wear for body composition test)

Please **DO NOT** eat or drink anything before this appointment.

Please follow the preliminary instructions for fitness testing as detailed above.

..... **Complete food diary (2 weekdays, 1 weekend day)**

..... **Collect 24-hour urine sample**

..... APPOINTMENT 3 (Day 2 of menstrual cycle)

Blood tests

Strength testing

BioDex (isokinetic dynamometer)

Given new urine collection container

Please bring: Running shoes

Clothes to exercise in

Completed food diary

Urine sample

Please **DO NOT** eat or drink anything before this appointment. The blood samples must be taken when you are in an overnight fasted state.
Please follow the preliminary instructions for fitness testing as detailed above.

..... **APPOINTMENT 4 (Day 7 of menstrual cycle)**

Blood tests
Repeated Wingate tests
BodPod

Please bring: Running shoes
Clothes to exercise in
Swimsuit

Please **DO NOT** eat or drink anything before this appointment. The blood samples must be taken when you are in an overnight fasted state.
Please follow the preliminary instructions for fitness testing as detailed above.

..... **Collect 24-hour urine sample**

..... **APPOINTMENT 5**

BioDex (isokinetic dynamometer)
Strength testing

Please bring: Running shoes
Clothes to exercise in
24 hour urine sample

Please **DO NOT** eat or drink anything before this appointment.
Please follow the preliminary instructions for fitness testing as detailed above.

APPENDIX IV: Instructions for completion of food diary

3 Day Food Diary

INTRODUCTION

This booklet is used to record your detailed daily food intake. It is meant to give researchers some idea of your *usual* dietary intake. Therefore, it is very important that you do not alter your eating habits while taking part in this study. In other words, do not let the fact that you are writing down what you eat influence your choice of foods. The names of the participants in this study will be kept confidential.

The usefulness of the results of this study depends on the accuracy with which you record your daily food intake. Please write down full details on all the food and drink that you consume each day.

INSTRUCTIONS

1. The purpose of this diary is to record all the food (including drinks) which you eat for a three day period.
2. Two pages are provided for each day of the three day period.
3. After each meal or snack that you eat, please write down in detail each separate food item you consumed – including the type of food (e.g. processed cheese) and the amount of food in household measures (e.g. 1 cup of cooked spaghetti). A meal will have to be listed by its separate parts (e.g. fried steak – 8 oz., french fries – 1 cup, coleslaw – 3 tbsp.).
4. The best way to record the information is by carrying this diary around with you wherever you go. Before going to sleep, you should look over the diary and check that you have not missed anything. Remember to include snacks!
5. If you eat fast food, you can just list the type of food you ate (e.g. 1 Big Mac, 1 large fries, 1 chocolate milkshake).
6. The following pages explain the use of household measures, and the description of foods. A sample day's diet sheet is given. Please take the time to read these pages as it will help you to make your diet record more accurate.

RECORDING IN THE DIARY

1. Please use household measures. For example:

Cup: vegetables, cereal, fruit, milk, beverages
Tablespoon: sauces, fats
Teaspoon: sugar, honey, drink mix
Slices: bread, bacon
Fractions: 1/6 pie

2. State the type of food eaten. For example:

Milk: homo, 2%, 1%, skim, goat's
Cheese: processed, swiss, spread
Bread: enriched white, 60% whole wheat, sweet cinnamon bun, bran muffin
Cereal: Sugar Pops, Miniwheats, granola, oatmeal
Meat: hamburger, fried chicken, scrambled eggs, cod fillets
Others: strawberry jam, Becel margarine, caesar dressing, oatmeal cookies

3. State the amount of food eaten. For example:

Cheese: 1" cube cheddar
3 tbsp lite cream cheese
¼ cup 2% creamed cottage cheese
Fruit: ½ cup canned peaches (in heavy syrup)
12 grapes
1 medium banana
Bread: 2 slices 100% whole wheat
1 large kaiser
Cereal: ¾ cup corn flakes
1 shredded wheat biscuit
Meat: 1 cup baked beans with pork
2 cups tuna casserole (tuna, cream of mushroom soup, noodles, peas)
4 thin slices roast beef
Vegetables: 2 slices cucumber
½ cup boiled cabbage

4. Include manner of cooking: fried, boiled, raw

5. Remember all alcoholic drinks

Here is a sample:

Date: Monday March 1, 1999

Time	Food Description	Amount	Code
9:30 a.m.	Waffles – white flour	3, 8” x 4”	
	Syrup – Aunt Jemima	½ cup	
	Yogurt - peach	125 mL	
	Coffee, tsp sugar	1 cup	
	Milk 2%	¼ cup	
10:30 a.m.	Chocolate chip cookies	3	
	Coffee, 1tsp sugar	1 cup	
	Milk (half and half – 10%)	¼ cup	
12:30 p.m.	Sandwich		
	- 2 slices whole wheat bread	2 slices	
	- mozzarella cheese (3”x1/4”x2”)	2 slices	
	- salami	4 slices	
	- lettuce	1 leaf	
	- butter	1 tsp	
	- mayonnaise	1 tsp	
5:30 p.m.	Spaghetti	1 cup	
	Meat sauce	½ cup	
	Garlic toast	2 slices	
	Milk 1%	2 cups	
8:30 p.m.	Sandwich		
	- 2 slices 60% whole wheat bread	2 slices	
	- peanut butter	1 tbsp	
	- honey	1 tbsp	
	Milk 2%	2 cups	
	* continue on next page if you need		
	* leave CODE column blank		

APPENDIX V: Physical Activity Readiness Questionnaire (PAR-Q)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO	
<input type="checkbox"/>	<input type="checkbox"/>	1. Has your doctor ever said that you have a heart condition <u>and</u> that you should only do physical activity recommended by a doctor?
<input type="checkbox"/>	<input type="checkbox"/>	2. Do you feel pain in your chest when you do physical activity?
<input type="checkbox"/>	<input type="checkbox"/>	3. In the past month, have you had chest pain when you were not doing physical activity?
<input type="checkbox"/>	<input type="checkbox"/>	4. Do you lose your balance because of dizziness or do you ever lose consciousness?
<input type="checkbox"/>	<input type="checkbox"/>	5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?
<input type="checkbox"/>	<input type="checkbox"/>	6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
<input type="checkbox"/>	<input type="checkbox"/>	7. Do you know of <u>any other reason</u> why you should not do physical activity?

**If
you
answered**

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active — begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal — this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever — wait until you feel better; or
- if you are or may be pregnant — talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____

DATE _____

SIGNATURE OF PARENT
or GUARDIAN (for participants under the age of majority) _____

WITNESS _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.



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APPENDIX VI: Physical Activity Readiness Medical Examination (PARmed-X)

PARmed-X PHYSICAL ACTIVITY READINESS MEDICAL EXAMINATION

The PARmed-X is a physical activity-specific checklist to be used by a physician with patients who have had positive responses to the Physical Activity Readiness Questionnaire (PAR-Q). In addition, the Conveyance/Referral Form in the PARmed-X can be used to convey clearance for physical activity participation, or to make a referral to a medically-supervised exercise program.

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. The PAR-Q by itself provides adequate screening for the majority of people. However, some individuals may require a medical evaluation and specific advice (exercise prescription) due to one or more positive responses to the PAR-Q.

Following the participant's evaluation by a physician, a physical activity plan should be devised in consultation with a physical activity professional (CSEP-Professional Fitness & Lifestyle Consultant or CSEP-Exercise Therapist™). To assist in this, the following instructions are provided:

PAGE 1: • Sections A, B, C, and D should be completed by the participant BEFORE the examination by the physician. The bottom section is to be completed by the examining physician.

PAGES 2 & 3: • A checklist of medical conditions requiring special consideration and management.

PAGE 4: • Physical Activity & Lifestyle Advice for people who do not require specific instructions or prescribed exercise.
• Physical Activity Readiness Conveyance/Referral Form - an optional tear-off tab for the physician to convey clearance for physical activity participation, or to make a referral to a medically-supervised exercise program.

This section to be completed by the participant

<p>A PERSONAL INFORMATION:</p> <p>NAME _____</p> <p>ADDRESS _____</p> <p>TELEPHONE _____</p> <p>BIRTHDATE _____ GENDER _____</p> <p>MEDICAL No. _____</p>	<p>B PAR-Q: Please indicate the PAR-Q questions to which you answered YES</p> <p><input type="checkbox"/> Q 1 Heart condition</p> <p><input type="checkbox"/> Q 2 Chest pain during activity</p> <p><input type="checkbox"/> Q 3 Chest pain at rest</p> <p><input type="checkbox"/> Q 4 Loss of balance, dizziness</p> <p><input type="checkbox"/> Q 5 Bone or joint problem</p> <p><input type="checkbox"/> Q 6 Blood pressure or heart drugs</p> <p><input type="checkbox"/> Q 7 Other reason: _____</p>
--	---

<p>C RISK FACTORS FOR CARDIOVASCULAR DISEASE: <i>Check all that apply</i></p> <p><input type="checkbox"/> Less than 30 minutes of moderate physical activity most days of the week.</p> <p><input type="checkbox"/> Currently smoker (tobacco smoking 1 or more times per week).</p> <p><input type="checkbox"/> High blood pressure reported by physician after repeated measurements.</p> <p><input type="checkbox"/> High cholesterol level reported by physician.</p> <p><input type="checkbox"/> Excessive accumulation of fat around waist.</p> <p><input type="checkbox"/> Family history of heart disease.</p> <div style="border: 1px solid red; padding: 2px; margin-top: 5px;"> <p><i>Please note: Many of these risk factors are modifiable. Please refer to page 4 and discuss with your physician.</i></p> </div>	<p>D PHYSICAL ACTIVITY INTENTIONS:</p> <p>What physical activity do you intend to do?</p> <p>_____</p> <p>_____</p> <p>_____</p>
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This section to be completed by the examining physician

<p>Physical Exam:</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 20%;">Ht</td> <td style="width: 20%;">Wt</td> <td style="width: 20%;">BP i) /</td> <td style="width: 20%;">/</td> </tr> <tr> <td></td> <td></td> <td>BP ii) /</td> <td>/</td> </tr> </table> <p>Conditions limiting physical activity:</p> <p><input type="checkbox"/> Cardiovascular <input type="checkbox"/> Respiratory <input type="checkbox"/> Other</p> <p><input type="checkbox"/> Musculoskeletal <input type="checkbox"/> Abdominal</p> <p>Tests required:</p> <p><input type="checkbox"/> ECG <input type="checkbox"/> Exercise Test <input type="checkbox"/> X-Ray</p> <p><input type="checkbox"/> Blood <input type="checkbox"/> Urinalysis <input type="checkbox"/> Other</p>	Ht	Wt	BP i) /	/			BP ii) /	/	<p>Physical Activity Readiness Conveyance/Referral:</p> <p>Based upon a current review of health status, I recommend:</p> <p><input type="checkbox"/> No physical activity</p> <p><input type="checkbox"/> Only a medically-supervised exercise program until further medical clearance</p> <p><input type="checkbox"/> Progressive physical activity:</p> <p style="margin-left: 20px;"><input type="checkbox"/> with avoidance of: _____</p> <p style="margin-left: 20px;"><input type="checkbox"/> with inclusion of: _____</p> <p style="margin-left: 20px;"><input type="checkbox"/> under the supervision of a CSEP-Professional Fitness & Lifestyle Consultant or CSEP-Exercise Therapist™</p> <p><input type="checkbox"/> Unrestricted physical activity—start slowly and build up gradually</p> <div style="border: 1px solid black; padding: 2px; margin-top: 5px;"> <p>Further Information:</p> <p><input type="checkbox"/> Attached</p> <p><input type="checkbox"/> To be forwarded</p> <p><input type="checkbox"/> Available on request</p> </div>
Ht	Wt	BP i) /	/						
		BP ii) /	/						

PARmed-X PHYSICAL ACTIVITY READINESS MEDICAL EXAMINATION

Following is a checklist of medical conditions for which a degree of precaution and/or special advice should be considered for those who answered "YES" to one or more questions on the PAR-Q, and people over the age of 69. Conditions are grouped by system. Three categories of precautions are provided. Comments under Advice are general, since details and alternatives require clinical judgement in each individual instance.

	Absolute Contraindications	Relative Contraindications	Special Prescriptive Conditions	
	Permanent restriction or temporary restriction until condition is treated, stable, and/or past acute phase.	Highly variable. Value of exercise testing and/or program may exceed risk. Activity may be restricted. Desirable to maximize control of condition. Direct or indirect medical supervision of exercise program may be desirable.	Individualized prescriptive advice generally appropriate: • limitations imposed; and/or • special exercises prescribed. May require medical monitoring and/or initial supervision in exercise program.	ADVICE
Cardiovascular	<input type="checkbox"/> aortic aneurysm (dissecting) <input type="checkbox"/> aortic stenosis (severe) <input type="checkbox"/> congestive heart failure <input type="checkbox"/> crescendo angina <input type="checkbox"/> myocardial infarction (acute) <input type="checkbox"/> myocarditis (active or recent) <input type="checkbox"/> pulmonary or systemic embolism—acute <input type="checkbox"/> thrombophlebitis <input type="checkbox"/> ventricular tachycardia and other dangerous dysrhythmias (e.g., multi-focal ventricular activity)	<input type="checkbox"/> aortic stenosis (moderate) <input type="checkbox"/> subaortic stenosis (severe) <input type="checkbox"/> marked cardiac enlargement <input type="checkbox"/> supraventricular dysrhythmias (uncontrolled or high rate) <input type="checkbox"/> ventricular ectopic activity (repetitive or frequent) <input type="checkbox"/> ventricular aneurysm <input type="checkbox"/> hypertension—untreated or uncontrolled severe (systemic or pulmonary) <input type="checkbox"/> hypertrophic cardiomyopathy <input type="checkbox"/> compensated congestive heart failure	<input type="checkbox"/> aortic (or pulmonary) stenosis—mild angina pectoris and other manifestations of coronary insufficiency (e.g., post-acute infarct) <input type="checkbox"/> cyanotic heart disease <input type="checkbox"/> shunts (intermittent or fixed) <input type="checkbox"/> conduction disturbances <ul style="list-style-type: none"> • complete AV block • left BBB • Wolff-Parkinson-White syndrome <input type="checkbox"/> dysrhythmias—controlled <input type="checkbox"/> fixed rate pacemakers	<ul style="list-style-type: none"> • clinical exercise test may be warranted in selected cases, for specific determination of functional capacity and limitations and precautions (if any). • slow progression of exercise to levels based on test performance and individual tolerance. • consider individual need for initial conditioning program under medical supervision (indirect or direct).
			<input type="checkbox"/> intermittent claudication	progressive exercise to tolerance
			<input type="checkbox"/> hypertension: systolic 160-180; diastolic 105+	progressive exercise; care with medications (serum electrolytes; post-exercise syncope; etc.)
Infections	<input type="checkbox"/> acute infectious disease (regardless of etiology)	<input type="checkbox"/> subacute/chronic/recurrent infectious diseases (e.g., malaria, others)	<input type="checkbox"/> chronic infections <input type="checkbox"/> HIV	variable as to condition
Metabolic		<input type="checkbox"/> uncontrolled metabolic disorders (diabetes mellitus, thyrotoxicosis, myxedema)	<input type="checkbox"/> renal, hepatic & other metabolic insufficiency <input type="checkbox"/> obesity <input type="checkbox"/> single kidney	variable as to status dietary moderation, and initial light exercises with slow progression (walking, swimming, cycling)
Pregnancy		<input type="checkbox"/> complicated pregnancy (e.g., toxemia, hemorrhage, incompetent cervix, etc.)	<input type="checkbox"/> advanced pregnancy (late 3rd trimester)	refer to the "PARmed-X for PREGNANCY"

References:

Arraix, G.A., Wigle, D.T., Mao, Y. (1992). Risk Assessment of Physical Activity and Physical Fitness in the Canada Health Survey Follow-Up Study. *J. Clin. Epidemiol.* 45:4 419-428.

Mottola, M., Wolfe, L.A. (1994). Active Living and Pregnancy. In: A. Quinney, L. Gauvin, T. Wall (eds.), **Toward Active Living: Proceedings of the International Conference on Physical Activity, Fitness and Health**. Champaign, IL: Human Kinetics.

PAR-Q Validation Report, British Columbia Ministry of Health, 1978.

Thomas, S., Reading, J., Shephard, R.J. (1992). Revision of the Physical Activity Readiness Questionnaire (PAR-Q). *Can. J. Sport Sci.* 17: 4 338-345.

