VITAMIN B12 STATUS AND ABSORPTION USING HOLO-TRANS CobalamIN IN YOUNG MEN AND WOMEN

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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2006
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by

Kristina von Castel-Roberts
To my father Gerard David von Castel-Dunwoody and my uncle Günter von Castel; they left this world too early but will live in my heart forever.
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I would like to thank my committee members, doctors Lynn B. Bailey, Gail P.A. Kauwell, Jesse F. Gregory III, and Lee McDowell for their guidance and support. I would particularly like to thank Dr. Bailey and Dr. Kauwell for their daily encouragement during this exciting endeavor. Their dedication and achievement in the field of science has given me high standards to follow and has driven me to push myself to the best of my potential and beyond. I would like to thank Dr. Gregory for the contribution of his scientific and technical knowledge and Dr. McDowell for making me aware of how my animal nutrition education can help me better understand human nutrition. I would like to express my gratitude to the members of our laboratory team, especially David Maneval, Amanda Brown, Claire Edgemon, and Dr. Karla Shelnutt. It was their combined effort that made it possible to successfully conduct two human studies, teaching me the value of teamwork.

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<td>5-methylterahydrofolate</td>
</tr>
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<td>AI</td>
<td>Adequate Intake</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Apo-HC</td>
<td>Free haptocorrin</td>
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<tr>
<td>Apo-TC</td>
<td>Free transcobalamin</td>
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<td>B12</td>
<td>Vitamin B12</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CH$_3$</td>
<td>Methyl</td>
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<tr>
<td>CN</td>
<td>Cyano</td>
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<td>d</td>
<td>day</td>
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<tr>
<td>DHQ</td>
<td>National Cancer Institute Dietary History Questionnaire</td>
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<tr>
<td>EAR</td>
<td>Estimated Adequate Requirement</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
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<td>HC</td>
<td>Haptocorrin</td>
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<tr>
<td>Holo-HC</td>
<td>Holo-haptocorrin</td>
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<td>Holo-TC</td>
<td>Holo-transcobalamin</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>Hcy</td>
<td>Homocysteine</td>
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<td>IF</td>
<td>Intrinsinc factor</td>
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<td>IM</td>
<td>Intramuscular</td>
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<td>mo</td>
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<tr>
<td>MS</td>
<td>Methionine synthase</td>
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<td>nmol/L</td>
<td>Nanomoles per liter</td>
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<tr>
<td>NTD</td>
<td>Neural tube defect</td>
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<td>OH</td>
<td>Hydroxyl</td>
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<td>OSC</td>
<td>Optimal Solutions Corporation</td>
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<td>pmol/L</td>
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<td>RDA</td>
<td>Recommended dietary allowance</td>
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<td>TC-R</td>
<td>Transcobalamin receptor</td>
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<td>s</td>
<td>Seconds</td>
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<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SST</td>
<td>Serum separator tube</td>
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<td>UL</td>
<td>Upper limit</td>
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<td>US</td>
<td>United States</td>
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<tr>
<td>µmol/L</td>
<td>Micromoles per liter</td>
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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

VITAMIN B12 STATUS AND ABSORPTION USING HOLO-TRANSCOBALAMIN IN ADULT MEN AND WOMEN

By

Kristina von Castel-Roberts

December 2006

Chair: Lynn B. Bailey
Major Department: Food Science and Human Nutrition

Vitamin B12 (B12) status of young adults has been considered adequate based on estimated intakes that met the RDA; however, few studies in the US have evaluated B12 status of young adults using a panel of B12 biomarkers. Vitamin B12 deficiency impairs neurological function and increases other health-related risks. Early detection and determination of whether B12 deficiency is due to dietary insufficiency, a genetic abnormality, or malabsorption are critical to effective treatment.

The aims of the first study were to compare B12 status using numerous biomarkers in young adult non-supplement users consuming vegetarian and omnivorous diets, determine the level of intake associated with optimal B12 status, and determine if the transcobalamin (TC) 776C → G polymorphism affected B12 metabolism. Blood samples were collected for determination of holo-TC, B12, methylmalonic acid (MMA), and homocysteine (Hcy) (n = 388). Dietary B12 intake was assessed using a food frequency questionnaire. A surprisingly high incidence of B12 deficiency was observed in both vegetarians and omnivores. Relative to omnivores, vegetarians had a higher rate of B12 deficiency, with lower B12 and higher MMA concentrations. Vitamin B12 status improved with B12 intake above the RDA. No differences were detected between TC 776C→G genotypes for any biomarkers.
In the second study the magnitude and patterns of post-absorption changes in several B12 biomarkers were assessed. Subjects (n = 21) had blood drawn at 17 intervals over three days with administration of three 9 µg doses of B12 at 6 hour intervals on day one. Mean B12, holo-TC, TC saturation, and the ratio of holo-TC to B12 increased significantly from baseline at hour 24 only.

In conclusion, a high incidence of impaired B12 status was observed in otherwise healthy young adults. The data suggest that further assessment of the adequacy of the B12 RDA is warranted. Measurement of multiple B12 biomarkers may provide a more accurate assessment of B12 status than measurement of one biomarker alone. Holo-transcobalamin appears to be a sensitive indicator of B12 absorption and a holo-TC based absorption test should involve measurement at 0 and 24 hours. No effect of the TC 776C→G polymorphism was detected.
CHAPTER 1
INTRODUCTION

Vitamin B12

History

Vitamin B12 (B12) is one of the thirteen essential vitamins that humans must obtain from their diet. Vitamin B12 was the last vitamin to be discovered, due in part to the lack of a suitable animal model in which to study the B12-related disease pernicious anemia (1). Pernicious anemia, which literally means fatal anemia, has been reported in medical records as far back as the early 1800’s, although the condition was likely responsible for deaths well before then. The earliest studies of patients with pernicious anemia led to the knowledge that the disease was due to some ailment of the stomach; although no treatment was available and most patients died from the disease (2). In the early 20th century, Minot and Murphy determined that feeding liver to patients with pernicious anemia improved their condition, a discovery for which they received the Nobel prize in 1934 (3). Castle conducted a series of experiments comparing the treatment of pernicious anemia patients with partially digested beef, or beef incubated in gastric juice, versus undigested beef. Patients receiving the pre-digested beef improved while those receiving undigested beef did not, suggesting that some (intrinsic) factor within gastric juice interacted with the unidentified (extrinsic) factor in the beef (4-7). The final identification of this extrinsic factor was delayed until 1945 when it was discovered that the anti-anemia substance was required by Lactobacillus locus, finally providing a useful laboratory model (8). Vitamin B12 was crystallized in 1948 and was quickly identified as the illusive “extrinsic” anti-anemia factor (9-12). After these discoveries, B12 research proceeded rapidly as did our understanding of the vitamin’s functions.
Chemistry

Vitamin B12 is a complex water-soluble molecule with a molecular weight of 1655.38 daltons. The molecule is comprised of a cobalt atom centered in a corrin ring, with two coordinating ligands. The 5,6-dimethylbenzimidazole component is linked to the \( \alpha \)-axial position of the cobalt, and a variable ligand is linked to the \( \beta \)-axial position (Figure 1-1) (13). Known ligands include CH\(_3\) (methylcobalamin), 5’-deoxyadenosyl (adenosylcobalamin), OH (hydroxylcobalamin), and CN (cyanocobalamin). Cyanocobalamin, the synthetic form of B12 included in vitamin supplements and fortified foods, is converted to methylcobalamin or adenosylcobalamin, the two coenzyme forms of the vitamin. Methylcobalamin is the primary form found in human plasma making up 60 to 80% of total cobalamins (14).

Nomenclature for B12 Binding Proteins

The nomenclature for B12 binding proteins in the gastrointestinal tract and plasma has changed over time, and both the new and old terms are used in current literature. The term R-protein was originally used to differentiate B12 binding proteins, which move rapidly upon electrophoresis, from intrinsic factor (IF), which moves slowly. Intestinal R-protein, now termed haptocorrin (HC) because of its ability to bind corrins other than B12, is also found in saliva, bile, and plasma. Transcobalamin (TC) I, II, and III were terms used to identify the plasma B12 binding proteins; however, further investigation proved that TC I and III were isoforms of HC, which differed only by carbohydrate content. The term transcobalamin II was used to identify the B12 binder that participated in delivery of B12 to cells, but now it is simply referred to as TC (15).
Absorption

The absorption of B12 is primarily an active receptor mediated process that uses several different transporters (Figure 1-2). Vitamin B12 is bound to proteins in foods and must be liberated by the action of pepsin and hydrochloric acid (HCL) in the stomach in order for absorption to occur. Reduced gastric pH, as often seen in adults over the age of 50 y and with chronic antacid use, impairs breakdown of the protein matrix and ultimately results in reduced B12 absorption (16, 17). Once B12 is released from the protein matrix, it binds to HC enabling it to travel to the duodenum where pancreatic proteases degrade HC. In the duodenum, free B12 binds to IF, a glycoprotein synthesized and secreted from gastric parietal cells. The IF-B12 complex is resistant to attack from pepsin, chymotrypsin, and intestinal bacteria, allowing the complex to travel to the ileum intact, where it is transferred across the ileal epithelium via-receptor mediated endocytosis. This ileal receptor (cubilin) only recognizes the IF-B12 complex, so that free B12 can not cross the membrane in this manner (18, 19). Although passive diffusion of B12 across the epithelium does occur at a rate of 1% of any B12 dose, B12 is primarily absorbed by active transport (14). Once in the enterocyte, IF is degraded by the lysosome. Transcobalamin plays an essential role in B12 absorption, binding B12 at some point after release from IF and appearance in the blood as holo-TC. The exact mechanism by which B12 binds to TC is under debate, however, it is hypothesized that binding occurs in the enterocyte (20-22). Holo-transcobalamin can be detected in the blood 3 hours after B12 intake, with maximum absorption occurring 8 to 12 hours after intake. Cellular uptake occurs within minutes (23, 24).

Transport and Cellular Uptake

Transport of B12 in circulatory system and into the cells of target tissues is dependent on two binding proteins, TC and HC. Each protein has only one binding site with a high affinity
(Kd = $10^{-10}$ to $10^{-17}$) for the various chemical forms of B12 (25). Transcobalamin is a 43 kDa non-glycosylated protein found in plasma and in various cells including endothelial cells (22). Numerous tissues contain TC mRNA, including kidney, heart, liver, and leukocytes; however the specific cell type in which TC is synthesized is unknown (26). Transcobalamin is required for transport of B12 into the cell since only the B12 bound to TC is taken up by cell surface receptors. For this reason, the holo-TC fraction of serum B12 is the only component that is considered biologically active (27-29). The TC receptor (TC-R) is a 50 kDa heavily glycosylated protein, that binds both holo-TC and apo-TC (TC with no B12 bound) (26). In the cytoplasm, lysosomes break down the holo-TC complex making free B12 available for metabolic processes. Holo-transcobalamin constitutes only 20% of plasma B12, the remaining 80% is bound to HC (13, 30). Haptocorrin is a heavily-glycosylated, 70 kDa protein found in various biological fluids including saliva, bile, and blood. Although the majority of plasma B12 is bound to HC (holo-HC), holo-HC cannot be used by the cells as there are no receptors for this complex (15). The function of HC is unclear, and is still debated (15, 31, 32).

**Storage and Turnover**

The main storage tissues for B12 are the liver and muscle, which contain approximately 60% and 30%, respectively, of the body’s total B12. High concentrations also are found in the pituitary, kidney, heart, spleen and brain. Interestingly, although B12 is enzyme bound in most tissues, the kidney maintains a pool of free B12, which can be used to maintain plasma B12. When B12 intake is high, uptake of B12 into the kidney increases; when plasma B12 concentrations are low, B12 is released first from the kidney (33, 34). Mean total body stores range from 2 to 5 mg with a half life of 340 to 400 days (14).

Vitamin B12 is excreted only in the free form in the urine and bile at a rate of 0.1 to 0.2% (2 to 5 µg) of total body reserves per day (35). Enterohepatic recirculation is very efficient and
helps reduce total loss of B12 (13). Up to 75% of biliary B12 is actively reabsorbed in the ileum, so that very little is excreted in the feces, effectively conserving this essential nutrient (14). Additionally, because the kidney is rich in TC-R, B12 stored in the kidney is efficiently reabsorbed back into circulation reducing urinary losses.

**Biochemical Reactions**

In humans and other higher animals, B12 serves as a coenzyme for two metabolic processes, the conversion of methylmalonyl-CoA to succinyl-CoA as adenosylcobalamin and the remethylation of homocysteine (Hcy) to methionine as methylcobalamin (13, 35). In succinyl-CoA synthesis, adenosylcobalamin undergoes homolytic cleavage by the action of L-methylmalonyl CoA mutase forming cob(II)alamin and a 5'-deoxyadenosyl radical. Radical formation allows the rearrangement of the L-methylmalonyl-CoA molecule to form succinyl-CoA.

The Hcy remethylation process is an important component of overall one carbon metabolism. Methionine synthase (MS) catalyzes the remethylation of Hcy to methionine with the associated B12 acting as a methyl carrier. Methionine synthase contains separate binding domains for Hcy, 5-methyltetrahydrofolate (5-CH3THF), B12, and S-adenosylmethionine (SAM). Vitamin B12 in its reduced active state as cob(I)alamin is remethylated by 5-CH3THF and the methyl group can again be donated to Hcy (Figure 1-3). Methionine is of great biological importance because it is the precursor of SAM the major methyl donor in over 100 biochemical reactions (36, 37).

**Daily Requirement**

The Dietary Reference Intakes (DRI) for essential nutrients are guidelines for estimating the average vitamin and mineral intake needed to maintain health (38). The DRIs include Estimated Average Requirement (EAR), Adequate Intake (AI), Recommended Dietary
Allowance (RDA) and Tolerable Upper Intake Level (UL). The RDA for nutrients is calculated from the EAR and is defined as the average daily intake required to meet the needs of most individuals in the defined age bracket (38). The daily requirement of B12 is relatively low in comparison to other essential vitamins, with an EAR and RDA for adult men and women (19 to 50 y) of 2 µg/d and 2.4 µg/d, respectively. Estimates for the EAR for adults are based on the level of intake needed to maintain normal hematological status and a serum B12 concentration above 150 pmol/L minus the amount conserved in daily B12 turnover. Data were gathered primarily from patients with pernicious anemia in remission who were receiving regular intramuscular (IM) injections of B12. Studies of patients with pernicious anemia reported that IM doses of 0.8 to 1.7 µg/d were sufficient to maintain normal hematological parameters. From these studies an average of 1.5 µg/d was estimated to be the B12 requirement. The final calculation of the EAR in healthy adults with normal B12 absorption was calculated as 1.5 µg/day minus 0.5 µg/d (the estimated amount of B12 reabsorbed) with a correction to account for an estimated bioavailability of 50% (38). The RDA was then calculated as the EAR (2.0 µg/d) plus twice the coefficient of variation (CV) for 97 to 98% of the population, or 120% of the EAR. Recommendations for children are based on B12 concentrations in milk for infants and are extrapolated down from adult requirements for children up to age 18 y. Adults over the age of 50 y have the same RDA as younger adults; however, due to an age-related reduction in gastric pH, it is recommended that older adults obtain most of the RDA from fortified foods and B12 supplements (23).

