A CANDIDATE GENE ANALYSIS OF VITAMIN B12 DEFICIENCY IN ELDERLY WOMEN AND THE NURSING IMPLICATIONS OF PERSONALIZED MEDICINE

by

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ABSTRACT

Functional vitamin B12 (cobalamin) deficiency is a subtle, progressive clinical disorder affecting 6-23% of elderly adults. Its symptoms, including fatigue, mood disturbances, and decreased strength, are vague and erroneously attributed to aging. Detection of cobalamin deficiency in elderly adults is confounded by clinical heterogeneity and lack of standardization in metabolic tests. Whereas some patients are asymptomatic with slightly altered metabolite profiles, others develop severe clinical outcomes. Better understanding of biologic factors contributing to cobalamin deficiency heterogeneity in older adults is needed.

This is a candidate gene association study evaluating the relationship between genetic variation in the cobalamin-transport molecules (transcobalamin II and its receptor) with cobalamin-related outcome parameters in 795 research participants of the Women's Health and Aging 1 and 2 Studies. Research participant DNA was whole genome amplified and genotyped using the iPLEX Sequenom mass spectroscopy platform. Relationships between genotypes and clinical parameters were assessed using two-way analysis of variance and two-way analysis of covariance, on the fixed factors, race and Single Nucleotide Polymorphism genotype.

Results of the dissertation research generated several genetic associations that are useful for further hypothesis testing and clinical validation research. In the transcobalamin II gene, two missense variants were associated with homocysteine

and methylmalonic acid levels (rs9621049, rs35838082), two intronic variants were associated with serum cobalamin and homocysteine levels (rs4820888, rs4820887), and one missense variant was associated with mean corpuscular volume (rs11801198). A cluster of SNPs in the promoter region of the transcobalamin II gene was associated with the physical performance parameters, hand grip strength, and walking speed. In the transcobalamin II-receptor gene, a missense coding SNP (rs2336573) was associated with mean serum cobalamin concentrations.

Scientific advances responsible for the technology used in this dissertation are being incorporated into healthcare. The tailoring of treatment to an individual's genetic make-up is termed Personalized Medicine. To assist nursing professionals in understanding and preparing for use of these technologies, four elements of Personalized Medicine are reviewed, including 1) discovery of novel biology that guides clinical translation mechanisms, 2) genetic risk assessment, 3) molecular diagnostic technology, and 4) pharmacogenetics and pharmacogenomics. Opportunities for nursing profession engagement are addressed.

For Andrew James Thoma

Shaka and Sheba

"If we're serious about preventive medicine, and using personalized genomics

to inform that, we're not going to change the genome.

It's the environment we're going to want to change."

-Francis S. Collins, MD, PhD

Personalized Medicine Coalition Address, 2009: Washington, D.C.

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CHAPTER 1

OVERVIEW OF DISSERTATION RESEARCH

Statement of Clinical Research Problem

Prevalence of metabolically confirmed cobalamin (vitamin B12) deficiency among community-dwelling elderly is between 6% and 23%, and depending upon definition criteria used—as high as 40.5% (Allen, 2009; Baik & Russell, 1999; Johnson et al., 2003; Lindenbaum, Rosenberg, Wilson, Stabler, & Allen, 1994; Pennypacker et al., 1992). Classic hematological and neurological manifestations include megaloblastic anemia, psycho-cognitive decline, and functional impairment. Less recognized is sub-clinical deficiency, involving subtle biochemical and clinical changes, resulting in unrecognized or misattributed diagnosis.

Manifestations of cobalamin deficiency are vague and include fatigue, decreased cognition, malaise, peripheral insensitivity, decreased strength, sleep, and mood disturbances, which present prior to grossly elevated metabolite profiles and hallmark presence of megaloblastic anemia. Long-term consequences of cobalamin deficiency in older adult individuals may increase the disability trajectory, resulting in increased frequency of hospital admissions, lengthier and more severe hospitalizations, and greater degrees of chronic disablement that significantly effect mobility and quality of life (Bartali et al., 2006).

Numerous challenges in detection, diagnosis, and treatment of cobalamin deficiencies in older adults exist secondary to lack of accurate laboratory assays and vast clinical heterogeneity. While some individuals are asymptomatic with low-normal cobalamin levels and slightly altered metabolite screening panels, others with similar profiles develop severe, permanent clinical outcomes (Carmel & Sarrai, 2006). For these reasons, relatively little progress has been made in the identification of cobalamin deficient individuals who would benefit most from pre-emptive supplementation of the nutrient.

Better understanding of the genetic factors contributing to clinical heterogeneity surrounding cobalamin deficiency, subclinical deficiency states, and treatment responses could enhance clinical care of elderly individuals. To identify possible factors contributing to the clinical heterogeneity in cobalamin deficiency, this study used a candidate gene approach to perform a secondary analysis of data and banked biologic samples from the Women's Health and Aging Studies. Because of their roles in cobalamin physiology and metabolism, the candidate genes for the dissertation research included the cobalamin carrier protein (transcobalamin II) and the cobalamin carrier protein receptor (transcobalamin II-receptor). There are different forms of cobalamin; for the purposes of this work, the terms cobalamin and vitamin B12 will be used interchangeably to denote all chemical forms of the nutrient unless otherwise specified through more exact terminology (methyl-, 5' deoxyadenosyl-, cyano-, etc.).

Dissertation Project Scientific Aims and Research Questions

The goal of this project was to determine if genetic variants, Single Nucleotide Polymorphisms (SNPs), in two candidate cobalamin metabolic genes were associated with clinical and biochemical parameters in a cohort of community-dwelling elderly women. Secondary scholarly aims were to orient the scientific data within a broader translation framework for the field of professional nursing, and thus relatable to the context of Personalized Medicine.

Aim 1

Accounting for folate status, Aim 1 was to determine if there are differences in the hematological vitamin B12 indicators, hemoglobin concentration and mean corpuscular volume (MCV) level, by race and SNP genetic variation in the transcobalamin II (vitamin B12 carrier molecule) and transcobalamin II-receptor (vitamin B12 carrier molecule receptor) genes.

Research Question 1.1

Do hemoglobin concentrations differ by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Research Question 1.2

Do MCV levels differ by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Aim 2

Accounting for renal function (questions 2.1-2.3), folate (question 2.2), and cobalamin status (questions 2.2-2.3), Aim 2 was to determine if there are concentration differences in the biochemical vitamin B12 indicators, serum cobalamin, homocysteine, and serum methylmalonic acid, by race and SNP genetic variation in the transcobalamin II and transcobalamin II-receptor genes.

Research Question 2.1

Accounting for renal function, are there differences in serum cobalamin concentrations by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Research Question 2.2

Accounting for renal function, folate, and cobalamin status, are there differences in homocysteine levels by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Research Question 2.3

Accounting for renal function and cobalamin status, are there differences in serum methylmalonic acid levels by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Aim 3

Aim 3 was to determine if there are differences in the neurologic vitamin B12 indicators, depression score and peripheral vibration sensitivity, by race and genetic variation within the transcobalamin II and transcobalamin II-receptor genes.

Research Question 3.1

Do depression scores differ by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Research Question 3.2

Do peripheral extremity vibratory sensation scores differ by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Aim 4

Aim 4 was to determine if there are differences in the functional performance vitamin B12 indicators, hand grip strength, and walking speed, by race and genetic variation within the transcobalamin II and transcobalamin II-receptor genes.

Research Question 4.1

Does hand grip strength differ by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Research Question 4.2

Accounting for standing height, does walking speed differ by race and SNPs in the transcobalamin II and transcobalamin II-receptor genes?

Aim 5

Aim 5 was to identify opportunities for the field of professional nursing in the area of genetics and genomics, or Personalized Medicine.

Research Question 5.1

What is the current state of genetic/genomic science as relevant for professional nurses in research, education, and practice settings?

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CHAPTER 2

THE MOLECULAR, PHYSIOLOGIC, GENETIC, AND CLINICAL BASIS OF VITAMIN B12 METABOLISM IN AGING INDIVIDUALS

History and Origins

The critical importance of vitamin B12 to human physiology has a rich history steeped in vigilant patient observation. The initial discovery of what is now known to be pernicious anemia can be traced to 1824 when James Scarfe Combe, a civil practice physician, described an anecdotal account of an individual whose symptoms included severe pallor, thirst, diarrhea, and excessive urination (Combe, 1824). The patient, Mr. Alexander Haynes, initially presented to Combe in July of 1821 at which point the symptoms progressively became worse until his death in February 1822.

Dr. Combe's report did not gain recognition as being significant until 1849, when English physician Thomas Addison submitted a description of anemia "commencing insidiously, and proceeding very slowly, so as to occupy a period of several weeks, or even months, before any serious alarm is taken by either the patient or by the patient's friends" (Addison, 1849). Addison's clinical observations outlined physiological characteristics unlike any other previous classification of anemia, most striking of which was the subtle and progressive clinical course of symptom development. A historical

account cites that in 1872, a German physician from Zurich, Dr. Biermer, noted the severe intractable progressive characteristics of the Addisonian anemia in 15 subjects and issued the title “pernicious anemia” (Sinclair, 2008).

Patients suffered over decades from pernicious anemia and its unsuccessful treatment; common therapeutic interventions for the condition included dietary modifications, exposure to sunlight, and even use of an arsenic supplementation known as “Fowler’s Solution” (Sinclair, 2008). Things would not change until 1925 when a team of three physicians, Drs. Whipple, Hooper, and Robscheit, researched hematopoeisis associated with chronic blood loss. Among the treatment interventions studied was the administration of beef liver to canines and significant findings were obtained; dogs who were bled and fed the uncooked liver were noted to experience rapid resolution of anemic symptoms (Whipple, 1925). Two prominent Boston-area physicians in the United States who learned of the work developed a protocol for human patients with pernicious anemia, and fed 45 patients a high protein daily diet incorporating 120-240 grams of raw liver (Minot, 1926). Within days of starting treatment, patients’ jaundice began to resolve, reticulocyte counts increased, and hemoglobin values normalized.

This discovery went on to be confirmed by many practicing physicians and in 1934, Drs. Whipple, Minot, and Murphy were the first Americans to receive the Nobel Prize in Medicine and Physiology. In 1948, the fraction responsible for this physiologic, dubbed vitamin B12, was purified from liver and kidney and shortly after, daily dietary intake requirements were established (Rickes, Brink, Koniuszy, Wood, & Folkers, 1948; E. Smith, 1948). As researchers identified cobalt as a key component of vitamin B12, its name was changed from vitamin B12 to cobalamin, and in 1955-1956, a scientific team

led by Dorothy Hodgkin used x-ray crystallography to identify and elucidate its crystalline 3-dimensional structure. Using electron density measurements, Hodgkin's landmark effort identified atomic positions of elements surrounding the central cobalt atom (Hodgkin et al., 1956; Hodgkin, Pickworth, Robertson, Trueblood, & Prosen, 1955). The discovery led to another Nobel Prize, this time in Chemistry, and was issued to Dr. Hodgkin in 1964.

Scientific progress from the late 1950s through the 1960s was notable for the identification of variable chemical isoforms of cobalamin and the key enzymes directing molecular rearrangements in metabolic reduction/oxidation reactions. In 1958, Barker, Weissbach, and colleagues discovered a key biologic role for vitamin B12 in the bacterial model system *Clostridium tetanomorphum*; the conversion of L-methylmalonyl-Coenzyme A required adenosylcobalamin to formulate succinyl-Coenzyme A (Barker, Smyth, Wawszkiewicz, Lee, & Wilson, 1958; Barker, Smyth, Wilson, & Weissbach, 1959; Barker, Weissbach, & Smyth, 1958). In 1962, Smith and colleagues created carbon-enriched methylcobalamin and found that it could serve as a cofactor for methionine synthase (Guest, Friedman, Woods, & Smith, 1962). Shortly after, Weissbach deducted the chemical reaction responsible for methionine synthesis, and proved methylcobalamin serves as the necessary cofactor for conversion of homocysteine to methionine (Weissbach & Taylor, 1966).

In the 1970s, scientists produced the total chemical synthesis of cobalamin, a monumental achievement. The effort was notable for 11 years' worth of chemical reaction calculations and required the skills of over 100 collaborating scientists (Woodward, 1973). Heading the project at Harvard University's Department of

Chemistry was Dr. Robert Woodward, who, in 1965, also received the Nobel Prize for Chemistry.

In the 1980s-1990s, scientists identified the stereochemistry and functional mechanisms responsible for cobalamin-dependent reduction/oxidation rearrangements. The piecing together of all the intricate steps of aerobic microbial cobalamin biosynthesis in 1993 was a capstone achievement spanning over 25 years of scientific research (Battersby, 1994). Elucidation of three-dimensional structures of the methionine synthase and methylmalonyl CoA enzymes provided understanding of how vitamin B12 reactions occur in both mammalian and microbial species (Dixon, Huang, Matthews, & Ludwig, 1996; Drennan, Huang, Drummond, Matthews, & Lidwig, 1994; Mancia et al., 1996). Recently, techniques and knowledge derived from fields such as genetics, molecular biology, and recombinant engineering are permitting not just discovery, but purposeful manipulation of both the aerobic and anaerobic cobalamin pathways to understand intermediate steps and biosynthetic processes across all life forms (Battersby, 1994; Warren, Raux, Schubert, & Escalante-Semerena, 2002).

Chemical Properties

The chemical activities of vitamin B12 vary temporally, spatially, and across numerous life forms. Their overall function can be broken down into three distinct categories where they can serve as 1) mutases, facilitating electron exchange between hydrogen and other atoms nested between two carbon atoms; 2) ribonucleotide reductases, reducing ribonucleotide triphosphate to 2'-deoxyribonucleotide phosphate via adenosylcobalamin; and 3) intermolecular methyl group transfers, shuttling of methyl

groups across chemical bonds and intermediaries as catalyzed by methylcobalamin (Green & Miller, 2007; Martens, Barg, Warren, & Jahn, 2002). In the animal kingdom, cobalamin is used for only two enzymatic reactions, which will be discussed in a later section.

Structure

The organometallic cobalamin molecule (chemical formula $C_{83}H_{88}O_{14}N_{14}PCo$; molecular weight 1355 daltons) is among the most structurally complex found in all of nature. There are two primary features in addition to its central cobalt atom, including (1) a planar corrin ring and (2) a nucleotide that lies perpendicular to the planar group. Comprising the nucleotide is a base, 5,6-dimethylbenzimidazole, and a phosphorylated sugar, ribose-3-phosphate. The 5,6-dimethylbenzimidazole base is exclusive to the cobalamin molecule in nature, and the ribose is unusually phosphorylated at carbon position 3.

The corrin ring contains a group of four pyrroles (5-member rings of $C_4H_4NCH_3$) with each N atom affixed and coordinated to the central cobalt atom. A fifth ligand extends from the central cobalt atom, where various functional (R) groups may attach and yield various biologic forms of cobalamin. The four primary groups in mammals include a 5'-deoxyadenosyl group (adenosylcobalamin), a hydroxyl group (hydroxocobalamin), a methyl group (methylcobalamin), and a glutathione group (glutathionylcobalamin). The two co-enzyme forms of cobalamin directly relevant to humans are adenosylcobalamin, found in mitochondrial membranes, and methylcobalamin in the cytosol, which is

clinically measurable in human plasma. Other forms of cobalamin exist in plants and bacteria.

Variable oxidation and reduction states of the cobalt atom yields greater complexity, where arrangement of electrons can result in varying forms of hydroxo-, adenosyl-, and methylcobalamin. For example, trivalent cob(III)alamin represents full oxidation capacity in the hydroxocobalamin form, but adenosylcobalamin and methylcobalamin contain divalent cob(II)alamin and monovalent cob(I)alamin states of the cobalt atom.

As the C-Co chemical bonds in cobalamin are extremely sensitive to degradation, in the presence of light and a cyanide source, all cobalamin forms are converted to cyanocobalamin. This is cobalamin's most stable form, and subsequently is the commercial preparation used for the majority of pharmaceutical and therapeutic applications in the U.S. (National Academy of Sciences, 1998). Regardless of the form, the cobalamin that is delivered to mammalian cells is enzymatically activated to either methylcobalamin or 5'-deoxyadenosylcobalamin (Scott, 1999).

Synthesis

Vitamin B12 is the most chemically complex vitamin; in biologic systems that produce it, the coordinated and functional integration of over 30 genes is required. Although cobalamin is required by humans and mammals, synthesis of vitamin B12 is exclusive to microorganisms, and even then—limited to members of the Archea and Eubacteria families (Raux, Schubert, & Warren, 2000). Plants and fungi are understood not to produce or use cobalamin (Benner, Ellington, & Tauer, 1989).

Microbial synthesis of vitamin B12 can be either aerobic or anaerobic, and begins along a complex branched pathway starting with uroporphyrinogen III. The formation of adenosylcobalamin from uroporphyrinogen III has three distinct steps including 1) synthesis of the corrin ring, 2) construction of the nucleotide base (right-angle) ligand, and 3) the piecing together of the corrin ring with the base ligand to produce the final coenzyme (Roth, Lawrence, Rubenfield, Kieffer-Higgins, & Church, 1993). Both aerobic and anaerobic pathways begin with uroporphyrinogen III, with precorrin biosynthetic intermediates of cobalamin successively carrying methyl groups across varying numbered carbon units (i.e., precorrin 1, precorrin 2, precorrin 3A, etc.) (Battersby, 1994). However, the most significant difference between the two biosynthetic pathways is the point of cobalt insertion; aerobic production features a late insertion whereas anaerobic production is characterized by early incorporation (Warren et al., 2002). Metabolism of cobalamin in mammalian cells will be discussed later in this chapter.

Food Sources and Daily Requirements

As cobalamin is produced exclusively by certain bacteria, natural sources of its production are found in microorganisms from soil, sewage, water, human and other mammalian intestines, and animal rumens (first stomach of plant-eating mammals such as cattle, sheep, and goats). Thus, human dependency on cobalamin is rooted in dietary intake of the animals ingesting microbially synthesized vitamin B12. Human food sources rich in vitamin B12 are animal-based protein, including liver, meat, seafood, shellfish, eggs, and dairy products.