The PAR-Q and PARmed-X were developed by the British Columbia Ministry of Health. They have been revised by an Expert Advisory Committee of the Canadian Society for Exercise Physiology chaired by Dr. N. Gledhill (2002).

No changes permitted. You are encouraged to photocopy the PARmed-X, but only if you use the entire form.

Disponible en français sous le titre
«Évaluation médicale de l'aptitude à l'activité physique (X-AAP)»

Continued on page 3...

	Special Prescriptive Conditions	ADVICE
Lung	<input type="checkbox"/> chronic pulmonary disorders	special relaxation and breathing exercises
	<input type="checkbox"/> obstructive lung disease <input type="checkbox"/> asthma	breath control during endurance exercises to tolerance; avoid polluted air
	<input type="checkbox"/> exercise-induced bronchospasm	avoid hyperventilation during exercise; avoid extremely cold conditions; warm up adequately; utilize appropriate medication.
Musculoskeletal	<input type="checkbox"/> low back conditions (pathological, functional)	avoid or minimize exercise that precipitates or exacerbates e.g., forced extreme flexion, extension, and violent twisting; correct posture, proper back exercises
	<input type="checkbox"/> arthritis—acute (infective, rheumatoid; gout)	treatment, plus judicious blend of rest, splinting and gentle movement
	<input type="checkbox"/> arthritis—subacute	progressive increase of active exercise therapy
	<input type="checkbox"/> arthritis—chronic (osteoarthritis and above conditions)	maintenance of mobility and strength; non-weightbearing exercises to minimize joint trauma (e.g., cycling, aquatic activity, etc.)
	<input type="checkbox"/> orthopaedic	highly variable and individualized
	<input type="checkbox"/> hernia	minimize straining and isometrics; strengthen abdominal muscles
	<input type="checkbox"/> osteoporosis or low bone density	avoid exercise with high risk for fracture such as push-ups, curl-ups, vertical jump and trunk forward flexion; engage in low-impact weight-bearing activities and resistance training
CNS	<input type="checkbox"/> convulsive disorder not completely controlled by medication	minimize or avoid exercise in hazardous environments and/or exercising alone (e.g., swimming, mountaineering, etc.)
	<input type="checkbox"/> recent concussion	thorough examination if history of two concussions; review for discontinuation of contact sport if three concussions, depending on duration of unconsciousness, retrograde amnesia, persistent headaches, and other objective evidence of cerebral damage
Blood	<input type="checkbox"/> anemia—severe (< 10 Gm/dl)	control preferred; exercise as tolerated
	<input type="checkbox"/> electrolyte disturbances	
Medications	<input type="checkbox"/> antianginal <input type="checkbox"/> antiarrhythmic <input type="checkbox"/> antihypertensive <input type="checkbox"/> anticonvulsant <input type="checkbox"/> beta-blockers <input type="checkbox"/> digitalis preparations <input type="checkbox"/> diuretics <input type="checkbox"/> ganglionic blockers <input type="checkbox"/> others	NOTE: consider underlying condition. Potential for: exertional syncope, electrolyte imbalance, bradycardia, dysrhythmias, impaired coordination and reaction time, heat intolerance. May alter resting and exercise ECG's and exercise test performance.
Other	<input type="checkbox"/> post-exercise syncope	moderate program
	<input type="checkbox"/> heat intolerance	prolong cool-down with light activities; avoid exercise in extreme heat
	<input type="checkbox"/> temporary minor illness	postpone until recovered
	<input type="checkbox"/> cancer	if potential metastases, test by cycle ergometry, consider non-weight bearing exercises; exercise at lower end of prescriptive range (40-65% of heart rate reserve), depending on condition and recent treatment (radiation, chemotherapy); monitor hemoglobin and lymphocyte counts; add dynamic lifting exercise to strengthen muscles, using machines rather than weights.

*Refer to special publications for elaboration as required

The following companion forms are available online: <http://www.csep.ca/forms.asp>

The **Physical Activity Readiness Questionnaire (PAR-Q)** - a questionnaire for people aged 15-69 to complete before becoming much more physically active.

The **Physical Activity Readiness Medical Examination for Pregnancy (PARmed-X for PREGNANCY)** - to be used by physicians with pregnant patients who wish to become more physically active.

For more information, please contact the:

Canadian Society for Exercise Physiology
202 - 185 Somerset St. West
Ottawa, ON K2P 0J2
Tel. 1-877-651-3755 • FAX (613) 234-3565 • Online: www.csep.ca

Note to physical activity professionals...

It is a prudent practice to retain the completed Physical Activity Readiness Conveyance/Referral Form in the participant's file.



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Canada

Continued on page 4...

PARmed-X PHYSICAL ACTIVITY READINESS MEDICAL EXAMINATION

Physical activity improves health.

Every little bit counts, but more is even better – everyone can do it!

Get active your way – build physical activity into your daily life...

- at home
- at school
- at work
- at play
- on the way ...that's active living!

Increase Endurance Activities

Increase Flexibility Activities

Increase Strength Activities

Reduce SITTING for long periods

Endurance
4-7 days a week
Continuous activities for your heart, lungs and circulatory system.

Flexibility
4-7 days a week
Gentle reaching, bending and stretching activities to keep your muscles relaxed and joints mobile.

Strength
2-4 days a week
Activities against resistance to strengthen muscles and bones and improve posture.

Starting slowly is very safe for most people. Not sure? Consult your health professional.

For a copy of the *Guide Handbook* and more information:
1-888-334-9769, or www.paguide.com

Eating well is also important. Follow *Canada's Food Guide to Healthy Eating* to make wise food choices.

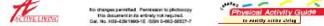
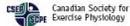
Get Active Your Way, Every Day – For Life!
Scientists say accumulate 60 minutes of physical activity every day to stay healthy or improve your health. As you progress to moderate activities you can cut down to 30 minutes, 4 days a week. Add-up your activities in periods of at least 10 minutes each. Start slowly... and build up.

Very Light Effort	Light Effort	Moderate Effort	Vigorous Effort	Maximum Effort
60 minutes	30-60 minutes	20-30 minutes		
• Strolling • Dusting	• Light walking • Volleyball • Stretching	• Brisk walking • Biking • Gardening • Swimming • Dancing • Water aerobics	• Aerobics • Jogging • Hockey • Basketball • Fast swimming • Fast dancing	• Sprinting • Racing
Range needed to stay healthy				

You Can Do It – Getting started is easier than you think

- Physical activity doesn't have to be very hard. Build physical activities into your daily routine.
- Walk whenever you can – get off the bus early, use the stairs instead of the elevator.
 - Reduce inactivity for long periods, like watching TV.
 - Get up from the couch and stretch and bend for a few minutes every hour.
 - Play actively with your kids.
 - Choose to walk, wheel or cycle for short trips.
 - Start with a 10 minute walk – gradually increase the time.
 - Find out about walking and cycling paths nearby and use them.
 - Observe a physical activity class to see if you want to try it.
 - Try one class to start – you don't have to make a long-term commitment.
 - Do the activities you are doing now, more often.

Benefits of regular activity:	Health risks of inactivity:
<ul style="list-style-type: none"> • better health • improved fitness • better posture and balance • better self-esteem • weight control • stronger muscles and bones • feeling more energetic • relaxation and reduced stress • continued independent living in later life 	<ul style="list-style-type: none"> • premature death • heart disease • obesity • high blood pressure • adult-onset diabetes • osteoporosis • stroke • depression • colon cancer



Source: Canada's Physical Activity Guide to Healthy Active Living, Health Canada, 1998 <http://www.hc-sc.gc.ca/hppb/paguide/pdf/guideEng.pdf>
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PARmed-X Physical Activity Readiness Conveyance/Referral Form

Based upon a current review of the health status of _____, I recommend:

- No physical activity
- Only a medically-supervised exercise program until further medical clearance
- Progressive physical activity
 - with avoidance of: _____
 - with inclusion of: _____
 - under the supervision of a CSEP-Professional Fitness & Lifestyle Consultant or CSEP-Exercise Therapist™
- Unrestricted physical activity — start slowly and build up gradually

Further information:
 Attached
 To be forwarded
 Available on request

Physician/clinic stamp:

NOTE: This physical activity clearance is valid for a maximum of six months from the date it is completed and becomes invalid if your medical condition becomes worse.

_____ M.D.

_____ 20____
 (date)

APPENDIX VII: Details of assay protocols

Homocysteine Assay, Siemens Medical Solutions Diagnostics, Deerfield, IL

IMMULITE® 2500 Homocysteine

English

Intended Use: For *in vitro* diagnostic use with the IMMULITE 2500 Analyzer — for the quantitative determination of L-homocysteine in human plasma or serum. This device can assist in the diagnosis and treatment of patients suspected of having hyperhomocysteinemia or homocystinuria.

Catalog Number: **L5KHO2** (200 tests)

Test Code: **HCY** Color: **Dark Gray**

Warning: Specimens from patients who are on drug therapy involving S-adenosyl-methionine may show falsely elevated levels of homocysteine.

Results on specimens obtained from patients taking methotrexate, carbamazepine, phenytoin, nitrous oxide, anti-convulsants, or 6-azauridine triacetate should be interpreted with caution as these substances interfere with homocysteine determination.

Summary and Explanation

Total homocysteine (tHcy) has emerged as an important risk factor in the assessment of cardiovascular disease.^{3-7,12} Hcy, a thiol-containing amino acid, is produced by the intracellular demethylation of methionine. Hcy thereby serves as a pool that can be later scavenged for use in the remanufacture of either methionine through the action of the folate-dependent enzyme methionine synthase or cysteine using the B6 dependent transsulphuration pathway^{1,2} Hcy in plasma is found primarily in a protein bound form but free, oxidized and disulfide forms are also present.

Highly elevated levels of tHcy are found in patients with homocystinuria, a rare genetic disorder of the enzymes involved in Hcy metabolism^{1,2,3} Homocystinuria patients exhibit arterial thromboembolism, mental retardation and early arteriosclerosis.¹ Less severe genetic

defects are also associated with moderate levels of Hcy.^{3,4,5}

Homocysteine has been identified as a indicator of cardiovascular disease. A meta-analysis of 27 epidemiological studies has suggested that a 5 µmol/L increase in tHcy could be associated with an odds ratio for coronary artery disease (CAD) of 1.6 for men and 1.8 for women, the same increase in risk as a 0.5 mmol/L increase in cholesterol.⁷ In addition, patients with chronic renal disease complicated by arteriosclerotic cardiovascular disease show elevated tHcy due to the inability of the kidney to remove Hcy from the blood.^{1,2,3}

Principle of the Procedure

IMMULITE 2500 Homocysteine is a solid-phase, competitive chemiluminescent enzyme immunoassay.

The IMMULITE 2500 performs an on-line one-cycle sample pretreatment of patient plasma or serum with S-adenosyl-L-homocysteine (SAH) hydrolase and dithiothreitol (DTT) solution in a reaction tube containing no bead. After a 30-minute incubation, the treated sample is transferred to a second reaction tube containing a SAH-coated polystyrene bead and an alkaline phosphatase-labeled antibody specific for SAH. During a 30-minute incubation, the converted SAH from the sample pretreatment competes with immobilized SAH for binding alkaline phosphatase-labeled anti-SAH antibody. Unbound enzyme conjugate is removed by centrifugal wash. Substrate is added and the procedure continues as described for typical immunoassays in the Operator's Manual.

Incubation: 60 minutes. 2 test positions per assay: 1 sample treatment cup; 1 immunoreaction cup.

Cycle 1: Release of bound homocysteine and conversion to SAH.

Cycle 2: Immunoreaction.

Specimen Collection

Heparinized and EDTA plasma are the samples of choice, but serum is also suitable for use. It is important to separate

the plasma or serum from the cells as soon as possible after collection, as synthesis of HCY will take place in red blood cells after sampling. **Samples should be stored on ice between the time of sampling and centrifugation.** Note that storage on ice makes the use of serum samples particularly difficult.

Centrifuging serum samples before a complete clot forms may result in the presence of fibrin. To prevent erroneous results due to the presence of fibrin, ensure that complete clot formation has taken place prior to centrifugation of samples. Some samples, particularly those from patients receiving anticoagulant therapy, may require increased clotting time.

Blood collection tubes from different manufacturers may yield differing values, depending on materials and additives, including gel or physical barriers, clot activators and/or anticoagulants. IMMULITE 2500 Homocysteine has not been tested with all possible variations of tube types. Consult the section on Alternate Sample Types for details on tubes that have been tested.

Volume Required: 15 μ L plasma or serum.

Storage: 14 days at 2–8°C, or 6 months at –20°C.

Warnings and Precautions

For *in vitro* diagnostic use.

Reagents: Store at 2–8°C. Dispose of in accordance with applicable laws.

Follow universal precautions, and handle all components as if capable of transmitting infectious agents. Source materials derived from human blood were tested and found nonreactive for syphilis; for antibodies to HIV 1 and 2; for hepatitis B surface antigen; and for antibodies to hepatitis C.

Sodium azide, at concentrations less than 0.1 g/dL, has been added as a preservative. On disposal, flush with large volumes of water to prevent the buildup of potentially explosive metal azides in lead and copper plumbing.

Chemiluminescent Substrate: Avoid contamination and exposure to direct sunlight. (See insert.)

Water: Use distilled or deionized water.

Materials Supplied

Components are a matched set. Labels on the inside box are needed for the assay.

Homocysteine Bead Pack (L2HO12)

With barcode. 200 beads, coated with S-adenosyl-L-homocysteine (SAH). Stable at 2–8°C until the expiration date.

L5KHO2: 1 pack.

Homocysteine Reagent Wedge (L2HOA2)

With barcode. One wedge, containing 3 reagents: 15.5 mL bovine S-adenosyl-L-homocysteine hydrolase in buffer, with preservative. 18.5 mL dithiothreitol (DTT) in buffer. 11.5 mL alkaline phosphatase (bovine calf intestine) conjugated to murine monoclonal anti-SAH in buffer. Stable at 2–8°C until expiration date.

L5KHO2: 1 wedge.

Before use, tear off the top of the label at the perforations, without damaging the barcode. Remove the foil seal from the top of wedge; snap the sliding cover down into the ramps on the reagent lid.

Homocysteine Adjustors (LHOL, LHOH)

Two amber vials (Low and High), 2.0 mL each, of synthetically-derived S-adenosyl-L-homocysteine (SAH) in a protein/buffer matrix. Stable at 2–8°C for 30 days after opening, or for 6 months (aliquotted) at –20°C.

L5KHO2: 1 set.

Before making an adjustment, place the appropriate Aliquot Labels (supplied with the kit) on test tubes so that the barcodes can be read by the on-board reader.

Kit Components Supplied Separately

Homocysteine Sample Diluent (L2HOZ)

For the on-board dilution of high samples. 25 mL of concentrated (ready-to-use) homocysteine-free protein/buffer matrix. Storage: 30 days (after opening) at 2–8°C or 6 months (aliquotted) at –20°C.

Barcode labels are provided for use with the diluent. Before use, place an appropriate label on a 16 × 100 mm test tube, so that the barcodes can be read by

the on-board reader.

L2HOZ: 3 labels

L2SUBM: Chemiluminescent Substrate

L2PWSM: Probe Wash

L2KPM: Probe Cleaning Kit

LRXT: Reaction Tubes (disposable)

L2ZT: 250 Sample Diluent Test Tubes
(16 × 100 mm)

L2ZC: 250 Sample Diluent Tube Caps

CCCM: A bi-level, nonhuman serum-based Cardiac Marker Control Module, containing homocysteine as one of four different constituents.

Also required

Distilled or deionized water; test tubes; controls.

Assay Procedure

Note that for optimal performance, it is important to perform all routine maintenance procedures as defined in the IMMULITE 2500 Operator's Manual.

See the IMMULITE 2500 Operator's Manual for: preparation, setup, dilutions, adjustment, assay and quality control procedures.

Recommended Adjustment Interval:
4 weeks.

Quality Control Samples: Use controls or plasma or serum pools with at least two levels (low and high) of homocysteine.

Expected Values

Homocysteine levels can vary with age, gender, geographical area and genetic factors, therefore it is important for laboratories to establish their own reference ranges based on their local populations. Literature suggests a reference range of 5 – 15 $\mu\text{mol/L}$ for adult males and females,¹¹ but also notes that men tend to have higher levels than women, and that postmenopausal women tend to have higher levels than premenopausal.¹²

The reference range values were generated on the IMMULITE 2000 analyzer.

Based on its relationship to IMMULITE 2000 Homocysteine (See Method Comparison 1) IMMULITE 2500 Homocysteine can be expected to have essentially the same reference range.