**Dietary and Supplemental Sources**

Vitamin B12 is synthesized only by microorganisms in bacteria rich environments such as the intestinal tracts of animals. Some species have sufficient microbial synthesis of B12 to meet
their biological need without any additional dietary B12. Although humans have B12 synthesizing bacteria in their intestinal tract, they are primarily found in the colon where little absorption takes place making B12 an essential nutrient for humans. Natural dietary sources of B12 are limited to foods of animal origin. The liver and kidney store large amounts of B12, therefore, these organ meats are the richest dietary sources of B12 (24 to 122 µg/100 g). Other more commonly consumed food sources are red meat (0.55 to 3.64 µg/100 g), poultry (0.32 to 0.379 µg/100 g), fish (1.9 to 21.2 µg/100 g), eggs (0.09 to 9.26 µg/100 g), and milk products (0.06 to 1.71 µg/100 g) (14). Vitamin B12 also can be obtained in the following: (a) supplements; (b) B12-fortified foods such as cereals and meal replacement bars and drinks; (c) B12-fortified vegetarian products such as soy milk; and (d) B12-fortified meat substitutes and frozen meal entrees made from wheat gluten or soybeans; and some fermented food products (39).

In the US, foods of animal origin are a common part of the diet, and the estimated average daily intake of B12 from food sources is 3 to 5 µg/d on average (38). The largest percentage of dietary B12 in the US diet comes from mixed foods (16 to 19%) which includes non-beef meats, poultry, and fish; a substantial portion also comes from beef (12 to 15%), and milk products (11 to 15%). In the case of vegetarians, who are estimated to represent up to 25% of US women of reproductive age, dairy and egg products for lacto-ovo-vegetarians and dairy products for lacto-vegetarians, are the sole sources of B12 if supplements or fortified foods are not consumed.

Individuals who exclude some or all animal-derived foods and do not add B12 fortified foods to their diet are at increased risk for developing B12 deficiency (40-42). Non-meat animal-derived sources of B12 including dairy products and eggs can contribute significantly to B12 intake (38), but are excluded from the diets of strict vegetarians (vegans). The extent to
which animal-derived foods are excluded from the diets of self-described vegetarians may determine the effect on B12 status. For example, one serving (4 oz) of beef can provide the RDA for B12 (2.4 μg/d), while one serving of chicken (4 oz) provides only 12% of the RDA. The B12 content of fish varies by species; 1 serving (4 oz) of grouper provides 25% of the RDA, while tuna and herring provide as much as 500% of the RDA per serving (1 to 4 oz). Dairy products also provide variable amounts of B12 with 8 to 50% the RDA per serving (2 to 8 oz) (39).

**Vitamin B12 Status Assessment**

**Vitamin B12 Concentration**

In clinical settings, serum B12 determination is the primary method for assessing B12 status (35). In the general US population, the mean serum B12 concentration for healthy individuals over four years of age is 381 pmol/L (43). Reliance on serum B12 as the sole diagnostic tool may lead to a false diagnosis since not all individuals with low values are deficient and a “normal” serum concentration may or may not indicate adequate B12 status (44). This is due in part to the manner in which B12 is metabolized and stored in the body. Because some tissues, such as the liver and kidney, can store a relatively large amount of B12, total body depletion takes years; however, some cells with lower storage capacity may become B12 deficient while circulating B12 is still in the low normal range. In these cells, B12 dependant enzyme function may become impaired causing elevated MMA and Hcy. Currently, clinical B12 deficiency is classified as serum concentrations < 148 pg/mL; however, a significant percentage of patients with clinical symptoms of B12 deficiency who respond to B12 therapy have serum B12 concentrations in the “low-normal” (148 to 221 pg/ml) range. To enhance the diagnostic value of serum B12 concentrations, additional status indicators should be evaluated.
**Holo-transcobalamin Concentration**

Measurement of holo-TC is considered a functional indicator of B12 status because only the B12 bound to TC can be taken up by cell receptors for use in intracellular metabolic reactions (27, 28, 45, 46). In contrast, serum B12 consists of holo-TC (~20%) and holo-HC (~80%), the latter can not be used by cells since it lacks known cellular receptors. Evidence supports the conclusion that holo-TC concentration responds more rapidly to changes in B12 intake than other indices of B12 status (28). Bor et al. (47) reported that oral B12 treatment (400 μg/d) resulted in a highly significant maximal increase (+54%) in holo-TC after 3 days, in contrast to serum B12, which responded with a smaller initial change (+28%) and a slower graded increase over time. Routine measurement of holo-TC as an index of B12 status is now possible since technical problems associated with the analytical procedure have been successfully addressed (Holo-TC RIA, Axis-Sheild) (45, 48). Loikas et al. (49) confirmed the suitability of the holo-TC RIA for use in a clinical laboratory, determined reference values for the method (37 to 171 pmol/L), and confirmed that low holo-TC concentrations (< 35 pmol/L) were associated with other biochemical indicators of low B12 status.

**Methylmalonic Acid Concentration**

When B12 status is low the conversion of methylmalonyl-CoA to succinyl CoA is impaired; as methylmalonyl-CoA accumulates, it is converted to methylmalonic acid. This alteration in metabolism results in a measurable increase in MMA. Methylmalonic acid concentration is a highly specific diagnostic indicator of B12 status because, unlike the MS reaction that requires both B12 and folate, no other nutrient is required for the methylmalonyl CoA mutase reaction (50-52). A normal serum MMA concentration is ≤ 271 nmol/L, with reported reference ranges for serum MMA concentration of ~50 to 400 nmol/L (53, 54). Serum MMA concentration also provides diagnostic information when it is obtained before and after
B12 supplementation in B12 deficient individuals. Moelby et al. (52), like previous investigators, reported a marked decline in serum MMA concentration to normal one month after treatment with B12 (55).

**Homocysteine Concentration**

Homocysteine concentration is inversely associated with B12 status, and may or may not be elevated in individuals with low B12 status. Individuals that have elevated Hcy due to a B12 deficiency will respond to B12 supplementation (55, 56). Traditionally the cut-off for normal Hcy concentration has been ≤ 14 μmol/L; however, due to the implementation of mandatory folate fortification in the US, Hcy concentrations within the population have decreased significantly (57, 58). In a population-based study, Selhub et al. (56) reported that plasma Hcy concentration was inversely associated with B12 status, and mean Hcy concentration was significantly higher in individuals in the lowest compared to the highest decile for plasma B12 concentration (15.4 and 10.9 μmol/L, respectively). Mezzano et al. (59) evaluated plasma Hcy concentrations and response to B12 therapy in a group of vegetarians with low B12 status (baseline mean serum B12 concentration 110 pmol/L) with elevated plasma Hcy concentration. Following intramuscular injection with B12, serum B12 concentration increased to 392 pmoL/L and mean plasma Hcy concentration dropped significantly (12.4 to 7.9 μmol/L). Unlike MMA, Hcy concentration is not a specific indicator of B12 status. Because the folate derivative, 5-CH₃-THF is the methyl donor in the conversion of Hcy to methionine, low folate status also can lead to an elevation in Hcy concentration.
Vitamin B12 Deficiency

Etiology

Vitamin B12 deficiency may occur due to dietary restriction, malabsorption, or disturbances in transport or cellular uptake. Malabsorption of B12 can be caused by several physiological and congenital defects. Pernicious anemia is a disease of the autoimmune system in which antibodies to parietal cells and IF develop leading to a complete lack of IF and an inability to absorb B12. Individuals with this condition can be given IM B12 injections to meet B12 requirements, bypassing the absorption process (35). The elderly population is at a higher risk of B12 malabsorption due to the age-related decrease in stomach acid. The acidic environment in the stomach is required for the release of B12 from food, and a significant decrease in hydrochloric acid can impair the process leading to increased excretion and decreased absorption. In such cases the daily B12 requirement must be met with supplemental B12 (35).

Clinical Abnormalities

Depleted B12 status may take years to develop in individuals with impaired absorption or inadequate intake and an individual may have marginal B12 status prior to developing severe clinical symptoms such as megaloblastic anemia and irreversible neurological damage (32). Development of B12 deficiency begins with depletion of serum B12, followed by cellular deficiency and biochemical changes including elevated Hcy and MMA concentrations (32). Neurological abnormalities affecting physical reflexes, stamina, and mental attributes including memory and behavioral changes may accompany a moderate B12 deficiency (60-62). In addition, the risk of inadequate B12 intake to a developing fetus, should pregnancy occur, is of great concern for women of reproductive age. Infants born to mothers with a B12 deficiency have been reported to suffer devastating symptoms including growth retardation, delayed
psychomotor development, and in some instances, permanent effects on the brain (63-66). A B12 deficiency may increase the risk for birth defects as illustrated by the well documented independent role for B12 in the etiology of neural tube defects (NTDs) (67-73). These studies have provided evidence that even small reductions in serum B12 concentrations within the normal range may be associated with a significantly increased risk for NTDs. Afman et al. (74) measured the plasma concentrations of B12, Hcy, and the apo- and holo- forms of TC in NTD case mothers and in control women. Low plasma holo-TC concentration was associated with a 3-fold increased risk for having a child with an NTD, while a low percentage of B12 bound to TC (TC saturation) was associated with a 5-fold increased risk.

Vegetarianism

Vegetarians are at increased risk for developing a B12 deficiency since B12 is only naturally present in animal-derived products (i.e., meat, eggs, dairy). There is much variability in the amount of B12 consumed by individuals characterized as “vegetarians”. This classification includes those who consume diets completely devoid of all animal-derived products (vegans), including meat, fish, dairy, and eggs, as well as those who exclude meat but consume either dairy products (lacto-vegetarians) or dairy and eggs (lacto-ovo-vegetarians) (75). Approximately 2.5% of the entire US adult population (4.8 million people) report consumption of vegetarian diets, and approximately 1% report consuming vegan diets (75-77). There is an increasing trend for the younger segment of the population to consume vegetarian diets (75). In a nationally-representative survey (75), the number of self-defined vegetarians who reported no meat consumption was highest in the 20 to 29 year age group and was two to three times higher than that of 50 to 59 and 60 to 69 year old individuals, respectively. This increasing trend in consumption of vegetarian diets is especially prevalent among young adult women of
reproductive age, documented by survey data indicating that 20 to 25% of this group follow some type of vegetarian diet (78).

Multiple reports provide evidence that all vegetarians including lacto-vegetarians and lacto-ovo-vegetarians are at increased risk for developing a B12 deficiency compared to omnivores (79-81), supporting the conclusion that a vegetarian does not have to be a strict vegan for B12 status to be impaired (32, 81-85). Impaired B12 status may lead to elevated Hcy concentrations and increased risk for cardiovascular disease, cancer and birth defect-affected pregnancies. A number of studies indicate that vegetarians have significantly higher Hcy concentrations than omnivores and that the consumption of a vegetarian diet may be associated with elevated Hcy concentrations (59, 80, 86). For example, Mezzano et al. (59) reported that the Hcy concentration was 41% higher in vegetarians than in omnivores and that Hcy was inversely related to serum B12 concentration. In a study comparing B12 status of Taiwanese vegetarians and non-vegetarians, Huang et al. (87) reported that vegetarians had higher plasma Hcy concentrations than non-vegetarians (13.2 vs. 9.8 μmol/L, respectively) and that serum B12 concentration was a strong predictor of plasma Hcy concentration. Another similar study conducted in a European population reported that Hcy concentration was significantly higher (11.6 μmol/L) in a group of vegetarians compared to omnivorous controls (9.8 μmol/L) and that the Hcy concentration increased as the vegetarian diet became more restrictive, with vegans having the highest values (86). In the only study reported to date in which the B12 status of young adult vegans in the US has been evaluated, Carmel et al. (88) found that elevated Hcy concentration associated with dietary inadequacy of B12 was a major problem in young Asian Indian medical students with hyperhomocysteinemia occurring in 25% of group. This study
illustrates that well-educated young adults are among the vegetarians in the US whose consumption of a B12-deficient diets has been associated with an elevation in Hcy concentration.

**Gene-Nutrient Interactions**

**Transcobalamin 776C→G**

The most common polymorphism affecting the TC B12 transport protein is a C→G base substitution in DNA at base pair 776, which results in the substitution of proline with arginine at codon 259 (74, 89). The estimated prevalence of the TC 776 CC, CG and GG genotypes are approximately 20%, 50%, and 30%, respectively (74, 89, 90). Our research group recently estimated the distribution (27% CC; 49% CG; 24% GG) of the TC 776 polymorphism in a large population group of young women for whom B12 status was previously reported, which is in agreement with previous studies (91).

The potential influence of the TC 776 C→G polymorphism on indices of B12 status has been investigated by several research groups (74, 89, 90). Afman et al. (30) found lower holo-TC, total TC, and holo-TC/total-TC ratios in individuals with either the TC 776 GG or CG genotypes compared to those with the CC genotype. Miller et al. (89) reported a lower mean holo-TC concentration, a lower percent of total B12 bound to TC, and a higher mean MMA concentration in elderly subjects with the TC 776 GG compared to the CC genotype. Our research group evaluated B12 status in young women with all three TC 776 C→G genotypes (CC, CG, GG) (91). Mean holo-TC concentration was significantly lower in TC 776 GG compared to CC genotypes, and individuals with low (< 35 pmol/L) holo-TC had a significantly higher mean Hcy concentration. Alternatively, some studies have reported no effect of the TC 776 C→G polymorphism on B12 status or metabolism (92-94).
Any reduction in B12 binding capacity, protein synthesis, or transport function caused by the TC 776 C→G polymorphism could impair functional B12 status, leading to reduced cellular availability of B12. If the polymorphism has a physiologically significant impact on B12 availability, it would likely be exacerbated by concurrently low B12 status due to insufficient dietary B12 intake. In a population where more individuals have marginal B12 status, such as in vegetarians, any negative effect of the TC 776 C→G polymorphism would be expected to be more apparent.

**Malabsorption of Vitamin B12**

The most common form of B12 malabsorption is often termed “food-bound malabsorption” (95). Because only free B12 can bind to the transport proteins and be taken up into the enterocyte or absorbed passively, any physiological condition that reduces the ability to free B12 from the protein matrix will lead to malabsorption and can lead to a B12 deficiency. Approximately 5 to 25% of adults over the age of 60 y are estimated to have some degree of food-bound B12 malabsorption due to an age-related decrease in stomach acid, or achlorhydria (96). Because achlorhydria is so prevalent in older adults it is recommended that the daily requirement of B12 be met by consuming supplemental forms of B12 (97). Vitamin B12 found in fortified foods and vitamin supplements is not protein bound and therefore, can be absorbed with normal efficiency even if gastric pH is high.