Vitamin B12 content for standard Western diets varies between 5-30 micrograms per day and fulfills average daily intake requirements of 7-8 micrograms per day for men, 4-5 micrograms per day for women, and 3-4 micrograms per day for children under age five (Beck, 2001). For adolescents, the recommended daily intake requirement for cobalamin is approximately 2 micrograms per day (The Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Subcomittee on Upper Reference Levels of Nutrients, Food and Nutrition Board, 1998). Intestinal microbes represent an additional nondietary source of vitamin B12 for humans, and account for absorption of 1-5 micrograms per day (Heyssel, Bozian, Darby, & Bell, 1966). Obligatory losses of the nutrient have been established at a rate of 0.1% of the body's circulatory cobalamin pool per day, and occurs independently from other factors such as total body storage amounts in tissues and the liver (Heyssel et al., 1966).

Physiologic Properties

Absorption

The normal absorption of vitamin B12 in mammals demonstrates both passive and active mechanisms (Rosenblatt & Fenton, 2001). Passive absorption accounts for 1-2% of oral intake, occurs rapidly throughout the entire gastrointestinal tract, and is extremely inefficient (Green & Miller, 2007). Active absorption is dependent upon the coordinated actions of binding proteins that attach to ingested dietary protein in the stomach and intestine, and facilitate its entry into the plasma (Herrmann, Obeid, Schorr, & Geisel, 2003; Rosenblatt & Fenton, 2001). Age-related changes in humans related to these processes are discussed in a later section in this chapter.

Intrinsic Factor

After food containing vitamin B12 enters the stomach, gastric parietal cells in the fundus and body of the stomach release pepsin and intrinsic factor (IF). In the acidic environment of the stomach, pepsin breaks down food/protein particles to release vitamin B12, which attaches to salivary R-binder proteins (haptocorrin family). Salivary R-binder/B12 complexes then pass through the duodenum, where the pancreatic enzyme trypsin splits them apart. This frees vitamin B12 to bind to IF. Binding of vitamin B12 to IF is dependent on specific folding interactions with the 5,6-dimethylbenzimidazole base and the corrin ring, after which the complex shrinks to close around the cobalamin molecule (Lien, Ellenbogen, Law, & Wood, 1974). This binding process protects both IF and vitamin B12; free IF is particularly susceptible to rapid degradation by pancreatic enzymes, and similarly in acid, free vitamin B12 is susceptible to side chain modifications of the corrin ring and removal of axial ligands (Kondo et al., 1982). In mammals, all forms of vitamin B12 (methylcobalamin, adenosylcobalamin, cyanacobalamin) are absorbed by the IF-dependent mechanism.

Cubulin and Megalin

IF/B12 complexes travel the small intestine until they come into contact with IF receptors embedded on the outer surface of the terminal ileum, and are endocytosed. Ileal mucosal receptors preferentially accept IF/B12 over free IF, and binding takes place in villous cells as opposed to crypts (Kapadia & Essandoh, 1988; Mathan, Babior, & Donaldson, 1974). There are two structural subcomponents to the IF receptors—cubulin and megalin. Cubulin is a peripherally attached glycoprotein on the intestinal brush

border, and megalin is a large, endocytotic transmembrane glycoprotein (Alpers, 2005; Birn et al., 1997; Christensen & Birn, 2002). On the external border of the enterocyte, cubulin uses calcium ions to recognize and bind the IF/B12 complex (Barth & Argraves, 2001; Birn, 2006). Upon cubulin recognition and ligand binding of the IF/B12 complex, megalin facilitates entry of IF/B12 into the ileal enterocyte via receptor-mediated endocytosis (Green & Miller, 2007).

Cubulin and megalin have broader physiologic functions than just the binding of IF/B12 complexes in the small intestine. Located in the plasma membranes and endocytoplasmic surfaces of cells across various types of epithelial tissue, cubulin and megalin colocalize for ligand binding in renal epithelium, visceral yolk sacs, and in the placental cytotrophoblast (Birn et al., 1997; Moestrup et al., 1998). Other ligands bound by the cubulin and megalin dual-receptor complex include albumin, vitamin-D binding protein, and hemoglobin (Birn et al., 2000; Cui, Verroust, Moestrup, & Christensen, 1996; Gburek et al., 2002; Nykjaer et al., 1999; Nykjaer et al., 2001). Categorized as part of the low-density lipoprotein family, megalin also binds ligands singularly (without cubulin) in a wider array of epithelial tissues. Present in lung alveoli, epididymis, endometrium, oviduct, inner ear, thryocytes, eye cilia, choroid plexus in cerebral ventricles, kidney, and parathyroid, megalin binds ligands, including retinol binding protein, lactoferrin, apolipoproteins (B, E, J, H), hormones, drugs, toxins, enzymes, immune, stress-response-related proteins, and B12 transcobalamin II complex during enterohepatic recirculation (Christensen & Birn, 2002).

Transport

Transcobalamin II

After an ileal enterocyte absorbs the IF/B12 complex, vitamin B12 is dissociated from IF in lysosomal compartments. It is then paired with the cobalamin transport protein transcobalamin II, released into the portal circulation, and carried to cells in target tissues throughout the body. After ingestion of food, the time required for absorption and binding to transcobalamin II is approximately 3-4 hours (Carkeet et al., 2006). Under normal physiologic conditions, transcobalamin II is 20-30% saturated, and represents 10-20% of the total circulatory cobalamin pool (Refsum, Johnston, Guttormsen, & Nexo, 2006). Also called holotranscobalamin (holoTC), it is the biologically active form of vitamin B12 in the body and has a half-life of approximately 2 hours (Lindgren, Kilander, Bagge, & Nexo, 1999). Upon arrival at target cells, the B12/transcobalamin II binds to the transcobalamin II-receptor embedded in a cell's plasma membrane, and vitamin B12 is endocytosed into an intracellular lysosome (Christensen & Birn, 2002).

Transcobalamin I (Haptocorrin)

In contrast to transcobalamin II-mediated transport of cobalamin (10-20%), the remaining 80-90% of circulating B12 in blood is bound to plasma transcobalamin I (Carmel, 1985). Transcobalamin I has a second isoform known as transcobalamin III. Both are encoded by a single gene. Transcobalamin III contains the same protein core as transcobalamin I but is glycosylated differently. These sugar moieties change the biophysical properties of the molecule but do not significantly alter vitamin B12 binding. For the purposes of this work, transcobalamin I will be used to denote both isoforms.

Part of the haptocorrin family, transcobalamin I belongs to a group of cobalamin-binding glyoproteins that are also found in saliva and gastric mucosa (R-binders), (Russell-Jones & Alpers, 1999). In the blood, transcobalamin I does not facilitate direct B12 uptake from a receptor-mediated mechanism; it binds to an asialoglycoprotein receptor in the liver and transports released hepatic cobalamin stores, recycling some into bile. Hypothesized functions of transcobalamin I include the removal of cobalamin analogs from the bloodstream (Burger, Schneider, Mehlman, & Allen, 1975; Hardlei & Nexo, 2009). Vitamin B12 analogs bound to the transcobalamin I protein turn over extremely slowly and demonstrate a half-life of approximately 10 days (Finkler & Hall, 1967). There is significant unsaturated binding capacity and up to 47.1% of transcobalamin I in plasma can be free of cobalamin analogs (Beck, 2001). Transcobalamin I receptors are not ubiquitously expressed in tissues; thus, in the blood, transcobalamin I's role as an effective transport protein is unclear.

Metabolism: Vitamin B12 Biochemistry

Vitamin B12 is required in all mammalian cells for one-carbon metabolism and cellular mitosis (Refsum et al., 2006). It plays important roles in two essential reactions, one mitochondrial and the other cytoplasmic (Figure 1). In the mitochondria, vitamin B12 (5'-deoxyadenosylcobalamin) is required for the enzyme methylmalonyl CoA mutase, which catalyzes conversion of methylmalonyl CoA to succinyl CoA. This conversion is critical for odd-chain fatty acid oxidation and ketogenic amino acid catabolism (Green & Miller, 2007).

In the cytoplasm, vitamin B12 (methylcobalamin) is used in the conversion of homocysteine to methionine, and simultaneously overlaps with folate-dependent methylation and carbon exchange (Allen, Stabler, Savage, & Lindenbaum, 1993). Methionine is necessary for methylation, proper protein synthesis, and DNA formation. Methylcobalamin catalyzes a two-substrate two-product reaction. The conversion of homocysteine to methionine and folate-dependent reactions co-occur, the latter being the conversion of 5-methylenetetrahydrofolate (5-methyl THF) to tetrahydrofolate (THF). The end result of cobalamin-folate one-carbon metabolism mechanism is DNA precursor formation, deoxythymidine monophosphate (dTMP) (Beck, 2001).

Recycling Mechanisms

Storage

Half of ingested B12 is delivered immediately to tissues by transcobalamin II while the other half is taken up by the liver (National Academy of Sciences, 1998). In healthy adults, total body cobalamin stores are between 2 and 4 milligrams, with total hepatic content between 1 and 1.5 milligrams (Grasbeck, 1959). The large majority of hepatic cobalamin stores (up to 70%) are comprised of adenosylcobalamin. Mobilization and liberation of vitamin B12 stores is hypothesized to occur via hepatic cell haptocorrin surface receptors (Burger et al., 1975).

Reabsorption

B12 undergoes enterohepatic recycling and between 0.5-9.0 micrograms per day is released into the gastrointestinal tract from biliary content (Grasbeck, 1959; Grasbeck,

Nyberg, & Reizenstein, 1958). Of the 0.5-9.0 micrograms, 65-70% is intestinally reabsorbed through the actions of intrinsic factor (Booth & Spray, 1960). Also classified as part of the enterohepatic circulatory process, vitamin B12 from sloughed intestinal epithelial cells is absorbed. It has been hypothesized that presence of bile may enhance cobalamin absorption from the intestine (Green, Jacobsen, Van Tonder, Kew, & Metz, 1982).

Excretion

Vitamin B12 bound to carrier proteins filters through renal glomeruli with tubular reabsorption to prevent excessive losses. In the renal tubular epithelium, colocalized cubulin and megalin absorb cobalamin/transcobalamin II via receptor-mediated endocytosis (Birn, 2006). Normal renal filtration uptake of the cobalamin/transcobalamin II complex is estimated at 1.5 micrograms (Lindemans, van Kapel, & Abels, 1986). Because very small amounts of vitamin B12 binding proteins are measurable in human urine, it is recognized that tubular reabsorption is very effective (Hall, 1964; Wahlstedt & Grasbeck, 1985). When circulating levels of the vitamin B12/transcobalamin II complex exceeds the rate-limited binding ability of megalin and cubulin, the excess is excreted into urine.

Cobalamin Transport Genetics and Molecular Biology

Individual Genes in Transport

The cobalamin transport system is categorized into three main groups of ligands and receptors and includes intrinsic factor (IF) and the IF receptor, transcobalamin II and

the transcobalamin II-receptor, and haptocorrin and the haptocorrin receptor (Seetharam & Yammani, 2003). This section provides an overview of key genetic and molecular biology principles of the genes studied in this dissertation, transcobalamin II and the transcobalamin II-receptor. Also highlighted is the significance of the biologic overlap of transcobalamin II with the other cobalamin transporters, as demonstrated by shared exonic sequences, interspecies conservation, and amino-acid homology (Russell-Jones & Alpers, 1999).

Transcobalamin II Gene

The transcobalamin II gene, located at 22q12.2, has nine exons, eight introns, and a total length of 19,887 base pairs. The final protein product is non-glycosylated, comprises 427 amino acid residues, and yields a molecular mass of 43 kDa (Seetharam & Li, 2000). In humans, expression occurs across many different tissue types, but at varying levels. Li and associates (1994) reported a single 1.9kb ^{32}P-labelled cDNA band present across heart, brain, placenta, lung, liver, muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocyte tissue samples using Northern blot analysis (Li, Seetharam, Rosenblatt, & Seetharam, 1994). Quadros and associates (1989) identified a similar band, but analyses identified amino acid differences at three codon positions, leading to the hypothesis that there are multiple isoforms of transcobalamin II (Quadros, Rothenberg, & Jaffe, 1989). Kidney tissue expresses highest levels of transcobalamin II mRNA and compared to this kidney baseline (100%), heart and pancreas tissues were at 15%, placenta, lung, liver, muscle, prostate, and ovary tissues at 7-9%, and all remaining tissues were at between 2-5% of kidney mRNA

expression. Regulation of gene expression occurs through a promoter demonstrating features including a 1kb 5'-flanking region that is GC-rich, absence of a TATA start-transcription motif, and presence of multiple transcription start sites such as Sp1, CF1, HIP1, Ets-1, and MED-1 elements (Li, Seetharam, & Seetharam, 1995, 1998). Transcobalamin II expression occurs through binding to a TGGTCC (5'-3') hexameric sequence that is located 121bp upstream from the transcription start site (Regec, Quadros, & Rothenberg, 2002).

Transcobalamin II-Receptor Gene

The transcobalamin II-receptor gene, located at 19p13.3, has five exons, four introns, and is 6,229 base pairs in length. The final protein product is heavily glycosylated with a molecular mass of 58,000 atomic mass units (Quadros, Sai, & Rothenberg, 1994).

Expression of the transcobalamin II-receptor gene has been identified in many human tissues. Bose and associates (1995) used immunoblotting experiments to ascertain presence of a noncovalent homodimerized 124 kDa protein band (Bose, Seetharam, & Seetharam, 1995). Quantitative evaluation showed expression in human kidney was the greatest (100%) followed by placenta at 28%, intestine at 18%, and liver at 2%. Subsequent monomeric purification of the 124 kDa fragment yielded a 62 kDa band bound to a phospholipid bilayer. Study of the purified 62 kDa fragment identified a single polypeptide with 27% carbohydrate content and four intermolecular disulfide bonds; these characteristics were thought to indicate that transcobalamin II-receptor's functional

importance extended beyond ligand binding to include Golgi-trafficking across plasma membranes (Bose & Seetharam, 1997).

Despite these novel purification findings, they proved incomplete and irreproducible until 2005, when the binding and functional properties of transcobalamin II were described by Quadros and associates (Quadros, Nakayama, & Sequeira, 2005). Results differed significantly from previously published work, where a 58 kDa band was observed and demonstrated binding specificity lacking in previous reports. Full purification and definitive identification of transcobalamin II-receptor's primary structure and gene sequence was published in 2009 (Quadros, Nakayama, & Sequeira, 2009). Because transcobalamin II-receptor is highly glycosylated, the true size and the conformations of the attached sugars were difficult to identify and likely contributed to ambiguous results between various laboratory efforts. Quadros and associates (2009) used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate a single homogenous band of 58-60 kDa that was 252 amino acid residues in length. Comprising the 252-amino acid sequence is an extracellular domain of 199 residues, a transmembrane domain of 21 residues, and a cytoplasmic domain of 32 residues. Within the molecule are 18 residues that comprise two low-density lipoprotein receptor-class A domains, indicating that disulfide bonding is likely.

Relationships Between Cobalamin Transport Genes

Since cobalamin is a highly polar and complex molecule, it cannot easily cross plasma membranes in physiologically significant amounts. All higher animals, including humans, use hydrophobic cobalamin transport proteins to bind dietary vitamin B12, and

facilitate its movement from the stomach and intestine (haptocorrin R-binders and intrinsic factor) into the plasma and cells (transcobalamin II), and across circulatory storage pools (transcobalamin I in the haptocorrin family).

Sequence Alignment

Although proteins across the three groups are distinct and function through specific receptors, they share biologic similarity in their capacity to bind cobalamin with high affinity (Seetharam, 1999). This insight was first appreciated when Hoedemaker and associates described presence of IF in the stomachs of multiple species (Hoedemaeker, Abels, Wachters, Arends, & Nieweg, 1966). Several decades later, molecular biologic approaches significantly advanced this understanding. Through techniques such as cloning, genetic sequence from multiple organisms was compared in genes across the three groups.

A 1988 analysis of genetic sequence (cDNA) for rat IF determined primary structural domains for cobalamin binding and outlined preliminary homologies to other biochemical proteins (Dieckgraefe, Seetharam, Banaszak, Leykam, & Alpers, 1988). Comparison of these genetic rat IF features to human transcobalamin II and transcobalamin I (haptocorrin) sequences yielded identification of homologous regions across the groups; transcobalamin II had 20% amino acid identity with both human transcobalamin I and rat IF (Platica et al., 1991). Simultaneous progress in porcine model systems produced additional alignments between rat IF, porcine haptocorrin, and human transcobalamin I (Hewitt, Seetharam, Leykam, & Alpers, 1990; Johnston, Bollekens, Allen, & Berliner, 1989). Collectively from these studies, overall protein alignment was

at 33% and found within them were six regions of high structural sharing, at 60-90% similarity in amino acid sequence. The most highly conserved of these six regions extends across 15 residues, from position 174 to 188 ($_{174}$SVDTAAMAGLAFTCL$_{188}$).

Ancestral Origins

The overlap across species for the cobalamin binding protein genes is significant because it indicated a likely common ancestral gene of evolutionary origin. Recent mammalian (rat, mouse, porcine, bovine, human) phylogenetic analysis of the highly conserved 15-amino acid residue showed an evolutionary relationship suggesting that transcobalamin II evolved earlier than other cobalamin binding proteins (Kalra, Li, Yammani, Seetharam, & Seetharam, 2004). The radial tree that was generated from this work pictured a relationship where transcobalamin II evolved independently and earlier than that of IF and haptocorrin, which co-evolved in a more dependent manner. The analysis was also significant for the convergence of mouse and rat transcobalamin II, with 95-98% sequence homology compared to 71-74% for human and bovine counterparts.

Pathophysiology of Cobalamin Deficiency

Since total-body stores of cobalamin are between 2 and 5 milligrams and daily requirements are at several micrograms per day, abrupt cessation of cobalamin dietary intake does not yield immediately clinically observable effects. It can take many years for classical cobalamin deficiency clinical symptoms to appear as tissues slowly release needed minute daily requirements. There are numerous causes of clinical cobalamin

deficiency that result from 1) insufficient intake from poor diet or mal-absorptive pathology, 2) increased metabolic needs (i.e., pregnancy), and 3) impaired use or vitamin activation in tissues such as that which occurs in genetic inborn errors of metabolism. The majority of documented clinical deficiencies arise from the first category stemming from varying states of impaired gastrointestinal absorption.