One hundred and twenty samples from apparently healthy adult male and female volunteers age 22–66 were analyzed using the IMMULITE 2000 Homocysteine procedure. The samples were drawn in heparinized plasma tubes and kept on ice prior to separation of the plasma from the cells. The median value was 7.7 $\mu\text{mol/L}$, with a central 95% range of

5.0 – 12 $\mu\text{mol/L}$.

Consider these limits as *guidelines* only. Each laboratory should establish its own reference ranges.

Limitations

Specimens from patients who are on drug therapy involving S-adenosyl-methionine may show falsely elevated levels of homocysteine.

Although analysis of the parent compounds of carbamazepine, phenytoin, 6-azauridine, and anthopterin indicate no crossreactivity, specimens obtained from patients treated with these drugs, as well as with methotrexate, nitrous oxide, and other anticonvulsants, should be interpreted with caution as these substances have been shown to interfere in some homocysteine assays.

Heterophilic antibodies in human serum can react with the immunoglobulins included in the assay components causing interference with *in vitro* immunoassays. [See Boscato LM, Stuart MC. Heterophilic antibodies: a problem for all immunoassays. Clin Chem 1988;34:27-33.] Samples from patients routinely exposed to animals or animal serum products can demonstrate this type of interference potentially causing an anomalous result. These reagents have been formulated to minimize the risk of interference; however, potential interactions between rare sera and test components can occur. For diagnostic purposes, the results obtained from this assay should always be used in combination with the clinical examination, patient medical history, and other findings.

Performance Data

See Tables and Graphs for data *representative* of the assay's performance. Results are expressed in $\mu\text{mol/L}$. (Unless

otherwise noted, all were generated on heparinized plasma samples.)

The IMMULITE 2500 assay employs the same reagent and bead formulation as IMMULITE 2000. All performance characteristics are equivalent between the two platforms.

All performance data except Method Comparison 1 were generated on the IMMULITE 2000 analyzer.

Calibration Range: 2 to 50 µmol/L.

The assay is traceable to an internal standard manufactured using qualified materials and measurement procedures.

Analytical Sensitivity: 0.5 µmol/L

Precision: Samples were repeatedly assayed in quadruplicate over the course of several days, for a total of 20 runs and 80 replicates. (See "Precision" table.)

Linearity: Samples were assayed under various dilutions. (See "Linearity" table for representative data.)

Recovery: Samples spiked 1 to 19 with three homocysteine solutions (125, 250, 500 µmol/L), were assayed. (See "Recovery" table for representative data.)

Specificity: The antibody is highly specific for homocysteine. (See "Specificity" table.)

Bilirubin: Presence of conjugated and unconjugated bilirubin in concentrations up to 200 mg/L has no effect on results, within the precision of the assay.

Hemolysis: Presence of hemoglobin in concentrations up to 512 mg/dL has no effect on results, within the precision of the assay.

Lipemia: Presence of lipemia in concentrations up to 3,000 mg/dL has no effect on results, within the precision of the assay.

Alternate Sample Type: To assess the effect of alternate sample types, blood was collected from 34 volunteers into plain, heparinized and EDTA vacutainer tubes. Equal volumes of the matched samples were spiked with various concentrations of homocysteine, to obtain values throughout the calibration range of the assay, and then assayed by the IMMULITE 2000 Homocysteine procedure.

(EDTA) = 0.98 (Heparin) + 0.85 µmol/L
r = 0.954

(Serum) = 1.02 (Heparin) – 0.53 µmol/L
r = 0.975

Means:
20.2 µmol/L (Heparin)
20.7 µmol/L (EDTA)
20.1 µmol/L (Serum)

To assess the effect of storage temperature, heparinized, EDTA and plain vacutainer tubes were collected from five volunteers for each tube type. Some tubes were stored at room temperature, while other tubes were kept on ice for various time periods prior to separation. The graphs below show the effect of storage time and temperature for heparin, EDTA and serum. (See graphs 1-3).

Method Comparison 1: IMMULITE 2500
Homocysteine was compared to IMMULITE 2000 Homocysteine on 51 patient samples. (Concentration range: approximately 3 to 40 µmol/L. See graph 1.) By linear regression:

(IML 2500) = 0.99 (IML 2000) + 0.9 µmol/L
r = 0.971

Means:
13.0 µmol/L (IMMULITE 2500)
12.1 µmol/L (IMMULITE 2000)

Method Comparison 2: IMMULITE 2000
Homocysteine was compared to a commercially available manual enzyme immunoassay (Kit A) on 168 plasma samples (Concentration range: approximately 4 to 43 µmol/L. See graph 2.) By linear regression:

(IML 2000) = 1.0 (Kit A) + 0.24 µmol/L
r = 0.966

Means:
12.9 µmol/L (IMMULITE 2000)
12.7 µmol/L (Kit A)

Method Comparison 3: IMMULITE 2000
Homocysteine was also compared to an in-house HPLC method in use at a reference laboratory in the United States on 95 heparinized plasma samples (Concentration range: approximately 4 to 44 µmol/L. See graph 3.) By linear regression:

(IML 2000) = 0.97 (HPLC) + 0.71 µmol/L
r = 0.974

Means:
13.4 µmol/L (IMMULITE 2000)
13.2 µmol/L (HPLC)

Method Comparison 4: IMMULITE 2000
Homocysteine was compared to another commercially available immunoassay (Kit B) on 113 plasma samples (Concentration range: approximately 4 to 23 µmol/L. See graph 4.) By linear regression:

$$(IML\ 2000) = 0.90 (Kit\ B) - 0.02\ \mu\text{mol/L}$$

$$r = 0.925$$

Means:

8.7 µmol/L (IMMULITE 2000)

9.6 µmol/L (Kit B)

References

1) Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by s-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am J Clin Nutr* 1992;55:131-8. 2) Jacques PF, Bostom AG, Williams RR, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase and plasma homocysteine concentrations. *Circulation* 1996;93:7-9. 3) Malinow MR. Plasma homocysteine and arterial occlusive diseases: A mini review. *Clin Chem*. 1995;40:173-76. 4) Clarke R, Daly L, Robinson K, Naughten E, et al. Hyperhomocysteinemia: An independent risk factor for vascular disease. *New Eng J Med* 1991;324:1149-55. 5) Deloughery TG, Evans A, Sadeghi A, et al. Common mutation in methylenetetrahydrofolate reductase: Correlation with homocysteine metabolism and late-onset vascular disease. *Circulation* 1996;94:3074-78. 6) Bostom AG, Selhub J. Homocysteine and arteriosclerosis: Subclinical and clinical disease associations. *Circulation* 1999;99:2361-63. 7) Boushey CJ, Beresford SAA, et al. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *JAMA* 1995;274(13):1049-57. 8) Guttormsen AB, Svarstad E, et al. Elimination of homocysteine in subjects with end-stage renal failure. *Irish J Med Sci* 1995;164:8. 9) Bostom AG, Lathrop, L. Hyper-homocysteinemia in end-stage renal disease (ESDR): Prevalence, etiology and potential relationship to arteriosclerotic outcomes. *Kidney Int* 1997;52:10-20. 10) Refsum H, Ueland PM. Clinical significance of pharmacological modulation of homocysteine metabolism. *TIPS* 1990;11:411-16. 11) Ueland PM, Refsum H, Stabler SP, et al. Total homocysteine in plasma or serum: Methods and clinical applications. *Clin Chem* 1993;39:1764-79. 12) Nehler MR, Taylor LM, Porter JM. Homocysteine as a risk factor for atherosclerosis: A Review. *Cardiovasc Pathol* 1997;6:1-9.

Technical Assistance

In the United States, contact Siemens Medical Solutions Diagnostics Technical

Services department. Tel: 800.372.1782 or 310.645.8200

Fax: 310.645.9999. IMMULITE:

Tel: 973.927.2828 Fax: 973.927.4101.

Outside the United States, contact your National Distributor.

The Quality System of Siemens Medical Solutions Diagnostics is certified to ISO 13485:2003.

Tables and Graphs

Precision (µmol/L)

	Mean ³	Within-Run ¹		Total ²	
		SD ⁴	CV ⁵	SD	CV
1	3.94	0.29	7.4%	0.41	10.4%
2	9.88	0.47	4.8%	0.75	7.6%
3	11.1	0.38	3.4%	0.46	4.1%
4	25.9	1.06	4.1%	1.31	5.1%

Specificity (µmol/L)

Compound ¹	Amount Added ² µmol/L	% Cross reactivity ³
Adenosine	5000	ND
S-adenosyl-L-methionine	500	0.6%
Cystathionine	500	6.1%
L-Cysteine	100000	ND
Glutathione	100000	ND

ND: Not detectable.⁴

Linearity (µmol/L)

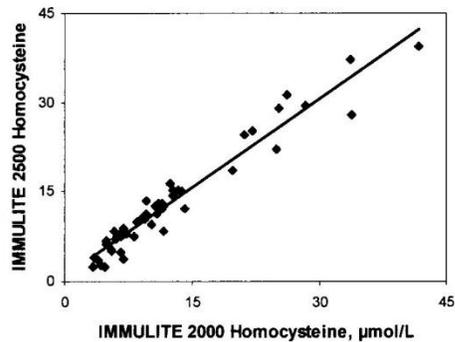
	Dilution ¹	Observed ²	Expected ³	%O/E ⁴
1	8 in 8 ⁵	19.2	—	—
	4 in 8	9.47	9.60	99%
	2 in 8	4.94	4.80	103%
2	1 in 8	2.11	2.40	88%
	8 in 8	25.2	—	—
	4 in 8	13.8	12.6	110%
	2 in 8	6.15	6.30	98%
	1 in 8	3.34	3.15	106%

	Dilution ¹	Observed ²	Expected ³	%O/E ⁴
3	8 in 8	31.6	—	—
	4 in 8	15.7	15.8	99%
	2 in 8	7.44	7.90	94%
	1 in 8	3.37	3.95	85%
4	8 in 8	47.5	—	—
	4 in 8	24.2	23.7	102%
	2 in 8	11.5	11.9	97%
	1 in 8	4.91	5.94	83%

Recovery (µmol/L)

	Solution ¹	Observed ²	Expected ³	%O/E ⁴
1	—	7.41	—	—
	A	13.3	13.3	100%
	B	20.2	19.5	104%
	C	34.4	32.0	108%
2	—	9.96	—	—
	A	14.7	15.7	94%
	B	22.6	22.0	103%
	C	36.2	34.5	105%
3	—	13.3	—	—
	A	16.9	18.9	89%
	B	26.0	25.1	104%
	C	32.0	37.6	85%
4	—	20.2	—	—
	A	27.7	25.4	109%
	B	33.0	31.7	104%
	C	44.5	44.2	101%

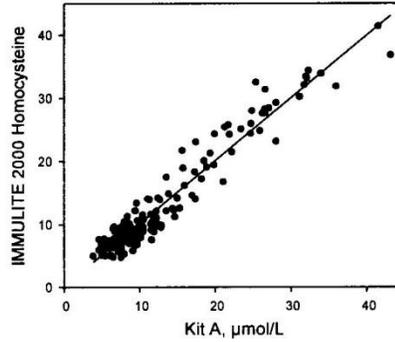
Method Comparison 1



$$(IML\ 2500) = 0.99 (IML\ 2000) + 0.9\ \mu\text{mol/L}$$

$$r = 0.971$$

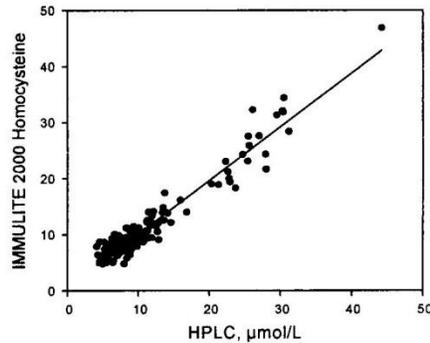
Method Comparison 2



$$(IML\ 2000) = 1.0 (Kit\ A) + 0.024\ \mu\text{mol/L}$$

$$r = 0.966$$

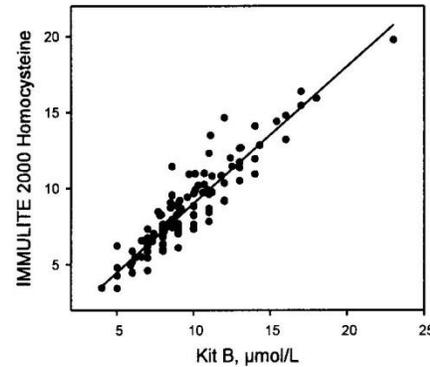
Method Comparison 3



$$(IML\ 2000) = 0.97c (HPLC) + 0.71\ \mu\text{mol/L}$$

$$r = 0.974$$

Method Comparison 4

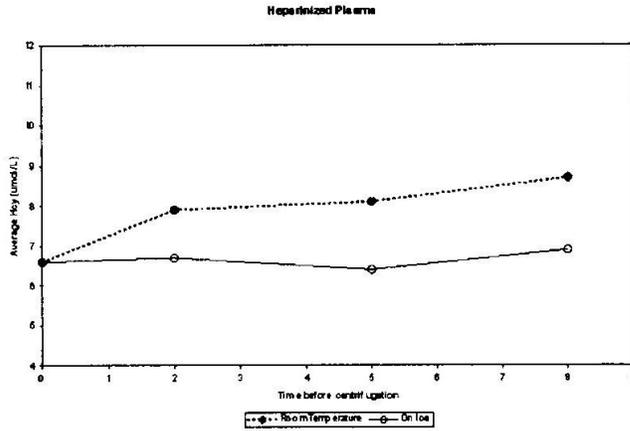


$$(IML\ 2000) = 0.90 (Kit\ B) - 0.02\ \mu\text{mol/L}$$

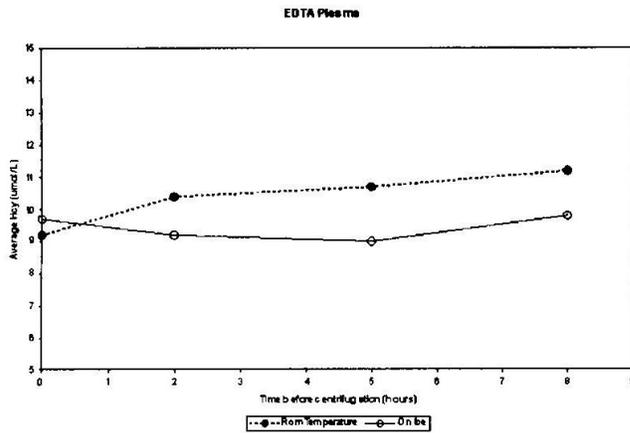
$$r = 0.925$$

Effect of Storage Temperature on Different Sample Types

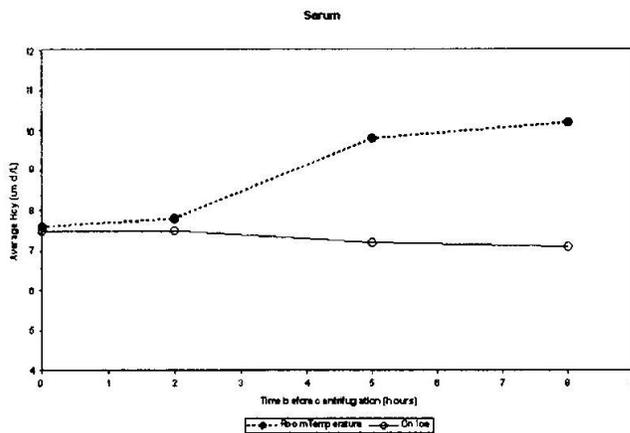
Graph 1: Heparin¹



Graph 2: EDTA



Graph 3: Serum



B₁₂ Assay, Abbot Laboratories, Abbott Park, IL

NAME

ARCHITECT B12

INTENDED USE

The ARCHITECT B12 assay is a Chemiluminescent Microparticle Intrinsic Factor assay for the quantitative determination of vitamin B12 in human serum and plasma.