Another less common, but often more severe form of B12 malabsorption, termed pernicious anemia, can occur in all age groups, although incidence does increase with age. Pernicious anemia is caused by a lack of IF resulting from an autoimmune response, atrophy of the gastric mucosa, chronic gastritis, and in rare cases a congenital defect in the gene for IF. Congenital defects may lead to synthesis of an altered, and therefore non-functional IF protein or a complete lack of synthesis. Both conditions cause B12 deficiency at an early age and have
been reported to be caused by a variety of genetic mutations and post-translational defects. The autoimmune-based B12 malabsorption condition is more prevalent in older adults but has been observed in all age groups (98). In this case, the body recognizes either the IF itself or the gastric parietal cells as foreign and synthesizes antibodies to the protein or cell eliciting an immune response. Destruction of IF or the parietal cells by this autoimmune response may occur to varying degrees resulting in variation in the severity of B12 malabsorption (98).

Currently the only available diagnostic tests for pernicious anemia are not clinically practical. The Schilling test, which involves ingestion of radioactively-labeled B12, a flushing dose of non-labeled B12, and collection of urine over a period of 24 hours requires meticulous adherence to protocol making it error prone and costly (99-101). Presence of IF or parietal cell antibodies can be measured to diagnose pernicious anemia; however, parietal cell antibodies can occur in other autoimmune diseases, and both tests are only clinically meaningful in a subgroup of patients with autoimmune conditions (102, 103). It has been hypothesized that changes in holo-TC in response to a supplemental dose of B12 may be used to assess B12 absorption (28, 47, 104). Bor et al (20) reported a significant increase in holo-TC and TC saturation 24 and 48 hours after receiving three 9 μg oral B12 doses. Since no blood was collected before 24 hours (post baseline), the magnitude and pattern of change of holo-TC during the first 24 hours could not be determined (47). In developing a clinical diagnostic test, it is important to know the optimal time post dose at which to draw blood.

**Overall Rationale**

Vitamin B12 plays a central role in Hcy metabolism, and B12 deficiency has been associated with numerous health risks, including birth defect-affected pregnancies. Few studies have been designed to evaluate the relationship between dietary exclusion or limitation of
specific animal products and B12 status in individuals who do not take B12 supplements or consume B12-fortified foods. The proposed study will evaluate the association between B12 status and intake of specific animal-derived food products among vegetarians who do not consume B12-containing supplements.

Several studies, including one conducted by our research group (91), indicate that the TC 776 GG genotype results in decreased holo-TC concentrations, and could therefore be a risk factor for a B12 deficiency. Although most studies have not found a correlation between TC 776 C→G genotype and Hcy or MMA, holo-TC and Hcy and MMA have been negatively correlated (89, 105). It is hypothesized that B12 transport, and thus metabolic function, may be impaired in individuals with the TC 776 GG genotype, and that an effect on Hcy and MMA concentrations due to the TC 776 GG genotype may be evident in individuals with low B12 intake and status. These data could be used for public health screening and intervention approaches for adults whose combined dietary choices and genetic make-up may put them at higher risk for certain diseases or poor pregnancy outcomes. Information generated from this study could benefit individuals who exclude B12-dense food sources from their diets for reasons related to health or personal choice rather than religion, culture, or the environment, as well as for individuals who consume strict vegan diets for religious/cultural or environmental reasons.

**Hypothesis # 1**

Moderate B12 deficiency will be more common in vegetarians not taking B12 supplements than in their omnivorous counterparts.

**Specific aim:** To determine if non-supplement taking young adults who exclude animal-based foods and are not taking B12-containing supplements are at a greater risk for a B12
deficiency than those who eat animal-based foods by comparing B12 status indices between groups.

**Hypothesis #2**

Vitamin B12 intake at the current RDA may not be sufficient to maintain normal B12 status.

**Specific aim:** To determine the level of B12 intake associated with optimal status as defined by normal B12 status biomarkers.

**Hypothesis #3**

Genotype status for the TC 776C→G polymorphism will have a greater physiological impact on individuals with low B12 status than those with normal B12 status.

**Specific aim:** To determine if genotype status for the TC 776C→G polymorphism further impairs B12 status in individuals with low B12 intake by comparing B12 status indices among genotype groups in individuals with low and normal B12 status.

**Hypothesis #4**

Holo-transcobalamin concentration can be used to assess B12 absorption.

**Specific aim:** To determine if holo-TC concentration increases measurably in response to B12 supplementation within a 24 hour time period.
Figure 1-1 Structure of vitamin B12. Modified from Stabler (35) p. 22
Figure 1-2 Overview of vitamin B12 (B12) absorption. (1) Food bound B12 is released in the acidic environment of the stomach. (2) Free B12 binds to haptocorrin and the complex travels to the duodenum. (3) Pancreatic proteases degrade HC. (4) Free B12 binds to intrinsic factor, which is synthesized in the gastric parietal cells. (5) The B12 – IF complex to travel to the ileum and is transferred across the ileal epithelium via receptor mediated endocytosis, along with 1% passive diffusion. (6) In the enterocyte, intrinsic factor is degraded by the lysosome. (7) Transcobalamin binds B12 at some point after release from intrinsic factor, this may occur in the enterocyte.
Figure 1-3  Role of vitamin B12 in the remethylation of homocysteine. THF = tetrahydrofolate; 5-CH3-THF = 5-methyltetrahydrofolate; MS = methionine synthase; SAM = s-adenosylmethionine; SAH = S-adenosylhomocysteine.
CHAPTER 2
VITAMIN B12 STATUS IS IMPAIRED IN A SUBGROUP OF HEALTHY YOUNG VEGETARIAN AND OMNIVOROUS ADULT MEN AND WOMEN

Naturally occurring dietary sources of B12 are limited to foods of animal origin, which if restricted in the diet may impair B12 status (40-42). Vitamin supplements and fortified foods can also contribute to B12 intake (97); however, it is estimated that ~70 % of the United States (US) population does not take supplements (106). Vegetarians, individuals who avoid some or all animal-derived foods, have limited dietary intake of B12 and may be at greatest risk for developing a B12 deficiency compared to omnivores. Few data are available on B12 status in young adult vegetarians in the United States, and further evaluation of B12 status in this subgroup of individuals is warranted to better determine relative risk of B12 deficiency and related disease. Clinical determination of B12 deficiency relies on the availability of specific and reliable biomarkers of B12 status. Biomarkers currently used to assess B12 status include serum B12, MMA, Hcy and holo-TC concentrations. Although holo-TC is not yet used clinically, holo-TC is reported to be more sensitive than serum B12 and may be comparable to MMA as a biomarker of B12 status. The objectives of this study were to determine if young adult vegetarians who do not take B12 supplements are at a greater risk for B12 deficiency than omnivores not taking B12 supplements, and to compare the various B12 status indicators within these groups.

Subjects and Methods

Subjects and Subject Recruitment

Healthy adults (n = 388) from the Alachua county, FL community including university students, faculty and staff were recruited by flyers and newspaper advertisements with simultaneous recruitment for “healthy adult vegetarians” and “healthy adults”. Subjects were screened by phone and selected based on the following exclusion criteria: (a) < 18 y & > 49 y (b)
major change in animal-product consumption (i.e. vegetarian or omnivore) habits during the past 3 years; (c) B12-containing supplement use within 6 mo of screening; (d) chronic alcohol consumption (>1 drink/d of any kind); (e) use of tobacco products; (f) use of prescription medications other than oral contraceptives; (g) personal history of chronic disease; (h) regular blood donations; and (i) pregnant or lactating women.

Potential subjects were asked about their meat consumption habits during the phone screening for initial classification as vegetarian or omnivore. Specifically subjects were asked “How often do you consume (a) beef, (b) chicken, (c) turkey, (d) pork, and (e) fish”. Subjects who responded “never” to all questions were temporarily classified as vegetarian. This study was approved by the University of Florida Institutional Review Board, and all subjects signed an informed consent prior to beginning the study.

**Study Design and Data Collection**

Between the hours of 7:00 am and 9:00 am subjects were scheduled for fasting blood sample collections. Subjects were called 24 hours prior to their scheduled appointment to remind them to fast overnight and the following morning. Following sample collection, subjects were given a small meal and a comprehensive information session explaining how to complete the National Cancer Institute Diet History Questionnaire (DHQ). Subjects were asked to complete the questionnaire at home and return it within two weeks and to contact a designated member of our recent team personnel if they had any questions or problems completing or returning the questionnaire. For any unreturned DHQs, individuals were contacted by phone or e-mail to determine if the questionnaires were lost in transit or if the subject had not had an opportunity to complete the DHQ instrument. Subjects that chose not to return the DHQ were not included in the final data analysis. The DHQ has been validated for the estimation and
quantification of dietary intake of all essential nutrients including B12 (107). Subjects were instructed to answer all questions based on their diet over the past 12 mo estimating the frequency of intake and portion size of 125 different food items. A total of 70 mL of blood were collected for analysis of the following indices: (a) serum holo-TC; (b) plasma B12; (c) serum MMA; (d) serum Hcy (e) serum folate, and (f) hematocrit.

Sample Processing

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) and serum separator clot activator (SST) tubes. EDTA tubes were centrifuged for 30 min at 2000 x g at 4°C to separate and collect plasma for B12 analyses. Serum separator tubes were centrifuged for 15 min at 650 x g at room temperature to separate and collect serum for holo-TC, MMA, Hcy, and folate determination. All samples were stored frozen at −30°C until analysis.

Competitive Binding Assays of Serum Holo-transcobalamin and Plasma B12.

Serum holo-TC concentration was determined by radioimmunoassay (holo-TC RIA reagent kit; Axis Shield, Ulvenveien, Oslo, Norway) based on the method of Ulleland et al. (45). Specifically, magnetic microspheres coated with anti-human TC monoclonal antibodies were incubated with each sample for a period of one hour to isolate both holo-TC and apo-TC. Once attached to the metal beads via antibody interaction, the TC protein and associated B12 were magnetically separated from the sample. Next, isolated TC was incubated with $^{57}$Co-labeled B12 tracer plus reducing agent followed by a denaturing agent to free B12 from the TC protein. Finally, each sample was incubated with IF to which unlabeled and labeled B12 bind competitively based on their relative concentrations. Remaining unbound B12 was removed and the relative radioactivity of each sample measured by gamma counter. Radioactivity of each
sample in counts per minute (CPM) was compared to a standard curve with serum holo-TC concentration being inversely associated with CPM.

Plasma B12 was determined by RIA using a commercially available kit (Quantaphase II, Bio-Rad). Specifically, samples were incubated with a $^{57}$Co labeled B12 tracer in a 100°C water bath to convert all forms of B12 to cyanocobalamin. Samples were brought to room temperature after boiling for 20 min, and then mixed with purified porcine IF bound to polymer beads and incubated for one hour. During incubation labeled and unlabeled B12 compete for binding to IF at rates that match their relative concentrations. Finally, samples were centrifuged, and supernatant containing unbound B12 was removed. Sample radioactivity was measured by gamma counter and B12 concentration was calculated using a standard curve on which the radioactivity was inversely related to B12 concentration.

**Measurement of Serum Homocysteine and Methylmalonic Acid.**

Serum Hcy and MMA concentrations were determined by gas chromatography – mass spectrometry (Metabolite Laboratories, Inc. Denver, Colorado) (108, 109).

**Diet Analysis**

Daily B12 intake was assessed based on data obtained from the DHQ, which was modified to include additional B12-containing foods including meats, mixed dishes, fortified foods and meat substitutes. The original DHQ is available online at http://appliedresearch.cancer.gov. The DHQ was scanned by Optimal Solutions Corporation (OSC), Lynbrook, New York. Dietary data obtained from scanning the questionnaires was sent to the University of North Carolina, Chapel Hill as an ASCII text file, and then analyzed using the Diet*Calc Analysis program. In order to analyze the modified questionnaire, the Diet*Calc program was updated to include nutrient values for all of the food items added based on data from the United States Department of
Agriculture (USDA) National Nutrient Database for Standard Reference and nutrition label information when data was not available from the former USDA database Diet*Calc is a freeware program and can be downloaded from the National Cancer Institute website (www.riskfactor.cancer.gov).

**Subject Dietary Intake Classification**

Individuals were classified as vegetarian or omnivore based on their responses to the DHQ. Individuals classified as vegetarians were those who reported no consumption of any meat products (i.e., beef, poultry, pork, lamb and seafood) or meat-based mixed dishes and who reported consuming dairy products and eggs never to daily. Omnivores were defined as individuals who consumed any meat, poultry or seafood products. In addition to the preexisting questions in the DHQ that asked about all types of meat consumption, a new question was added to more accurately classify subjects into specific dietary intake categories. This question required subjects to indicate the foods they never consumed including (a) beef, (b) chicken, (c) turkey, (d) pork, (e) fish, (f) dairy products, and (g) eggs.

**Statistical Methods.**

Vitamin B12 status based on the measured B12 indicators and dietary B12 intake were compared between groups using analysis of variance (ANOVA) with an alpha of 0.05. Dependent variables also were classified as “normal” vs. “abnormal” according to whether they were above or below established thresholds (B12, ≥ 148 pmol/L; holo-TC, ≥ 35 pmol/L; MMA ≤ 271 nmol/L; and ≤ Hcy 12 µmol/L) and comparisons with respect to dietary group were done by Pearson Chi-Square tests. The distributions of all possible combinations of normal and abnormal test results were calculated and the rate of each B12 indicator being abnormal when all others were normal or abnormal was compared using a Chi-square test. Age distributions were
compared between the two dietary groups by ANOVA, while race and gender for the two dietary
groups were compared by Pearson Chi-Square tests.

**Results**

One hundred and twenty one vegetarians and 181 omnivores completed the study (total n = 305). Of the 388 enrolled subjects, 62 were excluded due to reporting supplement use, and 23 did not complete the DHQ. All results are reported as mean ± SD unless otherwise noted. There was a significant difference in age and BMI between groups, with vegetarians being older and having a lower BMI. There were no significant differences in gender or ethnicity between diet categories (Table 2-1).

Total B12 intake (µg/d ± SD) and B12 intake expressed as µg/1000 kcals were significantly lower (P < 0.001) in vegetarians than in omnivores (Table 2-2). Plasma B12 concentration was significantly lower (P < 0.01) in vegetarians than omnivores (Table 2-2). Serum MMA concentration was significantly higher (P = 0.001) in vegetarians compared to omnivores (Table 2-2). Mean holo-TC and Hcy concentrations were not significantly different between groups (Table 2-2).