Traditional and common clinical definitions of the cobalamin deficiency include 1) presence of megaloblastic anemia and/or neuropsychiatric alterations that respond to supplemental cobalamin therapy, or 2) decreased total serum cobalamin with or without altered biochemical metabolites (Miller et al., 2006; Savage, Lindenbaum, Stabler, & Allen, 1994).

Megaloblastic Anemia

When there is decreased availability of dTMP coming from conversion of homocysteine to methionine, deoxyuridine monophosphate (dUMP) is erroneously incorporated into DNA. DNA repair enzymes subsequently recognize this misincorporated uracil and cleave out the base. Incomplete DNA repair leads to frequent gaps and breaks in DNA sequence (Goulian, Bleile, & Tseng, 1980). Improper DNA synthesis results in cellular derangement and premature cell death. Although required in all cells, vitamin B12 is needed most by dividing cells, such as those in the bone marrow. In absence of adequate dTMP, all hematopoietic precursors undergo abnormal DNA synthesis and yield delayed or halted cell division events observable via variable cell morphologies (Aster, 2005). As a result, up to 90% of an affected patient's red blood cell

precursors may be destroyed prior to their release into the blood, compared to 10-15% under normal circumstances (Babior & Franklin Bunn, 2005).

Destruction of red blood cell precursors on this scale will result in decreased hemoglobin concentration and elevated mean corpuscular volume (MCV); presence of both are classic hallmark hematologic indicators of megaloblastic changes (Andres et al., 2006). Normal hemoglobin values in adult women according to the World Health Organization are 12-16 g/dL, and levels under 12 g/dL are considered indicative of anemia (Aster, 2005; Chaves, Ashar, Guralnik, & Fried, 2002). MCV is used in anemia classification discerning microcytic (MCV below 83 fL), normocytic (MCV 83 to 103 fL), and macrocytic (MCV greater than 103 fL) categories (Williamson et al., 1995). Thus, macrocytic anemia is characterized by a low red blood cell count, decreased hemoglobin concentration, and abnormally large (macrocytic) red blood cells, and indicates presence of cobalamin or folate deficiency (Aster, 2005). Cobalamin's overlap with folate will be discussed later in this section.

In cobalamin deficiency, rapidly dividing granulocytic precursors demonstrate nuclear immaturity while protein assembly continues normally. Although cytoplasmic features are intact, nuclei demonstrate visible chromatin clumping, become abnormally large for the cell's size, and develop numerous, hyper-segmented granules (Chui et al., 2001). Malformed platelets, thrombocytopenia, hypercellular bone marrow, and decreased white blood cell counts can also occur (Allen et al., 1993).

Accompanying these cellular changes, symptoms commonly reported by patients include vertigo, light-headedness, palpitations, and chest pain. Physical examination may yield pallor, jaundice (secondary to red blood cell destruction), rapid heart rate, evidence

of cardiomegaly, and a holosystolic murmur. Insidiously, anemia impacts a person's perception and understanding of their own health, resulting in feelings of fatigue, decreased strength, and a poor sense of well-being (Eisenstaedt, Penninx, & Woodman, 2006; Guralnik, Eisenstaedt, Ferrucci, Klein, & Woodman, 2004; Penninx et al., 2004; Woodman, Ferrucci, & Guralnik, 2005). Life threatening hematological changes can also occur, including symptomatic pancytopenia, pseudo-thrombotic microangiopathy, and hemolytic anemia (Andres et al., 2006).

Neurologic Changes

Although anemic symptoms usually occur first in cobalamin deficiency, neurological symptoms can present prior to, in concordance with, or separately from hematological alterations (Carmel, 2000; Carmel, Green, Rosenblatt, & Watkins, 2003; Lindenbaum et al., 1988). The central nervous system and neural cells are dependent upon continuous supply of nutrients (Selhub, Bagley, Miller, & Rosenberg, 2000). For example, myelin sheaths experience frequent turnover and are dependent upon methylations of precursor proteins and essential fatty acid oxidations (Scott, 1999). When altered, the primary feature of neurologic pathology resulting from decreased cobalamin is demyelination that affects both central and peripheral neurons (Green & Kinsella, 1995).

There are two hypotheses on the pathophysiology that leads to demyelination: 1) in the absence of cobalamin, there is decreased synthesis of the methyl group donor S-adenosylmethionine, which prevents precursor myelin basic protein from being formed, (the S-adenosylmethionine hypothesis); and 2) in the absence of cobalamin,

mitochondrial precursor methylmalonyl CoA accumulates, becomes toxic, and disrupts odd-chain fatty acid metabolism in neurons, (the adenosylcobalamin hypothesis). The first of these hypotheses, the S-adenosylmethionine hypothesis, is favored to the second, adenosylcobalamin hypothesis.

The S-Adenosylmethionine Hypothesis

In the cytoplasm, methylcobalamin serves as a cofactor for the conversion of homocysteine to methionine. This enzymatic conversion feeds two cycles, the methylation cycle and the DNA replication cycle (Figure 2), (Scott, 1999). The universal methyl donor S-adenosylmethionine donates a methyl group to S-adenosylhomocysteine for which to use in methylation of proteins, DNA, lipids, and other needed substrates (Dinn et al., 1980). Without proper methylation, required substrates are not produced properly, including neurotransmitters, membrane phospholipids, and precursor proteins used in nerve conduction, such as myelin basic protein (Metz, 1992). Multiple nervous system components become affected by these derangements, including long tracts of white matter in the posterior and lateral columns of the spinal cord, sensory fibers responsible for vibration sensitivity and position sense, and motor fibers controlling movement.

The Adenosylcobalamin Hypothesis

The alternative hypothesis to neurodegenerative changes observed in cobalamin deficiency postulates that neurologic symptoms arise from insufficient conversion of methylmalonyl CoA to succinyl CoA in the mitochondria. There is evidence that suggests

that accumulated methylmalonic acid disrupts odd-chain fatty acid metabolism, leading to neurological damage (Frenkel, 1973). Although there are numerous studies that identify quantitative abnormalities of odd-chain and branched-chain fatty acids in vitamin B12-deficient spinal cord and peripheral nerve tissue, there is not a clear relationship between these measurements and clinical development of cobalamin neuropathy (Kishimoto, Williams, Moser, Hignite, & Biermann, 1973; Levy, Mudd, Schulman, Dreyfus, & Abeles, 1970; Ramsey, Scott, & Banik, 1977). Further evidence against the adenosylcobalamin hypothesis comes from children with inherited disorders yielding high methylmalonic acid levels; despite having extraordinarily high levels, the patients' neurologic features are inclusive of mental retardation and muscular hypotonia and not those of cobalamin deficiency neuropathy (Rosenblatt & Cooper, 1987).

Clinical Progression and Effects

When there is insufficient methylation for normal neurologic homeostasis, small vacuoles in the myelin sheath result in focal swelling of individual neuronal fibers (Pant, Asbury, & Richardson, 1968). The focal swellings expand in scope to develop larger foci; beginning at the cervicothoracic junction of the spinal cord, posterior columns are usually the first to be affected before spreading up and down the cord and into anterior segments. On magnetic resonant imaging, increased T2-weighted signal, decreased T1-weighted signal, and contrast enhancement of the posterior and lateral spinal cord columns in cervical and upper thoracic segments are observed (Locatelli, Laureno, Ballard, & Mark, 1999).

Clinical presentation of the focal neuronal swelling is initially mild and measurable only by electrophysiological methods (Carmel & Sarrai, 2006). Neuropsychiatric symptoms, including parasthesias, ataxia, memory loss, mood alterations, and extremity weakness, are common initial presentations. If untreated, these symptoms become more severe and progress to numbness and tingling of extremities, clonus, weakness, spasticity of extremities, ataxia, abnormal reflexes, and gait and visual disturbances (Babior & Franklin Bunn, 2005). Underlying these worsening symptoms is progressive demyelination that affects peripheral nerves, posterior and lateral columns of the spinal cord, and the cerebrum (Allen et al., 1993).

If continuing uncorrected, permanent pathology such as axonal degeneration and neuronal death occur (Babior & Franklin Bunn, 2005). Cerebellar involvement is inclusive of urinary and/or fecal incontinence, and cranial nerve decompensation, including visual disturbances and optic neuritis (Allen et al., 1993). Decreases in cognitive function, development of dementia, personality changes, and occurrence of depression and Parkinsonian symptoms have all been documented as part of the clinical neurologic vitamin B12 deficiency profile (Carmel, 2000; Carmel & Sarrai, 2006; Clarke et al., 2003).

Metabolite Abnormalities

Prior to development of hallmark anemic or neurologic symptoms, metabolite assays are a valuable tool in providing an indication of a patient's vitamin B12 status. In deficiency states, the cobalamin metabolic reaction precursors methylmalonic acid (mitochondrial indicator) and homocysteine (cytosolic indicator) are not metabolized and

accumulate in cells and tissues (Lindgren et al., 1999). Increased serum methylmalonic acid and total homocysteine correlate with hematological and neurologic symptoms of clinical vitamin B12 deficiency and decrease responsively with supplemental cobalamin therapy (Henning, Tepel, Riezler, & Naurath, 2001; Naurath et al., 1995; Rajan et al., 2002).

In clinical practice, normal vitamin B12 metabolic profiles include serum cobalamin at >258 pmol/liter, total homocysteine at 5.4-14.9 μmol/liter, and serum methylmalonic acid (MMA) at <280 nmol/liter (Carmel, 2000; Carmel et al., 2003; Carmel & Sarrai, 2006). Clinical vitamin B12 deficiency is diagnosed when serum cobalamin <148pmol/liter, total homocysteine >50 μmol/liter, or serum MMA >1000 nmol/liter. Less restrictive parameters are used for ascertaining presence of subclinical vitamin B12 deficiency, which is present when serum cobalamin is 185-258 pmol/liter, total homocysteine is 15-25 μmol/liter, or serum MMA is 280-999 nmol/liter. Altered metabolite levels in subclinical deficiency can be present even if hemoglobin concentration and MCV are normal. In addition to reflecting altered vitamin B12 status, abnormal metabolite concentrations have been hypothesized to affect risk for development of comorbid pathology, such as homocysteine and cardiac disease. For some time, elevated serum homocysteine was recognized as a risk factor for the occurrence of cardiovascular disease and thrombosis (Refsum, Ueland, Nygard, & Vollset, 1998). However this role is no longer clear, as a recent randomized evaluation of folic acid and vitamin B12 versus placebo on blood homocysteine failed to demonstrate beneficial effects in preventing myocardial infarction (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine Collaborative Group, 2010).

Elevated methylmalonic acid and homocysteine concentrations occur in over 90% of patients with vitamin B12 deficiency, are increased prior to development of overt clinical symptoms, and often accompany normal serum cobalamin levels (Herrmann et al., 2003; Stabler, Allen, Savage, & Lindenbaum, 1990). Both methylmalonic acid and homocysteine concentrations can be altered in other disease states, such as inborn errors of metabolism, folate deficiency, and renal insufficiency (Allen, Lindenbaum, & Stabler, 1996; Bostom & Lathrop, 1997; Herrmann et al., 2000; Manns et al., 1999; Metz et al., 1996; D. S. Rosenblatt & Cooper, 1987). Serum homocysteine can be elevated in folic acid deficiencies, but methylmalonic acid elevations are specific to cobalamin deficiency (Stabler, Lindenbaum, & Allen, 1997).

To better define subtle features of early clinical development of cobalamin deficiency, four stages of progressive metabolite alterations and clinical characteristics have been suggested: (1 and 2) depletion of plasma and cell stores; (3) incidence of functional imbalances as measured by decreased biologically active cobalamin in serum, increased homocysteine and/or methylmalonic acid; and (4) appearance of megaloblastic and neuropsychiatric clinical symptoms (Herrmann et al., 2003).

Measurement Challenges

Even in individuals with known cobalamin deficiency, normal circulating levels of vitamin B12 can be maintained at the cost of tissues for several years (Lindenbaum, Savage, Stabler, & Allen, 1990). Total serum cobalamin demonstrates poor sensitivity and specificity for ascertainment of when the body is “low” in cobalamin from a tissue perspective (Miller et al., 2006). Serum holotranscobalamin, the biologically active

cobalamin in serum that is bound to transcobalamin II, is recognized for providing improved detection of cobalamin status in individuals (Green, 2008; Lindgren et al., 1999). Although more precise measurement alternatives such as holotranscobalamin can provide clinicians better proof of deficiency, it is generally accepted that interpreting multiple testing analytes with clinical presentation symptoms yields accurate detection (Green & Miller, 2007; Herrmann et al., 2003; Herrmann et al., 2000; Obeid, Schorr, Eckert, & Herrmann, 2004).

Measurement of cobalamin metabolites can be challenging, as sophisticated techniques require specialized equipment and training. This is primarily because methylmalonic acid and homocysteine levels accumulating in cobalamin deficiency states are typically in the nanomolar to micromolar range, and accurately detected by gas chromatography-mass spectrometry (GC-MS) and high-pressure liquid chromatography (HPLC) in both serum and urine (Green, 1995; Miller et al., 2006). Due to their cost, many laboratories cannot afford the technology required for these specialized assays. For laboratories that do perform them, accurate interpretation can be affected by absence of standardized laboratory procedures, assay reagents, and testing approaches. For serum cobalamin, concentrations reported by laboratories often vary from those used in international measurement standards, contributing to clinician misinterpretation. Coupled with absence of consensus on what is considered ‘elevated’ or ‘decreased’ due to poor correlation with actual tissue level—clinicians can erroneously underestimate the complexity of commonly used cobalamin laboratory tests (Carmel et al., 2003).

Cobalamin Overlap with Folate and the Methyl-Folate Trap

As mentioned, megaloblastic anemia and elevated homocysteine clinical features can also be caused by decreased folate. This is because cobalamin metabolism overlaps with folate metabolism in the conversion of homocysteine to methionine (Figure 3). In the absence of adequate cobalamin, an intracellular backlog of 5-methylenetetrahydrofolate occurs. Since the 5,10-methylenetetrahydrofolate enzyme strongly favors one direction, it does not go backwards in the pathway; thus, the cell has plenty of folate but it is "trapped" in an unusable form for DNA synthesis (Scott & Weir, 1981; Scott & Weir, 1994). The resulting clinical presentation is identical to that of megaloblastic anemia caused by true deficiency of folate.

Synthetic folate (folic acid) enters downstream of the methionine synthase conversion, and as it is converted to tetrahydrofolate from dihydrofolate, can rescue DNA synthesis in cobalamin deficiency. However, it does not resolve elevated homocysteine levels, as cobalamin is required for conversion of homocysteine to methionine. If a well-meaning health care provider aims to treat megaloblastic anemia with folic acid without fully reviewing metabolic parameters, the patient experiences resolution of megaloblastic anemia as cobalamin deficiency continues, masked by this treatment (Pfeiffer, Caudill, Gunter, Osterloh, & Sampson, 2005). In such cases, it is not until progression of neurologic symptoms becomes significantly severe that cobalamin deficiency is suspected and more detailed metabolite profiles are checked. In the advent of widespread folate fortification, research into vitamin B12 and folate status has identified that low-cobalamin levels exist with high folate levels, and can compound risk of neurologic decline (Morris et al., 2005; Selhub, Morris, & Jacques, 2007).

Gerontologic Research Factors

Cobalamin Deficiency

Nutritional deficiencies in the elderly population are well characterized as functions of expected age-related physiological changes (Saltzman & Russell, 1998). At worst, 20-50% of the aging population experience some form of vitamin B12 deficit (Lindenbaum, Rosenberg, Wilson, Stabler, & Allen, 1994; Selhub et al., 2000; Stabler et al., 1997). Pernicious anemia (Type A atrophic gastritis) is the end-stage presentation of autoimmune gastritis and is the primary cause of frankly overt clinical vitamin B12 deficiency in North American populations (Baik & Russell, 1999). For every elderly adult female diagnosed with pernicious anemia, approximately five males are affected, and Caucasian and African American elderly adults shoulder increased prevalence of pernicious anemia compared to other racial demographic groups (Baik & Russell, 1999). Antibodies that attack H+/K+ ATPase pumps progressively destroy parietal cells, causing continued decline of vitamin B12 absorption and extraction from protein (Morris, Jacques, Rosenberg, & Selhub, 2007).

In contrast, Type B atrophic gastritis is a naturally occurring phenomenon of aging, stemming from decreased secretion of stomach acid, pepsin, and intrinsic factor (Saltzman & Russell, 1998). Concomitant with achlorydia stemming from all forms of gastric atrophy, microorganism overgrowth fostered by decreased gastric acid production can competitively consume ingested vitamin B12 in aging individuals (Suter, Golner, Goldin, Morrow, & Russell, 1991). Although more rare in elderly populations, poor diet, decreased dietary intake of foods containing vitamin B12, and strict vegetarianism can also result in dietary deficiency for aging adults.

Additional causative factors include side effects from medications and comorbid conditions that increase nutritional need for vitamin B12. Medications interfering with vitamin B12 absorption commonly prescribed in elderly individuals include antiepileptic agents, proton pump inhibitors, histamine receptor antagonists, the antidiabetic drug metformin, antibiotics, and cholestyramine (Wolters, Strohle, & Hahn, 2004). Comorbid conditions contributing to malabsorption or increasing nutritional requirements include intestinal diseases (Crohn's), gastric or ileal resections, alcohol intake, smoking, renal insufficiency, diabetes mellitus, and lymphoma (Wolters et al., 2004).

Subclinical Cobalamin Deficiency

Subclinical B12 deficiency is a burden largely shouldered by older adults because symptoms indicating its presence are often interpreted by clinicians to be associated with other disease states or nonspecific effects of aging (Andres et al., 2004). The subtle nature of weakness, fatigue, headache, depression, shortness of breath, malaise, vertigo, early dementia, and sensory parasthesias is common in aging patients and often improperly attributed to other causative mechanisms (Baik & Russell, 1999; Clarke et al., 2003)**.** Unrecognized deficiency or misattribution of symptoms to folate increases chance of progression to irreversible neurological changes. For elderly adults, permanent neurological changes commonly experienced by the elderly stemming from unrecognized cobalamin deficiencies include taste alterations, memory loss, parasthesias, spinal cord subacute degeneration, neuropathy, gait ataxia, dementia, anosmia, incontinence, impotence, decreased visual acuity, and psychosis (Baik & Russell, 1999; Carmel, 2000). For elderly individuals, the best primary defense against permanent neuropsychiatric

injury is maintaining a healthy awareness for subclinical cobalamin deficiency in the event of nonspecific symptom presentations.