SUMMARY AND EXPLANATION OF TEST

Vitamin B12 (B12), a member of the corrin family, is a cofactor for the conversion of methylmalonyl Coenzyme-A (CoA) to succinyl CoA. In addition, B12 is a cofactor in the synthesis of methionine from homocysteine, is implicated in the formation of myelin, and, along with folate, is required for DNA synthesis.^{1,2}

B12 is absorbed from food after binding to a protein called intrinsic factor which is produced by the stomach. Causes of vitamin B12 deficiency can be divided into three classes: nutritional deficiency, malabsorption syndromes, and other gastrointestinal causes. B12 deficiency can cause megaloblastic anemia (MA), nerve damage and degeneration of the spinal cord. Lack of B12, even mild deficiencies, damages the myelin sheath that surrounds and protects nerves, which may lead to peripheral neuropathy. The nerve damage caused by a lack of B12 may become permanently debilitating, if the underlying condition is not treated. People with intrinsic factor defects who do not get treatment eventually develop a MA called pernicious anemia (PA).²

The relationship between B12 levels and MA is not always clear in that some patients with MA will have normal B12 levels; conversely, many individuals with B12 deficiency are not afflicted with MA. Despite these complications, however, in the presence of MA (e.g., elevated mean corpuscular volume (MCV)) there is usually serum B12 or folate deficiency.^{2,3}

The true prevalence of B12 deficiency in the general population is unknown but increases with age. In one study,⁴ fifteen percent of adults older than 65 years old had laboratory evidence of vitamin B12 deficiency.

A serum B12 level below the normal expected range may indicate that tissue B12 levels are becoming depleted. However, a B12 level in the low normal range does not ensure that B12 levels are healthy and symptomatic patients should be further evaluated with tests for homocysteine and methylmalonic acid.^{5,6}

There are a number of conditions that are associated with low serum B12 levels, including iron deficiency, normal near-term pregnancy, vegetarianism, partial gastrectomy/ileal damage, celiac disease, oral contraception, parasitic competition, pancreatic deficiency, treated epilepsy, and advancing age.^{5,7-9,11} Disorders associated with elevated serum B12 levels include renal failure, liver disease, and myeloproliferative diseases.^{7,10}

BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The ARCHITECT B12 assay is a two-step assay with an automated sample pretreatment, for determining the presence of B12 in human serum and plasma using Chemiluminescent Microparticle Immunoassay (CMA) technology with flexible assay protocols, referred to as Chemillex.

Sample and Pre-Treatment Reagent 1, Pre-Treatment Reagent 2, and Pre-Treatment Reagent 3 are combined. An aliquot of the pre-treated sample is aspirated and transferred into a new RV. The pre-treated sample, assay diluent, and intrinsic factor coated paramagnetic microparticles are combined. B12 present in the sample binds to the intrinsic factor coated microparticles. After washing, B12 acridinium-labeled conjugate is added in the second step. Pre-Trigger and Trigger Solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs). An inverse relationship exists between the amount of B12 in the sample and the RLUs detected by the ARCHITECT *i* optical system.

For additional information on system and assay technology, refer to the ARCHITECT System Operations Manual, Section 3.

REAGENTS

Reagent Kit, 100 Tests/500 Tests

NOTE: Reagent Kit configurations vary based on order.

NOTE: Some kit sizes are not available in all countries or for use on all ARCHITECT *i* Systems. Please contact your local distributor.

ARCHITECT B12 Reagent Kit (6C09U)

- **CONJUGATE** 1 or 4 Bottle(s) (8.6 mL/27.0 mL) intrinsic factor (porcine) coated Microparticles in borate buffer with protein (bovine) stabilizers. Minimum Concentration: 0.1% solids. Preservative: antimicrobial agents.

- **CONJUGATE** 1 or 4 Bottle(s) (5.9 mL/26.3 mL) B12 acridinium-labeled Conjugate in MES buffer. Minimum concentration: 0.7 ng/mL. Preservative: antimicrobial agent.
- **ASSAY DILUENT** 1 or 4 Bottle(s) (10.0 mL/51.0 mL) B12 Assay Diluent containing borate buffer with EDTA. Preservative: antimicrobial agents.
- **PRE-TREATMENT REAGENT 1** 1 or 4 Bottle(s) (27.0 mL/50.4 mL) B12 Pre-Treatment Reagent 1 containing 1.0 N sodium hydroxide with 0.005% potassium cyanide.
- **PRE-TREATMENT REAGENT 2** 1 or 4 Bottle(s) (5.5 mL/25.9 mL) B12 Pre-Treatment Reagent 2 containing alpha monothiolglycerol and EDTA.
- **PRE-TREATMENT REAGENT 3** 1 or 4 Bottle(s) (5.5 mL/25.9 mL) B12 Pre-Treatment Reagent 3 containing cobinamide dicyanide in borate buffer with protein (avian) stabilizers. Preservative: Sodium Azide.

Assay Diluent

ARCHITECT *i* Multi-Assay Manual Diluent (7D92-50)

- **MULTI-ASSAY MANUAL DILUENT** 1 Bottle (100 mL) ARCHITECT *i* Multi-Assay Manual Diluent containing phosphate buffered saline solution. Preservative: antimicrobial agent.

Other Reagents

ARCHITECT *i* Pre-Trigger Solution

- **PRE-TRIGGER SOLUTION** Pre-Trigger Solution containing 1.32% (w/v) hydrogen peroxide.

ARCHITECT *i* Trigger Solution

- **TRIGGER SOLUTION** Trigger Solution containing 0.35 N sodium hydroxide.

ARCHITECT *i* Wash Buffer

NOTE: Bottle and volume varies based on order.

- **WASH BUFFER** Wash Buffer containing phosphate buffered saline solution. Preservative: antimicrobial agent.

WARNINGS AND PRECAUTIONS

- For *In Vitro* Diagnostic Use.
- Package insert instructions must be followed accordingly. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

Safety Precautions

CAUTION: This product requires the handling of human specimens. It is recommended that all human sourced materials be considered potentially infectious and handled with appropriate biosafety practices.

- The ARCHITECT Pre-Treatment Reagent 2 may cause eye irritation. If this solution comes in contact with eyes, rinse immediately with water. If irritation persists, seek medical attention.
- The ARCHITECT B12 Conjugate contains a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (3:1) which is a component of ProClin® and is classified per applicable European Community (EC) Directives as Irritant (Xi). The following are the appropriate Risk (R) and Safety (S) phrases.



- | | |
|-----|---|
| R43 | May cause sensitization by skin contact. |
| S24 | Avoid contact with skin. |
| S35 | This material and its container must be disposed of in a safe way. |
| S37 | Wear suitable gloves. |
| S46 | If swallowed, seek medical advice immediately and show this container or label. |

- The ARCHITECT B12 Pre-Treatment Reagent 1 (6C09U) contains sodium hydroxide and is classified per applicable European Community (EC) Directives as Corrosive (C). The following are the appropriate Risk (R) and Safety (S) phrases.



- | | |
|-----------|---|
| R34 | Causes burns. |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. |
| S35 | This material and its container must be disposed of in a safe way. |
| S36/37/39 | Wear suitable protective clothing, gloves, and eye/face protection. |
| S45 | In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). |

- This product contains sodium azide; for a specific listing, refer to the REAGENTS section. Contact with acids liberates very toxic gas. This material and its container must be disposed of in a safe way. For information on the safe disposal of sodium azide and a detailed discussion of safety precautions during system operation, refer to the ARCHITECT System Operations Manual, Section 8.
- For product not classified as dangerous per European Directive 1999/45/EC as amended - Safety data sheet available for professional user on request.

Handling Precautions

- Do not use reagent kits beyond the expiration date.
- Do not mix reagents from different reagent kits.
- Prior to loading the ARCHITECT B12 Reagent Kit on the system for the first time, the microparticle bottle requires mixing to resuspend microparticles that have settled during shipment. For microparticle mixing instructions, refer to the PROCEDURE, Assay Procedure section of this package insert.
- Septums **MUST** be used to prevent reagent evaporation and contamination and to ensure reagent integrity. Reliability of assay results cannot be guaranteed if septums are not used according to the instructions in this package insert.
 - Prolonged exposure of B12 Pre-Treatment Reagent 1 to air may compromise performance.
- To avoid contamination, wear clean gloves when placing a septum on an uncapped reagent bottle.
- Prior to placing the septum on an uncapped reagent bottle, squeeze the septum in half to confirm that the slits are open. If the slits appear sealed, continue to gently squeeze the septum to open the slits.
- Once a septum has been placed on an open reagent bottle, do not invert the bottle as this will result in reagent leakage and may compromise assay results.
- Over time, residual liquids may dry on the septum surface. These are typically dried salts which have no effect on assay efficacy.
- For a detailed discussion of handling precautions during system operation, refer to the ARCHITECT System Operations Manual, Section 7.

Storage Instructions

-  The ARCHITECT B12 Reagent Kit must be stored at 2-8°C and may be used immediately after removal from 2-8°C storage.
- When stored and handled as directed, reagents are stable until the expiration date.
- The ARCHITECT B12 Reagent Kit may be stored onboard the ARCHITECT i System for a maximum of 30 days. After 30 days, the reagent kit must be discarded. For information on tracking onboard time, refer to the ARCHITECT System Operations Manual, Section 5.
- Reagents may be stored on or off the ARCHITECT i System. If reagents are removed from the system, store them at 2-8°C (with septums and replacement caps) in an upright position. For reagents stored off the system, it is recommended that they be stored in their original trays and boxes to ensure they remain upright. If the microparticle bottle does not remain upright (with a septum installed) while in refrigerated storage off the system, the reagent kit must be discarded. After reagents are removed from the system, you must initiate a scan to update the on-board stability timer.

Indications of Reagent Deterioration

When a control value is out of the specified range, it may indicate deterioration of the reagents or errors in technique. Associated test results may be invalid and may require retesting. Assay recalibration may be necessary. For troubleshooting information, refer to the ARCHITECT System Operations Manual, Section 10.

INSTRUMENT PROCEDURE

- The ARCHITECT_B12 assay file (assay number 715) must be installed on the ARCHITECT i System from the ARCHITECT i Assay CD-ROM prior to performing the assay. For detailed instructions on assay file installation and on viewing and editing assay parameters, refer to the ARCHITECT System Operations Manual, Section 2.
- ARCHITECT maintenance procedure 604r Daily Maintenance (version 5 or higher) must be installed on the ARCHITECT i System prior to performing the assay. For information on installing and deleting maintenance procedures, refer to the ARCHITECT System Operations Manual Section 2.

- ARCHITECT maintenance procedure 604r Daily Maintenance (version 5 or higher) must be run a minimum of once every 24 hours. For laboratories processing a higher volume of B12 and Folate tests on a single module, this procedure must be run more than once in a 24 hour period.
 - If B12 but no Folate is run on a single module and > 100 B12 tests are run in 24 hours, perform the 604r Daily Maintenance procedure (version 5 or higher) after every 100 B12 tests run.
 - If B12 and Folate are run on a single module and > 100 B12 or > 100 Folate tests are run in 24 hours, perform 604r Daily Maintenance procedure (version 5 or higher) after every 100 B12 or 100 Folate tests run.
 - To protect the integrity of HBsAg and/or HBsAg Confirmatory test results, one of the following preventive measures should be taken:
 - Run HBsAg and/or HBsAg Confirmatory assays on a separate module from that used for B12 testing. As a precaution, replace the sample probe on the module intended for HBsAg and/or HBsAg Confirmatory testing, or
 - If HBsAg and/or HBsAg Confirmatory assays are run on the same module as B12, perform retesting in duplicate of initial reactive HBsAg specimens and HBsAg Confirmatory testing after performing the sample probe 604r Daily Maintenance procedure (version 5 or higher). The samples must be retested in ascending order of IU/mL.
 - Refer to LIMITATIONS OF THE PROCEDURE for additional information.
- For information on printing assay parameters, refer to the ARCHITECT System Operations Manual, Section 5.
- For a detailed description of system procedures, refer to the ARCHITECT System Operations Manual.
- The default result unit for the ARCHITECT B12 assay is pg/mL. An alternate result unit, pmol/L, may be selected for reporting results by editing assay parameter "Result concentration units", to pmol/L. The conversion factor used by the system is 0.7378.

SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

- Human serum (including serum collected in serum separator tubes) or plasma collected in tripotassium EDTA may be used in the ARCHITECT B12 assay. Other anticoagulants have not been verified for use with the ARCHITECT B12 assay. Follow the tube manufacturer's processing instructions for serum or plasma collection tubes.
- The ARCHITECT i System does not provide the capability to verify specimen type. It is the responsibility of the operator to verify the correct specimen types are used in the ARCHITECT B12 assay.
- Use caution when handling patient specimens to prevent cross contamination. Use of disposable pipettes or pipette tips is recommended.
- For optimal results, inspect all samples for bubbles. Remove bubbles with an applicator stick prior to analysis. Use a new applicator stick for each sample to prevent cross contamination.
- For optimal results, serum and plasma specimens should be free of fibrin, red blood cells or other particulate matter.
- Ensure that complete clot formation in serum specimens has taken place prior to centrifugation. Some specimens, especially those from patients receiving anticoagulant or thrombolytic therapy may exhibit increased clotting time. If the specimen is centrifuged before a complete clot forms, the presence of fibrin may cause erroneous results.
- If testing will be delayed more than 24 hours, remove serum or plasma from the clot, serum separator or red blood cells. Specimens may be stored for up to 7 days at 2-8°C prior to being tested. If testing will be delayed more than 7 days, specimens should be stored at -20°C or colder. Specimens stored at -20°C or colder for 78 days showed no performance differences.
- Multiple freeze-thaw cycles of specimens should be avoided. Specimens must be mixed THOROUGHLY after thawing, by LOW speed vortexing or by gently inverting, and centrifuged prior to use to remove red blood cells or particulate matter to ensure consistency in the results.
- When shipped, specimens must be packaged and labeled in compliance with applicable state, federal and international regulations covering the transport of clinical specimens and infectious substances. Specimens may be shipped at -20°C or colder. Prior to shipment, it is recommended that specimens be removed from the clot, serum separator or red blood cells.

PROCEDURE

Materials Provided:

- 6C09 ARCHITECT B12 Reagent Kit

Materials Required but not Provided:

- ARCHITECT *i* System
- ARCHITECT *i* [ASSAY CDROM]
- 6C09-02 ARCHITECT B12 Calibrators
- 6C09-10 ARCHITECT B12 Controls
- 7D82-50 ARCHITECT *i* Multi-Assay Manual Diluent
- ARCHITECT *i* [PRE-TRIGGER SOLUTION]
- ARCHITECT *i* [TRIGGER SOLUTION]
- ARCHITECT *i* [WASH BUFFER]
- ARCHITECT *i* [REACTION VESSELS]
- ARCHITECT *i* [SAMPLE CUPS]
- ARCHITECT *i* [SEPTUM]
- ARCHITECT *i* [REPLACEMENT CAPS]

- For information on materials required for maintenance procedures, refer to the ARCHITECT System Operations Manual, Section 9.
- Pipettes or pipette tips (optional) to deliver the volumes specified on the patient or control order screen.

Assay Procedure

- Before loading the ARCHITECT B12 Reagent Kit on the system for the first time, the microparticle bottle requires mixing to resuspend microparticles that have settled during shipment:
 - Invert the microparticle bottle 30 times.
 - Visually inspect the bottle to ensure microparticles are resuspended. If microparticles are still adhered to the bottle, continue to invert the bottle until the microparticles have been completely resuspended.
 - Once the microparticles have been resuspended, remove and discard the cap. Wearing clean gloves, remove a septum from the bag. Squeeze the septum in half to confirm that the slits are open. Carefully snap the septum onto the top of the bottle.
 - If the microparticles do not resuspend, DO NOT USE. Contact your local Abbott representative.
- Order tests.
- Load the ARCHITECT B12 Reagent Kit on the ARCHITECT *i* System. Verify that all necessary assay reagents are present. Ensure that septums are present on all reagent bottles. Refer to ARCHITECT Operations Manual, section 5 for details on how to load reagents.
- The minimum sample cup volume is calculated by the system and is printed on the Order List Report. No more than 10 replicates may be sampled from the same sample cup. To minimize the effects of evaporation verify adequate sample cup volume is present prior to running the test.
 - Priority: 125 μ L for the first B12 test plus 75 μ L for each additional B12 test from the same sample cup
 - \leq 3 hours onboard: 150 μ L for the first B12 test plus 75 μ L for each additional B12 test from the same sample cup
 - > 3 hours onboard: additional sample volume is required. Refer to the ARCHITECT System Operations Manual, Section 5 for information on sample evaporation and volumes.
 - If using primary or aliquot tubes, use the sample gauge to ensure sufficient patient specimen is present.
 - To obtain the recommended volume requirements for the ARCHITECT B12 Calibrators and Controls, hold the bottles vertically and dispense 6 drops of each calibrator or 6 drops of each control into each respective sample cup.
- Load samples
 - For information on loading samples, refer to the ARCHITECT System Operations Manual, Section 5
- Press RUN. The ARCHITECT *i* System performs the following functions:
 - Moves the sample to the aspiration point
 - Loads a reaction vessel (RV) into the process path
 - Aspirates and transfers sample into the RV
 - Advances the RV one position and adds Pre-Treatment Reagent 1, Pre-Treatment Reagent 2, and Pre-Treatment Reagent 3 to the sample
 - Mixes and incubates
 - Transfers an aliquot of the pretreated sample to a new RV
 - Advances the RV one position and transfers microparticles and assay diluent into the RV
 - Mixes, incubates and washes the reaction mixture
 - Adds conjugate to the RV
 - Mixes, incubates and washes the reaction mixture
 - Adds Pre-trigger and Trigger Solutions

- Measures chemiluminescent emission to determine the quantity of B12 in the sample
- Aspirates contents of RV to liquid waste and unloads RV to solid waste
- Calculates the result
- For information on ordering patient specimens, calibrators and controls, and general operating procedures refer to the ARCHITECT System Operations Manual, Section 5.
- For optimal performance, it is important to follow the routine maintenance procedures defined in the ARCHITECT System Operations Manual, Section 9. If your laboratory requires more frequent maintenance, follow those procedures.