Vitamin B12 deficiency, based on having a value outside the normal range for one or more of the B12 status indicators, was twice as prevalent (P < 0.001) in vegetarians than omnivores (42% and 23%, respectively). Impaired B12 status based on concentrations of plasma B12, serum holo-TC and serum MMA combined also was significantly greater in vegetarians compared to omnivores. Specifically, more than twice as many (P < 0.05) vegetarians had low serum holo-TC (< 35 pmol/L), plasma B12 (< 148 pmol/L), and elevated serum MMA (> 270 nmol/L) concentrations than omnivores (Figure 2-1). There was no significant difference in the percentage of vegetarians versus omnivores with elevated Hcy (> 12 µmol/L).
Subjects were cross-tabulated by B12 status as defined by having a value within (+) or outside (-) the normal range for B12, holo-TC and MMA singly and in combination (Table 2-3). Because of the small numbers of subjects within each of the resulting 8 categories, statistical analysis was not done; however, the likelihood of one B12 status indicator being abnormal while the remaining tests were normal was conducted. In this analysis, B12, holo-TC and MMA were more likely to be abnormal when one or more of the other indicators were abnormal (23, 38, and 36 % of the time, respectively) compared to when all others were normal (6, 11, 10 % of the time, respectively) (Figure 2-2).

Discussion

The primary objective of this study was to assess and compare the B12 status and intake of young adult vegetarians and omnivores who do not take B12-containing vitamin supplements to determine if vegetarians are at greater risk for developing a B12 deficiency than their omnivorous counterparts. Although long term adherence to a vegetarian diet can provide substantial health benefits (110), limitation of most or all animal-based foods, particularly when B12-fortified foods or supplements are not added to the diet, can increase the risk for developing a B12 deficiency.

It has been estimated that B12 intake in the general US population is adequate (111), however, data from this study suggest that a subgroup of healthy young, non-supplement using adults may not be consuming adequate B12. Within the vegetarian groups, 43% were determined to be potentially B12 deficient based on having a value outside the normal range for one or more B12 status indicators, 61% of whom had elevated MMA, indicating metabolic impairment. Surprisingly, of the omnivores, 23% were potentially B12 deficient, with 48% of them having an elevated MMA concentration, suggesting that even a meat-containing diet may not provide sufficient B12.
This is of particular concern for young women of childbearing age, who might chose to avoid meat in an attempt to lower fat and cholesterol intake, but do not consume other sources of B12. Nutrient availability is crucial in the first 180 days of pregnancy, during a portion of which a woman might not even know she is pregnant (112). This issue has been addressed in relation to folic acid; however unlike folic acid, it is not well recognized that a B12 deficiency is an independent risk factor for neural tube defects (57, 113, 114).

The DHQ used to assess dietary B12 intake asks subjects to recall dietary intake over the past 12 mo and answer questions based on their best estimate of food intake. Full analysis of DHQ data was the focus of an investigation conducted by another member of the laboratory group and will be reported separately. The data obtained from the analyses conducted for this study were used to group individuals within a similar range of overall B12 intake and to identify foods eaten or excluded by each subject. This allowed for a very strict definition of “vegetarian” subjects who reported no meat consumption and “omnivore” subjects who reported some degree of meat consumption. Previous studies, the majority of which were conducted in Europe, used a similar approach to classify individuals as vegetarian. Vegetarians have previously been sub-grouped into lacto-ovo-vegetarians, lacto-vegetarian and vegan categories, with the greatest deficiency associated with the vegan diet relative to other vegetarian sub-groups (81). It is widely believed in the US, that a B12 deficiency is not a problem in healthy young adults who consume at least some animal-based products. The data from the present study do not support this perception.

In addition to the moderate deficiency that was detected in the vegetarian and omnivorous groups, a small subset (including both omnivores and vegetarians) was determined to be severely B12 deficient as evidenced by reports of neurological problems including numbness and a
tingling sensation in the extremities. Of these subjects none had previously sought out medical attention for these symptoms, which illustrates that B12 deficiencies may go undetected among seemingly healthy young adult men and women. Early detection of a B12 deficiency is the most effective way to prevent progression to serious health complications. Recent studies suggest that measurement of holo-TC is superior to serum B12 because only holo-TC is taken up into cells, and therefore only that portion (≤ 20%) is biologically active (115). Additionally, holo-TC has been reported to be more sensitive to changes in B12 intake than total serum B12 (28). Miller et al. (116) reported that the use of holo-TC and serum B12 together as a ratio may be superior to the use of either alone. Their data suggest (116) that use of combined holo-TC and serum B12 measurement could lead to three possible diagnoses; normal, possible deficiency (only 1 low indicator), and deficient (both indicators low). Finally MMA is still considered by many researchers to be the gold standard (54, 117). In the current study, we cross tabulated subjects by B12 status as defined by having a value within or outside the normal range for B12, holo-TC and MMA singly and in combination with each other. Statistical analysis could not be conducted due to limited sample size; however, a combined analysis of the two diet groups indicated that it is more likely that when the value of one biomarker is outside the normal range at least one other indicator is more likely to also be outside the normal range. Additionally, the length of time an individual adheres to a B12-insufficient diet will have differential effects on specific B12 biomarkers (32). Considering the differences in the primary biomarkers of B12 status, holo-TC may be initially affected followed by a decrease in serum B12 (once B12 stores have been depleted), and finally an elevation in MMA indicating impaired cellular B12-dependant enzyme function. The data from the current study do not definitively support one B12 biomarker as
being superior to another; however, in a clinical setting the more biomarkers that are outside the normal range, the more likely a B12 deficiency exists.

In conclusion, the high incidence of impaired B12 status observed in these otherwise healthy young adults was unexpected. These data indicate that dietary intake alone may not be meeting the B12 needs of non-supplement using adults, especially vegetarians. Further research focusing on B12 status and intake in individuals consuming both vegetarian and low-meat containing diets is warranted. Assessment of B12 status by a combination of biomarkers may provide a more definitive diagnostic approach prior to treatment.

### Table 2-1 Characteristics of study groups

<table>
<thead>
<tr>
<th></th>
<th>Vegetarians (n = 121)</th>
<th>Omnivores (n = 181)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y; mean ± SD)</td>
<td>28 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>BMI&lt;sup&gt;a&lt;/sup&gt; (mean ± SD)</td>
<td>22.9 ± 3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.9 ± 4.1</td>
</tr>
<tr>
<td>Gender (count)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>67</td>
<td>98</td>
</tr>
<tr>
<td>Male</td>
<td>54</td>
<td>83</td>
</tr>
<tr>
<td>Race/Ethnicity (count)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>69</td>
<td>119</td>
</tr>
<tr>
<td>African American</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Asian</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Asian Indian</td>
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<td>4</td>
</tr>
<tr>
<td>Hispanic</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Body mass index (BMI); <sup>b</sup> Significantly different from omnivores (P < 0.05)(ANOVA)
Table 2-2  Mean (± SD) dietary vitamin B12 intake and status of omnivorous and vegetarian adults.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Vegetarians (n = 121)</th>
<th>Omnivores (n = 181)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total B12 intake (µg/d)</td>
<td>3.39 ± 2.97</td>
<td>6.80 ± 4.04</td>
</tr>
<tr>
<td>B12 intake (µg/1000 Kcal/d)</td>
<td>1.92 ± 1.87</td>
<td>3.31 ± 1.88</td>
</tr>
<tr>
<td>B12 (pmol/L)</td>
<td>280 ± 146</td>
<td>313 ± 124</td>
</tr>
<tr>
<td>Holo-TC (pmol/L)</td>
<td>83 ± 84</td>
<td>87 ± 55</td>
</tr>
<tr>
<td>MMA (nmol/L)</td>
<td>260 ± 229</td>
<td>195 ± 116</td>
</tr>
<tr>
<td>Hcy (µmol/L)</td>
<td>7.7 ± 2.7</td>
<td>7.3 ± 2.5</td>
</tr>
</tbody>
</table>

*Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy); b Different from vegetarians (P < 0.001); c Different from vegetarians (P < 0.01) (ANOVA)

Figure 2-1  Percent of vegetarian (n = 121) and omnivorous (n = 181) adults (18 to 49 y) with concentrations outside the normal range for holo-transcobalamin (holo-TC, < 35 pmol/L), serum vitamin B12 (B12, < 148 pmol/L), methylmalonic acid (MMA, > 270 nmol/L), and homocysteine (Hcy, > 12 µmol/L). * Different from omnivores (P < 0.05) (ANOVA)
Table 2-3  Cross-tabulation of vitamin B12 status of subjects based on select biomarker combinations

<table>
<thead>
<tr>
<th>Status by biomarker measured&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diet group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vegetarian</td>
<td>Omnivore</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>B12 ≥ 140 pmol/L</td>
<td>n = 121</td>
<td>n = 181</td>
<td>n = 302</td>
<td></td>
</tr>
<tr>
<td>Holo-TC ≥ 35 pmol/L</td>
<td>68</td>
<td>138</td>
<td>206</td>
<td></td>
</tr>
<tr>
<td>MMA ≤ 270 nmol/L</td>
<td>5</td>
<td>7</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>4</td>
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<td></td>
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<tr>
<td></td>
<td>7</td>
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<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> + Yes; - No; vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA)

Figure 2-2  Frequency of single versus combined vitamin B12 (B12) status biomarkers being outside the normal range including plasma B12, serum holo-transcobalamin (holo-TC) and serum methylmalonic acid (MMA). *Significantly different from group with all others normal (P < 0.001) (Pearson’s Chi-squared test)
CHAPTER 3
VITAMIN B12 INTAKE AT THE CURRENT RDA LEVEL IS NOT OPTIMAL

The current RDA for B12 was established based on data from patients who were being treated for pernicious anemia. Specifically, the amount of B12 required in an injectable form to normalize serum B12 in patients diagnosed with pernicious anemia was determined to be the daily B12 requirement for adults. The B12 RDA (2.4 μg/d) was derived by adjusting the estimated B12 requirement for bioavailability, enterohepatic recirculation, and the CV for 97 to 98% of the population. It has been suggested that the RDA for B12 is not optimal, and that B12 status is improved with intakes up to 6 μg/d (118, 119). The objective of this analysis was to determine the level of dietary B12 intake associated with optimal B12 status as defined by B12 status biomarkers within the normal range.

Subjects and Methods

Subjects and Subject Recruitment

Healthy adults (n = 302) were recruited from the Alachua county, FL community including university students, faculty and staff. Specifically, subjects were screened by phone and selected based on the following inclusion criteria: (a) 18 to 49 y (b) no change in meat consumption habits over the past 3 years; (c) no B12-containing supplement use within the past 6 mo; (d) limited chronic alcohol consumption (<1 drink/d of any kind); (e) no use of tobacco products; (f) no chronic use of prescription medications other than oral contraceptive agents; (g) no history of chronic disease; (h) no chronic blood donations; and (i) non-pregnant and non-lactating. All 302 qualified subjects from the first part of the current study were included in this analysis. The approved institutional review board informed consent form signed by the subjects at the beginning of the study included consent for all aspects of studies described in the manuscript.

Study Design and Data Collection
Subjects were called the day before their scheduled study day to remind them to fast overnight (8 hours) and the following morning prior to having their blood drawn. Between the hours of 7:00 am and 9:00 am qualified subjects were scheduled for blood sample collection followed by a comprehensive information session explaining how to complete the National Cancer Institute Diet History Questionnaire (DHQ) used to assess dietary intake. A total of 70 mL of blood were collected for analysis of the following indices: (a) serum holo-TC; (b) plasma B12; (c) serum MMA; (d) serum homocysteine (Hcy) (e) serum folate, and (f) hematocrit.

Sample Processing and Analysis

Blood samples were collected in EDTA and SST clot activator tubes. EDTA tubes were centrifuged at 2000 x g at 4°C for 30 min to obtain plasma for B12 analyses. SST tubes were centrifuged at 650 x g at room temperature for 15 min to obtain serum for determination of holo-TC, MMA, Hcy, and folate concentrations. Samples were stored at −30°C until analysis. Serum holo-TC concentration was determined by radioimmunoassay (holo-TC RIA reagent kit; Axis Shield, Ulvenveien, Oslo, Norway) based on the method of Ulleland et al. (45) using magnetic microspheres coated with anti-transcobalamin monoclonal antibodies to isolate both holo-TC and apo-TC, and 57Co-labeled B12 as a tracer. Plasma B12 concentration was determined by RIA using a commercially available kit (Quantaphase II, Bio-Rad). Serum Hcy and MMA concentrations were determined by gas chromatography – mass spectrometry (Metabolite Laboratories, Inc. Denver, Colorado) (108, 109).

Diet Analysis

Daily B12 intake was estimated based on data obtained from the DHQ, which was modified to include an extensive list of B12-containing foods including meat containing mixed dishes, fortified foods, and meat substitutes. The unmodified DHQ is available for review online.
at http://appliedresearch.cancer.gov. The DHQ was scanned by Optimal Solutions Corporation (OSC), Lynbrook, New York. Once scanned OSC sent the dietary data as an ASCII text file to the University of North Carolina, Chapel Hill (UNC), where the data were analyzed using the Diet*Calc Analysis program modified for this version of the DHQ. This freeware program can be downloaded from the NCI website (www.riskfactor.cancer.gov). The B12 content of the food items added to the DHQ was obtained from the USDA National Nutrient Database for Standard Reference and nutritional labels (39).

**Statistical Analysis**

Results are reported in the text as mean ± SD with an alpha = 0.05. The dependent variables B12, holo-TC and MMA concentrations were classified as “normal” vs. “abnormal” according to falling above or below an established threshold (B12, 148 pmol/L; holo-TC, 35 pmol/L; MMA 271 nmol/L; and Hcy 12 µmol/L) and comparisons with respect to dietary B12 intake were performed using the Pearson Chi-Square test. Subjects were divided into dietary B12 intake quintiles, and B12 status based on plasma B12, serum holo-TC, MMA, and Hcy concentrations were compared between groups using ANOVA with an alpha of 0.05. The “Least Significant Difference” (LSD) method of multiple comparisons was used for assessment of differences between quintiles. The LSD ensures every target population paired difference in means will be within +/- LSD of the corresponding difference in sample means with 95% confidence. The data were analyzed using EXCEL (Microsoft, Redmond, WA) and PRISM software (Graph-Pad Software Inc. El Camino, CA).