Although measuring cobalamin metabolite levels helps greatly in ascertaining presence of deficiency in older adults, several clinical factors are known to affect accurate detection. Similar to trends in other age groups, erroneous attribution of megaloblastic anemia to folate in the absence of a full metabolic workup can obscure diagnosis (Allen & Casterline, 1994; Pfeiffer et al., 2005). Common in elderly individuals, renal insufficiency is known to cause increases in both homocysteine and methylmalonic acid (Herrmann, Obeid, Schorr, & Geisel, 2005; Herrmann, Schorr, Geisel, & Riegel, 2001; Obeid, Kuhlmann, Kirsch, & Herrmann, 2005). Molecular etiology for accumulations of homocysteine and methylmalonic acid stemming from renal dysfunction are largely unknown. However, elevations in either serum homocysteine or methylmalonic acid from renal insufficiency are modest compared to those that occur in true cobalamin deficiency states (Savage et al., 1994; Stabler, Lindenbaum, & Allen, 1996).

Renal Function Assessment

In clinical practice, serum creatinine is a common measure of renal function and is the most widely used method of assessing an individual's renal status. However, it is recognized that relying on serum creatinine measurement alone is limiting, as it does not correlate with actual physiologic glomerular filtration rates (Shemesh, Golbetz, Kriss, & Myers, 1985; Stevens, Coresh, Greene, & Levey, 2006). In older adults, renal function slowly declines and tubular secretion of creatinine increases; thus, elevations in serum

creatinine are not seen until more than half of total glomerular filtration is lost (Giannelli et al., 2007). In addition, decreased muscle mass in older adults is associated with decreased creatinine production. If kidney problems in older adults are not diagnosed, renal disease can progress and result in additional costly comorbidities, clinical complications, and drug toxicities. In a recent study of 660 elderly adults with normal serum creatinine values, alternative renal-estimation equations identified that up to 39% of them could be classified as having renal impairment (Giannelli et al., 2007).

A common measure of renal-function estimation used in elderly individuals that employs age, weight, and serum creatinine, is the Cockcroft-Gault estimation equation (Cockcroft & Gault, 1976). For females, the Cockcroft-Gault formula incorporates a constant of 0.85 to account for 15% less muscle mass as compared to males. The Cockcroft-Gault has been used in many studies of older adults, and although reported to underestimate creatinine clearance, is considered by many experts a more valid and reliable method than if using serum creatinine alone (Froissart, Rossert, Jacquot, Paillard, & Houillier, 2005; Lamb et al., 2005).

Role of Cobalamin in Cognition

There is a significant and storied history of the suspected role of cobalamin in cognition, with much clinician speculation focused on delineating cause and effect between altered homocysteine levels and dementia in Alzheimer's disease (McCaddon, 2006). However, the concept that deficits in nutritional status would precipitate neuropsychiatric decline was not fully acknowledged until 1990, when it was reported that low-normal concentrations of cobalamin were associated with cognitive impairment

(Bell et al., 1990). The chart review by Bell et al. of 102 geriatric inpatients found that the 3.7% of patients who were vitamin B12 deficient were more likely to have significantly lower Mini-Mental State Examination scores. Shortly later, Rosenberg and Miller's review of dietary factors and neuro-cognitive health concluded that elderly individuals were extremely susceptible to cognitive decline from subtle and progressive forms of subclinical nutritive deficiencies (Rosenberg & Miller, 1992). An unintended consequence of this position was the assumption that vitamin B12 indeed contributed to pathologic progression of cognitive decline in elderly patients, without there being presence of strong prospective controlled clinical evidence to confirm it.

Since that time, numerous research studies have reported variable efficacy of vitamin B12 on the protection and maintenance of cognitive function (Smith & Refsum, 2009). Total homocysteine and cognitive measures were inversely related in 2,096 elderly adult dementia and stroke-free participants (>60 years) of the Framingham Offspring Study (Elias et al., 2005). A 3-year prospective evaluation of the association between dietary B-vitamin intake and cognitive decline in a subset of 321 men in the Veterans Affairs Normative Aging Study revealed that the association of poor cognitive status with low vitamin B12 and high homocysteine was only applicable to performance on a construction praxis spatial copying score (Tucker, Qiao, Scott, Rosenberg, & Spiro, 2005). A 6-year prospective observational study of 3,718 community-dwelling elderly individuals over 65 indicated that high dietary B12 intake was associated with slower cognitive decline, but only in the oldest individuals (Morris et al., 2005). Authors of a recent Cochrane Collaboration meta-analysis concluded that there is no evidence that folic acid with or without vitamin B12 improves cognitive function of elderly individuals

with or without dementia; however, long-term supplementation may be protective for healthy elders with high homocysteine levels (Malouf & Grimley Evans, 2008). This is likely due to the great clinical and research heterogeneity across studies in the literature. Variability in cognitive assessment tools, clinical outcome parameters, quasi-experimental study designs, laboratory metabolite measurements, and deficiency threshold cutoffs contribute to poor-quality evidence from which to draw clinical conclusions (Raman et al., 2007).

The Dissertation's Clinical Measurements of Altered Cobalamin Status

The dissertation research uses multiple measures of cobalamin status for elderly adult women subjects. Measures previously discussed as related to pathophysiology, diagnosis, and clinical management of vitamin B12 deficiency include hemoglobin, mean corpuscular volume, serum cobalamin, and the biochemical metabolites homocysteine and methylmalonic acid. However, the dissertation also explores relationships to other clinical outcomes, including depression, peripheral neuropathy, and functional decreases in strength and speed of ambulation.

Depression

Patients experiencing alterations in mood, primarily depression, are commonly reported to also have abnormal cobalamin and folate laboratory values (Bell et al., 1990; Carney et al., 1990; Lindenbaum et al., 1988; Savage & Lindenbaum, 1995). Previous work in the Women's Health and Aging Study found a twofold increase in risk of severe

depression in patients who experienced clinical cobalamin deficiency as measured by serum cobalamin, homocysteine, and methylmalonic acid levels (Penninx et al., 2000). A longitudinal association study of depressive symptoms in 3,503 older adults over 7 years identified that for each 10 additional micrograms of vitamin B12 intake, there was a 2% lower odds of depressive symptoms per year (Skarupski, Tangeny, Li, Ouyang, Evans & Morris, 2010).

Although the association between vitamin B12 intake and depression in older adults is reported in multiple observation studies, causation of its presence has not been able to be determined. Hypothesized molecular origins of the connection may stem from insufficient cobalamin and folate to drive the conversion of homocysteine to methionine, preventing methylation of key neurotransmitters in the brain, including the monoamines dopamine and serotonin (Bottiglieri et al., 2000; Weir & Scott, 1999). However, many behavioral factors that accompany depressive symptomology in aging individuals, such as decreased appetite and socioeconomic status, likely contribute to this association as well (Donini, Savina, & Cannella, 2003).

Depression is a challenging phenomenon to measure; it displays lack of agreement between clinicians and researchers on the concepts that comprise it, and classification mechanisms across numerous settings and groups vary widely according to patient subpopulation and clinical care specialty (Pasacreta, 2004). Depression contains both affective and somatic components, which results in significant overlap with comorbid symptomology (i.e., fatigue, decreased appetite) obscuring accurate research measurement. Common to elderly populations, dementia is a common confounder where

psychomotor retardation and passive responses to examiner questions are misinterpreted as depressive pathology.

The Geriatric Depression Scale (GDS) was derived for use in geriatric populations so that somatic depression manifestations and dementia presence do not threaten validity of accurate detection (Feher, Larrabee, & Crook, 1992; Yesavage et al., 1982). Comprised of 30 items requiring a yes/no answer, higher GDS scores indicate depression severity. Generally accepted cutoffs are no depression (scores less than or equal to 9), mild depression (scores 10-13), and severe depression (scores greater than or equal to 14) (Ferrucci, Kittner, Corti, & Guralnik, 1995; Lyness et al., 1997; Norris, Gallagher, Wilson, & Winograd, 1987). Criterion validity using psychiatric evaluation of DSM III diagnostic standards for mild depression on GDS was 89%, and for severe symptoms was 78%. Specificity values were similar at 73% for mild depression and 86% for severe depression (Norris et al., 1987). Although GDS measurements are reliable and valid in older persons with respect to research diagnostic criteria and DSM-IV criteria, presence of mild or severe depression using the screening scale is not necessarily indicative of a clinical diagnosis.

Peripheral Neuropathy

Abnormal sensation is a widely reported indicator of presence or development of vitamin B12 deficiency pathology (Carmel et al., 2003). Abnormal sensation is also considered a key subtle clinical manifestation indicative of subclinical vitamin B12 deficiency (Carmel & Sarrai, 2006). As discussed in previous pathophysiology sections, aberrated methylation for myelin basic protein precursors from altered conversion of

homocysteine to methionine disrupts neuronal signaling. The neurologic syndrome of cobalamin deficiency commonly starts with peripheral paresthesias in the feet before progressing to more severe pathology (Beck, 2001; Green & Miller, 2007). In a study of neurologic aspects of cobalamin deficiency in 369 cobalamin deficient patients, it was found that paresthesia was the most common neurologic finding at the time of diagnosis on physical examination (Healton, Savage, Brust, Garrett, & Lindenbaum, 1991). Comprising 87.7% of the paresthesia symptom profiles, the most common abnormality was found to be significant diminishment of vibration sensitivity in the feet, or feet and legs up to the knees. Less commonly, diminished abnormality also extended up from the feet to include the iliac crest, lower thoracic area, midthoracic area, hands and elbows, and shoulders.

Alterations in vibration sensitivity stemming from large-fiber peripheral nerve function provide an objective measurement for declining nerve function (Ferrucci, Kittner, et al., 1995). Vibratory perception sensory thresholds in older adults are reproducible and reliable indicators of polyneuropathy (de Neeling, Beks, Bertelsmann, Heine, & Bouter, 1994). Vibration perception testing (VPT) protocols involve serial application of quantified vibration and assessing if subjects are able to feel it or not. Depending upon a patient's response, the strength of stimulus is incrementally adjusted until an individual can no longer sense vibration. VPT can be administered via electronic or mechanical clinical measurement tools, such as vibrometers and tuning forks. Although reliability is well established for varying measurement modalities individually, measurement methods are not interchangeable due to slight physiological differences in how sensory neurons transmit mechanical stimuli (tuning fork) as compared to energy-

based stimuli (voltage or current) (Temlett, 2009). Key advantages to using voltage- and current-based modalities include the removal of variability coming from inconsistent techniques of generating and applying the tuning fork's blade intensity.

VPT in the Women's Health and Aging Studies (WHAS) 1 and 2 were assessed using different techniques, WHAS 1 with a vibrometer modality and WHAS 2 with a tuning fork. In WHAS 1, the VPT used was modeled after a diabetic neuropathy protocol, where vibration measures are considered valid if 18 or less stimulation attempts are made, and not more than one error occurs in the first eight attempts (Ferrucci, Kittner, et al., 1995; Maser et al., 1989). The amplitude of vibration stimulus is converted to a micron unit measurement, with higher values indicating that a stronger stimulus was required to elicit a correct sensory response from the patient (Resnick et al., 2000; Volpato et al., 2003). Accepted neuropathic functional micron unit cutoffs include normal function at less than 3.43 units, mild dysfunction at 3.44-4.87 units, moderate dysfunction at 4.88-6.31 units, and severe dysfunction at over 6.31 units (Resnick, Vinik, Heimovitz, Brancati, & Guralnik, 2001; Volpato, Leveille, Blaum, Fried, & Guralnik, 2005)**.**

Functional Indicators

As reviewed in earlier sections, 5' adenosylcobalamin serves as the coenzyme for methylmalonyl-coenzyme A mutase, which facilitates conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A in the mitochondria. This conversion is necessary for catabolism of odd-chain fatty acids and some amino acids, and results in maintenance of normal energy metabolism.

In the absence of this conversion, methylmalonyl-coenzyme A and its precursor, propionic acid, accumulates and diffuses out of the mitochondria into the cytoplasm and disrupts normal metabolism (Brass, Tahiliani, Allen, & Stabler, 1990). The cellular accumulation of propionic acid and methylmalonic acid (acyl-coenzyme A thioesters) has been shown to inhibit gluconeogenesis from pyruvate, pyruvate oxidation, fatty acid oxidation, and ureogenesis (Brass, 1986; Glasgow & Chase, 1976; Walajtys-Rode, Coll, & Williamson, 1979; Walajtys-Rode & Williamson, 1980). In cobalamin deficiency, the combined effects of deranged energy production processes in addition to accumulated methylmalonic, propionic acids, disrupts energy homeostasis and impairs the action of multiple critical enzymes (Depeint, Bruce, Shangari, Mehta, & O'Brien, 2006; Kolker & Okun, 2005). The clinical effects of these metabolic impairments can be severe, such as that which is demonstrated by individuals diagnosed with methylmalonic acidurias.

As a diagnostic class, methylmalonic acidurias are a group of autosomal recessive genetic disorders that offer valuable biologic insight to the broader mechanisms of cobalamin deficiency. Methylmalonic acidurias are caused by autosomal recessive mutations in genes that code for methylmalonyl-coenzyme A mutase or the cobalamin complementation groups, which synthesize 5’ adenosylcobalamin for use in the mitochondria (Chandler et al., 2007; Coelho et al., 2008; Rosenblatt & Fenton, 2001). Inherited methylmalonic acidurias produce severe clinical symptoms that are often fatal. Because of their severity, clinical study of organ-system derangements from drastically excessive methylmalonic acid levels is limited to animal studies, observations of affected newborn infants, postmortem analyses, and children with less severe defects who have matured into adolescence and young adulthood. Common symptoms experienced by

individuals with methylmalonic acidurias include lactic acidosis, decreased muscle strength, muscular hypotonia, lethargy, and failure to thrive (Coelho et al., 2008; Rosenblatt & Cooper, 1987; Tanpaiboon, 2005). In a recent characterization of the methylmalonic aciduria phenotype, investigators examined correlations of murine and human disease characteristics and found that altered metabolism in skeletal muscle was a significant source of pathology (Chandler et al., 2007).

Although the autosomal recessive methylmalonic acidurias do not present in older adults and thus cannot be generalized to the geriatric population, they may offer biologic insight into concomitant metabolic processes when methylmalonic acid concentrations are significantly elevated. For example, methylmalonic acidurias in neonates and children produce drastically elevated methylmalonic acid levels at approximately 1000 nmol/L, a level which is similar to that of severe cobalamin deficiency in older adults. Although these metabolic concentrations may be similar, a key limitation in understanding the relationship between elevated methylmalonic acid concentration and poor functional status in elderly individuals is that it is relatively unexplored in the area of cobalamin metabolism.

Available data is limited to several separate investigations of neuromuscular effects of cobalamin deficiency and its possible genetic influences. In a focused observational study of 153 cases of cobalamin deficiency in older adults, 16 individuals experienced weakness in limbs and 28 individuals experienced difficulties walking (Healton et al., 1991). Descriptive data on the neurophysiologic profiles of older individuals with cobalamin deficiency identify ataxia as a common clinical presentation, with confirmatory decreases in motor neuron action potentials (Fine, Soria, Paroski,

Petryk, & Thomasula, 1990). Recent investigations of the Women's Health and Aging Study cohorts have identified that genetic variation in cobalamin metabolism genes influences methylmalonic acid concentrations and functional status (Matteini et al., 2008; Matteini et al., 2010). The dissertation study more fully explored these relationships by examining the functional performance measures hand grip strength and 4-meter walking speed.

Hand Grip Strength

Hand grip strength measurements are an indication of basic upper extremity function and measure total force of upper limb muscles (Rantanen, Era, & Heikkinen, 1994). Although it is a measurement of isometric strength in the upper body, hand grip strength has been found to also correlate with other skeletal muscle groups in the body (Rantanen, Pertti, Kauppinen, & Heikkinen, 1994). For this reason, hand grip strength is commonly used as an estimate of overall body strength.

Hand grip strength has consistently demonstrated value in clinical and research settings as a reliable and valid measurement technique. It is a sensitive predictor of progressive disability, and morbidity, mortality in elderly adults (Blake et al., 1988; Kerr et al., 2006; Phillips, 1986; Rantanen, Era et al., 1994; Rantanen et al., 1999). Additionally, it has been found to be a useful clinical assessment parameter in screening individuals for nutritional deficiencies (Klidjian, Archer, Foster, & Karran, 1982; Matos, Tavares, & Amaral, 2007). Crucial for work with elderly patient populations, the hand dynamometers that measure grip strength force are portable, inexpensive, rapid, simple to use, and ideal for clinical assessments in home and community health settings.

Various studies identify use of grip strength as a valuable measurement technique, since it demonstrates both low intra- and interobserver variability and high clinical reproducibility (Bohannon, 2006; Schaubert & Bohannon, 2005; Windsor & Hill, 1988). However, it is recommended that use of dynamometers in research and clinical settings is consistent with respect to device manufacturer (Guerra & Amaral, 2009)**.** A recent comparison report identified that although accuracy of dynamometers is high in elderly adults, measurements do not correlate well across manufacturers.

Walking Speed

An indication of strength, mobility, coordination, proprioception, reflex control, and balance, the ability to walk provides significant information about a patient across numerous functional parameters (Ferrucci, Guralnik, et al., 1995). One of these parameters, lower extremity muscle strength, is determined by the speed at which an individual is able to walk (Holloszy, 1995; Schwartz, 1997). Walking speed, or gait velocity, in combination with other key lower extremity functional health assessments can predict disability, mortality, and nursing home admission across diverse elderly populations (Fried, Bandeen-Roche, Chaves, & Johnson, 2000; Guralnik, Ferrucci, Simonsick, Salive, & Wallace, 1995; Guralnik et al., 1994; Ostir, Markides, Black, & Goodwin, 1998; Seeman et al., 1994). When used singularly, gait speed is a relatively accurate proxy for full lower extremity performance battery examination, and predicts incident disability up to 6 years (Guralnik et al., 2000). Furthermore, longitudinal evaluations identify that changes in lower extremity strength over time are linearly

associated with meaningful changes in gait speed for sedentary older adults (Purser, Pieper, Poole, & Morey, 2003).