Specimen Dilution Procedures

Specimens with a B12 value exceeding 2000 pg/mL, are flagged with the code " >2000" and may be diluted with either the Automated Dilution Protocol or the Manual Dilution Procedure.

- If using the Automated Dilution Protocol, the system performs a 1:3 dilution. The system will use the dilution factor to automatically calculate the concentration of the sample before dilution. This will be the reported result.
- Manual dilutions should be performed as follows:
 - The suggested dilution for B12 is 1:4.
 - For a 1:4 dilution, add 100 μ L of the patient specimen to 300 μ L of ARCHITECT *i* Multi-Assay Manual Diluent (7D82-50).
 - The suggested dilution for specimens that generate repeated (2 or more) "3250 Unable to process test-aspiration error for (Sample Pipetter) at (RV 24)" errors is 1:2.
 - For a 1:2 dilution, add 100 μ L of the patient specimen to 100 μ L of ARCHITECT *i* Multi-Assay Manual Diluent (7D82-50).
 - The operator must enter the dilution factor in the patient or control order screen. The system will use this dilution factor to automatically calculate the concentration of the sample before dilution. This will be the reported result. The result (before dilution factor is applied) should be greater than 60 pg/mL.
- For detailed information on ordering dilutions, refer to the ARCHITECT System Operations Manual, Section 5.

Calibration

- To perform an ARCHITECT B12 calibration, test Calibrators A through F in duplicate. A single sample of all levels of B12 controls must be tested to evaluate the assay calibration. Ensure that assay control values are within the concentration ranges specified in the control package insert. Calibrators should be priority loaded.
- Calibration Range: 0 - 2000 pg/mL.
- Once an ARCHITECT B12 calibration is accepted and stored, all subsequent samples may be tested without further calibration unless:
 - A reagent kit with a new lot number is used
 - Controls are out of range
- For detailed information on how to perform an assay calibration, refer to the ARCHITECT System Operations Manual, Section 6.

QUALITY CONTROL PROCEDURES

The recommended control requirement for the ARCHITECT B12 assay is a single sample of all control levels tested once every 24 hours each day of use. If the quality control procedures in your laboratory require more frequent use of controls to verify test results, follow your laboratory-specific procedures. Ensure that assay control values are within the concentration ranges specified in the control package insert.

Verification of Assay Claims

For protocols to verify package insert claims, refer to the ARCHITECT System Operations Manual, Appendix B. The ARCHITECT B12 assay belongs to method group 1.

RESULTS

The ARCHITECT B12 assay utilizes a 4 Parameter Logistic Curve Fit data reduction method (4PLC, Y weighted) to generate a calibration curve.

Alternate Result Units

- The default result unit for the ARCHITECT B12 assay is pg/mL. When the alternate result unit, pmol/L, is selected, the conversion factor used by the system is 0.7378.
- Conversion Formula: (Concentration in pg/mL) x (0.7378) = pmol/L

Flags

- Some results may contain information in the Flags field. For a description of the flags that may appear in this field, refer to the ARCHITECT System Operations Manual, Section 5.

LIMITATIONS OF THE PROCEDURE

- For diagnostic purposes, results should be used in conjunction with other data; eg., symptoms, results of other tests, clinical impressions, etc.
- The diagnosis of B12 deficiency cannot be solely based on serum or plasma B12 levels. Further testing for folic acid, intrinsic factor blocking antibodies, homocysteine, and/or methylmalonic acid is suggested for symptomatic patients with hematological or neurological abnormalities.^{5,6} See the Expected Values section for additional information.
- If the B12 results are inconsistent with clinical evidence, additional testing is suggested to confirm the result.
- Specimens containing R protein (transcobalamin [III]; haptocorrin) concentrations greater than 1600 pg/mL of total cobalamin-binding capacity (TBBC) may depress ARCHITECT B12 assay results and should be quantified using the Automated Dilution Protocol or Manual Dilution Procedure.
- Specimens containing above normal protein concentrations may generate repeated (2 or more) *3350 Unable to process test- aspiration error for (Sample Pipetter) at (PV 24)* errors and should be quantified using the Automated Dilution Protocol or Manual Dilution Procedure (1:2).
- Accumulation of denatured protein from the pretreatment step in the sample probe may impact results of other assays on the ARCHITECT System. ARCHITECT maintenance procedure 804r Daily Maintenance (version 5 or higher) must be run to eliminate this effect. If HBsAg and/or HBeAg Confirmatory assays are run on the same module as B12, preventive measures should be taken to protect integrity of ARCHITECT HBsAg and HBeAg Confirmatory results. Refer to the INSTRUMENT PROCEDURE section for instructions.

EXPECTED VALUES

B12 Normals

A study was performed based on guidance from NCCLS document C28-A2.¹² Serum specimens from 262 individuals with normal mean corpuscular volume, homocysteine, and folate results were assayed for B12 using the ARCHITECT B12 assay. The median B12 concentration for this population was 423 pg/mL with a range of 77 to 1553 pg/mL. The central 95% of the sample population defined the expected range of 189 to 883 pg/mL.

Normal	189 - 883 pg/mL (129-651 pmol/L)
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It is recommended that each laboratory establish its own range, which may be unique to the population it serves depending upon geographical, patient, dietary, or environmental factors.

B12 Deficients

Levels below 100 pg/mL are usually associated with B12 deficiency.⁶

B12 Indeterminates

Levels above 300 or 400 pg/mL are rarely associated with B12 deficiency induced hematological or neurological diseases respectively. Further testing is suggested for symptomatic patients with B12 levels between 100 to 300 pg/mL (hematological abnormalities), and between 100 and 400 pg/mL (neurological abnormalities).^{5,6}

SPECIFIC PERFORMANCE CHARACTERISTICS

Precision

The ARCHITECT B12 is designed to have a precision of ≤ 10 total %CV for concentrations in the range of the low, medium, and high controls.

A study was performed for the ARCHITECT B12 assay based on the guidance from the National Committee for Clinical Laboratory Standards (NCCLS) Protocol EP5-T2.¹³ A four-member panel was used consisting of:

- buffered protein based panel members (1 and 3)
- a processed human serum panel member (2)
- a reconstituted processed human serum panel (4)

Each panel member was assayed, in replicates of two at two separate times per day, for 20 days on two instruments using one lot of reagents and a single calibration for each instrument. Data from this study are summarized in the following table.

Panel Member	Reagent Lot	Instrument	n	Mean Conc. Value (pg/mL)	Within Run SD	Within Run %CV	Total SD	Total %CV
1	1	1	80	204	12.1	5.9	15.7	7.7
1	1	2	80	195	11.6	5.9	15.0	7.7
2	1	1	80	374	21.6	6.4	25.0	6.4
2	1	2	80	355	20.6	6.6	22.6	6.2
3	1	1	80	751	32.9	4.4	40.1	5.3
3	1	2	80	727	28.6	3.7	45.8	6.3
4	1	1	80	220	15.1	6.9	19.3	8.8
4	1	2	80	215	15.5	7.2	20.4	9.5

Recovery

The ARCHITECT B12 assay is designed to have an average recovery within $\pm 10\%$ of the spiked level when analyzing samples with known amounts of B12.

In a representative study, known concentrations of cyanocobalamin were added to five human serum specimens. The concentration of cyanocobalamin was determined using the ARCHITECT B12 assay and the resulting percent recovery was calculated.

Sample	Endogenous B12 Conc. (pg/mL)	B12 Added (pg/mL)	Observed B12 Conc. (pg/mL)	Percent Recovery
1	300	213	630	95.8
	300	627	951	105.8
	300	950	1306	108.1
2	379	213	704	105.8
	379	627	1052	107.2
	379	950	1391	108.5
3	488	213	647	114.7
	488	627	1174	108.4
	488	950	1555	112.2
4	319	213	648	104.5
	319	627	965	107.8
	319	950	1323	105.7
5	670	213	927	104.5
	670	627	1259	92.5
	670	950	1726	111.4

Average Recovery: 105.8

$$\% \text{ Recovery} = \frac{\text{Observed B12 Conc. (pg/mL)} - \text{Endogenous B12 Conc. (pg/mL)}}{\text{B12 Added (pg/mL)}} \times 100$$

Analytical Sensitivity

The ARCHITECT B12 assay is designed to have an analytical sensitivity of ≤ 60 pg/mL.

Analytical sensitivity is defined as the concentration at two standard deviations from the mean FLU value of the ARCHITECT B12 Calibrator A (0 pg/mL), and represents the lowest measurable concentration of vitamin B12 that can be distinguished from zero. In a representative study, the analytical sensitivity of the ARCHITECT B12 assay was calculated to be 39 pg/mL (n = 22 runs).

Specificity

The ARCHITECT B12 assay is designed to have less than 0.0001% cross reactivity with cobinamide, a B12 analogue.

The specificity of the ARCHITECT B12 assay was determined by studying the cross reactivity with cobinamide. Three human serum specimens were supplemented with cobinamide at 9000 pg/mL and the resulting cross reactivity was undetectable.

Interference

The ARCHITECT B12 assay is designed to have a mean potential interference of $\leq 10\%$ difference in concentration for patient samples with triglycerides (3000 mg/dL), bilirubin (20 mg/dL), hemoglobin (400 mg/dL), and $\leq 11\%$ difference with protein (2 - 10 g/dL).

In a representative study, the potential interference from hemoglobin, bilirubin, triglycerides, and protein was evaluated in the ARCHITECT B12 assay. Human serum specimens containing endogenous vitamin B12 were supplemented with potentially interfering compounds. The ARCHITECT B12 assay demonstrated the following interferences.

- Hemoglobin < 10% at 400 mg/dL
- Bilirubin < 10% at 20 mg/dL
- Triglycerides < 10% at 3000 mg/dL
- Protein < 11% at 2 g/dL and 10 g/dL

Accuracy by Correlation

The ARCHITECT B12 6-point calibration assay is designed to have a slope of 1.0 ± 0.1 and a correlation coefficient (r) of ≥ 0.95 within the range of 60 - 2,000 pg/mL when compared to the ARCHITECT B12 2-point calibration assay.

A study was performed where specimens were tested using the ARCHITECT B12 6-point calibration assay and the ARCHITECT B12 2-point calibration assay. The results of the specimen testing are shown in the following table.*

Method	Number of Specimens	Intercept	Slope	Correlation Coefficient
Least Squares Linear Regression	465	22	0.99	0.996
Passing-Bablok Linear Regression**	465	12	0.99	0.996

In this evaluation, serum specimens tested ranged from 76 to 1944 pg/mL by the ARCHITECT B12 6-point calibration assay, and from 77 to 1998 pg/mL by the ARCHITECT B12 2-point calibration assay.

In a study, the ARCHITECT B12 2-point assay was compared to a commercially available diagnostic kit for the determination of B12. The results of the specimen testing are shown in the following table.*

Method	Number of Specimens	Intercept	Slope	Correlation Coefficient
Least Squares Linear Regression	442	17	0.99	0.990
Passing-Bablok Linear Regression**	442	12	0.99	0.990

In this evaluation, serum specimens tested ranged from 77 to 1210 pg/mL by the ARCHITECT B12 assay, and from 73.8 to 1177.0 pg/mL by the AxSYM B12 assay.

* Representative data; results in individual laboratories may vary from these data.

** A linear regression method with no special assumptions regarding the distribution of the samples and the measurement errors.¹⁴

BIBLIOGRAPHY

1. Lee DSC, Griffiths BW. Human Serum Vitamin B12 Assay Methods - A Review. *Clin Biochem* 1985; 18:261-6.
2. Chararin I. Megaloblastic Anemia, Cobalamin and Folate. *J Clin Pathol* 1987; 40:978-84.
3. Beuerlein FJ. Testing Strategies for Anemias. *Lab Mgmt* 1988; Dec:23-9.
4. Pennypacker LC, Allen RH, Kelly JP et al. High Prevalence of Cobalamin Deficiency in Elderly Outpatients. *J Am Geriatr Soc* 1992;40:1197-204.
5. Klee GC. Cobalamin and Folate Evaluation: Measurement of Methylmalonic Acid and Homocysteine vs. Vitamin B12 and Folate. *Clin Chem* 46; 2000:1277-83.
6. Snow CF. Laboratory Diagnosis of Vitamin B12 and Folate Deficiency: A Guide for the Primary Care Physician. *Arch Intern Med*. 1999; 159:1289-98.
7. Beck WS. Biological and Medical Aspects of Vitamin B12. In: Dolphin D, editor. B12 Volume 2, Biochemistry and Medicine. New York: Wiley-Interscience, 1982.
8. Carethers M. Diagnosing Vitamin B12 Deficiency, A Common Geriatric Disorder. *Geriatrics* 1988; 43(3):89-112.
9. Herbert V. Five Possible Causes of All Nutrient Deficiency: Illustrated by Deficiencies of Vitamin B12 and Folic Acid. *Am J Clin Nutr* 1973; 26:77-86.
10. Pratt JJ, Woldring MG. Radioassay of Vitamin B12 and Other Corrinoids. *Methods Enzymol* 1982; 84:369-406.
11. Dehele A, Ghosh S. Vitamin B12 Deficiency in Untreated Celiac Disease. *Am J Gastroenterol*. 2001; 96:745-50.

12. National Committee for Clinical Laboratory Standards. *How to Define and Determine Reference Intervals in the Clinical Laboratory. Approved Guideline - Second Edition*. NCCLS Document C28-A2. Wayne, PA:NCCLS, 2000.
13. National Committee for Clinical Laboratory Standards. *Evaluation of Precision Performance of Clinical Chemistry Devices; Second Edition; Tentative Guideline*. NCCLS Document EP5-T2. Villanova, PA:NCCLS, March 1992.
14. Passing H, Bablok W. A New Biometrical Procedure for Testing the Equality of Measurements from Two Different Analytical Methods. *J Clin Chem Clin Biochem*. 1983; 21:709-20.

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Folate Assay, Abbott Laboratories, Abbott Park, IL

NAME

ARCHITECT® Folate

INTENDED USE

The ARCHITECT Folate assay is a Chemiluminescent Microparticle Folate Binding Protein assay for the quantitative determination of folate in human serum, plasma, and red blood cells on the ARCHITECT r System.

SUMMARY AND EXPLANATION OF TEST

Folates are a class of vitamin compounds related to pteroylglutamic acid (PGA), which serve as cofactors in the enzymatic transfer of single carbon units in a variety of metabolic pathways.^{1,2} Folate mediated one-carbon metabolism represents one of the most important biochemical reactions that occur in cells. Folates are necessary for nucleic acid and mitochondrial protein synthesis, amino acid metabolism, and other cellular processes that involve single carbon transfers. Folates can serve as carbon donors or acceptors. Since different metabolic pathways require carbon groups with different levels of oxidation, cells contain numerous enzymes that change the oxidation state of carbon groups carried by folates³ resulting in different metabolically active forms of folate. The predominant form of circulating folate is 5-methyltetrahydrofolic acid (5-mTHF). A methyl group is transferred from 5-mTHF to cobalamin in the pathway that links metabolism of folic acid and vitamin B₁₂.³

Folate deficiency can be caused by low dietary intake, malabsorption due to gastrointestinal diseases, inadequate utilization due to enzyme deficiencies or folate antagonist therapy, drugs such as alcohol and oral contraceptives, and excessive folate demand, such as during pregnancy and cellular proliferation disorders.⁴ Because deficiencies of both vitamin B₁₂ and folate can lead to megaloblastic (macrocytic) anemia, appropriate treatment requires differential diagnosis of the deficiency; thus, both vitamin B₁₂ and folate values are needed. Low serum folate levels reflect the first stage of negative folate balance, and precede tissue depletion.⁵ Low red-blood-cell folate values reflect the second stage of negative folate balance, and more closely correlate with tissue levels and megaloblastic anemia.

BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The ARCHITECT Folate assay is a two-step assay to determine the presence of folate in human serum, plasma, and red blood cells (RBC) using Chemiluminescent Microparticle Immunoassay (CMA) technology with flexible assay protocols, referred to as Chemiflex™. Two pre-treatment steps mediate the release of folate from endogenous folate binding protein. In Pre-Treatment Step 1, sample and Pre-Treatment Reagent 2 (Dithiothreitol or DTT) are aspirated and dispensed into a reaction vessel (RV). In Pre-Treatment Step 2, an aliquot of sample/Pre-Treatment Reagent 2 mixture is aspirated and dispensed into a second RV. Pre-Treatment Reagent 1 (potassium hydroxide or KOH) is then added. An aliquot of the pretreated sample is transferred into a third RV, followed by the addition of Folate Binding Protein (FBP) coated paramagnetic microparticles and assay specific diluent. Folate present in the sample binds to the FBP coated microparticles. After washing, pteric acid-acridinium labeled conjugate is added and binds to unoccupied sites on the FBP coated microparticles. Pre-Trigger and Trigger Solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs). An inverse relationship exists between the amount of folate in the sample and the RLUs detected by the ARCHITECT r optical system.

- * In the Folate RBC assay, a manual pre-treatment step converts RBC-bound folate to measurable folate, after which these samples are processed as described above.

For additional information on system and assay technology, refer to the ARCHITECT System Operations Manual, Section 3.

REAGENTS

Reagent Kit, 100 Tests/500 Tests

NOTE: Some kit sizes are not available in all countries, please contact your local distributor.

ARCHITECT Folate Reagent Kit (6C12-20, 6C12-25, 6C12-30)

- * **[MICROPARTICLES]** 1 or 4 Bottles (6.6 mL per 100 test bottles/27.0 mL per 500 test bottles) Anti-Folate Binding Protein (mouse, monoclonal) coupled to microparticles affinity bound with Folate Binding Protein (bovine), in TRIS buffer with protein stabilizers (human albumin [donor units of which are nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2] and caprine). Minimum concentration: > 0.1% solids. Preservative: Antimicrobial Agents.
- * **[CONJUGATE]** 1 or 4 Bottles (29.0 mL per 100 test bottles/29.0 mL per 500 test bottles) Pteric Acid (PTA)-acridinium labeled conjugate in MES buffer with protein stabilizer (porcine). Minimum concentration: 4 ng/mL. Preservative: Antimicrobial Agents.
- * **[ASSAY SPECIFIC DILUENT]** 1 or 4 Bottles (5.7 mL per 100 test bottles/25.8 mL per 500 test bottles) Folate Assay Specific Diluent containing borate buffer. Preservative: Antimicrobial Agents.

- * **[PRE-TREATMENT REAGENT 1]** 1 or 4 Bottles (50.2 mL per 100 test bottles/50.2 mL per 500 test bottles) Folate Pre-Treatment Reagent 1 containing potassium hydroxide.
- * **[PRE-TREATMENT REAGENT 2]** 1 or 4 Bottles (6.6 mL per 100 test bottles/27.0 mL per 500 test bottles) Folate Pre-Treatment Reagent 2 containing dithiothreitol (DTT) in acetic acid buffer with EDTA.
- * **[SPECIMEN DILUENT]** 1 or 4 Bottles (5.5 mL per 100 test bottles/25.0 mL per 500 test bottles) Folate Specimen Diluent containing TRIS buffer with protein stabilizer (human albumin [donor units of which are nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2]). Preservative: Sodium Azide.

Manual Diluent

ARCHITECT Folate Manual Diluent (6C12-50)

- * **[MANUAL DILUENT]** 1 Bottle (4 mL) Folate Manual Diluent containing TRIS buffer with protein stabilizer (human albumin [donor units of which are nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2]). Preservative: Sodium Azide.

Other Reagents

ARCHITECT Folate RBC Lysis Diluent (6C12-40)

- * **[RBC LYSIS DILUENT]** 1 Bottle (12.5 mL) Folate RBC Lysis Diluent containing citric acid and guanidine hydrochloride. Preservative: Antimicrobial Agent.

Folate Lysis Reagent (9C13-60)

- * **[LYSIS REAGENT]** 4 Bottles (285-385 mg each) Folate Lysis Reagent containing ascorbic acid and guanidine hydrochloride.

ARCHITECT r Pre-Trigger Solution

- * **[PRE-TRIGGER SOLUTION]** Pre-Trigger Solution containing 1.32% (w/v) hydrogen peroxide.

ARCHITECT r Trigger Solution

- * **[TRIGGER SOLUTION]** Trigger Solution containing 0.35N sodium hydroxide.

ARCHITECT r Wash Buffer

- * **[WASH BUFFER]** Wash Buffer (concentrated or diluted) containing phosphate buffered saline solution. Preservative: Antimicrobial Agent.

WARNINGS AND PRECAUTIONS

- * **[IVB]** For *In Vitro* Diagnostic Use.
- * Package insert instructions must be followed accordingly. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

Safety Precautions

- * **CAUTION:** This product contains human sourced and/or potentially infectious components. For a specific listing, refer to the REAGENTS section of this package insert. Donor units used to make these components have been tested and found to be nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2 by FDA licensed tests. No known test method can offer complete assurance that products derived from human sources or inactivated microorganisms will not transmit infection. Therefore, all human sourced materials should be considered potentially infectious. It is recommended that these reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens,⁶ Biosafety Level 2⁷ or other appropriate biosafety practices^{8,9} should be used for materials that contain or are suspected of containing infectious agents.
- * Folate Pre-Treatment Reagent 1 contains potassium hydroxide (KOH) and is classified per applicable European Community (EC) Directives as: Corrosive (C). The following are the appropriate Risk (R) and Safety (S) phrases.



- R34 Causes burns.
- S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- S33 This material and its container must be disposed of in a safe way.
- S36/37/39 Wear suitable protective clothing, gloves, and eye/face protection.
- S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

- This product contains sodium azide; for a specific listing, refer to the REAGENTS section. Contact with acids liberates very toxic gas. This material and its container must be disposed of in a safe way.

- ARCHITECT i Trigger Solution contains sodium hydroxide (NaOH) and is classified per applicable European Community (EC) Directives as: Irritant (Xi). The following are the appropriate Risk (R) and Safety (S) phrases.



R61	Risk of serious damage to eyes.
S25	Avoid contact with eyes.
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S36	This material and its container must be disposed of in a safe way.
S36/37	Wear suitable protective clothing and eyewear protection.
S46	If swallowed, seek medical advice immediately and show this container or label.

- For product not classified as dangerous per European Directive 1999/45/EC - Safety data sheet available for professional user on request.
- For information on the safe disposal of sodium azide and a detailed discussion of safety precautions during system operation, refer to the ARCHITECT System Operations Manual, Section 8.

Handling Precautions

- Do not use reagent kits beyond the expiration date.
- Do not mix reagents from different reagent kits.
- Prior to loading the ARCHITECT Folate Reagent Kit on the system for the first time, the microparticle bottle requires mixing to resuspend microparticles that have settled during shipment. For microparticle mixing instructions, refer to the PROCEDURE, Assay Procedure section of this package insert.
- Septa **MUST** be used to prevent reagent evaporation and contamination and to ensure reagent integrity. Reliability of assay results cannot be guaranteed if septa are not used according to the instructions in this package insert.
- Prolonged exposure of Folate Pre-Treatment Reagent 1 to air without septum in place may compromise performance.
- To avoid contamination, wear clean gloves when placing a septum on an uncapped reagent bottle.
- Prior to placing the septum on an uncapped reagent bottle, squeeze the septum in half to confirm that the slits are open. If the slits appear sealed, continue to gently squeeze the septum to open the slits.
- Once a septum has been placed on an open reagent bottle, do not invert the bottle as this will result in reagent leakage and may compromise assay results.
- Over time, residual liquids may dry on the septum surface. These are typically dried salts which have no effect on assay efficacy.
- For a detailed discussion of handling precautions during system operation, refer to the ARCHITECT System Operations Manual, Section 7.

Storage Instructions

-  The ARCHITECT Folate Reagent Kit, Folate RBC Lysis Diluent, and Folate Manual Diluent must be stored at 2-8°C and may be used immediately after removal from 2-8°C storage. Refer to the Handling Precautions section in this package insert for additional information.

NOTE: The ARCHITECT Folate Reagent Kit is shipped cold and should be stored at 2-8°C after receipt. Refer to the Handling Precautions section in this package insert for additional information.

- Unreconstituted Folate Lysis Reagent must be stored at 15-30°C. After reconstitution, store the Folate Lysis Reagent at 2-8°C for up to 7 days. Write the expiration date of the reconstituted Folate Lysis Reagent on the space provided on the bottle. Do not exceed the expiration date printed on the bottle.
- When stored and handled as directed, reagents are stable until the expiration date.
- The ARCHITECT Folate Reagent Kit may be stored on board the ARCHITECT i System for a maximum of 30 days. After 30 days, the reagent kit must be discarded. For information on tracking onboard time, refer to the ARCHITECT System Operations Manual, Section 5.
- Reagents may be stored on or off the ARCHITECT i System. If reagents are removed from the system, store them at 2-8°C (with septa and replacement caps) in an upright position. For reagents stored off the system, it is recommended that they be stored in their original trays and boxes to ensure they remain upright. If the microparticle bottle does not remain upright (with a septum installed) while in refrigerated storage off the system, the reagent kit must be discarded. After reagents are removed from the system, you must initiate a scan to update the onboard stability timer.

Indications of Reagent Deterioration

When a control value is out of the specified range, it may indicate deterioration of the reagents or errors in technique. Associated test results may be invalid and may require retesting. Assay recalibration may be necessary. For troubleshooting information, refer to the ARCHITECT System Operations Manual, Section 10.

INSTRUMENT PROCEDURE

- The ARCHITECT Folate (assay number 71) and/or Folate RBC (assay number 73) assay file(s) must be installed on the ARCHITECT i System from the ARCHITECT Assay CD ROM prior to performing the assay. For detailed information on assay file installation and on viewing and editing assay parameters, refer to the ARCHITECT System Operations Manual, Section 2.
 - ARCHITECT maintenance procedure 804r Daily Maintenance (version 5 or higher) must be installed on the ARCHITECT i System prior to performing the assay. For information on installing and deleting maintenance procedures, refer to the ARCHITECT System Operations Manual Section 2.
 - ARCHITECT maintenance procedure 804r Daily Maintenance (version 5 or higher) must be run at a minimum once every 24 hours. For laboratories processing a higher volume of Bt 2 and Folate tests on a single module, this procedure must be run more than once in a 24 hour period.
 - If you run Bt 2 and Folate on a single module and you run > 100 Bt 2 or > 100 Folate tests in 24 hours, perform the 804r Daily Maintenance procedure (version 5 or higher) after every 100 Bt 2 or 100 Folate tests run.
 - Refer to LIMITATIONS OF THE PROCEDURE for additional information.
- NOTE: If you run Folate but no Bt 2, 804r Daily Maintenance is only required once every 24 hours regardless of the number of Folate tests run.

- When configuring the host for the Folate RBC assay, set the appropriate default dilutions:
 - If running whole blood specimens or whole blood controls, configure the default dilution as "RBC DIL".
 - If running controls other than whole blood controls, configure the default dilution as "UNDILUTED".
- For information on printing assay parameters, refer to the ARCHITECT System Operations Manual, Section 5.
- For a detailed description of system procedures, refer to the ARCHITECT System Operations Manual.
- The default result unit for the ARCHITECT Folate assay is ng/mL. An alternate result unit, nmol/L, may be selected for reporting results by editing assay parameter "Result concentration units", to nmol/L. The conversion factor used by the system is 2.285.

SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

- Human serum, plasma, or whole blood specimens to be tested for folate should be protected from light.^{11,12}
- Serum or plasma specimens should be collected from fasting individuals. Recent food intake may appreciably increase the folate concentration.¹¹
- The ARCHITECT i System does not provide the capability to verify specimen type. It is the responsibility of the operator to verify the correct specimen types are used when running the ARCHITECT Folate or Folate RBC assay(s).
- Use caution when handling patient specimens to prevent cross contamination. Use of disposable pipettes or pipette tips is recommended.
- Do not use hemolyzed specimens. Serum or plasma specimens that are hemolyzed will give falsely elevated folate levels.
- For optimal results, serum or plasma specimens should be free of fibrin, red blood cells or other particulate matter. Serum or plasma specimens containing red blood cells may give falsely elevated folate levels.
- For optimal results, inspect all samples for bubbles. Remove bubbles with an applicator stick prior to analysis. Use a new applicator stick for each sample to prevent cross contamination.
- Multiple freeze/thaw cycles of specimens should be avoided. Specimens must be mixed THOROUGHLY after thawing, by LOW speed vortexing or by gently inverting, and centrifuged prior to use to remove particulate matter, if present, to ensure consistency in the results.

- When shipped, specimens must be packaged and labeled in compliance with applicable state, federal, and international regulations covering the transport of clinical specimens and infectious substances. Specimens must be shipped frozen at -10°C or colder and protected from light. Prior to freezing, it is recommended that:
 - serum be removed from the clot or serum separator tube.
 - plasma be separated from the red blood cells.

Serum Specimens

- Human serum (including serum collected in serum separator tubes) may be used in the ARCHITECT Folate assay (assay number 71). Follow the manufacturer's processing instructions for serum collection tubes.
- Ensure that complete clot formation in serum specimens has taken place prior to centrifugation. Some specimens, especially those from patients receiving anticoagulant or thrombolytic therapy may exhibit increased clotting time. If the specimen is centrifuged before a complete clot forms, the presence of fibrin may cause erroneous results.
- Remove serum from clot as soon as possible after complete clot formation. If testing will not be performed immediately, serum specimens may be stored either at 2-8°C for up to 7 days or frozen (-10°C or colder) for up to 30 days prior to being tested.

Plasma Specimens

- Human plasma (collected in lithium heparin tubes) may be used in the ARCHITECT Folate assay (assay number 71). Follow the manufacturer's processing instructions for plasma collection tubes. Other anticoagulants have not been verified for use with the ARCHITECT Folate assay.
- Do not use human plasma collected in tripotassium EDTA tubes.
- Remove plasma from red blood cells as soon as possible upon receipt. If testing will not be performed immediately, plasma specimens may be stored either at 2-8°C for up to 24 hours or frozen (-10°C or colder) for up to 7 days prior to being tested.

Whole Blood Specimens

- Whole blood (collected in tripotassium EDTA tubes) may be used in the ARCHITECT Folate RBC assay (assay number 73). Follow the manufacturer's processing instructions for collection tubes. Other anticoagulants have not been verified for use with the ARCHITECT Folate RBC assay.
- Do not use whole blood collected in lithium heparin tubes.
- If testing will not be performed immediately, whole blood specimens may be stored at 2-8°C for up to 24 hours or frozen (-10°C or colder) for up to 7 days prior to being tested.
- For red blood cell folate measurements, determine the hematocrit of each specimen prior to storage and prepare a red blood cell hemolyate as described in Preparation of Red Blood Cell Hemolyate in the PROCEDURE section of this package insert.

PROCEDURE

Materials Provided

- eC12-20, eC12-25, eC1 2-30 ARCHITECT Folate Reagent Kit

Materials Required but not Provided

- ARCHITECT i Assay CD-ROM
- eC12-01 ARCHITECT Folate Calibrators
- eC1 2-10 ARCHITECT Folate Low and Medium Controls
- eC12-11 ARCHITECT Folate High Control
- eC1 2-40 ARCHITECT Folate RBC Lysis Diluent
- eC12-50 ARCHITECT Folate Manual Diluent
- eC13-60 Folate Lysis Reagent
- ARCHITECT i **PRE-TRIGGER SOLUTION**
- ARCHITECT i **TRIGGER SOLUTION**
- ARCHITECT i **WASH BUFFER**
- ARCHITECT i **REACTION VESSELS**
- ARCHITECT i **SAMPLE CUPS**
- ARCHITECT i **SEPTUM**
- ARCHITECT i **REPLACEMENT CAPS**
- For information on materials required for maintenance procedures, refer to the ARCHITECT System Operations Manual, Section 9.
- Pipettes or pipette tips (optional) to deliver the volumes specified on the patient or control order screen.