**Results**

Three hundred and two healthy young adult (18 to 48 y) men and women were included in this analysis. Subject characteristics are listed in Table 3-1. Seventy six subjects (25 %) had an intake below the RDA of 2.4 µg/d. Dietary B12 intake was significantly correlated (P < 0.05)
with B12, Hcy, and MMA concentrations, but not with holo-TC concentration (Table 3-2). Individuals with low plasma B12 (< 148 pmol/L) or holo-TC (< 35 pmol/L) concentrations and those with elevated serum MMA (> 270 nmol/L) or Hcy (> 12 μmol/L) concentrations had significantly lower dietary B12 intake than those with normal concentrations (Figure 3-1).

To further evaluate the influence of B12 intake on B12 status, subjects were ranked and grouped by quintile of B12 intake. The mean concentration for each B12 status indicator was plotted against the mean B12 intake for each quintile group (Figure 3-2). Mean holo-TC, B12, MMA and Hcy concentrations were significantly different (P < 0.05) among B12 intake quintile groups (Figure 3-1). Specifically, mean holo-TC increased (P < 0.01) from quintile 1 through 3 and then maintained approximately the same value from quintiles 3 through 5, which was associated with a mean B12 intake of \( \geq 4.3 \) μg/d. Mean plasma B12 concentration increased (P < 0.001) from quintile 1 through 4, reaching a plateau from quintile 4 through 5 corresponding to a B12 intake \( \geq 6.7 \) μg/d. Mean MMA decreased (P < 0.001) from quintile 1 through 2 then reached a plateau, which was associated with a B12 intake of \( \geq 2.7 \) μg/d. Homocysteine changed to the smallest degree, but decreased (P < 0.05) from quintile 1 through 3 maintaining this approximate value thought quintile 5, which corresponded to a B12 intake > 4.3 μg/d.

In the case of holo-TC, B12, and Hcy concentrations, the means across all quintiles of B12 intake were in the normal range; mean MMA concentration was elevated in quintile 1 and within the normal range for all subsequent quintiles. The proportion of subjects with B12 deficiency as defined by abnormal biomarkers within each quintile group (i.e. low B12, low holo-TC, elevated MMA or elevated Hcy) decreased significantly from the lowest to highest B12 intake quintile for all indices measured (Table 3-3). Specifically, in the group of subjects who consumed > 3.4 μg/d of B12 there was a significantly smaller percentage of subjects with low holo-TC or
elevated MMA concentrations than those consuming $\leq 3.4$ $\mu$g/d. In the group of subjects who consumed at least the RDA for B12, there was a significantly lower number of individuals with a low plasma B12 concentration than in the group of individuals who consumed less than the RDA (Table 3-3).

**Discussion**

In this study, the relationship between estimated B12 intake and a panel of B12 status biomarkers were assessed in order to evaluate the adequacy of the current RDA for B12. It has been suggested by another research group that a B12 intake of 6 $\mu$g/d was associated with improved concentrations of all B12 biomarkers compared to an intake of 2.4 $\mu$g/d (118). Although the findings in the current study vary depending on the specific biomarker, the data indicate that an intake greater than the current RDA is associated with normal B12 status. Overall, no clear conclusion can be drawn from these data as to a specific intake level of B12 that might result in normalization of all B12 biomarkers; however, the data suggest that the current RDA may not be optimal. In the first investigation of this study group, subjects were classified as vegetarian or omnivore, and inadequate B12 status was detected in a surprising number of individuals in both groups. Specifically, 40% of vegetarians and 11% of omnivores were determined to have abnormal values for one or more of the four B12 biomarkers. The mean B12 intake of both groups exceeded the RDA. In the current analysis, the third quintile corresponded best to the current RDA for B12 with a mean and range of B12 intake at 2.7 $\mu$g/d and 2.0 to 3.4 $\mu$g B12, respectively. Beyond the second quintile, the mean concentrations of holo-TC and B12 increased; mean Hcy decreased and overall rate of deficiency decreased significantly. This suggests that B12 status improves with intakes above the second quintile,
which in this study was represented as an intake level of 3.4 μg/d. Little or no change was observed in subsequent quintiles, suggesting that an intake level above 3 μg/d may be required.

One limiting factor of the current study is the use of a FFQ to estimate B12 intake rather than a 7-day weighed food record as was used in the study by Bor et al. (118). Because the DHQ relies on subject recall and estimation of intake over the past 12 mo, it is more prone to error and less precise than a direct measure. In addition, neither the one week weighed food record nor a FFQ gives an estimate of duration of a particular diet, and because B12 status is slow to change relative to changes in B12 intake, estimated intake over one week or even over one year may not always correlate well with status at a given time. In a very large study using data of from the Framingham Offspring population, which also used an FFQ to assess B12 intake, improvements in B12 status were observed for intakes up to 10 μg/d (119). Therefore, data from the current study in addition to that from two previous studies agree with the conclusion that the RDA for B12 is inadequate to maintain normal B12 status (39, 118). Further investigations focusing specifically on changes in B12 status with increasing B12 intake are warranted to address this issue and derive an estimate of B12 intake that is consistent with maintenance of normal B12 status. Because the current RDA was established using data from research conducted with patients who had pernicious anemia and who were injected with B12 rather than in healthy individuals consuming dietary B12, there is clear justification for conducting controlled feeding studies to obtain pertinent data necessary to revise the current RDA. The RDAs are not intended to be therapeutic recommendations for individuals with disease conditions such as pernicious anemia. Recommended intake of B12 for the adult population should apply to a majority of the population, potentially with additional recommendations for some sub-groups such as vegetarian
groups and the elderly. In future studies, consideration must be made for potential differences in bioavailability of B12 from foods and fortified products consumed by healthy young adults.

In conclusion, the previous assumption that the general US population has adequate B12 status is not supported by data from this investigation in young healthy adult men and women who consume either omnivorous or vegetarian diets. The data from this study support that from two previous investigations including one in the US indicating that the current RDA for B12 is inadequate to maintain normal B12 status in healthy men and women. Further investigation of the changes in B12 status in response to controlled levels of B12 intake is warranted to provide data to support a revised RDA.

Table 3-1 Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Mean (± SD)</th>
<th>Range</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>26 ± 8</td>
<td>18 - 49</td>
<td></td>
</tr>
<tr>
<td>BMIb</td>
<td>24 ± 4</td>
<td>16 - 48</td>
<td></td>
</tr>
<tr>
<td>B12 intakea (µg/d)</td>
<td>5.4 ± 3.9</td>
<td>0.4 - 22.67</td>
<td></td>
</tr>
<tr>
<td>B12a (pmol/L)</td>
<td>300 ± 134</td>
<td>40 - 937</td>
<td>148 - 444</td>
</tr>
<tr>
<td>Holo-TCa (pmol/L)</td>
<td>85 ± 69</td>
<td>6 - 576</td>
<td>35 - 150</td>
</tr>
<tr>
<td>MMAa (nmol/L)</td>
<td>221 ± 173</td>
<td>81 - 1866</td>
<td>80 - 270</td>
</tr>
<tr>
<td>Hcya (µmol/L)</td>
<td>7.5 ± 2.6</td>
<td>3.5 - 29.6</td>
<td>4.5 - 12.0</td>
</tr>
</tbody>
</table>

a Body mass index (BMI); vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy)

Table 3-2 Correlations (r) between vitamin B12 intake and concentrations of B12 status biomarkers

<table>
<thead>
<tr>
<th></th>
<th>B12a</th>
<th>Holo-TCa</th>
<th>MMAa</th>
<th>Hcya</th>
</tr>
</thead>
<tbody>
<tr>
<td>B12 intakea</td>
<td>0.23</td>
<td>&lt; 0.0001</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>MMAa</td>
<td>-0.17</td>
<td>0.004</td>
<td>-0.12</td>
<td>0.04</td>
</tr>
</tbody>
</table>

a Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy)
Figure 3-1  Total daily vitamin B12 (B12) intake (mean ± SD) in individuals with concentrations outside the normal range for holo-transcobalamin (holo-TC; normal > 35 pmol/L), plasma B12 (normal > 148 pmol/L), serum homocysteine (Hcy; normal < 12 μmol/L), and serum methylmalonic acid (MMA; normal < 270 nmol/L). The current RDA for B12 is represented (-----). *Different from normal (P < 0.01) (ANOVA, Chi-square test)
Figure 3-2  Relationship between vitamin B12 (B12) intake and status. Mean B12 (± SD) intake for each quintile (n = 60 for quintiles 1, 3 and 5; n = 61 for quintiles 2, and 4; respectively), is plotted against concentrations (mean ± SD) of B12, holo-transcobalamin (holo-TC), methylmalonic acid (MMA), and homocysteine (Hcy). Values with different superscript letters are significantly different (P < 0.001 for B12 and MMA, P < 0.01 for holo-TC and P < 0.05 for Hcy). The current RDA for B12 is represented in each graph (----).
Table 3-3  Proportion (%) of individuals with concentrations outside the normal range for select vitamin B12 status biomarkers

<table>
<thead>
<tr>
<th>Biomarker(^a)</th>
<th>Total n</th>
<th>1 (≤ 2.0 μg/d)</th>
<th>2 (≤ 3.4 μg/d)</th>
<th>3 (≤ 5.3 μg/d)</th>
<th>4 (≤ 8.5 μg/d)</th>
<th>5 (≤ 22.67 μg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 148 pmol/L)</td>
<td>50</td>
<td>25(^b)</td>
<td>8</td>
<td>10</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Normal (≥ 148 pmol/L)</td>
<td>248</td>
<td>75</td>
<td>92</td>
<td>90</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td>Holo-TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 35 pmol/L)</td>
<td>33</td>
<td>35(^c)</td>
<td>23(^d)</td>
<td>13(^e)</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Normal (≤ 35 pmol/L)</td>
<td>269</td>
<td>65</td>
<td>77</td>
<td>86</td>
<td>95</td>
<td>93</td>
</tr>
<tr>
<td>MMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated (&gt; 270 nmol/L)</td>
<td>52</td>
<td>32(^c)</td>
<td>23</td>
<td>10</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Normal (≤ 270) nmol/L</td>
<td>248</td>
<td>68</td>
<td>77</td>
<td>90</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td>Hcy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated (&gt; 14 μmol/L)</td>
<td>10</td>
<td>6(^d)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal (≤ 14 pmol/L)</td>
<td>290</td>
<td>90</td>
<td>97</td>
<td>97</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\)Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy); data was not available for all subjects for some biomarkers; \(^b\)Significantly different from Q2, Q3, Q4, Q5; \(^c\)Significantly different from Q3, Q4, Q5; \(^d\)Significantly different from Q4, Q5; \(^e\)Significantly different from Q4. (P < 0.05) (Chi-square test)
CHAPTER 4
GENOTYPE FOR THE TRANSCOBALAMIN 776C→G POLYMORPHISM IS NOT ASSOCIATED WITH ABNORMAL VITAMIN B12 STATUS BIOMARKERS IN HEALTHY ADULTS

Transcobalamin (TC), the B12 transport protein required for cellular uptake is essential to maintain B12 metabolic function (26, 120). A common genetic polymorphism for TC (TC 776 C→G) may impair the metabolic role of this protein (74, 89). It is hypothesized that B12 transport and thus metabolic function will be impaired in individuals with the homozygous variant genotype (GG) for the TC 776 C→G polymorphism. The metabolic and health-related risks associated with this polymorphism are predicted to be exacerbated by the consumption of low-B12 vegetarian diets that exclude specific animal-derived foods. The primary goals of this study were to evaluate the effects of the TC 776 C→G polymorphism on B12 metabolism in young adult men and women who consume a low B12 diet compared to those consuming adequate B12.

Subjects and Methods
Subjects and Subject Recruitment

Healthy adults (n = 302) were recruited from the Alachua county, FL community including university students, faculty and staff. Subjects were initially screened by phone and selected for the study based on the following inclusion criteria: (a) ≥ 18 y & ≤ 49 y (b) no change in meat consumption habits during the last 3; (c) no B12 containing supplement use within the past 6 mo; (d) limited chronic alcohol consumption (<1 drink/d of any kind); (e) no use of tobacco products; (f) no chronic use of prescription medications other than oral contraceptive agents; (g) no history of chronic disease; (h) no chronic blood donations; and (i) non-pregnant and non-lactating. All subjects from the first part of the research described in the manuscript were included in this
analysis after a second informed consent form approved by the University of Florida Institutional 
Review Board that was specific for genetic analysis.

**Study Design and Data Collection**

Subjects were called the day before their scheduled study day to remind them to fast 
overnight (8 hours) and the following morning prior to having their blood drawn. Qualified 
subjects were scheduled for fasting blood sample collection to be performed between the hours 
of 7:00 am and 9:00 am followed by a comprehensive information session explaining how to 
complete the National Cancer Institute Diet History Questionnaire (DHQ), which was used to 
assess dietary intake. A total of 70 mL of blood were collected for analysis of serum holo-TC; 
plasma B12; serum MMA; serum homocysteine (Hcy), serum folate, and DNA extraction.

**Sample Processing and Analysis**

Blood samples were collected in EDTA and SST clot activator tubes. EDTA tubes were 
centrifuged at 2000 x g at 4°C for 30 min to obtain plasma for B12 analyses. SST tubes were 
centrifuged at 650 x g at room temperature for 15 min to obtain serum for holo-TC, MMA, Hcy, 
and folate determination. Samples were stored at −30°C until analysis. Serum holo-TC was 
determined by radioimmunoassay (holo-TC RIA reagent kit; Axis Shield, Ulvenveien, Oslo, 
Norway) based on the method of Ulleland et al. (45) using magnetic microspheres coated with 
anti-transcobalamin monoclonal antibodies to isolate both holo-TC and apo-TC, and 57Co-
labeled B12 as a tracer. Plasma B12 was determined by RIA using a commercially available kit 
(Quantaphase II, Bio-Rad). Serum Hcy and MMA concentrations were determined by gas 
**Genotype Determination**

DNA was extracted from blood as previously described (121) using a commercial kit (Quantum Prep, BioRad, Hercules, CA) and standard laboratory procedures. Genotypes of potential subjects were determined using Dynamic Allele Specific Hybridization (DASH) performed by DynaMetrix (Stockholm, Sweden). Briefly, a short PCR product was created spanning the polymorphic position. One PCR primer was 5’-labeled with biotin for attachment of the amplified targets to streptavidin-coated 96-well microtiter plates. Following denaturation and a wash to remove the unbound strand, an allele-specific probe was hybridized to the bound target DNA strand at low temperature in the presence of the double-strand specific intercalating dye Sybr Green. Finally, the temperature was steadily increased while recording the probe-target duplex melting temperature, as monitored by diminution of Sybr Green fluorescence with a quantitative PCR analysis device.

Properly designed matched target-probe duplexes have higher melting temperatures than those with single-base mismatches, enabling unambiguous allele discrimination. Heterozygous samples show two separate phases of denaturation. For analysis, the negative derivatives of the melting curves are plotted. A single peak at a lower temperature indicates homozygous allelic mismatch to the probe, and a single peak at a higher temperature, a homozygous match. A double peak is generated from a heterozygous sample (Figure 4-1).