In addition to their predictive capacity for adverse outcomes, walking speed measurements are simple, reliable, and inexpensive to obtain. Commonly identified factors known to affect their accurate measurement include height and gender (Samson et al., 2001). Presence of dual tasking, simultaneously performing another task while an individual is walking, has also been recognized to affect gait measurement and its interference correlates to the difficulty of the concurrent task (Ble et al., 2005; Springer et al., 2006). Environmental and contextual factors, such as familiarity with surroundings, lighting, smooth walking surface, and use of assistive medical equipment, are also recognized to modify walking speed assessments (Hoenig et al., 2006; Richardson, Thies, DeMott, & Ashton-Miller, 2004a, 2004b).

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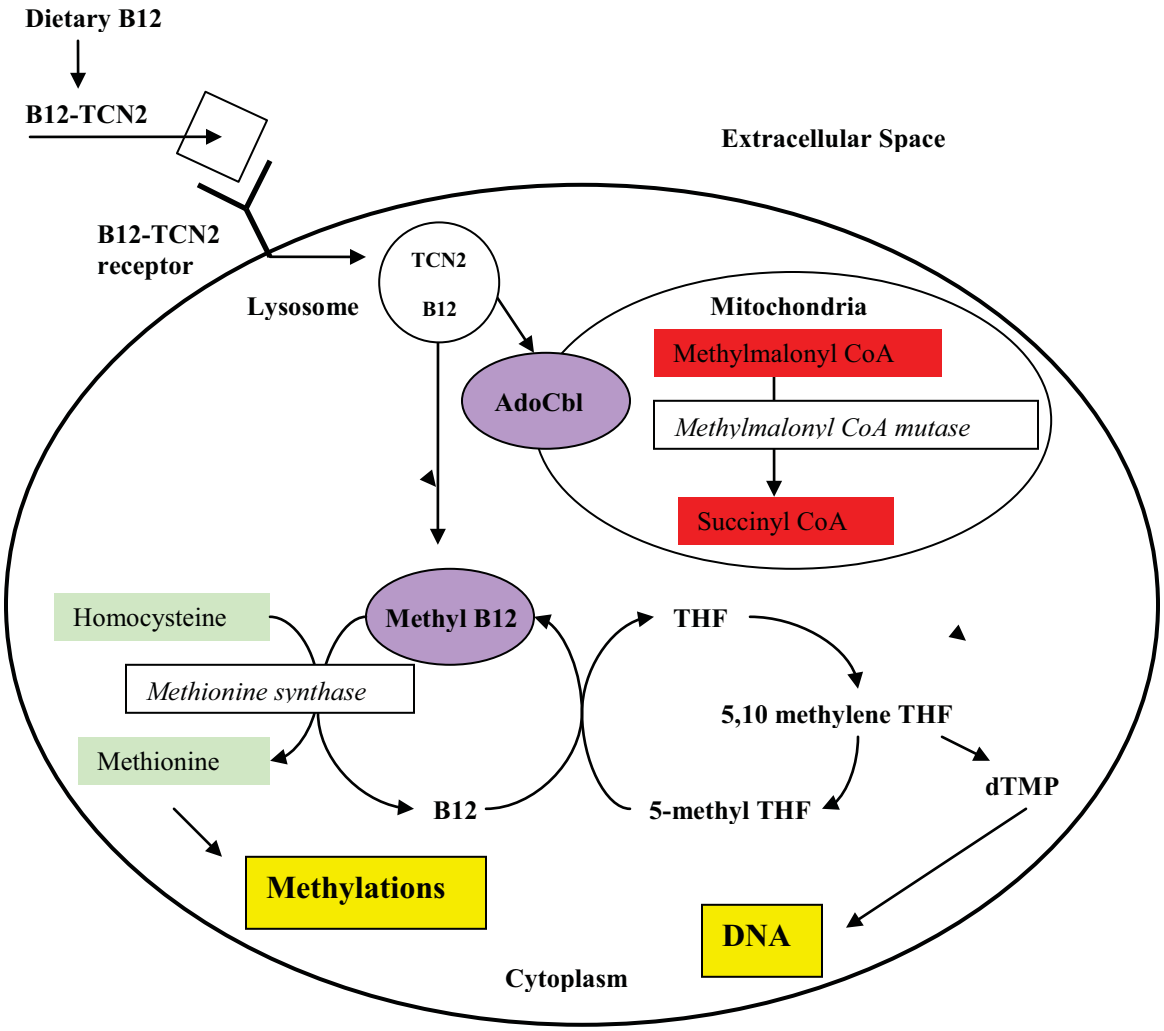


Figure 1. The Biochemical Role of Cobalamin (Vitamin B12) in the Cell.

Vitamin B12 participates in 2 essential reactions: (1) methylcobalamin in folate-dependent conversion of homocysteine to methionine (cytoplasm) for DNA synthesis and methylation, (2) adenosylcobalamin in conversion of methylmalonyl coenzyme-A to succinyl coenzyme-A (mitochondrial). *Abbreviations:* TCN2 (Transcobalamin II), methyl B12 (methylcobalamin), THF (tetrahydrofolate), dT (deoxythymidine).

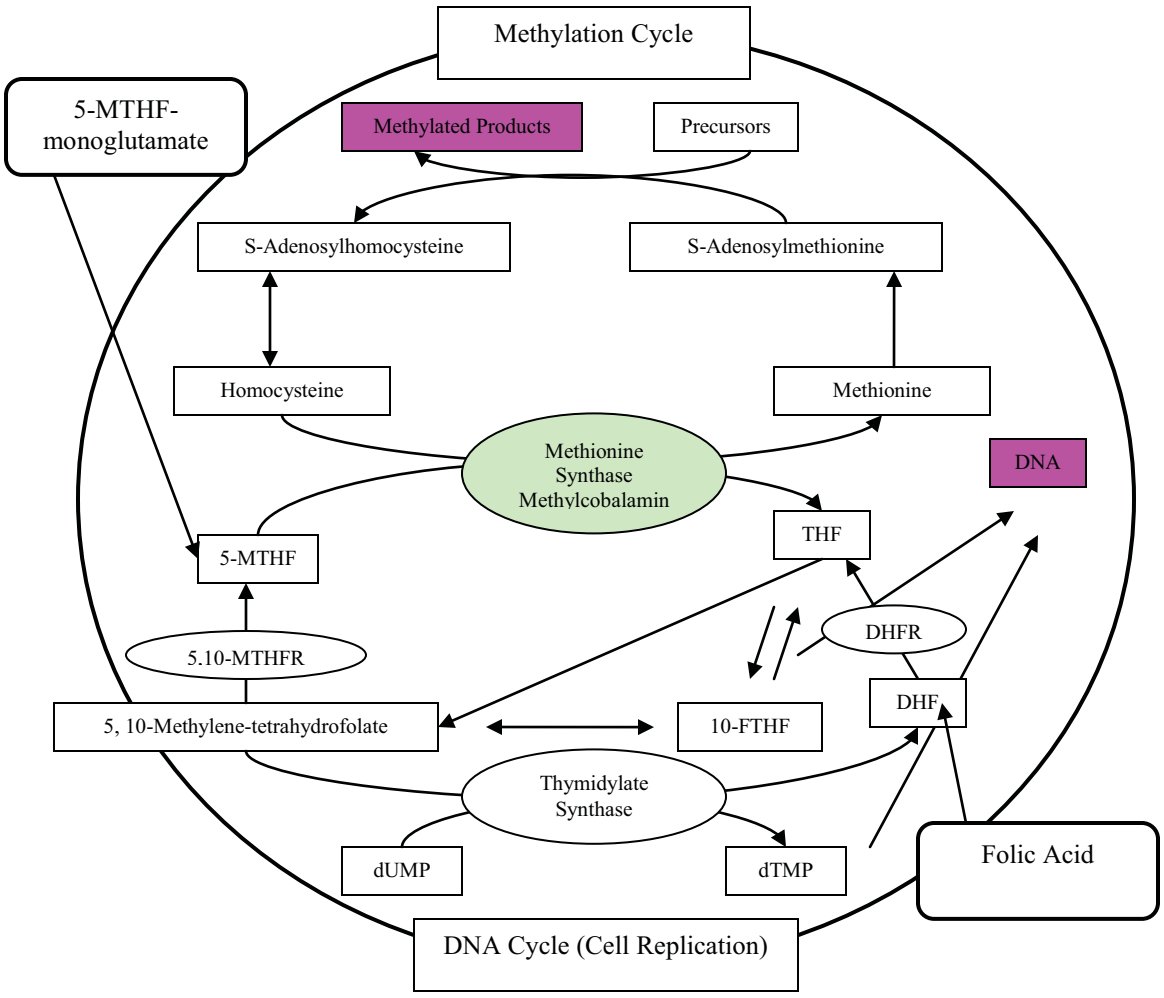
Figure 2. The Metabolic Pathway Overlap of Cobalamin and Folate in the Cell.

The metabolic pathways that involve cobalamin and folate are shown. Their point of overlap encompasses the vitamin B12-dependent conversion of homocysteine to methionine, which drives 1) methylation (lipids, myelin basic protein, DNA, others) and 2) the 5-methyl-THF conversion to THF that produces DNA precursors.

For methylation to occur, S-adenosylmethionine donates a methyl group to S-adenosylhomocysteine. In the absence of sufficient vitamin B12, this transfer stops. Folic acid supplementation bypasses this path.

Abbreviations: 5,10-MTHFR (5,10-methylene-tetrahydrofolate reductase), 5-MTHF (5-methyl-tetrahydrofolate), THF (tetrahydrofolate), DHFR (dihydrofolate reductase), 10-FTHF (10-formyl-tetrahydrofolate), dUMP (deoxyuridine monophosphate), dTMP (deoxythymidylate monophosphate), DNA (deoxyribonucleic acid).

Extracellular Space



CHAPTER 3

STUDY DESIGN, MEASUREMENT, METHODS, AND ANALYSIS

Introduction

The Women's Health and Aging Study (WHAS) 1 and 2 cohorts offer a unique and valuable research opportunity to evaluate cobalamin metabolism through the lens of a population-based approach. The WHAS study methods were designed to capture functional health heterogeneity existing in the community-dwelling elderly, including presence of disability and its complex modifiers (Kasper, Shapiro, Guralnik, Bandeen-Roche, & Fried, 1999). The WHAS 1 and 2 initiatives are a rich data source, cataloguing numerous clinical, psychosocial, and demographic measures over several years in order to assess trajectories of aging and disability. For example, several hundred peer-reviewed publications stem from WHAS 1 and 2 data.

Study Design

This dissertation project is a candidate-gene association study using previously collected data from the National Institute on Aging's WHAS 1 and 2 research initiatives. WHAS 1 and 2 were cross-sectional research cohorts of low-, mid-, and high-functioning community-dwelling elderly women. Primary objectives of both WHAS initiatives were to better understand the causes and trajectories of physical disability in elderly women

(Chaves et al., 2006; Ferrucci et al., 1995; Guralnik, Fried, Simonsick, Bandeen-Roche, & Kasper, 1995). Subjects in WHAS 1 were active study participants from 1992-1995 and for WHAS 2 from 1994-1996.

Sampling and Recruitment, Data Collection

Sampling for both WHAS cohorts was conducted by randomly selecting subjects from 32,538 Health Care Financing Administration Medicare eligibility lists in 12 contiguous Baltimore zip codes (Fried, Bandeen-Roche, Chaves, & Johnson, 2000; Guralnik, Fried, Simonsick, Kasper, & Lafferty, 1995). Patients who were unable to speak English, physically unable to complete the screening, or who were too cognitively impaired to respond to questions were excluded from participation in both cohorts. Participants in WHAS 2 had to be well functioning enough to participate in a 1-day clinic evaluation.

Sampling and Recruitment: WHAS 1, 1992-1995

From the 32,538 Health Care Financing Administration (HCFA) Medicare records, 6,521 age stratified elderly women (65-74, 75-84, and over 85) were randomly selected for screening and possible enrollment (Ferrucci et al., 1995). An introductory letter describing the WHAS 1 study was sent to the 6,521 potential participants, which was followed 2 weeks later by a second letter inviting participation from Dr. Linda Fried. A study interviewer contacted women and administered a screening instrument in each patient's home. Women who lived in nursing homes or were not able to be contacted by

study personnel were not eligible for screening, resulting in the final total of 3,841 participants screened.

The objectives of WHAS 1 recruitment were to select the community-dwelling elderly adult women who were most disabled. To do this, population-based disability screening was used to select WHAS subjects that were the one-third most disabled in the Baltimore area. Each screened research participant was evaluated on 15 tasks across four physical disability domains: 1) mobility and exercise tolerance, 2) upper extremity function, 3) higher function tasks (instrumental activities of daily living), and 4) basic self care (high function tasks that are nonmobility dependent). Using the combination approach of 4 domains allowed many more women to participate in WHAS than if using one singular criterion (i.e., such as self care disability). Previous factor analyses and piloting efforts demonstrated validity in using this type of prospective population-based functional screening (Branch, Katz, Kniepmann, & Papsidero, 1984; Fried, Ettinger, Lind, Newman, & Gardin, 1994; Kasper et al., 1999).

To conduct the disability screening, trained study personnel conducted 20-30 minute home interviews to collect basic demographic information, self-reported health status, and medical history (Guralnik et al., 1995). Data collected for the physical disability screening included a participant's ability to perform basic tasks, their adaptation to disability, difficulty, and/or dependency, prescription and nonprescription medication use, presence of medical diagnoses, physical symptoms for over 20 chronic medical conditions, psychological health, social networks and support, health behaviors, and health care utilization practices (Ferrucci et al., 1995).

Women who reported difficulty with two, three, or four physical disability domains and had a Mini-Mental State Examination (MMSE) score of 18 or higher were invited to participate in WHAS 1 (n = 1,409) (Fried, Kasper, Guralnik, & Simonsick, 1995; Guralnik et al., 1995). MMSE is a global measure of cognition and surveys multiple domains in order to comprehensively assess for presence of impairment (Folstein, Folstein, & McHugh, 1975). Total possible score is 30, and anything under 24 indicates presence of cognitive impairment (Kaufman, Weinberger, Strain, & Jacobs, 1979). The final WHAS 1 sample size eligible for WHAS and who consented to participation was 1,002 (Guralnik et al., 1995). A further subset of 762 elderly women consented to research involving phlebotomy. Missing data are discussed later in this chapter.

Sampling and Recruitment: WHAS 2, 1994-1996

Following shortly after WHAS 1 was WHAS 2, a companion study that focused on the higher functioning group in Baltimore's community-dwelling elderly women population. Primary aims for WHAS 2 included prospective observation and analysis of the transition from predisability states to functional impairment (Chaves, Garrett, & Fried, 2000). WHAS 2 participants were the two-thirds least disabled community-dwelling elderly women. Although using the same sampling frame as WHAS 1 (HCFA records in 12 Baltimore zip codes), a new random sample was selected from 3,592 age-stratified (70-74, 75-79 years) HCFA Medicare Enrollee records. A total of 2,541 elderly women were randomly selected for physical disability screening, with a total of 1,630 women undergoing initial screening (Fried et al., 2000).

Women who were not living in extended care facilities were contacted by a trained study interviewer who evaluated a third of the patients directly in their home, and the remaining two-thirds via telephone (secondary to cost). Of the same 15 tasks across 4 domains, women who reported difficulty in zero or one domain of functioning, and demonstrated an MMSE score of 24 or higher were eligible for WHAS 2 (Chaves et al., 2006; Fried et al., 2000; Fried, Young, Rubin, & Bandeen-Roche, 2001). For participants screened over the phone, the MMSE was abbreviated to exclude irrelevant questions (i.e., location of examination center) and questions requiring in-person assessment. Screened individuals answering at least 80% of abbreviated MMSE questions correctly via telephone were eligible for WHAS 2 study inclusion. A total of 1,630 patients were screened for eligibility, n = 880 were invited to participate, with final WHAS 2 sample size participation at n = 436 (Fried et al., 2000). Similar to WHAS 1, a subset of the consented WHAS 2 patients also consented to additional phlebotomy (n = 405). Missing data is discussed later in this chapter.

Data Collection: WHAS 1, 1992-1995

Approximately 2 weeks after eligibility screening, a trained nurse conducted a standardized 4-5 hour examination in the homes of each study participant (Ferrucci et al., 1995). Reported medical conditions were validated and their severities ascertained. A comprehensive examination was completed and included: physical assessments, vital signs, anthropomorphic data, electrocardiography, joint photographs, audiometry, pulmonary function, and various other strength, agility, and endurance performance measures. Medical records underwent ongoing surveillance and participants' primary

care physicians provided confirmation of self-reported information. Phlebotomy was performed on 762 of the 1,002 participants who signed a secondary consent. Prospective data collection occurred in the form of in-home follow up visits that were conducted every 6 months for a total of 3 years (Volpato, Leveille, Blaum, Fried, & Guralnik, 2005). Updated information on participants' health status was collected, including new diagnoses of any major illnesses, medication changes, surgeries, hospitalizations, and acute decompensations stemming from chronic conditions. After the 3-year prospective follow-up was completed, two more phone interviews were done to perform vital status assessment, yielding a total follow-up time span of 5 years (Chaves et al., 2004).

Data Collection: WHAS 2, 1994-1996

After screening, eligible participants were scheduled for a 5-6 hour baseline examination at the John's Hopkins Functional Laboratory (Fried et al., 2000). Participants completed the same standardized questionnaires as WHAS 1 subjects. Detailed demographic information, information on functional capacity, and self-report of medical diagnoses for 11 chronic diseases were among data collected. Many tests of functional capacity were administered, and phlebotomy was performed on 369 of the 436 participants who signed a secondary consent. A 4-5 hour follow-up examination (also at the John's Hopkins Functional Laboratory) was performed for each participant 18 months after baseline to ascertain change in functional status. For the majority of laboratory and clinical measurements, WHAS 2 methods of data collection were standardized to WHAS 1 instrumentation and techniques (Semba, Garrett, Johnson, Guralnik, & Fried, 2000). Exceptions to this rule were due to time and cost constraints.

Data Collection Differences Between WHAS 1 and 2

For the dissertation study's laboratory, clinical outcome, and covariate measurements, all data collection and measurement methods used the same laboratory services, clinical instrumentation, and data collection protocols except for the peripheral extremity vibration sensitivity testing. WHAS 1 employed a vibrometer (voltage/current stimuli) measurement protocol while WHAS 2 used a tuning fork measurement protocol. For several reasons, dissertation analysis of peripheral sensitivity scoring was restricted to include only WHAS 1 vibrometer data (n = 498).