Determination of Folate in Red Blood Cells (RBC)

Reconstitution of Folate Lysis Reagent

- Reconstitute one bottle of the Folate Lysis Reagent using 30 mL distilled or deionized water.

- Cap the reagent and mix by inversion until dissolved. The reconstituted lysis reagent is stable for 7 days at 2-8°C not to exceed the expiration date printed on the bottle.
- Write the expiration date of the reconstituted lysis reagent on the space provided on the bottle label.

Preparation of Red Blood Cell Hemolyate: Same Day Procedure

- Mix whole blood tube by inversion or vortexing to assure a homogenous sample.
- Pipette 100 µL of whole blood sample to 1.0 mL of reconstituted Folate Lysis Reagent.
- Mix by inversion or vortexing and allow to stand at room temperature (15-30°C) for 60 minutes (± 5 minutes).
- Add 100 µL ARCHITECT Folate RBC Lysis Diluent to 100 µL of hemolyzed sample and initiate assay within 2 hours.

Assay Procedure (Folate and Folate RBC Assays)

- Before loading the ARCHITECT Folate Reagent Kit on the system for the first time, the microparticle bottle requires mixing to resuspend microparticles that have settled during shipment.
 - Invert the microparticle bottle 30 times.
 - Visually inspect the bottle to ensure microparticles are resuspended. If microparticles are still adhered to the bottle, continue to invert the bottle until the microparticles have been completely resuspended.
 - Once the microparticles have been resuspended, remove and discard the cap. Wearing clean gloves, remove a septum from the bag.
 - Squeeze the septum in half to confirm that the slits are open. Carefully snap the septum onto the top of the bottle.
 - If the microparticles do not resuspend, DO NOT USE. Contact your Abbott representative.
- Order calibration, if necessary.
 - For information on ordering calibrations, refer to the ARCHITECT System Operations Manual, Section 6.
- Order tests.
 - Select the appropriate assay protocol.
 - If running a serum or plasma specimen/control, select Folate (assay number 71, "UNDILUTED").
 - If running an automated dilution on a serum or plasma specimen, select the 1:2 protocol of Folate (assay number 71, "1:2").
 - If running a whole blood specimen or whole blood control, select Folate_RBC (assay number 73, "RBC DIL").
 - If running controls other than whole blood controls with the Folate RBC assay, select the undiluted protocol of Folate_RBC (assay number 73, "UNDILUTED").
 - For additional information on ordering patient specimens, calibrators, and controls and for general operating procedures, refer to the ARCHITECT System Operations Manual, Section 5.
- Load the ARCHITECT Folate Reagent Kit on the ARCHITECT i System. Verify that all the necessary assay reagents are present. Ensure that septums are present on all reagent bottles. Refer to ARCHITECT Operations Manual, section 5 for details on how to load reagents.
- The minimum sample cup volume is calculated by the system and is printed on the Orderlist report. No more than 10 replicates may be sampled from the same sample cup. To minimize the effects of evaporation, verify adequate sample cup volume is present prior to running the test.
 - Priority: 85 µL for the first ARCHITECT Folate test plus 35 µL for each additional ARCHITECT Folate test from the same sample cup.
 - ≤ 3 hours on board: 150 µL for the first ARCHITECT Folate test plus 35 µL for each additional ARCHITECT Folate test from the same sample cup.
 - If using primary or aliquot tubes, use the sample gauge to ensure sufficient patient specimen is present.
- Prepare calibrators and controls.
 - Refer to the appropriate ARCHITECT Folate calibrator and control package inserts before using the Folate Calibrators, Low and Medium Controls, and High Control.
 - To obtain the recommended volume requirements for the ARCHITECT Folate calibrators and controls, hold the bottles vertically and dispense 6 drops of each calibrator or 6 drops of each control into each respective sample cup.
 - NOTE: It is very important to return the ARCHITECT Folate Calibrators, Low and Medium Controls, and High Control to the correct storage conditions immediately after use, as follows:

Return calibrators to their carton and store at -10°C or colder.
 Return low and medium controls to their carton and store at 2-8°C.
 Return the high control to 2-8°C storage, if the expiration date written on the vial at the time of thawing has not been exceeded.

- Load samples.
 - For information on loading samples, refer to the ARCHITECT System Operations Manual, Section 5.
- Press RUN. The ARCHITECT System performs the following functions:
 - Moves the sample to the aspiration point
 - Loads a reaction vessel (RV) into the process path
 - Aspirates and transfers sample into the RV
 - Advances the RV one position and adds Pre-Treatment Reagent 2 to the sample
 - Mixes and incubates
 - Transfers an aliquot of sample/Pre-Treatment Reagent 2 to a second RV
 - Advances the RV one position and adds Pre-Treatment Reagent 1 into the RV
 - Mixes and incubates
 - Transfers an aliquot of the pretreated sample to a third RV
 - Advances the RV one position and transfers microparticles and assay specific diluent into the RV
 - Mixes, incubates, and washes the reaction mixture
 - Adds conjugate to the RV
 - Mixes, incubates and washes the reaction mixture
 - Adds Pre-trigger and Trigger Solutions
 - Measures chemiluminescent emission to determine the quantity of folate in the sample
 - Aspirates contents of RV to liquid waste and unloads RV to solid waste
 - Calculates the result
- For optimal performance, it is important to follow the routine maintenance procedures defined in the ARCHITECT System Operations Manual, Section 9. If your laboratory requires more frequent maintenance, follow these procedures.

Specimen Dilution Procedures (for folate serum or plasma determinations only)

Specimens with a folate serum or plasma value exceeding 20.0 ng/mL are flagged with the code "> 20.0" and may be diluted using either the Automated Dilution Procedure or the Manual Dilution Procedure.

Automated Dilution Procedure

- If using the Automated Dilution Protocol (assay number 71, 1:2 Protocol), the system performs a 1:2 dilution. The system will use the dilution factor to automatically calculate the concentration of the sample before dilution. This will be the reported result.

Manual Dilution Procedure

- The suggested dilution for ARCHITECT Folate is 1:2. It is recommended dilutions not exceed 1:4.
- For a 1:2 dilution, add 100 µL of the patient specimen to 100 µL of ARCHITECT Folate Manual Diluent (6C12-53).
- The operator must enter the dilution factor in the Patient or Control order screen. The system will use this dilution factor to automatically calculate the concentration of the sample before dilution. This will be the reported result. The dilution should be performed so that the diluted result reads ≥ 0.8 ng/mL.
- For detailed information on ordering dilutions, refer to the ARCHITECT System Operations Manual, Section 5.

Calibration

- The ARCHITECT Folate and ARCHITECT Folate RBC assays require separate calibrations.
- To perform an ARCHITECT Folate calibration, test Calibrators A through F in duplicate. A single sample of all levels of folate controls must be tested to evaluate the assay calibration. Ensure that assay control values are within the concentration ranges specified in the control package inserts. Calibrators should be priority loaded.
- Calibration Range: 0.0-20.0 ng/mL.
- Once an ARCHITECT Folate calibration is accepted and stored, all subsequent samples may be tested off the appropriate calibration curve without further calibration unless one or more of the following occur:
 - A reagent kit with a new lot number is used.
 - Controls are out of range.
- For detailed information on how to perform an assay calibration, refer to the ARCHITECT System Operations Manual, Section 6.

QUALITY CONTROL PROCEDURES

The recommended control requirement for the ARCHITECT Folate assay is that a single sample of all control levels be tested once every 24 hours each day of use. If the quality control procedures in your laboratory require more frequent use of controls to verify test results, follow your laboratory-specific procedures. Ensure that assay control values are within the concentration ranges specified in the control package insert.

Verification of Assay Claims

For protocols to verify package insert claims, refer to the ARCHITECT System Operations Manual, Appendix B. The ARCHITECT Folate assay belongs to method group 1.

RESULTS

The ARCHITECT Folate assay utilizes a 4 Parameter Logistic Curve fit (4PLC, Y weighted) data reduction method to generate a calibration curve.

Alternate Result Units

- The default result unit for the ARCHITECT Folate assay is ng/mL. When the alternate result unit, nmol/L, is selected, the conversion factor used by the system is 2.265.

- Conversion Formula: (concentration in ng/mL) x (2.265) = nmol/L

Calculation of Red Blood Cell Folate Concentration (for Folate RBC Assay only):

Sample results obtained with the Folate RBC assay (assay number 73 utilizing "RBC DIL" protocol) are multiplied by a factor of 22. The reported result therefore is automatically corrected for dilutions required in the preparation of red blood cell hemolysate.

To calculate the RBC folate concentration from the ARCHITECT Folate RBC reported test result, use the following formula:

$$\text{RBC Folate Concentration (ng/mL)} = \frac{\text{ARCHITECT RBC Reported Test Result (ng/mL)} \times 100}{\% \text{ Hematocrit}}$$

Example:

ARCHITECT Folate RBC Reported Test Result = 11 0.0 ng/mL

% Hematocrit = 47

$$\frac{110.0}{47} \times 100 = 234.0 \text{ ng/mL}$$

Calculation of Corrected Red Blood Cell Folate Concentration (for Folate RBC Assay only):

Folate concentrations from serum or plasma are very small as compared to RBC folate concentrations, in most cases. It is possible for the serum or plasma folate concentration to be within or above the expected normal range while the RBC folate concentration is below the expected normal range. The following calculation will correct for serum or plasma folate concentrations:

Corrected RBC Folate Concentration (ng/mL) =

$$\text{RBC Folate Concentration (ng/mL)} \times \left[\frac{100 - \% \text{ Hematocrit}}{\% \text{ Hematocrit}} \right]$$

* Serum or Plasma Result

Example:

RBC Folate Concentration = 234.0 ng/mL

ARCHITECT Folate Test Result = 0.5 ng/mL,

% Hematocrit = 47

$$234.0 \text{ ng/mL} \times \left[0.5 \times \left[\frac{100-47}{47} \right] \right] = 223.3 \text{ ng/mL}$$

Formulas and examples indicate ng/mL as the result unit. If the chosen ARCHITECT Folate result is nmol/L, the final result would be in nmol/L.

LIMITATIONS OF THE PROCEDURE

- For diagnostic purposes the ARCHITECT Folate assay result should be used in conjunction with other data; e.g. other clinical testing, symptoms, clinical impressions, etc.
- If the folate level is inconsistent with clinical evidence, additional testing is suggested to confirm the result.
- Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show either falsely elevated or depressed values when tested with assay kits that employ mouse monoclonal antibodies.^{12,13}
- Heterophilic antibodies in human serum can react with reagent immunoglobulins, interfering with in vitro immunoassays.¹⁴ Patients routinely exposed to animals or to animal serum products can be prone to this interference and anomalous values may be observed.
- Serum or plasma containing red blood cells may give falsely elevated folate levels. These samples should be centrifuged prior to use. Serum or plasma samples that are hemolyzed will give falsely elevated folate levels.

- Serum and plasma specimens from patients with renal impairment or failure (including dialysis patients) may exhibit varying degrees of falsely depressed ARCHITECT Folate values, which could give dilution over-recovery. Therefore, to evaluate folate patients with renal impairment or failure, it is recommended that ARCHITECT Folate values below the expected range be confirmed by an alternate folate method such as the ARCHITECT Folate RBC assay.
- Methotrexate, aminopterin, and folic acid (Leucovorin) are chemotherapeutic agents whose molecular structures are similar to folate. These agents cross react with folate binding protein in folate assays.¹⁴
- Samples to be tested for folate should be protected from light.¹⁵ Light accelerates the degradation of folate.
- Representative performance data are given in the EXPECTED VALUES and SPECIFIC PERFORMANCE CHARACTERISTICS sections. Results obtained in individual laboratories may vary.
- Accumulation of denatured protein from the pretreatment step in the sample probe may impact results of other assays on the ARCHITECT System. ARCHITECT maintenance procedure 8041 *Daily Maintenance* (version 5 or higher) must be run to eliminate this effect. Refer to the INSTRUMENT PROCEDURE section for instructions.

Expected Values

Folate Normals

The ARCHITECT Folate assay was used to evaluate two sets of specimens. One set consisted of 203 serum specimens from fasting individuals. These serum specimens had normal folate concentrations, as determined by a commercially available folate assay. The other consisted of 244 whole blood specimens collected from individuals where fasting was not required. These whole blood specimens had normal folate concentrations, as determined by a commercially available folate radioassay.

The median ARCHITECT Folate concentration obtained in this study from the serum population was 12.9 ng/mL with a range of 1.8 to 70.8 ng/mL. The central 95% interval of this serum population defined an expected normal range of 2.7 to 34.0 ng/mL. The median ARCHITECT Folate RBC concentration obtained in this study from the whole blood population was 305.6 ng/mL with a range of 101.1 to 974.8 ng/mL. The central 95% interval of this whole blood population defined an expected normal range of 159.8 to 713.6 ng/mL.

Folate Deficients

The ARCHITECT Folate assay was used to evaluate 16 serum specimens that were considered deficient in folate concentration, as determined by a commercially available folate assay. The mean ARCHITECT Folate concentration for this population was 1.7 ng/mL and the median ARCHITECT Folate concentration was 1.6 ng/mL. The concentrations obtained from this study ranged from 0.9 to 2.3 ng/mL. The 97.5 percentile of these specimens defined the upper limit of the expected deficient range to be 2.3 ng/mL.

The ARCHITECT Folate assay was not used to evaluate RBC specimens that were considered deficient in folate. However, literature cites deficient folate levels in tissue as usually associated with RBC folate levels of < 100 ng/mL.¹⁶

It is recommended that each laboratory establish its own normal and deficient ranges, which may be unique to the population it serves depending upon geographical, patient, dietary, or environmental factors.

SPECIFIC PERFORMANCE CHARACTERISTICS

Precision

The ARCHITECT Folate assay was evaluated for precision by following the National Committee for Clinical Laboratory Standards (NCCLS) Protocol EPS-A.¹⁷ A three-member buffered protein based panel (1, 2, and 3) and a two-member processed human serum panel (4 and 5) were assayed in replicates of two at two separate times of day, for 20 days on two instruments using two lots of reagent and a single calibration for each lot.

The results obtained from this study demonstrated the ARCHITECT Folate assay exhibits total imprecision of < 10% across the assay's calibration range. Data from this study are summarized in the following table:

Panel Member	Reagent Lot	Instrument	n	Mean Conc. Value (ng/mL)	Within Run SD	Run %CV	Total SD	%CV
1	1	1	60	2.7	0.10	3.9	0.13	4.8
1	2	1	60	2.7	0.08	3.0	0.15	5.3
1	1	2	60	2.5	0.18	6.2	0.20	8.1
1	2	2	60	2.6	0.14	5.2	0.20	7.6
2	1	1	60	7.2	0.19	1.8	0.19	2.7
2	2	1	60	7.4	0.14	1.9	0.21	2.9
2	1	2	60	7.0	0.20	2.9	0.32	4.5
2	2	2	60	7.2	0.19	2.7	0.34	4.7
3	1	1	60	15.2	0.39	2.2	0.43	2.8
3	2	1	60	15.2	0.39	2.6	0.47	3.1
3	1	2	60	15.0	0.34	2.3	0.77	5.2
3	2	2	60	15.0	0.38	2.5	0.64	4.3
4	1	1	60	4.1	0.11	2.6	0.27	6.4
4	2	1	60	4.2	0.13	3.0	0.28	6.5
4	1	2	60	3.9	0.18	4.6	0.32	8.3
4	2	2	60	4.0	0.17	4.3	0.38	9.5
5	1	1	60	12.7	0.18	1.4	0.56	4.4
5	2	1	60	12.9	0.20	2.2	0.56	4.4
5	1	2	60	12.9	0.33	2.7	0.72	5.9
5	2	2	60	12.8	0.32	2.5	0.78	6.2

Dilution Linearity

The ARCHITECT Folate assay was evaluated for dilution linearity by diluting 10 human serum specimens, which had undiluted values that ranged between 10.0 and 20.0 ng/mL. These specimens were diluted using the Automated Dilution Procedure (1:2 dilution by the ARCHITECT System) and the Manual Dilution Procedure. In the Manual Dilution Procedure, 1:2 and 1:4 dilutions of specimens were made in ARCHITECT Folate Manual Diluent. All specimens and dilutions were tested by the ARCHITECT Folate assay. In this study, the ARCHITECT Folate assay exhibited an average dilution recovery of within 20% of the expected results with both dilution methods. The results from this study are summarized in the following tables.