**Diet Analysis**

Daily B12 intake was estimated based on data obtained from the DHQ, which was modified to be inclusive of an extensive list of B12-containing foods including meat containing mixed dishes, fortified foods, and meat substitutes. The unmodified DHQ is available for review online at http://appliedresearch.cancer.gov. The DHQ was scanned by Optimal Solutions Corporation (OSC), Lynbrook, New York. Once scanned, OSC sent the dietary data as an
ASCII text file to the University of North Carolina, Chapel Hill (UNC). Data was analyzed using the Diet*Calc Analysis program modified for this version of the DHQ. This freeware program can be downloaded from the NCI website (www.riskfactor.cancer.gov). The B12 content of foods added to the DHQ were based on up-to-date information from the USDA National Nutrient Database for Standard Reference and nutrition labels (39).

Statistical Methods

Results are reported in the text as mean ± SD with an alpha = 0.05. Vitamin B12 status, based on measurement of plasma B12, serum holo-TC, the ratio of holo-TC to B12, MMA, and Hcy, was compared among genotype groups with and without an adjustment for B12 intake, using ANOVA with an alpha of 0.05. Dependent variables also were classified as “normal” vs. “abnormal” according to falling above or below an established threshold (B12 ≥ 148 pmol/L; holo-TC ≥ 35 pmol/L; MMA ≤ 271 nmol/L; and Hcy ≤ 12 µmol/L) and comparisons with respect to genotype were performed using a Pearson Chi-Square test. Qualitative data including gender, age, and ethnicity were compared using a Pearson Chi-Square test. The data were analyzed using Statistical Analysis System Software (SAS Institute Inc. Cary, NC) and PRISM software (Graph-Pad Software Inc. El Camino, CA).

Results

No significant differences were detected among genotype groups for gender, age, or BMI, however there were differences in ethnic distribution (Table 4-1). There were no significant differences among genotype groups, with or without adjustment for B12 intake, for holo-TC, MMA, and Hcy concentration whether considered alone (Table 4-2) or in combination with low B12 status (Table 4-3). There was no significant difference in B12 among genotype groups, though there was a trend for higher B12 in the TC 776 GG group (Table 4-3; P < 0.01). In addition, there were no significant differences among genotype groups in the number of
individuals with values outside the normal range for plasma B12, holo-TC, MMA, or Hcy (Table 4-4).

Individuals with the TC 776 GG genotype had a significantly lower (P < 0.05) ratio of holo-TC to plasma B12 than individuals with the CC genotype (Table 4-2). Total TC was significantly lower (P < 0.001) in the TC 776 GG genotype group compared to both the CG and CC genotype groups (Table 4-2). Transcobalamin saturation was not significantly different among groups.

Discussion

Studies investigating the effect of the TC 776 C → G polymorphism have resulted in conflicting findings. In a previous study by our laboratory, significant differences were detected in holo-TC concentration among the TC 776 genotype groups, however in the present larger study, which included a wider range of B12 intake by subjects, a significant difference was not detected. In addition, there were no significant differences in Hcy or MMA concentration among the genotype groups, further suggesting no real physiological impact of this single base pair mutation of the TC gene on biochemical indexes of B12 metabolism. Even when considering the combined influence of the polymorphism and low B12 status, there were no significant differences in any B12 status biomarkers among the genotype groups, indicating no effect on B12 metabolism in B12 impaired individuals.

Interestingly, some significant differences were found among genotype groups, suggesting a moderate effect of the polymorphism on TC protein synthesis or catabolism. Specifically, total-TC concentration was lower in subjects with the TC 776 GG genotype. Transcobalamin saturation, however, was not different among genotype groups, suggesting no effect on the ability of TC to bind B12. The ratio of holo-TC to B12 also was significantly lower in subjects with the TC 776 GG genotype compared to the CC genotype, though there was no significant
difference in mean holo-TC among the groups. Because there was a significant difference in total-TC among genotype groups but not in TC saturation there must have been some difference in holo-TC as well, because TC saturation is the ratio of holo-TC to total-TC. Although the differences were not significant, holo-TC was somewhat lower and TC saturation somewhat higher in subjects with the TC 776 GG genotype compared to the CC genotype. Additionally, because there was no difference in MMA concentration among genotype groups, even in combination with low B12 status, the reduced concentration of total-TC in the TC 776 CC genotype likely has no important physiological effect on B12 metabolism or functional status.

Previous studies focusing on other higher risk groups, such as the individuals with low B12 intake included in this study, also have not detected a significant effect of the TC 776 C\rightarrow G polymorphism (92, 93, 122). Fodinger et al (93) reported no significant difference in holo-TC or Hcy concentration in end-stage renal disease patients with the TC 776 GG or CC genotypes. Wans et al (92) compared holo-TC, B12, Hcy and MMA concentrations in elderly subjects with the TC 776 CC and GG genotypes, and reported a lower holo-TC concentration in subjects with the TC 776 GG genotype compared to the CC genotype, but no difference in B12 or MMA concentrations. Comparing the absolute difference in mean values in the study by Wans et al. to the current study, the differences were - 4 pmol/L versus 51 pmol/L, respectively, for B12 (GG mean – CC mean); and - 22 pmol/L versus - 6 pmol/L respectively for holo-TC (GG mean - CC mean). Differences in initial B12 status could account for the discrepancies seen in the many studies examining the relationship between genotype status for the TC 776 C\rightarrow G polymorphism and B12 status. Because such small differences in B12 status may overcome any negative effect of the polymorphism, and because changes in metabolic indicators of B12 status such as Hcy and
MMA are not consistently observed, it is unlikely that any B12-related metabolic change related to this polymorphism is of clinical concern.

It is important to note that the developing embryo may be at risk of negative consequences of metabolic changes associated with genetic polymorphisms that coexist with suboptimal nutrient intake. A polymorphism affecting a key folate enzyme, methylenetetrahydrofolate reductase (MTHFR 776 C→T), is associated with a significant increase in risk for neural tube defects, and the risk is exacerbated when folate intake is low (123-125). Again, reports of the effect of the TC 776 C→G polymorphism on birth defect risk have been mixed (30, 94, 126, 127), but most results suggesting an increased risk for pregnant women with the TC 776 GG genotype are not definitive. Potentially, combined effects of several polymorphisms that might interfere with B12 absorption or metabolism could be physiologically important and further investigation may be warranted based on findings from recent studies that considered several types of birth defects (128-130).

Figure 4-1 Melting curve plots for Dynamic Allele Specific Hybridization analysis of polymorphism the TC 776C→G polymorphism. Negative derivatives of Sybr Green fluorescence vs. time plots are shown for two samples of each allele combination. Single peak at a lower temperature (↓) indicates homozygous allelic mismatch to the preferred probe; single peak at a higher temperature (↓↓), a homozygous match; double peaks, a heterozygous sample.
Table 4-1  Demographic distribution of subjects by genotype

<table>
<thead>
<tr>
<th>TC 776 C→G genotype</th>
<th>CC (n = 94)</th>
<th>CG (n = 139)</th>
<th>GG (n = 65)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Male</td>
<td>44</td>
<td>66</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>73</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>20 ± 10</td>
<td>26 ± 7</td>
<td>26 ± 7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>BMIa</td>
<td></td>
<td></td>
<td></td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>White</td>
<td>56</td>
<td>87</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>5</td>
<td>22</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>17</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>12</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

a Body mass index (BMI)

Table 4-2  Mean (± SD) concentrations of selected vitamin B12 biomarkers in all subjects

<table>
<thead>
<tr>
<th>TC 776 C→G genotype</th>
<th>Biomarkera</th>
<th>CC (n = 94)</th>
<th>CG (n = 139)</th>
<th>GG (n = 65)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12 (pmol/L)</td>
<td>280 ± 119</td>
<td>298 ± 137</td>
<td>331 ± 145</td>
<td></td>
</tr>
<tr>
<td>Holo-TC (pmol/L)</td>
<td>87 ± 56</td>
<td>87 ± 68</td>
<td>81 ± 85</td>
<td></td>
</tr>
<tr>
<td>Total-TC (pmol/L)</td>
<td>849 ± 181</td>
<td>763 ± 136</td>
<td>668 ± 144a</td>
<td></td>
</tr>
<tr>
<td>Holo-TC/B12</td>
<td>0.34 ± 0.18</td>
<td>0.31 ± 0.22</td>
<td>0.25 ± 0.17a</td>
<td></td>
</tr>
<tr>
<td>TC Saturation</td>
<td>0.10 ± 0.07</td>
<td>0.11 ± 0.08</td>
<td>0.13 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>MMA (nmol/L)</td>
<td>229 ± 179</td>
<td>221 ± 187</td>
<td>205 ± 128</td>
<td></td>
</tr>
<tr>
<td>Hcy (µmol/L)</td>
<td>7.6 ± 3.1</td>
<td>7.3 ± 1.8</td>
<td>7.6 ± 3.3</td>
<td></td>
</tr>
</tbody>
</table>

a Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy)

Table 4-3  Mean (± SD) concentrations of selected B12 biomarkers in subjects with vitamin B12 deficiency

<table>
<thead>
<tr>
<th>TC 7776C→G genotype</th>
<th>Biomarkera</th>
<th>CC n</th>
<th>CG n</th>
<th>GG n</th>
</tr>
</thead>
<tbody>
<tr>
<td>B12 (pmol/L)</td>
<td>&lt; 148 pmol/L</td>
<td>105 ± 28</td>
<td>10</td>
<td>112 ± 30</td>
</tr>
<tr>
<td>Holo-TC (pmol/L)</td>
<td>&lt; 35 pmol/L</td>
<td>28 ± 8</td>
<td>14</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>MMA (nmol/L)</td>
<td>&gt; 270 nmol/L</td>
<td>505 ± 280</td>
<td>17</td>
<td>461 ± 340</td>
</tr>
</tbody>
</table>

a Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy)
Table 4-4  Percentage of individuals within each TC 776 C→G genotype group with concentrations outside the normal range for select B12 biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>CC</th>
<th>CG</th>
<th>GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma B12 &lt; 148 pmol/L</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Holo-TC &lt; 35 pmol/L</td>
<td>15</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>MMA &gt; 270 nmol/L</td>
<td>18</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Hcy &gt; 12 µmol/L</td>
<td>4</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

*a Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy)*
CHAPTER 5
HOLO-TRANSOCBALAMIN IS AN INDICATOR OF VITAMIN B12 ABSORPTION IN
HEALTHY ADULTS WITH NORMAL VITAMIN B12 STATUS

Circulating B12 is bound to one of two carrier proteins, haptocorrin (HC) or
transcobalamin (TC). Although the majority of B12 (~80%) is bound to HC (holo-HC), only TC
bound B12 (holo-TC) can be taken up by body cells (26). Depletion of total body B12 occurs
slowly, and is often a result of malabsorption, which is difficult to diagnose clinically (13, 81,
131, 132). Currently the only available diagnostic tests for vitamin B12 absorption are not
clinically practical. It has been hypothesized that changes in holo-TC in response to a
supplemental dose of orally administered B12 may be used to assess B12 absorption (28, 47,
104). Bor et al (20) reported a significant increase in holo-TC and TC saturation 24 and 48 hours
after receiving three 9 μg oral B12 doses. Since no blood was collected before 24 hours (post
baseline), the magnitude and pattern of change of holo-TC during the first 24 hours could not be
determined (47). In developing a clinical diagnostic test, it is important to know the optimal time
post dose at which to draw blood. The objective of this study was to evaluate the post-absorption
response of holo-TC to oral B12 relative to other indicators of B12 status.

Subjects and Methods

Subjects

Twenty one healthy adult men (n = 13) and women (n = 8) (18 to 49 y) from the
Gainesville, Florida community were selected based on the following inclusion criteria: (a)
serum B12 concentration > 350 pmol/L at time of screening; (b) no B12-containing supplement
use or B12 injections during past year; (c) no use of tobacco products; (d) no history of chronic
disease; (e) non-pregnant and non-lactating; (f) non-anemic (Hgb ≥11 g/dL [7.4 mmol/L],
females; ≥12 g/dL, [8.1 mmol/L] males); (g) normal blood chemistry profile; (h) BMI between
18 and 29; and (i) no blood donations within 30 days of the study.
Study Design and Data Collection

All participants signed an informed consent form approved by the University of Florida Institutional Review Board prior to the initiation of the study. Individuals had a fasting blood sample drawn at the University of Florida Shands General Clinical Research Center (GCRC). Subjects’ heights and weights were measured and a medical history questionnaire was completed. Blood analyses included serum B12, blood chemistry profile, hematological indices, and a pregnancy test for women.

Eligible subjects were admitted to the GCRC the evening before (day 0) the intervention. The following morning (day 1) after an overnight fast, an indwelling catheter was inserted for all blood collections during day 1. Blood samples were collected a total of 17 times starting on day 1 through day 3, and three 9 μg B12 doses were orally administered at six hour intervals on day 1 beginning after the baseline blood draw (Figure 1). Immediately after taking each B12 dose, subjects consumed a piece of bread and 236 ml (8 oz) of juice to improve absorption efficiency. In addition to the bread and juice consumed with each B12 dose, subjects were given a mid-morning snack and lunch at 2 hours and 3.5 hours, respectively after dose 1. Dinner was fed 4 hours after dose 2, and an evening snack was provided 3 hours after dose 3. The RDA for B12 was provided in the diet on day 1 and on day 2. Take-home meals were provided on day 2 of the study. Water and non-caffeinated, non-caloric beverages were allowed ad libitum. Subjects remained in the GCRC overnight and were allowed to leave on pass following the collection of a fasting blood sample the morning of day 2. Subjects returned on the morning of day 3 at which time a final fasting blood sample was drawn.

Biochemical Analysis

At each blood collection, holo-TC, total-TC, B12, and plasma albumin concentrations were determined. The ratios of holo-TC concentration to total-TC concentration (TC saturation) and
holo-TC concentration to B12 concentration (holo-TC/B12) were determined to assess changes in these indicators in relation to one another. Additionally methylmalonic acid (MMA), creatinine, serum folate, and homocysteine (Hcy) concentrations were measured at baseline. The B12 supplement (9 μg cyanocobalamin) was prepared by Westlab Pharmacy (Gainesville, FL). The B12 content of the supplement was validated by an independent laboratory (Analytical Research Laboratories, Oklahoma City, OK).

Sample Processing and Analysis

Blood samples were collected in EDTA and SST clot activator tubes. EDTA tubes were centrifuged at 2000 x g at 4°C for 30 min to obtain plasma for B12 analyses. SST tubes were centrifuged at 650 x g at room temperature for 15 min to obtain serum for holo-TC, MMA, Hcy, and folate determination. Samples were stored at -80°C in the GCRC until analysis.