Reasons for restricting analyses to WHAS 1 included the following: 1) reliability of results would be threatened if using measurements from both instruments since the protocols were not interchangeable; 2) there was potential for variability coming from tuning fork stimulus application that could not be adjusted for (i.e., could not ensure clinician hit the tuning fork the same way in the same location for all WHAS 2 subjects); and 3) there was a much smaller sample size for WHAS 2 subjects with tuning fork data, which did not meet power analysis estimate requirements.

Subject Demographics

Numerous investigations have pooled WHAS 1 and 2 subject data for a strengthened approach representative of the community-dwelling elderly (Bandeen-Roche et al., 2006; Chaves, Ashar, Guralnik, & Fried, 2002; Chaves et al., 2005; Leng, Xue, Tian, Walston, & Fried, 2007; Semba et al., 2000; Semba et al., 2005; Walston et al., 2005). Key differences in the two WHAS studies include an increased proportion of

individuals with higher education in WHAS 2, and greater representation of African-Americans in WHAS 1. Both cohorts have minimal inclusions for other racial minorities.

Outcome Variable Measurement

Hematological measurements used in the dissertation include hemoglobin concentration and mean corpuscular volume (MCV). Cobalamin-related biochemical measurements used in the dissertation include serum cobalamin and the metabolites homocysteine and methylmalonic acid. Neurologic measurements used in the dissertation include depression scores (Geriatric Depression Scale) and vibratory sensitivity. Functional performance measurements include grip strength and 4-meter walking speed.

Phlebotomy and WHAS Sample Processing, Storage

The dissertation research includes measurements from WHAS 1 and 2 participants who consented to phlebotomy. WHAS 1 venipuncture was performed in subjects' homes by a certified phlebotomist who followed a standardized study protocol. The blood samples were nonfasting and after venipuncture, taken for processing and aliquoting at the Core Genetics Laboratory in Johns Hopkins University School of Medicine. WHAS 2 venipuncture was performed during clinic visits by a certified phlebotomist following the same standardized protocol as used in WHAS 1. The blood samples were also nonfasting and processed at the Johns Hopkins University School of Medicine. Frozen aliquots were sent on dry ice to Quest Diagnostics (formerly Corning Clinical Laboratories and MetPath) in Teterboro, NJ for analysis that included complete blood count, and biochemical, hormonal serum measurements. Remaining aliquots were

stored at -80 degrees Celsius in the Core Genetics Laboratory at the Johns Hopkins University School of Medicine.

Hematologic Measures

Hemoglobin

WHAS 1 and 2 blood samples were collected for hemoglobin assessment in sterile, vacuum, ethylenediaminetetraacetic acid (EDTA) tubes. At Quest Diagnostics, hemoglobin levels were measured via the standard cyanmethemoglobin method, a calorimetric approach that determines hemoglobin concentration through spectrophotometry (Chaves et al., 2006; Williamson et al., 1995).

Mean Corpuscular Volume (MCV)

The MCV is a calculated value of the hematocrit percentage (proportion of erythrocyte volume to whole blood volume in a sample) divided by the red blood cell count number, and is reported in femtoliters (fL), (Williamson et al., 1995). Performed as part of the complete blood count for WHAS subjects, the MCV levels were collected at the same time and in the same tubes as blood drawn for hemoglobin.

Biochemical Metabolites

Serum Cobalamin

At Quest Diagnostics (Teterboro, NJ), serum cobalamin concentrations were determined using a competitive intrinsic factor protein-binding assay according to the method of Ciba-Corning Diagnostics Corporation in Medfield, MA (Stabler et al., 1999).

Homocysteine and Methylmalonic Acid

A subset of WHAS 1 and 2 sample aliquots were shipped on dry ice from the Core Genetics Laboratory at John's Hopkins University School of Medicine to the University of Colorado Health Sciences Center (UCHSC) for measurement by WHAS collaborator, Dr. Sally Stabler (Penninx et al., 2000; Stabler et al., 1999). Both total homocysteine and methylmalonic acid levels were obtained using stable-isotope dilution and capillary gas chromatography-mass spectrometry with selected ion monitoring (Stabler, Marcell, Podell, Allen, & Lindenbaum, 1986; Stabler et al., 1988).

Neurologic Measures

Geriatric Depression Scale (GDS)

WHAS 1 and 2 subjects were administered the GDS. It is a straightforward depressive examination scale consisting of 30 items requiring approximately 10 minutes to administer (Yesavage et al., 1982). Questions require a yes/no answer from participants; of 30 items, only one concerns presence of somatic (physical) symptomology, which was of special interest for WHAS applications where on average participants had at least one chronic condition (Kasper et al., 1999).

Questions in many generalized depression screening tools involve assessing presence of symptoms that are also present in other disease states, such as "do you have low energy"? A false positive can easily result in elderly individuals suffering from a chronic condition such as chronic obstructive pulmonary disease, as they would experience low energy due to difficulty breathing and not depression. The GDS was specifically designed to assess depression symptoms in the elderly to reduce the

occurrence of these false positives from the aging process or other common comorbid conditions, by removing focus from physical symptoms (Norris, Gallagher, Wilson, & Winograd, 1987). GDS scores under 10 indicate absence of depression, 10-13 mild depression, and scores over 14 indicating moderate and severe depression (Norris et al, 1987).

Vibratory Sensitivity

As indicated in Chapter 2 and earlier in this chapter, vibration sensitivity was not measured similarly between WHAS 1 and WHAS 2 subjects due to cost and time limitations. Because the measurement methods did not use the same instruments and protocols, only WHAS 1 subjects' vibration sensitivity data were analyzed in the dissertation study.

In WHAS 1, vibration perception testing (VPT) was conducted using a vibrometer. Thresholds were measured and established by applying voltage/current based stimuli called 'vibration units' to participants' lower extremities with the Vibratron II (Physitemp Instrument, Inc., Clifton, NJ). Women were asked to indicate when vibration was felt after stimulation of the lower surface of their right big toe (yes/no), using a two-alternative forced choice procedure. Progressive stimulation intensity decreases in 10% increments were performed until subjects could no longer detect vibration. Upon error, the intensity was increased by 10%, with progressive incremental decreases continued until a total of five errors were made. After conversion of vibration units to microns, mean thresholds were identified by taking the five errors and five lowest correct scores,

eliminating the highest and lowest of each, and obtaining the mean of the remaining values.

Functional Measures

Grip Strength

Grip strength was measured for study subjects using a JAMAR hand dynamometer (Model #BK-7498; Fred Sammons Inc, Burr Ridge, IL). WHAS 1 and 2 subjects were asked to grasp the dynamometer and squeeze as hard as possible three times on each hand. The best measure in the stronger hand was recorded and reported in kilograms.

Four-Meter Timed Walk

WHAS 1 and 2 participants were asked to walk over a 4-meter course, two times at their usual speed and once, as fast as possible. For some participants, 4 meters was not available in their homes and a distance of 3 meters was used instead. Beginning at a starting line, walking and timing of the walk did not begin until the command to start was given by the interviewer. Using the faster of the two usual-pace walks, average walking speed was calculated by dividing the length of the walk (in meters) by the time in seconds required to complete it. Subjects were permitted to use a cane, walker, or walking aid, but not assistance from an additional person.

Covariate Variable Measurement

Renal Function

The dissertation research analyzes biochemical metabolite data that is adjusted for a participant's renal function. An essential component of several renal-estimation calculations, serum creatinine levels were obtained for WHAS 1 and 2 subjects who participated in venipuncture. Performed by Quest Diagnostics in Teterboro, NJ, serum creatinine concentrations were analyzed via the conventional Jaffe method (Semba et al., 2009). Incorporating serum creatinine, age, weight, and a female muscle mass constant, the Cockcroft-Gault formula used in the dissertation research to estimate WHAS 1 and 2 subjects renal function (mL/min) is:

$$\text{Creatinine Clearance} = \frac{(140 - \text{age in years}) \times (\text{mass in kg}) \times 0.85}{72 \times \text{serum creatinine (mg/dL)}}$$

Folate

In addition to serum cobalamin levels, the central laboratory of Corning Clinical Laboratories in Teterboro, NJ also determined serum folate concentrations for WHAS 1 and 2 subjects. Serum folate concentrations were determined using a competitive folate-binding protein assay according to the method of Ciba-Corning Diagnostics Corporation in Medfield, MA (Penninx et al., 2000).

Standing Height

During in-home screenings for WHAS 1 subjects and clinic visits for WHAS 2 subjects, standing height (in centimeters) was measured by trained study staff accordant with study protocol.

Genotyping Methods

Isolation and Preparation of WHAS Subject DNA

Blood Processing

At the Core Genetics Laboratory in Johns Hopkins School of Medicine, WHAS 1 and 2 subject DNA was extracted from whole blood using the Puregene DNA Purification Kit from GentraSystems, Inc. DNA samples were then stored in a -80 (Celsius) freezer at the Johns Hopkins University. Approximately 50 nanograms of genomic DNA was provided for genotyping the 794 WHAS 1 and 2 subjects. It was not known how many freeze/thaw cycles WHAS subject DNA underwent prior to their receipt for this project; however, genetic research using WHAS material dates from published reports in 2005.

Whole Genome Amplification

Large-scale genotyping methods, such as that used in this dissertation research, require several micrograms of DNA. The small amount of available starting material (50 nanograms) would be quickly consumed, severely limiting investigative capacity. In the case of limited starting DNA, a method that can be used to provide ample supply of genomic material is Whole Genome Amplification (WGA). Relatively new, WGA

reproduction of small amounts of DNA is becoming a widely utilized technique in conducting high-throughput SNP genotyping (Berthier-Schaad et al., 2007; Dean et al., 2002).

There are several WGA methodologies and they are categorized into polymerase chain reaction (PCR)-based, and non-PCR-based approaches. A non-PCR-based method, multiple displacement amplification, is the WGA technique that was used in this dissertation research to amplify the limited starting quantity of WHAS DNA into more substantiative working material for genotyping. Multiple displacement amplification is based on the rolling circle amplification mechanisms used by plasmids and viral vectors during DNA replication—but is adapted to use genomic DNA instead (Dean et al., 2002). In multiple displacement amplification, a highly processive bacteriophage enzyme (Φ29 DNA polymerase) displaces DNA strands, replicates copies, and proofreads their assembly. Compared to *Taq Polymerase*, Φ29 DNA polymerase demonstrates a significantly lower error rate and faithfully amplifies DNA across the entire genome (Lovmar & Syvanen, 2006). Accuracy and fidelity of multiple displacement amplification is dependent upon the amount and quality of genomic DNA that is used as starting material in the reaction; at least 10 nanograms of genomic DNA input is required for SNP genotyping (Bergen, Qi, Haque, Welch, & Chanock, 2005).

The dissertation research used Qiagen's 100-reaction REPLI-g Midi Kit. The protocol required 10 nanograms of starting genomic material to yield over 40 micrograms of final high-molecular weight material in 10-100kb stretches (Appendix A). Although use of multiple displacement amplification produces accurate results, the Φ29 DNA polymerase can randomly amplify one of the patient's alleles and drop out the remaining

counterpart (Bergen et al., 2005). As a result, heterozygotes are susceptible to "allele drop-out," often causing heterozygotes to be erroneously interpreted as homozygotes. To correct for incidence of heterozygote dropouts, the dissertation research performed two separate and independent rounds of multiple displacement amplification, which were pooled together into a common solution for genotyping.

Candidate Gene SNP Selection

The Haplotype Map, or HapMap, was a research initiative completed in 2003 that provides public access to a catalogue of human genetic variation for racial subgroups: Caucasian, African American, Chinese, and Japanese (The International HapMap Consortium, 2003). Following successful dissertation proposal defense in May 2008, the most current version of HapMap (2.0) was used to select SNPs for the dissertation research (The International HapMap Consortium, 2007). Within HapMap 2.0, Haploview and Tagger were used to visualize population SNP alleles, genomic regions, and overall SNP selection. Conceptualized and released by Broad Institute, Haploview is a freely accessible public program that can be used to visualize linkage disequilibrium plots. A function that exists as part of the Haploview program, Tagger identifies similarity among SNPs as a function of their proximity to one another. SNPs closest together offer the same information as that contained by its neighbors (linkage disequilibrium), (Ardlie, Kruglyak, & Seielstad, 2002). The population statistic that is used to discern similarity among SNPs is r^2 and for the dissertation research, a conservative threshold ($r^2 = 0.9$) was used to eliminate redundancies (Wall & Pritchard, 2003).

In addition to HapMap SNPs, other variants that were likely to have functional implications were explored and included. Searched for in the public catalogue of SNPs (dbSNP), additional selected candidates included variants within exons, promoters, conserved sequences across species, and those reported in literature that were associated with clinical characteristics and biochemical parameters being explored in this research. In sum, a total of 51 SNPs incorporating both Caucasian and African American ethnic ancestries were selected for the dissertation study—29 SNPs in the transcobalamin II gene and 22 SNPs in the transcobalamin II-receptor gene (Tables 1 and 2).

SNP Genotyping for Whole Genome Amplified WHAS Material

There are many high-throughput SNP genotyping systems available for laboratory use, and they vary according to throughput capacity, accuracy, cost, and scale. The Sequenom MassARRAY iPLEX Platform is well known for its ability to incorporate high-throughput features for reasonable multistep system/reagent use and costs (Gabriel, Ziaugra, & Tabbaa, 2009).

The Sequenom MassARRAY platform allows for accurate custom SNP genotyping by using a homogeneous reaction format with two progressive phases of specificity comprising: 1) a locus-specific PCR reaction to obtain a 100-base pair region where a SNP is located, and 2) a locus-specific primer extension reaction that produces the SNP genotype. A single extension primer can generate allele-specific results with unique masses, and multiplexed SNP extension reactions can be performed with a single termination mix (iPLEX) and universal reaction conditions. Following extension, samples are analyzed by MALDI-TOF mass spectroscopy.

A singular Sequenom system is capable of generating over 100,000 genotypes per day (Gabriel et al., 2009). There are multiple preparatory phases and reaction steps that were required to obtain genotypes for plates of whole genome amplified WHAS DNA, including pre-PCR sample and assay preparation, PCR amplification, post-PCR cleanup, iPLEX primer extension, primer extension cleanup, and spotting extension products onto SpectroChips.

Pre-PCR: WHAS Sample and Assay Preparation

After WHAS DNA was whole genome amplified and the amplification rounds were pooled in 96-well plates, samples were diluted to create working stocks at a concentration of 10ng/ul. Four groups of 96-well plates were concatenated onto a single 384-well plate and stored in desiccators. In sum, there were nine unique master 384-well WHAS plates for the dissertation research, from which small volumes of sample were aliquoted and stamped into genotyping stockpiles using automated liquid transfer machinery (BeckmanCoulter's Biomek; Tomtec's Quadra tower). Labeled genotyping plates (384 wells) had approximately 5-20 ng of whole genome amplified DNA placed into each well. Negative blank and water control wells were created prior to automated transfer and were spatially unique to each master WHAS plate.

An automated approach was used to design PCR assays and to select oligonucleotide primers. Using sequence in publicly available bioinformatics databases, DNA sequence (~100 base pairs 3'-5') surrounding each selected SNP was copied and pasted into an Excel Spreadsheet and brackets were inserted around SNP sites. The final Excel spreadsheet was converted into a text file and run through Sequenom's

MassARRAY Designer software. The software automatically designed forward and reverse PCR primers, and extension iPLEX primers, and grouped all the assays into chemically optimized assay pools. Through this pooling, many different individual SNP loci can be analyzed simultaneously within the same sample well. The WHAS dissertation project had a total of 5 pools (also called "plexes") and were broken down according to the following: Plex 1 with 19 SNP assays, Plex 2 with 18 SNP assays, Plex 3 with 28 SNP assays, Plex 4 with 23 SNP assays, and Plex 5 with 17 SNP assays. Thus, when prepping a 384-well plate, reactants and primers would be mixed and aliquoted into each of the wells according to which Plex was being run.

PCR Amplification of Target Loci

PCR primers from Sequenom's MassARRAY Designer software output file were ordered from Integrated DNA Technologies (IDT) and were unmodified with standard purification at 25 nanomolar concentration. The following reactants were mixed and added to the 384-well plates of WHAS whole genome amplified DNA (1X reaction): 2.85 ul water, 0.625 ul of 10X PCR Buffer, .325 ul of 25 mM $MgCl_2$, 0.1 ul of 25mM dNTPs, 1 ul of 500 nM F/R PCR Primer Mix, and 0.1 ul of 0.5U/ul HotStart Taq Polymerase. Automated fluid transfer from a dispenser holding the PCR reaction mix into each of the 384 wells was performed through the automated Biomek (Beckman Coulter) in a pre-PCR area of the laboratory. PCR thermocycler conditions for the 384-well plates were as follows: initial denaturation at 94 degrees C for 15 minutes; 45 cycles of denaturing at 94 degrees C for 20 seconds, annealing at 56 degrees C for 30 seconds, and extension at 72 degrees C for 60 seconds; followed by final extension at 72 degrees C for

3 minutes; and held at 4 degrees C for infinity until removed from the thermocycler block.

Post-PCR Cleanup Using SAP

Following the PCR reaction, there are many remaining nonincorporated dNTPs from amplification products in each well of the 384-well plate. If allowed to remain in the wells, functional dNTPs can extend in the primer extension reactions, causing erroneous contaminant peaks to occur, which greatly detriments data analysis and interpretation. Treatment with the enzyme shrimp alkaline phosphatase (SAP) is performed to remove any remaining and unincorporated dNTPs from the PCR amplification products. SAP acts by dephosphorylating the unincorporated dNTPs and cleaves available phosphate groups from the 5' termini. The following reactants were mixed and added to the 384-well plates of WHAS whole genome amplified DNA following PCR (1X reaction): 1.53 ul water, 0.17 ul SAP buffer, and 0.30 ul SAP. Automated fluid transfer from a dispenser holding the SAP reaction mix into each of the 384 wells was performed through the automated Multimek in a post-PCR area of the laboratory. SAP thermocycler conditions for the 384-well plates included SAP activation treatment at 37 degrees C for 20 minutes, SAP deactivation at 85 degrees C for 5 minutes, and held at 4 degrees C for infinity until removed from the thermocycler block.