Sample ID	Mean Undiluted Value (ng/mL)	Dilution Method	Mean Expected Value (ng/mL)	Mean Observed Value (ng/mL)	Percent Recovery
1	11.0	1:2 Auto Dilute	5.5	5.1	92.4
		1:2 Manual Dilute	5.5	5.2	94.8
		1:4 Manual Dilute	2.7	2.8	102.1
2	11.2	1:2 Auto Dilute	5.6	5.0	89.0
		1:2 Manual Dilute	5.6	5.1	90.8
		1:4 Manual Dilute	2.8	2.8	98.5
3	11.4	1:2 Auto Dilute	5.7	5.4	95.0
		1:2 Manual Dilute	5.7	5.6	97.4
		1:4 Manual Dilute	2.9	3.0	103.0
4	10.7	1:2 Auto Dilute	5.3	5.3	98.8
		1:2 Manual Dilute	5.3	5.4	100.8
		1:4 Manual Dilute	2.7	2.9	106.8
5	17.3	1:2 Auto Dilute	8.5	8.9	104.7
		1:2 Manual Dilute	8.5	8.8	103.1
		1:4 Manual Dilute	4.3	4.3	100.0
6	12.8	1:2 Auto Dilute	6.4	6.1	95.0
		1:2 Manual Dilute	6.4	5.8	91.4
		1:4 Manual Dilute	3.2	3.0	93.0
7	13.8	1:2 Auto Dilute	6.9	6.7	96.9
		1:2 Manual Dilute	6.9	6.6	95.9
		1:4 Manual Dilute	3.4	3.4	97.8
8	17.5	1:2 Auto Dilute	8.7	9.7	111.1
		1:2 Manual Dilute	8.7	10.1	115.3
		1:4 Manual Dilute	4.4	4.9	111.5
9	11.2	1:2 Auto Dilute	5.6	5.1	91.3
		1:2 Manual Dilute	5.6	5.4	96.1
		1:4 Manual Dilute	2.8	2.7	96.5
10	15.3	1:2 Auto Dilute	6.8	6.9	99.5
		1:2 Manual Dilute	6.8	6.9	99.4
		1:4 Manual Dilute	3.3	3.2	96.5

Dilution Method	Average Percent Recovery*	Recovery Range
1:2 Auto Dilute	97.0%	89.0 - 111.1%
1:2 Manual Dilute	97.8%	89.4 - 111.5%
1:4 Manual Dilute	101.2%	95.0 - 111.5%

$$* \% \text{ Recovery} = \frac{\text{Mean Observed Value (ng/mL)}}{\text{Mean Expected Value (ng/mL)}} \times 100$$

Analytical Sensitivity

The analytical sensitivity of the ARCHITECT Folate assay was evaluated by repeated testing of the ARCHITECT Folate Calibrator A (n = 24 runs). The analytical sensitivity is defined as the concentration at two standard deviations from the mean FLU value of the ARCHITECT Folate Calibrator A (0.0 ng/mL) and represents the lowest measurable concentration of folate that can be distinguished from zero. In this study, the analytical sensitivity was calculated to be ≤ 0.8 ng/mL at a 95% confidence level.

Specificity

The specificity of the ARCHITECT Folate assay was evaluated by testing cross-reactivity with aminopterin, folic acid, and methotrexate in processed human serum containing endogenous folate. Therapeutic levels of these drugs can greatly exceed the levels tested in this study and are expected to interfere with the ARCHITECT Folate assay.¹³ The results from this study are shown in the following table:

ARCHITECT Folate Cross-Reactivity

Potential Cross-Reactant	Concentration (ng/mL)	Cross-Reactivity (%)
Aminopterin	48	1.2
Folic Acid	90	-0.3
Methotrexate	100	1.1

Interference

Potential interference from bilirubin, triglycerides, and protein were evaluated in the ARCHITECT Folate assay, as described in the NCCLS Protocol EP7-P.¹⁴ Human serum specimens containing endogenous folate in the normal range were supplemented with the potentially interfering compounds. In this study, the ARCHITECT Folate assay demonstrated the percent interferences as follows:

- Bilirubin < 1.0% at 20 mg/dL
- Triglycerides < 1.0% at 3000 mg/dL
- Protein < 10% at 2 g/dL
- Protein < 10% at 12 g/dL

Accuracy by Correlation

The ARCHITECT Folate assay was compared to a commercially available folate radioassay. Two evaluations were performed, one with serum specimens and a second with whole blood specimens. The results from these evaluations are shown in the following tables.⁴

ARCHITECT Folate vs. a Commercially Available Folate Radioassay

Method	Specimen Type	n	Intercept	Slope	Correlation Coefficient
Least Squares Linear Regression	Serum	241	1.4	0.93	0.904
Passing-Bablok Linear Regression	Serum	241	-0.0	1.05	0.904
Least Squares Linear Regression	Whole Blood	244	-54.7	1.05	0.904
Passing-Bablok Linear Regression	Whole Blood	244	-59.9	1.10	0.904

† A linear regression method with no special assumptions regarding the distribution of the samples and measurement errors.¹⁴

Concentration Ranges of Specimens Tested by ARCHITECT Folate and a Commercially Available Folate Radioassay

Assay	Specimen Type	n	Concentration Range
ARCHITECT Folate	Serum	241	3.0 - 20.0 ng/mL
Radioassay Folate	Serum	241	3.0 - 19.4 ng/mL
ARCHITECT Folate	Whole Blood	244	101.1 - 974.8 ng/mL
Radioassay Folate	Whole Blood	244	113.0 - 899.9 ng/mL

* Folate results in individual laboratories may vary from these.

BIBLIOGRAPHY

1. Steinberg SE. Mechanisms of folate homeostasis. *Am J Physiol* 1984; 246(3):G319-34.
2. Appling DR. Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. *FASEB J* 1991; 5(12):2845-51.
3. Burtis CA, Ashwood ER, eds. *Tietz Textbook of Clinical Chemistry*. 3rd ed. Philadelphia, PA: WB Saunders; 1990:1803-5.

4. Bagg A, Jacobson RJ. Pernicious anemia and other megaloblastic anemias. In: Conn RG, ed. *Current Diagnosis*. Philadelphia, PA: WB Saunders; 1984:527-34.
5. Konas R. Folic acid, 1991: an update, with new recommended daily allowances. *South Med J* 1990; 83(12):1454-8.
6. US Department of Labor, Occupational Safety and Health Administration. 29 CFR Part 1910.1030, Occupational exposure to Bloodborne Pathogens.
7. US Department of Health and Human Services. *Biosafety in Microbiological and Biomedical Laboratories*, Fourth Edition. Washington, DC: US Government Printing Office, May 1990.
8. World Health Organization. *Laboratory Biosafety Manual*. Geneva: World Health Organization; 2004.
9. Clinical and Laboratory Standards Institute. *Protection of Laboratory Workers from Occupationally Acquired Infections: Approved Guideline - Third Edition*. CLSI Document M29-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2005.
10. Mastropaola CS, Wilson MA. Effect of light on serum B₁₂ and folate stability. *Clin Chem* 1993; 39(5):913.
11. Burtis CA, Ashwood ER, eds. *Tietz Textbook of Clinical Chemistry*. 2nd ed. Philadelphia, PA: WB Saunders; 1994:2056.
12. Primus FJ, Kelley EA, Hansen HJ, Goldbary DM, et al. "Sandwich"-type immunoassay of carcinoembryonic antigen in patients receiving murine monoclonal antibody therapy. *Clin Chem* 1988; 34:281-4.
13. Schroff HW, Foon KA, Beatty SM, Okham RK, Morgan AC Jr, et al. Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res* 1985; 45:879-85.
14. Boscatto LM, Stuart MC. Heterophilic antibodies: a problem for all immunoassays. *Clin Chem* 1985; 34:27-33.
15. D.S. Young. *Effects of drugs on clinical lab tests*, 5th ed. AACCC Press, Washington D.C. 2000; 1:3335-3336.
16. Jacobs and DeMott. *Laboratory Test Handbook*, 5th ed. Hudson, OH: Lexi-Comp, Inc. 2001:435.
17. National Committee for Clinical Laboratory Standards. *Evaluation of Precision Performance of Clinical Chemistry Devices - Approved Guideline*. NCCLS Document EP5-A. Villanova, PA: NCCLS, February 1990.
18. National Committee for Clinical Laboratory Standards. *Interference Testing in Clinical Chemistry: Proposed Guideline*. NCCLS Document EP7-P. Villanova, PA: NCCLS, 1988.
19. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. *J Clin Chem Clin Biochem* 1983; 21:709-20.

The following US Patents are relevant to the ARCHITECT r System or its components. There are other such patents and patent applications in the United States and worldwide.

5,488,846 5,543,524 5,545,739 5,565,570 5,880,819 5,783,600

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Abbott Laboratories
Diagnostics Division
Abbott Park, IL 60064 USA

November, 2005

NAME

ARCHITECT® Folate

INTENDED USE

The ARCHITECT Folate assay is a Chemiluminescent Microparticle Folate Binding Protein assay for the quantitative determination of folate in human serum, plasma, and red blood cells on the ARCHITECT *i* System.

SUMMARY AND EXPLANATION OF TEST

Folates are a class of vitamin compounds related to pteroylglutamic acid (PGA), which serve as cofactors in the enzymatic transfer of single carbon units in a variety of metabolic pathways.^{1,2} Folate mediated one-carbon metabolism represents one of the most important biochemical reactions that occur in cells. Folates are necessary for nucleic acid and mitochondrial protein synthesis, amino acid metabolism, and other cellular processes that involve single carbon transfers. Folates can serve as carbon donors or acceptors. Since different metabolic pathways require carbon groups with different levels of oxidation, cells contain numerous enzymes that change the oxidation state of carbon groups carried by folates² resulting in different metabolically active forms of folate. The predominant form of circulating folate is 5-methyltetrahydrofolic acid (5-mTHF). A methyl group is transferred from 5-mTHF to cobalamin in the pathway that links metabolism of folic acid and vitamin B₁₂.³

Folate deficiency can be caused by low dietary intake, malabsorption due to gastrointestinal diseases, inadequate utilization due to enzyme deficiencies or folate antagonist therapy, drugs such as alcohol and oral contraceptives, and excessive folate demand, such as during pregnancy and cellular proliferation disorders.⁴ Because deficiencies of both vitamin B₁₂ and folate can lead to megaloblastic (macrocytic) anemia, appropriate treatment requires differential diagnosis of the deficiency; thus, both vitamin B₁₂ and folate values are needed. Low serum folate levels reflect the first stage of negative folate balance, and precede tissue depletion.⁵ Low red-blood-cell folate values reflect the second stage of negative folate balance, and more closely correlate with tissue levels and megaloblastic anemia.

BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The ARCHITECT Folate assay is a two-step assay to determine the presence of folate in human serum, plasma, and red blood cells (RBC) using Chemiluminescent Microparticle Immunoassay (CMIA) technology with flexible assay protocols, referred to as Chemiflex™. Two pre-treatment steps mediate the release of folate from endogenous folate binding protein. In Pre-Treatment Step 1, sample and Pre-Treatment Reagent 2 (Dithiothreitol or DTT) are aspirated and dispensed into a reaction vessel (RV). In Pre-Treatment Step 2, an aliquot of sample/Pre-Treatment Reagent 2 mixture is aspirated and dispensed into a second RV. Pre-Treatment Reagent 1 (potassium hydroxide or KOH) is then added. An aliquot of the pretreated sample is transferred into a third RV, followed by the addition of Folate Binding Protein (FBP) coated paramagnetic microparticles and assay specific diluent. Folate present in the sample binds to the FBP coated microparticles. After washing, pteric acid-acridinium labeled conjugate is added and binds to unoccupied sites on the FBP coated microparticles. Pre-Trigger and Trigger Solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs). An inverse relationship exists between the amount of folate in the sample and the RLUs detected by the ARCHITECT *i* optical system.

* In the Folate RBC assay, a manual pre-treatment step converts RBC-bound folate to measurable folate, after which these samples are processed as described above.

For additional information on system and assay technology, refer to the ARCHITECT System Operations Manual, Section 3.

REAGENTS

Reagent Kit, 100 Tests/500 Tests

NOTE: Some kit sizes are not available in all countries, please contact your local distributor.

ARCHITECT Folate Reagent Kit (6C12-20, 6C12-25, 6C12-30)

- **MICROPARTICLES** 1 or 4 Bottles (6.6 mL per 100 test bottles/27.0 mL per 500 test bottles) Anti-Folate Binding Protein (mouse, monoclonal) coupled to microparticles affinity bound with Folate Binding Protein (bovine), in TRIS buffer with protein stabilizers (human albumin [donor units of which are nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2] and caprine). Minimum concentration: > 0.1% solids. Preservative: Antimicrobial Agents.
- **CONJUGATE** 1 or 4 Bottles (29.0 mL per 100 test bottles/29.0 mL per 500 test bottles) Pteric Acid (PTA)-acridinium labeled conjugate in MES buffer with protein stabilizer (porcine). Minimum concentration: 4 ng/mL. Preservative: Antimicrobial Agents.
- **ASSAY SPECIFIC DILUENT** 1 or 4 Bottles (5.7 mL per 100 test bottles/25.3 mL per 500 test bottles) Folate Assay Specific Diluent containing borate buffer. Preservative: Antimicrobial Agents.

- **PRE-TREATMENT REAGENT 1** 1 or 4 Bottles (50.2 mL per 100 test bottles/50.2 mL per 500 test bottles) Folate Pre-Treatment Reagent 1 containing potassium hydroxide.
- **PRE-TREATMENT REAGENT 2** 1 or 4 Bottles (6.6 mL per 100 test bottles/27.0 mL per 500 test bottles) Folate Pre-Treatment Reagent 2 containing dithiothreitol (DTT) in acetic acid buffer with EDTA.
- **SPECIMEN DILUENT** 1 or 4 Bottles (5.5 mL per 100 test bottles/25.9 mL per 500 test bottles) Folate Specimen Diluent containing TRIS buffer with protein stabilizer (human albumin [donor units of which are nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2]). Preservative: Sodium Azide.

Manual Diluent

ARCHITECT Folate Manual Diluent (6C12-50)

- **MANUAL DILUENT** 1 Bottle (4 mL) Folate Manual Diluent containing TRIS buffer with protein stabilizer (human albumin [donor units of which are nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2]). Preservative: Sodium Azide.

Other Reagents

ARCHITECT Folate RBC Lysis Diluent (6C12-40)

- **RBC LYSIS DILUENT** 1 Bottle (12.5 mL) Folate RBC Lysis Diluent containing citric acid and guanidine hydrochloride. Preservative: Antimicrobial Agent.

Folate Lysis Reagent (9C13-60)

- **LYSIS REAGENT** 4 Bottles (285-385 mg each) Folate Lysis Reagent containing ascorbic acid and guanidine hydrochloride.

ARCHITECT *i* Pre-Trigger Solution

- **PRE-TRIGGER SOLUTION** Pre-Trigger Solution containing 1.32% (w/v) hydrogen peroxide.

ARCHITECT *i* Trigger Solution

- **TRIGGER SOLUTION** Trigger Solution containing 0.35N sodium hydroxide.

ARCHITECT *i* Wash Buffer

- **WASH BUFFER** Wash Buffer (concentrated or diluted) containing phosphate buffered saline solution. Preservative: Antimicrobial Agent.

WARNINGS AND PRECAUTIONS

- **IVD For In Vitro Diagnostic Use.**
- Package Insert Instructions must be followed accordingly. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

Safety Precautions

- **CAUTION:** This product contains human sourced and/or potentially infectious components. For a specific listing, refer to the REAGENTS section of this package insert. Donor units used to make these components have been tested and found to be nonreactive for HBsAg, HIV-1 Ag, anti-HCV, and anti-HIV-1/HIV-2 by FDA licensed tests. No known test method can offer complete assurance that products derived from human sources or inactivated microorganisms will not transmit infection. Therefore, all human sourced materials should be considered potentially infectious. It is recommended that these reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens,⁶ Biosafety Level 2⁷ or other appropriate biosafety practices^{8,9} should be used for materials that contain or are suspected of containing infectious agents.
- Folate Pre-Treatment Reagent 1 contains potassium hydroxide (KOH) and is classified per applicable European Community (EC) Directives as: Corrosive (C). The following are the appropriate Risk (R) and Safety (S) phrases.



- | | |
|-----------|---|
| R34 | Causes burns. |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. |
| S35 | This material and its container must be disposed of in a safe way. |
| S36/37/39 | Wear suitable protective clothing, gloves, and eye/face protection. |
| S45 | In case of accident or if you feel unwell, seek medical advice immediately (show label where possible). |