Serum B12 and folate concentrations were assayed on the Advia Centaur automated immunoassay system (Bayer A/S, Germany) with a total imprecision below 10%. Total TC concentration was determined by a sandwich ELISA with a total imprecision of 4 to 6% (intra-assay imprecision ~3%) (133). After removal of the apo-TC with B12 coated beads, holo-TC was measured by the TC ELISA. The total imprecision for measurement of holo-TC was ~8% (48), and the intra-assay imprecision was ~4% (134). Albumin and creatinine were measured on the Cobas Integra 800 (Roche Diagnostics, Indianapolis). Total imprecision was ~2 % for albumin and <3 % for creatinine.

Homocysteine concentration was measured by the immunological method on the IMMUNLITE 2000 (Diagnostic Products Corporation, California) (total imprecision <6%) (135) and MMA concentration was measured by slightly modified stable-isotope-dilution capillary gas chromatography mass-spectrometry (total imprecision <8%) (136).
Statistical Methods

Results are reported as mean ± SD with an alpha = 0.05 unless otherwise noted. The overall p-value for time was obtained by the F-test, which tests the null hypothesis that the distribution of the dependent variable was the same at all time points. The Tukey method (137) of multiple comparisons was utilized for assessment of differences between time periods. A “Least Significant Difference” (LSD), as defined by the Tukey procedure, ensures that simultaneously, in every target population, paired difference in means will be within +/- LSD of the corresponding difference in sample means with 95% confidence.

Results

Mean baseline values for all analytes were within normal ranges, although some individuals had values outside the normal range (Table 1). Plasma albumin concentration fluctuated throughout the intervention period suggesting a change in hydration status throughout day one and between the mornings of days 1, 2 and 3 (data not shown). Holo-transcobalamin, B12, and total-TC concentrations are reported as a ratio to albumin to adjust for diurnal changes in overall body protein concentration due to changes in hydration status. Unadjusted means for holo-TC, B12, and total-TC concentrations are reported in Table 2. All time-points are reported relative to baseline. Of all of the status indicator analytes, only holo-TC and TC saturation changed significantly on day 1.

Mean holo-TC concentration increased steadily after baseline and fluctuated throughout day 1. There were statistically significant increases in mean holo-TC concentration during the first 24 hours of the intervention; however, these small increases were not maintained. Mean holo-TC concentration reached a maximum value at hour 24, which was a significant increase relative to baseline and all other time points (Figure 2A). The mean percent increase from baseline also was greater at hour 24 than at all other all time points with a 49% increase relative
to baseline, and a 29% increase relative to hour 12 (Figure 3). This peak at hour 24 was observed for almost all subjects, with an increase of 22% or greater (22 to 85%) for all but one subject. By hour 48, mean holo-TC concentration decreased significantly relative to hour 24 (33%); however, it was still significantly greater than baseline (Figure 5-2A).

Mean serum B12 concentration did not increase significantly relative to baseline on day 1, although there were fluctuations in concentration throughout the day. At hour 24, mean serum B12 concentration was significantly greater than baseline (Figure 5-2B). Overall, the percent change in B12 concentration was smaller than for holo-TC throughout the intervention period with ranges of -2 to 15% and -1 to 50%, respectively.

Mean total-TC concentration did not change significantly during the study varying less than 6% from baseline at all time points (data not shown). Mean TC saturation began to increase significantly relative to baseline at hour 12.5, with the most significant increase at hour 24 (Figure 2C). As observed with holo-TC concentration, the mean TC saturation and percent change at hour 24 were significantly greater than at all other time-points with 48% and 15% increases from baseline and hour 12.5, respectively (Figure 5-4). Among all subjects, the percent change from baseline ranged from 7 to 104% with 19 of 21 subjects having a value of 22% or greater. The ratio of holo-TC to B12 did not increase significantly until hour 24 with absolute and percent increases of 0.15 and 32%, respectively. The range for percent change in this ratio among all subjects was -7 to 109% with 15 of 21 subjects having an increase of 23% or greater at hour 24.

**Discussion**

In this intervention study the changes in markers of B12 status were measured on an hourly basis during and following administration three 9 μg oral doses of B12. In previous studies the
changes in response to similar B12 doses were measured after 24 hours; however, no data were collected prior to this time-point (28, 47, 104). The data from the present study indicate that a series of three 9 µg doses of oral B12, given over 12 hours, led to small fluctuations in holo-TC concentration during the day 1 of the study followed by the previously observed maximum increase in holo-TC concentration 24 hours after the first B12 dose was given. There is a similarity in the overall pattern of change in holo-TC, B12 and TC saturation, with a gradual increase over the first day and the most pronounced increase occurring 24 hours after the initial B12 dose and 13 hours after the final B12 dose.

The timing of B12 absorption and metabolism may explain the pattern of change observed in holo-TC concentration during the first 12 hours of the intervention. An increase in holo-TC concentration is first measurable in the blood after 3 to 4 hours after ingestion and holo-TC can be taken up by cells within minutes (23). It is hypothesized that until cells are saturated with holo-TC, most of it is taken up so quickly that no major changes in blood levels would be observed initially. When intake is sufficient to saturate the cells with B12, significant changes in holo-TC can then be measured.

The absolute and percentage increases in B12 concentration were smaller, occurred later, and were maintained longer than those for holo-TC. This finding is not surprising as total serum B12 consists primarily of holo-HC, and the slower rate of HC metabolism relative to TC metabolism leads to a slower overall turnover of serum B12 and a slower response to changes in intake (26, 138). When comparing these two measures among the individual subjects, holo-TC had the most consistent pattern with only 1 subject not having a change of 20% or greater at hour 24. Additionally, the mean percent change at hour 24 was three times that of B12. Holo-TC concentration is clearly a more sensitive indicator of change in B12 intake and absorption than
serum B12 concentration since it increases earlier after supplementation, increased relatively more than serum B12 and decreased earlier post-supplementation ceased.

Total-transcobalamin concentration did not change significantly during the intervention period. Transcobalamin saturation increased in a similar manner to holo-TC (Figure 5-4). Both holo-TC concentration and TC saturation had comparable results even when considering individual subjects. Of all subjects, 95% and 90% had increases of at least 22% at hour 24 for holo-TC and TC saturation, respectively. In a previous study, a larger change in TC saturation (at hour 24) than for holo-TC was observed, which was due to a drop in total TC at this time point (47). No such conclusion can be made from our data since no significant difference was observed. Since TC saturation is a calculated rather than a direct measure, the potential error in this value is greater than that for holo-TC concentration. Therefore holo-TC concentration may be the better indicator of B12 absorption.

This is the first study to monitor hourly changes in holo-TC concentration in response to oral B12 intake. The most significant change in holo-TC concentration occurred at hour 24, indicating this is the optimal time post-dose at which to measure holo-TC. The three 9 µg oral vitamin dose sequence used in this study was used to minimize passive absorption and maximize the amount of actively absorbed B12 (47, 104). This aspect of the protocol would be important in a clinical B12 absorption test, because it is the capacity to actively absorb B12 that is being assessed. Further studies evaluating the necessity of three doses and the exact timing of the doses are warranted.

In conclusion, holo-TC increases measurably in response to administration of oral B12 within six hours with a maximum peak at 24 hours. Our results indicate that a B12 absorption
test based on measurement of holo-TC following three oral doses of 9 µg B12 should run for 24 hours.

Figure 5-1 Intervention protocol timeline
<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (± SD)</th>
<th>Range</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holo-TC (pmol/L)</td>
<td>85.2 ± 38</td>
<td>41 – 208</td>
<td>40-150</td>
</tr>
<tr>
<td>B12 (pmol/L)</td>
<td>406.9 ± 118</td>
<td>241 – 710</td>
<td>148-444</td>
</tr>
<tr>
<td>Transcobalamin saturation</td>
<td>0.12</td>
<td>0.05 – 0.27</td>
<td>0.05-0.20</td>
</tr>
<tr>
<td>Holo-TC/B12</td>
<td>0.22</td>
<td>0.08 – 0.44</td>
<td>0.15-0.51</td>
</tr>
<tr>
<td>Hcy (µmol/L)</td>
<td>6.6 ± 1.4</td>
<td>3.9 – 9.3</td>
<td>4.5-11.9</td>
</tr>
<tr>
<td>MMA (µmol/L)</td>
<td>0.134 ± 0.060</td>
<td>0.08 – 0.32</td>
<td>0.08-0.28</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>32.7 ± 7.3</td>
<td>22.2 – 54.4</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>69 ± 11.7</td>
<td>48 – 87</td>
<td>50 – 100</td>
</tr>
</tbody>
</table>

*Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA); homocysteine (Hcy)*
Table 5-2  Mean (± SD) concentrations of vitamin B12 status indicators at scheduled intervals

<table>
<thead>
<tr>
<th>Variable</th>
<th>0.5</th>
<th>1.5</th>
<th>2.5</th>
<th>3.5</th>
<th>4.5</th>
<th>5.5</th>
<th>6.0</th>
<th>7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holo-TC&lt;sup&gt;a&lt;/sup&gt; (pmol/L)</td>
<td>84 ± 38</td>
<td>85 ± 39</td>
<td>89 ± 43</td>
<td>91 ± 43</td>
<td>96 ± 43</td>
<td>97 ± 41</td>
<td>99 ± 45</td>
<td>97 ± 42</td>
</tr>
<tr>
<td>B12 (pmol/L)</td>
<td>395 ± 113</td>
<td>397 ± 109</td>
<td>409 ± 114</td>
<td>421 ± 113</td>
<td>431 ± 126</td>
<td>414 ± 109</td>
<td>423 ± 117</td>
<td>426 ± 117</td>
</tr>
<tr>
<td>Total-transcobalamin (pmol/L)</td>
<td>688 ± 134</td>
<td>706 ± 135</td>
<td>723 ± 136</td>
<td>743 ± 138</td>
<td>746 ± 147</td>
<td>763 ± 144</td>
<td>752 ± 141</td>
<td>755 ± 128</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>8.0</th>
<th>9.0</th>
<th>10.0</th>
<th>11.0</th>
<th>11.5</th>
<th>12.5</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holo-TC (pmol/L)</td>
<td>95 ± 41</td>
<td>96 ± 38</td>
<td>96 ± 38</td>
<td>97 ± 39</td>
<td>99 ± 41</td>
<td>100 ± 39</td>
<td>124 ± 46</td>
<td>102 ± 37</td>
</tr>
<tr>
<td>B12 (pmol/L)</td>
<td>424 ± 102</td>
<td>428 ± 102</td>
<td>432 ± 116</td>
<td>423 ± 114</td>
<td>429 ± 112</td>
<td>411 ± 107</td>
<td>456 ± 110</td>
<td>456 ± 115</td>
</tr>
<tr>
<td>Total-transcobalamin (pmol/L)</td>
<td>773 ± 139</td>
<td>772 ± 144</td>
<td>757 ± 143</td>
<td>738 ± 154</td>
<td>739 ± 136</td>
<td>739 ± 139</td>
<td>715 ± 715</td>
<td>758 ± 145</td>
</tr>
</tbody>
</table>

<sup>a</sup>Vitamin B12 (B12); holo-transcobalamin (holo-TC); methylmalonic acid (MMA)
Figure 5-2 Change in vitamin B12 (B12) biomarkers during the 48 hour study period. (A) Mean (± LSD) holo-transcobalamin (■, holo-TC) concentration relative to albumin at scheduled intervals after oral B12 intake (n = 21). Holo-TC increased from baseline at hours 6 to 7 and 11 to 48 (p < 0.001). Holo-TC increased significantly from all other time-points at hour 24 (p < 0.001). (B) Mean (± LSD) B12 (●) concentration relative to albumin at scheduled intervals after oral B12 intake (n = 21). Vitamin B12 increased significantly from baseline at hour 24 (p < 0.001). (C) Mean (± LSD) transcobalamin (TC) saturation (▼) at scheduled intervals after oral B12 intake (n = 21). Transcobalamin saturation increased significantly at hours 12.5 – 48 relative to baseline, and hour 24 relative to all other time-points (p < 0.001). (ANOVA, Tukey test)
Figure 5-3 Mean (± LSD) percent change in holo-transcobalamin (holo-TC; ■) and vitamin B12 (B12; ●) concentrations relative to albumin at scheduled intervals after oral B12 intake (n = 21). The increases in holo-TC and B12 from baseline to hour 24 were significantly larger than changes at all other time-points (p < 0.001) (ANOVA, Tukey’s test)
Figure 5-4 Mean (± LSD) percent change in transcobalamin (TC) saturation (▼), and holo-transcobalamin to vitamin B12 ratio (holo-TC/B12) (▲) at scheduled intervals after oral B12 intake (n = 21). There was a significantly larger percent increase in TC saturation and holo-TC/B12 at hour 24 relative to all other time-points compared to baseline (p < 0.001). (ANOVA, Tukey’s test)
Vitamin B12 is an essential water soluble vitamin functioning as a coenzyme for two metabolic processes, the conversion of methylmalonyl-CoA to succinyl-CoA as adenosylcobalamin and the remethylation of Hcy to methionine as methylcobalamin (13, 35). Absorption and utilization of B12 are dependent on adequate gastric HCl production to release food-bound B12, IF in the intestine for active transport of B12 into the enterocyte, and TC for uptake into body tissues. A deficiency of any of these components can impair B12 metabolism and lead to deficiency even if dietary intake is sufficient (139).

The RDA for B12 is 2.4 μg for adults (140). Older adults (> 60 y) have an increased risk for B12 malabsorption due to an age related increased risk for achlorhydria and auto-immune based pernicious anemia. The RDA for B12 in older adults is also 2.4 μg/d, but it is recommended that synthetic B12 provided by supplements or fortified foods be the primary source (140). Individuals with pernicious anemia are generally treated with IM B12 injections, although it has been reported that passive absorption of megadoses of oral B12 may be sufficient to meet dietary needs (141-143).

Vitamin B12 is synthesized by microorganisms, present in the intestinal microflora and is found naturally only in animal-derived foods. Consequently, individuals who restrict their intake of some or all animal-derived foods limit their chances of consuming a diet that provides an adequate amount of vitamin B12. Consumption of B12-fortified foods or B12-containing vitamin supplements can provide sufficient B12 for these individuals; however, it is estimated that ~ 60 % of the US population does not take supplements (144). Although B12 is required in relatively small amounts, long term adherence to a B12-deficient diet can lead to a B12 deficiency and even moderate B12 deficiency can seriously impair health. Of greatest concern...
are individuals who consume diets with restricted intakes of animal-based foods and who do not take B12-containing vitamin supplements or consume B12 fortified foods. Studies comparing the B12 status of vegetarians and omnivores have led to the conclusion that vegetarians are at greater risk for developing a B12 deficiency compared to omnivores (40, 81, 86, 145); however the majority of these studies have been conducted in Europe and therefore may not be applicable to the US population. Additionally they have included both supplement users and nonusers, making it difficult to interpret the effect of dietary B12 intake alone on status.