Primer Extension Using iPLEX Chemistry

In comparison to previous Sequenom genotyping technologies, the use of iPLEX extension assays is what allows for routine multiplexing of up to 30-36 SNPs in one

sample well. In the iPLEX extension assay, primers and amplified target DNA are incubated with dideoxynucleotide terminators that are mass modified. The extension into the SNP site proceeds according to the sequence at the variant loci, and is a single and complementary terminator base. Before iPLEX, the masses of the A, C, G, T terminators that were incorporated at the SNP site were very close together and difficult for the mass spectrometer to discern between; only several assays could be run simultaneously (i.e., 1-6 SNP assays). Sequenom's development of mass-modified dideoxynucleotide terminators provides clearer allele signals, reducing allele-bias and providing for easy discernment by the mass spectrometer (Table 3) (Oeth et al., 2006).

For the dissertation's iPLEX reaction in the lab, unmodified extension primers from Sequenom's MassARRAY Designer software output file were ordered from Integrated DNA Technologies (IDT) featuring standard purification at 250 nanomolar concentration. Because there is an inverse relationship between mass spectrometer peak intensity and the mass of the SNP analyte, all extension primers were optimized so that they were as equal in intensity as possible. After optimization, the following reactants were mixed and added to the 384-well plates of WHAS whole genome amplified DNA (1X reaction): 0.619 ul water, 0.2 ul 10X iPLEX buffer, 0.2 ul iPLEX terminator, 0.94 ul of extension primer mix, and 0.041 ul of 0.655U/ul iPLEX extension enzyme. Automated fluid transfer from a dispenser holding the iPLEX extension reaction mix into each of the 384 wells was performed through the automated Multimek in a post-PCR area of the laboratory. Extension thermocycler conditions for the 384-well plates included initial denaturation of 94 degrees C for 30 seconds, 40 cycles of denaturing at 94 degrees C for 5 seconds, annealing at 52 degrees C for 5 seconds, and extension at 80 degrees.

Embedded in each of the 40 cycles are 5 repeat cycles between annealing at 52 degrees C for 5 seconds and extension at 80 degrees for 5 seconds. Following the 30 cycles, a final extension at 72 degrees for 3 minutes occurs before being held at 4 degrees C for infinity (until taken out of thermocycler block).

Post-iPLEX Resin Cleanup

Following primer extension, there are many salts left over from the iPLEX extension reaction products. If left in the sample wells untreated, salts including Na^{+}, K^{+}, and Mg^{2+} ions can cause a great deal of background noise in the spectra, preventing the mass spectrometer from making genotype calls. Sequenom's SpectroCLEAN is a sand-like resin that is pretreated with acid reagents, and is added directly to the primer extension products in each of the 384 wells. To make the slurry, 80ul of water is added to 12 mg of resin in a 96-well PCR plate; the Multimek transfers 16 ul of the resin/water slurry to each of the 384 wells. After automated addition of the slurry, the 384-well plate is slowly rotated for 10-30 minutes to ensure that samples are thoroughly mixed with cation removal resin.

Spotting of iPLEX Products on SpectroChips

After rotation of the 384-well plates, the plates were centrifuged. Clear fluid on top of the settled resin contained the extended/desalted SNP genotype analyte products. After the plate was laid flat in an automated spotting machine, a pinned robotic spotting head transferred ~25 nL of the sample analyte solution onto a silica chip (SpectroChips). Each of the 384 spots on the SpectroChips was comprised of 3-hydroxypicolinic acid, the

appropriate matrix for MALDI TOF mass spectroscopy. In addition to the 384 sample matrix spots, 10 additional reference matrix spots were reserved for application of Sequenom's MassARRAY mass spectrometry calibrant solution.

Calling the Genotypes

Mass Spectrometry Detection of SNP Genotype

Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry can differentiate SNP densities and ascertain a patient's genotype at a SNP locus for one of three combinations: heterozygous alleles (A/T) or homozygous alleles (A/A and T/T). The laser is measuring the time-of-flight that genotype analyte fragments on the SpectroChip's matrix take to travel from one end of the mass spectrometer to the other. Each allele or allelic combination has an expected time-of-flight, which correlates to a specific weight in daltons. Longer times indicate heavier products and shorter times indicate lighter products.

A total of 80 SpectroChips were analyzed for the dissertation research. After spotting, each chip was placed into the mass spectrometer (Sequenom; Bruker Instruments), placed under vacuum pressure, and the spots were sequentially shot with a laser. The 3-hydroxypicolinic acid matrix comprising each spot played an important role in the MALDI-TOF; it absorbed the laser light energy and caused part of the illuminated analyte substrate to vaporize. The vaporized matrix plume expanded after the laser fired, and as it passed into the vacuum, became ionized. After vaporization and ionization, the genotype analyte fragments were electrostatically transferred into the time-of-flight chamber and separated from matrix ions. The genotypes were then detected (or "called")

based on this mass-to-charge characteristic; ion detection at the end of the flight chamber was directly proportional to the square root of the postvapor plume mass-to-charge ratio.

MassARRAY Typer 4.0 and Manual Calling

After spectra and calls for each chip were generated, Typer 4.0 Software was used to visualize plate results and the spectral curves. The Typer 4.0 system allowed visualization of an assay's performance for the chip, and inspection of automated calls by the software. MassARRAY had difficulties in automatically calling genotypes of WHAS material due to the whole genome amplification process. For example, heterozygote calls for many assays were scattered widely from the x-axis to the y-axis, as opposed to the typical tight cluster formation at the 45-degree demarcation area between the axes. MassARRAY often erroneously called these spread-out heterozygotes as homozygotes, since heterozygotes demonstrated a greater range than would normally be expected due to the whole genome amplified process. Subsequently, all genotype calls in the dissertation research were inspected manually, and approximately 40-50% of all WHAS genotypes were issued manually following the creation of a validated calling algorithm (Appendix B). To ensure manual calling did not introduce unacceptable levels of variability, all plexes (1 through 5) of WHAS DNA underwent three to five independent rounds of genotyping and calling, and irreconcilable discordant genotypes were discarded from analyses.

Protection of Human Subjects

For both WHAS 1 and 2, protection of human subjects was obtained through Institutional Review Board approval at the John's Hopkins University and the National Institutes of Health's National Institute on Aging. To obtain human subjects approval for the genotyping phase of this research, I submitted an application to the University of Utah IRB. A review panel concluded that the genotyping in my dissertation research did not meet the definitions of Human Subjects Research according to Federal regulations. Therefore, IRB oversight was not required (Appendix C).

To ensure maximum confidentiality, WHAS samples were anonymized and clinical phenotype data were not linkable to patients without a master code file, which was available only to approved study staff at Johns Hopkins University and at the National Institute on Aging. For the dissertation research, I catalogued WHAS subject DNA samples by anonymous ID numbers in an Excel spreadsheet on a heavily fire-walled NIH intramural network. Each record had a unique identification number that was not connected to any traceable personally identifiable information such as date of birth, social security number, address, etc. Presently, many of the WHAS 1 and 2 participants are deceased.

Analysis

The independent variable in this study across all aims and research questions was the transcobalamin II and transcobalamin II-receptor SNP genotype, according to three ordered categories: homozygous minor allele (AA), heterozygous (AB), and homozygous major allele (BB). The dependent outcome variables are continuous, and include

hemoglobin concentration, MCV, serum cobalamin and cobalamin metabolites, depression scores, vibration sensitivity, grip strength, and walking speed measurements. Genetic data was analyzed using Haploview 4.0 and linkage disequilibrium plots were constructed for the transcobalamin II and transcobalamin II-receptor genes for Caucasians and African American subjects.

Using SPSS v12.0, missing clinical phenotype data and descriptive statistics were analyzed, with summary statistics (means, medians, standard deviations, ranges, quartiles) reported for each outcome measure. Histograms and trend plots were evaluated to assess data normality, and Kolmogorov-Smirnoff tests were conducted to assess degree of normality violation. Extreme outliers that were greater than 3 standard deviations away from the mean were identified, evaluated, and removed from analyses if they inappropriately skewed sample population means or greatly affected normality tests. Pearson correlations between the dependent variables were evaluated for collinearity. Genotype and allele frequencies were counted for each SNP in both Caucasian and African American cohorts, and Hardy-Weinberg statistics were calculated. Hardy-Weinberg uses a χ^2 test (Pearson goodness-of-fit) to ascertain presence of equilibrium and demonstrates a χ^2 probability distribution under the null hypothesis (Balding, 2006). A conventional significance level of $\alpha = 0.05$ was designated for all analyses. Using Cohen's small effect size (.25σ), two-tailed significance of .05, and desired power of 0.90, the minimum sample size necessary for adequate strength was $n = 171$. Overall differences in socio-demographic, clinical, and biochemical variables between African Americans and Caucasians, and between WHAS 1 and WHAS 2 cohorts, were assessed using independent *t*-tests.

To assess differences in outcome variable means across race and SNP genotype, an analysis of variance or covariance was performed for each outcome measure. Homogeneity of variance was assessed through the Levene's test of equality of error variances. For all analysis of covariance tests, linearity was assessed between the covariates and the dependent variable, covariate reliability was ensured, and homogeneity of regression was evaluated by exploring slope inequality between the dependent variable and the covariate.

Significant interaction (race) and main (genetic) effect *F* tests from the analysis of variance or covariance were explored further with traditional post-hoc comparison tests. Statistical interactions between Caucasian and African American subjects across genotype groups were assessed by evaluating plotted graphs in SPSS. Post-hoc testing for significant main effects was conducted with the Tukey procedure to identify where mean differences between groups existed. Post-hoc testing for simple main effects following significant interactions was conducted with the Least Significant Difference procedure. In the presence of extreme normality violations or a violated Levene's test, nonparametric post-hoc comparison tests were used. The Bonferroni adjustment was used to reduce alpha inflation stemming from performing multiple statistical tests. At nine outcome measures and 27-29 SNPs per measure, the Bonferroni adjustment was designated at p=(0.05/243)=0.0002. Linkage disequilibrium plots to assess tagging success were constructed using Haploview 4.0 analysis program tools.

Aim 1

Research Question 1.1

Hemoglobin concentrations were continuous data. Differences in means were analyzed using a 2-way analysis of covariance (ANCOVA) on the fixed factors, race (African American, Caucasian) and SNP genotype, and accounted for serum folate variance. Post-hoc analyses for significant *F* tests were conducted by using simple tests of pairwise comparison for main effects and evaluating graphical interactions on profile plots.

Research Question 1.2

Mean corpuscular volume (MCV) concentrations were continuous data and differences in means were analyzed using a 2-way ANCOVA on the fixed factors, race (African American, Caucasian) and SNP genotype, and adjusted for serum folate variance. Post-hoc analyses for significant *F* tests were conducted by using simple tests of pairwise comparison for main effects and evaluating graphical interactions on profile plots.

Aim 2

Research Question 2.1

Serum cobalamin concentrations were continuous data. To account for the effects of a subject's renal function (creatinine clearance estimation per Cockcroft-Gault), differences in mean cobalamin concentration were analyzed using a 2-way ANCOVA on the fixed factors, race (African American, Caucasian) and SNP genotype. Post-hoc

analyses for significant *F* tests were conducted by using simple tests of pairwise comparison for main effects and evaluating graphical interactions on profile plots.

Research Question 2.2

Serum homocysteine concentrations were continuous data. To account for the effects of a subject's renal function (creatinine clearance estimation per Cockcroft-Gault), serum folate, and serum cobalamin, a 2-way ANCOVA was used to ascertain presence of association between homocysteine concentration and the fixed factors, race (African American, Caucasian) and SNP genotype. Post-hoc analyses for significant *F* tests were conducted by using simple tests of pairwise comparison for main effects and evaluating graphical interactions on profile plots.

Research Question 2.3

Serum methylmalonic acid concentrations were continuous data. Accounting for renal function (creatinine clearance estimation per Cockcroft-Gault) and serum cobalamin, a 2-way ANCOVA was used to ascertain presence of association between methylmalonic acid concentration and the fixed factors, race (African American, Caucasian) and SNP genotype. Post-hoc analyses for significant *F* tests were conducted by using simple tests of pairwise comparison for main effects and evaluating graphical interactions on profile plots.

Aim 3

Research Question 3.1

Geriatric Depression Scores (GDS) scores were continuous, interval-level data. Differences in GDS means were analyzed using a 2-way ANOVA on the fixed factors, race (Caucasian or African American) and SNP genotype. Post-hoc analyses for significant *F* tests were conducted by using the Tukey test and evaluating graphical interactions on profile plots.

Research Question 3.2

Vibrometer sensitivity measurements were continuous data. Differences in means were analyzed using a 2-way ANOVA on the fixed factors, race (African American, Caucasian) and SNP genotype. Post-hoc analyses for significant *F* tests were conducted by using the Tukey test and evaluating graphical interactions on profile plots.

Aim 4

Research Question 4.1

Hand grip strength dynamometer measurements were continuous data. Differences in hand grip strength means were analyzed using a 2-way ANOVA on the fixed factors, race (African American, Caucasian) and SNP genotype. Post-hoc analyses for significant *F* tests were conducted by using the Tukey test and evaluating graphical interactions on profile plots.

Research Question 4.2

Three and 4-meter walking speed were continuous data. Differences in mean walking speeds were analyzed using a 2-way ANCOVA on the fixed factors, race (African American, Caucasian) and SNP genotype, adjusted for an individual's standing height. Post-hoc analyses for significant *F* tests were conducted by using simple tests of pairwise comparison for main effects and evaluating graphical interactions on profile plots.

Missing Data

Data Availability, Transfer Procedures, and File Merging

Although a total sample of 1,167 women consented to phlebotomy in WHAS 1 and 2, blood was no longer available for all subjects at the onset of the dissertation study in 2008. DNA availability for WHAS 1 was $n = 536$ and for WHAS 2 was $n = 253$, yielding a total available patient sample of $n = 789$ for the dissertation's genetic analysis. A file provided by Johns Hopkins University study staff (Dr. Amy Matteini) in November 2009 contained selected health, clinical, and socio-demographic measurements for the total documented 1,438 WHAS 1 and 2 participants and included data on subjects who did not provide blood samples.

In December 2009, a combined data file was generated that restricted content to include only the WHAS 1 and 2 research subjects with corresponding genotype data generated in the laboratory ($n = 789$). Missing data within this pool of 789 subjects were analyzed (genotype field entries plus social and clinical characteristics) for nonrandom patterns through the SPSS 'Missing Data' function and cleaned. No outstanding patterns

of nonrandom missing data were identified; however, restricting analyses to WHAS 1 and 2 subjects with available genomic DNA likely affected result generalizability, in that it represented 54.7% (789/1,438) of the original sampling study frame. The 789 available DNA samples represented 67.6% of WHAS 1 and 2 subjects consenting to phlebotomy (789/1,167).

Collectively, missing data for the dissertation research in the n = 789 sample population were observed across two categories: genotyping failures (including noninformative monomorphic alleles) and unavailable clinical data in the WHAS 1 and 2 dataset.

Missing Genetic Data

Genotyping failures included 6 noninformative SNPs that were monomorphic in both African American and Caucasian subjects. A total of 16 of the 51 total SNPs did not meet quality control thresholds in the laboratory and were removed from analysis. For the remaining 29 SNPs, missing genotype data ranged from n = 11 to n=63 depending on the assay. On average each SNP was missing 40 genotypes, which corresponded to 5% of the dissertation study sample, which met the National Human Genome Research Institute's standard laboratory threshold metrics for minimally required data.

Missing Laboratory and Clinical Data

For each of the dissertation study's biochemical and clinical outcome variables, there were varying degrees of missing data. For example, not all laboratory tests were successful for each study participant and not all clinical parameters (depression, walking

speeds, vibration testing) were successfully measured in patient homes or during clinic appointments. Missing data for each of the dissertation study's outcomes for the 789 WHAS subjects include the following: n = 37 hemoglobin, n = 38 mean corpuscular volume, n = 12 cobalamin, n = 25 homocysteine, n = 25 methylmalonic acid, n = 0 depression score, n = 39 peripheral sensitivity score, n = 70 hand grip strength, and n = 21 walking speed. Missing data for each of the dissertation study's covariates for the 789 WHAS subjects include the following: n = 9 folate, n = 53 estimated creatinine clearance, and n = 41 standing height.

Outliers

For many of the laboratory traits, there were severe outliers that were either zero (representing an error in laboratory reporting) or greater than 3 standard deviations away from the mean. These values were coded as missing data and removed from analysis to mitigate their skewing effect on outcome variable sample means, distributions, and normality tests (n = 6 cobalamin, n = 6 total homocysteine, n = 10 serum methylmalonic acid, and n = 7 MCV). Final exact sample sizes for each genetic marker that was genotyped, and mean values for each WHAS outcome analyzed in this study, are listed for reader reference in Appendix D.

Impact of Missing Data

To meet the stated objectives of the dissertation's research aims and questions, research subjects had to have laboratory values and clinical parameter measurements in addition to a SNP genotype result to be included in the analyses of variance and

covariance statistical evaluation. Chapters 4 and 5 report *F* statistics and post-hoc test analyses incorporating this requirement. However, the generalized assessments of means and standard deviations for the WHAS subjects' biochemical and clinical traits in Chapters 4 and 5 (delineated by race and cohort) were conducted separately from this requirement in order to assess the impact of missing data.

Outcome means and clinical mean parameter profiles obtained from the dissertation sample were compared with previously published WHAS 1 and 2 data using the full sample population. Across all clinical outcome and covariate parameters, the means and standard deviations in the n =789 WHAS dissertation sample corresponded closely with previously published results for both the combined and separated WHAS cohort literature (Chaves et al., 2006; Fried et al., 2000; Guralnik, Fried, Simonsick, Kasper et al., 1995; Resnick et al., 2000; Resnick, Vinik, Heimovitz, Brancati, & Guralnik, 2001; Penninx et al., 2000; Rantanen et al., 1998; Stabler et al., 1999).