It is estimated that B12 intake in the US exceeds the current RDA (2.4 μg/d) leading to the conclusion that B12 dietary inadequacy is not a problem in the US (111, 146). The position of the American Dietetic Association is that “appropriately planned vegetarian diets are healthful, nutritionally adequate, and provide health benefits in the prevention and treatment of certain diseases” (110). The key to this statement is that a meat-free diet must be well planned to ensure that vitamin and mineral needs are met. The data from the current study, in addition to those from the Framingham Offspring study, and an investigation by Bor at al. suggest that consumption of the current RDA is insufficient to maintain normal B12 status in a significant percentage of young healthy adults (118, 119). Although these data do suggest that the current RDA is inadequate to maintain normal B12 status, they are insufficient to provide a definitive estimation of a new RDA. In the current study, a FFQ was used to estimate B12 intake. While data generated from FFQs are adequate for obtaining information on relative frequency of consumption of nutrients, contribution of food categories to overall intake, and estimating intake of key nutrients, FFQs do not generate data precise or specific enough to estimate a nutrient requirement. Future controlled metabolic studies designed to estimate the quantity of B12 intake
at which B12 status is optimal are needed since controlled metabolic studies have proven to be highly useful in estimating other nutrient requirements (147, 148).

Future studies assessing B12 status need to measure multiple indicators of B12 status. One strength of the current series of studies was that numerous biomarkers were used to assess B12 status, rather than just one. Although the assessment of B12 status has traditionally been based on plasma or serum B12, approximately 5 to 10% of individuals with a plasma B12 concentration between 148 to 221 pmol/L, have been reported to have hematological or neurological abnormalities that responded to B12 supplementation (44, 97). Assessment of vitamin B12 status based on serum holo-TC concentration, a relatively new B12 status indicator, has been reported to be an earlier marker of changes in B12 status than total plasma B12 concentration. It has been suggested that measurement of B12 and holo-TC concentrations in combination may be superior to either alone (27, 28, 81, 116, 149). Plasma homocysteine and serum MMA concentrations are functional indicators of B12 status and are inversely related to B12 concentration; however, only MMA concentration is specific for B12 status and is considered by some to be the most reliable B12 status indicator (35, 54). There is no clear consensus as to which particular B12 biomarker might be used as a “gold standard”; however, data from the current set of studies suggest that a panel of B12 biomarkers is preferable to any one status indicator for B12 status assessment. Additionally measurements at multiple time points over several days could help confirm a possible diagnosis of B12 deficiency, particularly in the case of holo-TC, which has been reported to be highly sensitive to changes in dietary B12 intake.

The sensitivity of holo-TC has also led to the hypothesis that it could be used to assess B12 absorption (47, 104). In a previous study conducted by Bor et al. (104), it was reported that
measurement of holo-TC 24 hours after administration of a series of three 9 µg doses of oral B12 identified individuals with B12 malabsorption. Individuals defined as B12 malabsorbers based on the Schilling test for B12 absorption had no significant change in holo-TC in contrast to a significant increase observed in normal controls. In the current set of studies, changes in holo-TC and other markers of B12 status were measured hourly with administration of three 9 µg oral doses of B12, to determine whether any significant changes occur before 24 hours. A clear peak in holo-TC concentration was observed at hour 24 for all but one of the 22 subjects with only small fluctuations in holo-TC prior to that. This was the first study to monitor hourly changes in holo-TC in response to oral B12 suggesting a test of B12 absorption utilizing holo-TC should involve measurement of holo-TC at baseline and 24 hours later. A limitation of the current study was that only healthy individuals with normal B12 status were included in this investigation. It is possible that saturation of cells might be necessary before an increase in holo-TC can be measured even in an individual with no B12 absorption problems. Therefore, individuals with low B12 status may need more oral B12 and may have a later peak increase in holo-TC compared to individuals with normal B12 status. It is important to note that a B12 malabsorption test would only be run in an individual with B12 deficiency; therefore, future studies evaluating holo-TC as a measure of B12 absorption needs to compare the efficacy of changes in holo-TC as an index of B12 absorption in individuals with deficient versus normal B12 status.

A final objective of this series of studies was to determine the effect of specific gene-nutrient interactions on B12 metabolism. Rare congenital defects known to impair B12 metabolism and status include various mutations and post-translational changes that result in altering IF and TC protein structure or a total lack of protein synthesis. Congenital errors in IF or TC lead to pernicious anemia; however errors evolving IF or TC lead to pernicious anemia; however errors evolving
absorption and can be treated by lifelong IM administration of B12, while errors involving TC lead to death early in life because B12 transport and uptake into body cells can not occur (150, 151). Perhaps less apparent than these severe genetic defects, are polymorphisms that also may alter protein structure enough to impair function. One such polymorphism investigated in the present investigation was the TC 776G→G polymorphism. In a previous study by our research group a significant effect of the polymorphism on holo-TC concentration was observed but no difference was detected in the current study. The small differences found between genotype groups in total-TC but not TC saturation suggest some small effect of the polymorphism however, there is likely to be no physiological impact of this polymorphism Previous studies focusing only on the effect of the polymorphism on a developing fetus have resulted in mixed findings, though continued investigations related to the potential association of B12-related polymorphisms and health-related consequences are warranted (128-130).

In conclusion, based on data from this series of investigations it is clear that healthy individuals who do not take supplements may not be consuming adequate B12 to meet biological requirements, particularly those limiting some or all animal-based foods. Although moderate B12 deficiency may not result in overt symptoms, the associated increased risk for disease and birth defect-affected pregnancies provide an impetus for continued research focusing on determining the optimal B12 intake to maintain normal status. Early findings of the potential negative effect of the TC 776C→G polymorphism on B12 metabolism were not confirmed by the current data, and any future investigations should focus on the combined effects of multiple polymorphisms in genes involved in B12 metabolism. Accurate detection and diagnosis of a B12 deficiency and its cause will help in the prevention of related health problems including abnormal pregnancy outcomes. Although there is yet no consensus on a single “gold standard”
test of B12 status, simultaneous measurement of two or more B12 biomarkers at several time points may be the best diagnostic approach. If existence of a B12 deficiency is established, further testing to determine if it is due to dietary insufficiency or malabsorption will aid in determining an appropriate treatment, including changes in dietary B12 intake and/or supplementation. Data from the current investigations support the use of holo-TC as an indicator of B12 absorption though further research is needed before a clinically reliable test could be developed.
APPENDIX A
SUBJECT PHONE SCREENING FORM

**Introduction**

I am calling in regard to your interest in our nutrition study; do you have a few minutes right now?

This is a UF Nutrition department study and involves coming in one morning for about 1 hour for a fasting blood sample, we take about 1 ½ ounces of blood, and you only need to fast 8 hours. We will give you a breakfast snack right afterward, and then give a brief explanation of a food frequency questionnaire you will be taking home. You will be asked to mail it back in the provided envelope, and once we receive the questionnaire you would get paid the $50. I just have to ask you some questions to see if you are eligible for our study and to get background information, OK?

How old are you? Must be 18-49

Do you smoke? Must answer no

Are you pregnant or breastfeeding? Must answer no

Do you take any prescription medications other than oral contraceptives? Must answer no

If not within the age range or if they answer yes to any above questions end call with:

I am very sorry, but you do not meet our exclusion criteria, but thank you for your interest.

Now I just have a few questions about your diet to see what specific category of our study you would fit in to. Please answer as best you can, estimates are okay and consider all instances of when you might eat the items I will ask about, even if only occasionally.

Do you take a multi-vitamin, complex, red star nutritional yeast, or any other supplement or additive ever?

If they take a multivitamin, B complex, red star nutritional yeast, complete the session through all diet info only. Conclude by confirming their name and saying “This has been a preliminary screening call, your information will be reviewed by the principal investigator based on need, and our selection criteria at this time. If you are chosen you will be called again to schedule an appointment over the next two weeks. Thank you very much for your interest and your time.

Do you eat breakfast cereals? (If so) What Kind do you eat mostly?

If they eat a 100% fortified cereal or eats a 50% cereal daily complete the through all diet info but do not record. Conclude by confirming their name and saying “This has been a preliminary screening call, your information will be reviewed by the principal investigator based on need, and our selection criteria at this time. If you are chosen you will be called again to schedule an appointment over the next two weeks. Thank you very much for your interest and your time.

If the interviewee fulfills all selection criteria continue with the questionnaire, record info on moderate/non-fortified cereal consumption
Do you eat breakfast cereals?
  o Yes
  o No

<table>
<thead>
<tr>
<th>Name/Brand</th>
<th>Quantity</th>
<th>Frequency</th>
</tr>
</thead>
</table>

Are you a vegan, vegetarian or meat eater?
Vegan – this means you eat NO animal derived foods intentionally (if they eat small amt like in cake then OK)
Vegetarian – this means you eat NO beef, chicken, turkey, pork, or fish
How often do you eat …

<table>
<thead>
<tr>
<th></th>
<th>Never</th>
<th>Rarely</th>
<th>Occasionally</th>
<th>Frequently</th>
<th>Always</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(&lt;1 x/mo)</td>
<td>(1-4 x/mo)</td>
<td>(2-4 x/wk)</td>
<td>(5-7 x/wk)</td>
<td></td>
</tr>
</tbody>
</table>

Beef
Chicken
Turkey
Pork
Fish
Eggs
Cheese
Cow’s Milk
Yogurt
Other Dairy

Do you follow a restricted diet such as

  o No red meat
  o Lactose-free
  o Kosher
  o Weight loss
  o Weight gain
  o Low salt
  o Low fat
  o Low cholesterol
  o Low carbohydrate
  o Hypoallergenic

(If so) How long have you consumed this type of diet?
________________________________________________________________________

Have you made any major dietary changes within the last 3 years?

  o No
  o Yes; How long ago did you make changes and what changes did you make?
________________________________________________________________________

Do you consume alcoholic beverages?

  o No
  o How often/quantity

Health Information
I am going to ask you a few questions about your health to determine if you are eligible for our study. I will be recording this information, but it will be kept confidential and is this ok with you? ______

Height: ___________ Weight: ___________

Have you do you currently have any of the following?
- Alcoholism
- Anemia
- Blood clots
- Bronchitis
- Cystic Fibrosis
- Dermatitis
- Diabetes
- Eating disorders/Chronic nausea or vomiting
- Food allergy
- Gall bladder disease
- GI problems/ Lactose intolerance
- Gout
- Migraines
- Hemorrhoids
- Hepatitis/Liver disease
- Heart disease/High cholesterol/High blood pressure
- HIV
- Kidney disease
- Neurological disorder
- Obesity
- Seizures/Stroke
- Thyroid problem
- Tumors/Cancer
- Ulcers
- Other

Have you been hospitalized within the last 5 years? ___________

Do you have a history of more than 1 miscarriage?
- Yes
- No

If you are selected to participate in this study are you willing to sign an informed consent understanding we have access to medical information on you?
- Yes
- No

Demographic Information

What is your birth date? _______ / _______ / ________
How would you describe your race or ethnic background?
- White
- Black or African American
- American Indian or Alaska Native
- Hispanic or Latino
- Asian
- Native Hawaiian or Other Pacific Islander
- Other _________________________________________________

What is the highest level of school or training that you have completed? [Circle only one response]

<table>
<thead>
<tr>
<th>Grade school</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
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<th>07</th>
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<td>10</td>
<td>11</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Technical school or college</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Graduate or professional</td>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20+</td>
<td></td>
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<td>Don’t know X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Marital status?
- Single/never married
- Married
- Separated
- Divorced
- Widowed

Are you a full-time or part-time student? Are you employed?
- Full time
- Part time
- Not a student
- Yes
- No
- Student employee
Contact Information

Name

M / F

Address

Last

First

Middle

Street

Apt. #

City

Zip code

Phone

Day

Evening

Cell

E-mail

Name of person and phone number to call in case of an emergency if you are invited to participate in this study:

______________________________________________________________________________

If we need to contact you, and can not reach you where/with who can a message be left?

______________________________________________________________________________

How did you hear about our study? _______________________________________________
APPENDIX B
INTERVENTION DIET

Diet – B12 Intervention Study

DAY 1
Breakfast
- Juice (apple or cranberry)
- Bread

Snack
- Graham crackers
- Peanut butter
- Apple
- Coffee/ tea

Lunch
- Grilled cheese sandwich
- Fruit cocktail
- Pudding
- Beverage*

Dinner
- Bean burrito
- Brown rice
- Salad with dressing
- Pears
- Beverage*

Snack
- Sherbet
- Pound cake
- Beverage*

DAY 2
Breakfast
- Scrambled egg
- Toast with jelly
- Coffee/ tea
- Juice (apple or cranberry)

Snack
- Graham crackers
- Peanut butter
- Apple
- Beverage*

Lunch
- Pita sandwich with hummus and veggies
- Corn chips
- Pineapple
- Beverage*

Dinner
- Cheese tortellini with spaghetti sauce
- Green beans
- Mandarin oranges
- Jello
- Beverage*

Snack
- Pudding
- Shortbread cookies
- Beverage*

*Beverage may be Crystal Light, non-caffeinated soda, Gatorade, Hawaiian Punch (selection to be made with the help of research staff)

Non-caffeinated, non-caloric beverages (Crystal Light, diet soda, water) available throughout the day as desired
LIST OF REFERENCES


7. Castle WB. Observations on etological relationship of achyilia gastrica to pernicious anemia III. Nature of reaction between normal human gastric juice and beef muscle leading to clinical improvement and increased blood formation similar to effect of liver feeding. Am. J. M. Sc. 1930;180:305-35.


Afman LA, Van Der Put NMJ, Thomas CMG, Trijbels JMF, Blom HJ. Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects. QJM 2001;94:159-66.


118. Bor MV, Lydeking-Olsen E, Moller J, Nexo E. A daily intake of approximately 6 micrograms vitamin B-12 appears to saturate all the vitamin B-12-related variables in Danish postmenopausal women. Am J Clin Nutr 2006;83:52-8.


BIOGRAPHICAL SKETCH

Kristina M von Castel - Roberts was born in Camden, New Jersey in 1975. She lived in Glenside and Penllyn, Pennsylvania from 1981 until she graduated from Springside School for girls in 1993. She graduated from the University of Florida in 2000 with a bachelor of science degree in animal science, with a specialization in animal biology. She was employed by the University of Florida Racing Laboratory until she entered her Ph.D. program in nutritional sciences in the fall of 2002 under the Davis Alumni fellowship. During her doctoral program she studied under the tutelage of Dr. Lynn B. Bailey in the field of folate and vitamin B12 nutrition and metabolism. Upon graduation she will continue her career in academia.