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Table 1

Transcobalamin II Gene SNPs

Reference Sequence #	Gene Position	Target Population	Identified From
rs12159600	intron 3	AA	HapMap
rs16988838	intron 1	AA	HapMap
rs11912238	intron 5	AA	HapMap
rs7286107	intron 1	CEPH	HapMap
rs4820021	intron 6	CEPH	HapMap
rs3178000	exon 8	CEPH	HapMap
rs2301955	intron 7	both	HapMap
rs2267163	intron 5	both	HapMap
rs2072194	intron 8	both	HapMap
rs740234	intron 2	both	HapMap
rs4820889	exon 8	both	HapMap
rs4820886	intron 7	both	HapMap
rs2301958	intron 7	both	HapMap
rs7289549	intron 1	both	HapMap
rs11703570	intron 3	both	HapMap
rs16988828	intron 1	both	HapMap
rs4820887	intron 7	both	HapMap
rs4820888	intron 7	both	HapMap
rs9606756	exon 2	both	HapMap
rs35915865	exon 3	CEPH	dbSNP
rs35838082	exon 5	both	dbSNP
rs1801198	exon 6	both	dbSNP
rs9621049	exon 7	both	dbSNP
rs1131603	exon 8	CEPH	dbSNP
rs11557600	exon 3	AA	literature
rs17849434	exon 5	n/a	literature
rs2301956*	intron 7	CEPH	HapMap
rs2267162*	intron 4	both	HapMap
rs740233*	intron 2	both	HapMap

Note. Dropped assays marked by asterisk (*), AA=African American, CEPH=Caucasian.

Table 2

Transcobalamin II-Receptor Gene SNPs

Reference Sequence #	Gene Position	Target Population	Identified From
rs3760680	3' near gene	AA	HapMap
rs250510	exon 5 (3' UTR)	AA	HapMap
rs36263	3' near gene	AA	HapMap
rs2232772	5' UTR	AA	HapMap
rs2232779	intron 1	AA	HapMap
rs2232788	exon 5 (3' UTR)	AA	HapMap
rs2232768	5' near gene	CEPH	HapMap
rs2927707	intron 1	both	HapMap
rs2227288	intron 4	both	HapMap
rs2232775	exon 1	AA	dbSNP
rs2232787	exon 5	no data	dbSNP
rs2336573	exon 4	both	dbSNP
rs2232786	exon 5	AA	dbSNP
rs173665	3' near gene	both	Brody lab hit
rs12461677*	intron 1	AA	HapMap
rs2232766*	near gene	AA	HapMap
rs2227266*	5' near gene	AA	HapMap
rs250511*	intron 4	both	HapMap
rs2232765*	near gene	both	HapMap
rs2232774*	exon 1	no data	dbSNP
rs5719548*	exon 2	no data	dbSNP

Note. Dropped assays marked by asterisk (*), AA=African American, CEPH=Caucasian.

Table 3

Dalton Differences in iPLEX Dideoxynucleotide Mass-Modified Terminators

Terminator	A	C	G	T
A	0.0	-24.0	16.0	55.9
C	24.0	0.0	40.0	79.9
G	-16.0	-40.0	0.0	39.9
T	-55.9	-79.9	-39.9	0.0

Note. Chemically modified dNTPs for iPLEX extension reaction (Oeth et al., 2006).

CHAPTER 4

ASSOCIATION OF TRANSCOBALAMIN II AND TRANSCOBALAMIN II-RECEPTOR GENETIC VARIATION WITH COBALAMIN METABOLITE LEVELS IN ELDERLY WOMEN

Abstract

Functional cobalamin deficiency, a subtle progressive clinical disorder, affects 6-23% of individuals >60 years old. This deficiency arises due to age-related decreases in the ability of the GI tract to extract and absorb cobalamin. Better understanding of biologic and genetic factors that contribute to the high prevalence of cobalamin deficiency observed in older adults would allow for targeted care and improved functional quality of life in advanced age. This candidate gene association study examined the association of genetic variation in the transcobalamin II and transcobalamin II-receptor genes on the concentration of cobalamin, homocysteine, and methylmalonic acid in the blood of 789 participants of the Women's Health and Aging Studies. A total of four SNPs were identified for additional hypothesis testing. In the transcobalamin II gene, a missense coding SNP (rs9621049) was associated with mean homocysteine concentrations, a missense coding SNP (rs35838082) was associated with mean homocysteine and methylmalonic acid levels, and two SNPs in intron 7 were associated with serum cobalamin (rs4820888) and homocysteine (rs4820887) concentrations. A

final SNP in the transcobalamin II-receptor gene, a missense coding SNP (rs2336573), was associated with mean serum cobalamin concentrations.

Introduction

Cobalamin, commonly known as vitamin B12, is a critical nutrient. Humans are dependent upon adequate dietary intake or supplementation of cobalamin for survival, since it is only synthesized by microorganisms. In the human body, cobalamin is used as enzymatic cofactors for only two biochemical reactions. The first is in the cytoplasm, where methylcobalamin serves as a cofactor for methionine synthase. This enzyme uses folate to convert homocysteine to methionine, which is necessary for one-carbon metabolism that fuels essential cell processes, including methylation and DNA synthesis. The second is in the mitochondrion where 5'-deoxyadenosylcobalamin catalyzes conversion of methylmalonyl coenzyme-A to succinyl coenzyme-A, which is essential for odd-chain fatty acid and amino acid catabolism (Beck, 2001). In the event of decreased cobalamin availability, the metabolites homocysteine and methylmalonyl coenzyme-A (methylmalonic acid) accumulate. Additionally, deficit of one-carbon intermediates produces a range of clinical disorders, including megaloblastic anemia and neurologic impairment (Lindenbaum et al., 1988). Because clinical symptoms can exist and progress in individuals with normal serum cobalamin concentrations, homocysteine and methylmalonic acid are commonly used as deficiency indicators in the assessment of an individual's cobalamin nutrition status (Green, 2008).

Prevalence of cobalamin deficiency in elderly adults is reported to range from 6% to 23%, and depending on diagnostic criteria used—even as high as 40.5% (Allen, 2009;

Baik & Russell, 1999; Johnson et al., 2003; Lindenbaum, Rosenberg, Wilson, Stabler, & Allen, 1994; Pennypacker et al., 1992). Elder adults are especially susceptible to cobalamin deficiency due to a combination of factors, including decreased dietary intake, age-related decline in gastric absorption, use of interfering medications, and presence of comorbid conditions (Baik & Russell, 1999; Wolters, Strohle, & Hahn, 2004). Unrecognized cobalamin deficiency in older adults may result in an exaggerated disability trajectory involving higher frequency of hospital admissions, lengthier and more severe hospitalizations, or greater degrees of chronic disablement significantly affecting mobility and quality of life (Bartali et al., 2006).

To assist in the development of strategies to prevent or delay the disablement process, better understanding of the biologic factors contributing to altered cobalamin status is needed. For example, low cobalamin concentrations inside cells can also result from disturbances in transport and cellular uptake processes. Following intestinal absorption, cobalamin is bound to the carrier molecule transcobalamin II in the plasma for nutrient delivery to target cells. From the circulation, the cobalamin-transcobalamin II complex is endocytosed into lysosomal compartments via the transcobalamin II-receptor (Quadros, Nakayama, & Sequeira, 2009; Seetharam & Li, 2000). Structural variations in the transcobalamin II carrier molecule and its receptor can affect binding efficiency characteristics, resulting in decreased cellular availability for essential metabolic reactions and influencing susceptibility to deficiency (Miller, Ramos, Garrod, Flynn, & Green, 2002; Quadros et al., 2010). To explore these factors more broadly, this study examined if transcobalamin II and transcobalamin II-receptor genetic variation was associated with biochemical cobalamin parameters in elderly participants of the Women's

Health and Aging Study 1 and 2 cohorts. Parameters of interest included serum cobalamin, homocysteine, and methylmalonic acid concentrations.

Methods

Study Design and Population

This project is a candidate gene association study using genetic material from research subjects who participated in the Women's Health and Aging Study (WHAS) 1 and 2 cohorts. WHAS 1 (1992-1995) and WHAS 2 (1994-1996) were prospective, observational research initiatives that examined the trajectories and sources of physical disability in community-dwelling elderly women in 12 Baltimore, MD area zip codes. WHAS 1 was designed to sample the one-third most disabled women in the Baltimore community, and as a complementary companion study, WHAS 2 was designed to sample the two-thirds least disabled women. Numerous investigations have pooled WHAS 1 and 2 subject data for a strengthened approach representative of the community-dwelling elderly (Bandeen-Roche et al., 2006; Chaves, Ashar, Guralnik, & Fried, 2002; Chaves et al., 2005; Leng, Xue, Tian, Walston, & Fried, 2007; Semba, Garrett, Johnson, Guralnik, & Fried, 2000; Semba et al., 2005; Walston et al., 2005). Although summarized briefly in this report, detailed descriptions of the sampling, screening, recruitment, and data collection procedures employed in WHAS 1 and 2 are fully described elsewhere (Fried, Bandeen-Roche, Chaves, & Johnson, 2000; J. Guralnik, Fried, Simonsick, Kasper, & Lafferty, 1995). The data collection protocols utilized in WHAS 1 and 2 were approved by the Johns Hopkins Medical Institutions institutional review board. The genotyping

portion of this research was approved by the Office of Human Subjects Research at the National Institutes of Health and the University of Utah's institutional review board.

WHAS 1 (1992-1995)

From 32,538 Health Care Financing Administration (HCFA) Medicare enrollee records in Baltimore, MD, 6,521 age-stratified elderly women (65-74, 75-84, and >85) were randomly selected for screening and possible enrollment (Ferrucci et al., 1995). A total of 3,841 individuals were evaluated for physical disability across four domains: 1) mobility and exercise tolerance, 2) upper extremity function, 3) high function tasks (activities of daily living), and 4) basic self care. Women who reported difficulty with two, three, or four physical disability domains and had a Mini-Mental State Examination (MMSE) score of 18 or higher were invited to participate in WHAS 1 (n = 1,409), (Fried, Kasper, Guralnik, & Simonsick, 1995; Guralnik, Fried, Simonsick, Bandeen-Roche, & Kasper, 1995). Of the 1,409 individuals eligible, 1,002 consented to participate in WHAS 1. Trained interviewers administered several health questionnaires and conducted physical performance measures and a standardized physical examination in homes of consented subjects. Part of a secondary consent, 762 of the 1,002 total WHAS 1 participants agreed to phlebotomy and provided blood samples for research. DNA that was still available for genetic analysis used in this study was for 536 WHAS 1 research participants.

WHAS 2 (1994-1996)

From the same 32,538 Health Care Financing Administration (HCFA) Medicare enrollee records in Baltimore, MD, another sample of elderly women (ages 70-79) was randomly selected for screening and possible enrollment. A total of 1,630 individuals were evaluated for physical disability across the same four functional disability domains. Women who reported difficulty with zero or one disability domains and had a Mini-Mental State Examination (MMSE) score of 24 or higher were invited to participate in WHAS 2 (n = 880). In WHAS 2, some subjects were screened by telephone, and an abbreviated MMSE was used; individuals who correctly answered at least 80% of questions were eligible for study inclusion. Of the 880 eligible individuals, 436 consented to WHAS 2 participation. The consented subjects participated in a clinic examination visit where trained study personnel administered the same standardized questionnaires, physical performance measures, and standardized physical examination as WHAS 1 subjects. Also part of a secondary consent, 405 of the 436 total WHAS 2 participants agreed to phlebotomy and provided blood samples. The DNA that was still available for genetic analysis used in this study was for 253 WHAS 2 research participants.

Blood Assay Measurement

Nonfasting blood samples from WHAS 1 and 2 subjects were processed, aliquoted, and frozen (-80 degrees Celsius) at the Core Genetics Laboratory in the Johns Hopkins University School of Medicine.

Serum Cobalamin, Folate, Creatinine

For determination of serum cobalamin, folate, and creatinine concentrations, frozen aliquots were sent from Johns Hopkins University School of Medicine to Quest Diagnostics (formerly Corning Clinical Laboratories and MetPath) in Teterboro, NJ. At Quest Diagnostics, serum cobalamin and folate concentrations were determined using competitive intrinsic factor protein-binding and folate-binding protein assays, according to the methods of Ciba-Corning Diagnostics Corporation in Medfield, MA. The normal range of serum cobalamin and folate was reported at 148-664 pmol/L and 6.8-36.0 nmol/L. Also at Quest Diagnostics, serum creatinine concentrations were analyzed via the conventional Jaffe method with normal ranges reported at 0.6-1.1 mg/dL for females. Cobalamin, folate, and creatinine laboratory values were available for the WHAS participants, including n = 772 cobalamin concentrations, n = 780 folate concentrations, and n = 769 creatinine concentrations.

Homocysteine and Methylmalonic Acid

A subset of WHAS 1 and 2 sample aliquots were shipped on dry ice from the Core Genetics Laboratory at John's Hopkins University School of Medicine to the University of Colorado Health Sciences Center for measurement. Homocysteine and methylmalonic acid levels were obtained using stable-isotope dilution and capillary gas chromatography-mass spectrometry with selected ion monitoring (Penninx et al., 2000; Stabler et al., 1999). Normal homocysteine and methylmalonic acid concentrations are 5.4-13.9 μmol/L and 73-271 nmol/L per previous WHAS 1 and 2 research reports (Penninx et al., 2000; Stabler et al., 1999). Homocysteine and methylmalonic acid

laboratory assay values were available for the WHAS participants, including *n* = 758 homocysteine concentrations and *n* = 754 methylmalonic acid concentrations.

Renal Function Assessment

Previous studies of elderly individuals demonstrate that modest elevations in homocysteine and methylmalonic acid can occur from renal insufficiency (Lindenbaum et al., 1994; Pennypacker et al., 1992; Stabler, Lindenbaum, & Allen, 1996). However, in older adults, singular use of serum creatinine as a kidney function assessment is recognized to significantly underascertain presence of impaired renal status (Giannelli et al., 2007). Thus, this study used serum creatinine to estimate creatinine clearance for WHAS 1 and 2 subjects through the Cockcroft-Gault formula: creatinine clearance (mL/s) = weight (kg) x [140 – age (years)] / [72 x serum creatinine (mg/dL) x 0.85]**,** (Cockcroft & Gault, 1976)**.** According to guidelines established by the National Kidney Foundation's Kidney Disease Outcome Quality Initiative (NKF-KDOQI), a calculated estimated value of over 90 mL/min indicates normal glomerular filtration (Stage 1), 60-89 mL/min indicates mild renal impairment (Stage 2), 30-59 mL/min indicates moderate renal impairment (Stage 3), 15-29 mL/min indicates severe kidney impairment (Stage 4), and under 15 mL/min indicates presence of kidney failure requiring dialysis (Stage 5) (National Kidney Foundation, 2002). Estimated creatinine clearance values were available for *n* = 736 WHAS participants.

Genetic Variant Measurement

SNP Selection

Single Nucleotide Polymorphisms (SNPs) were selected for the study's candidate genes using publicly available databases HapMap (Phase 1 and 2 full dataset), dbSNP (build 125), and NCBI (build 35). For both Caucasian and African American ancestral groups, Haploview (4.0) Tagger was used to tag SNPs in the candidate genes and including 10kb flanks on both ends of the genes. Because both candidate genes were small, at 19 kilobases and 6 kilobases, and there was high likelihood of dropped assays due to working with whole genome amplified material, the r^2 statistic threshold was set at 0.9 and minor allele frequency set at 0.05. Additional selected candidates included variants within exons, promoters, conserved sequences across species, and those reported in literature associated with clinical characteristics and biochemical parameters of interest. A total of 51 SNPs were selected—29 SNPs in the transcobalamin II gene and 22 SNPs in the transcobalamin II-receptor gene.

Whole Genome Amplification

At the Core Genetics Laboratory in Johns Hopkins School of Medicine, DNA from WHAS 1 and 2 subjects was extracted from whole blood using the Puregene DNA Purification Kit from GentraSystems, Inc. DNA samples for WHAS subjects were plated at Johns Hopkins University and 50 ng was sent on dry ice to the National Human Genome Research Institute for genotyping. Because of limited starting quantity of genetic material, 10 ng of each participant's DNA was whole genome amplified using Qiagen's REPLI-g Midi Kit (Product # 150045_100 Rx). This kit uses the multiple displacement

amplification method via a highly processive Φ29 DNA polymerase. To correct for possible allele dropout, two independent reactions of whole genome amplification for each WHAS subject were performed and pooled for use in high-throughput genotyping. Hardy-Weinberg calculations in pilot genotyping analyses for whole genome amplified material matched that of nonamplified WHAS genomic material. All 789 WHAS participant genomic samples were successfully amplified.

SNP Genotyping

Genotyping of the WHAS 1 and 2 whole genome amplified samples was performed at the National Human Genome Research Institute using the Sequenom MassArray iPLEX platform (San Diego, CA). The genotyping reaction is characterized by two phases: 1) a locus-specific PCR reaction to produce a 100-base pair region that contains the SNP of interest, and 2) a locus-specific primer extension reaction that produces a mass-modified product for each allele of the SNP. Following amplification and extension reactions, the mass-modified products are resolved using MALDI-TOF mass spectroscopy. Genotype data were transferred to a local database for analysis.

Statistical Analysis

Descriptive and inferential statistics were analyzed using the SPSS program (version 12.0). Summary statistics, frequency distributions, and independent *t*-tests were evaluated for sociodemographic and health characteristics, and for the study's three outcome variables, serum cobalamin, homocysteine, and methylmalonic acid. Missing data were assessed for nonrandomness and WHAS subjects missing SNP genotypes,

study outcome parameters, or covariate values were removed from analysis. Assessment of the outcome and covariate variables for linearity, homogeneity of variance, homogeneity of regression slopes, and measurement reliability indicated no violations of statistical assumptions. For the study's three biochemical outcome parameters, extreme outliers that were zero or greater than 3 standard deviations away from the mean were removed from analysis (serum cobalamin $n = 6$, homocysteine $n = 6$, methylmalonic acid $n = 10$).

Hardy-Weinberg statistics, allele, and genotype frequencies were calculated for transcobalamin II and transcobalamin II-receptor SNPs in African American and Caucasian WHAS subjects. To test for association between cobalamin concentration and the two independent variables, race and SNP, while also accounting for variability produced by altered renal function, a two-way analysis of covariance was conducted. To ascertain presence of association between mean homocysteine concentration and the independent variables while also accounting for variability produced from known physiologic mediators, a two-way analysis of covariance was conducted on the fixed factors, race and SNP genotype, with folate, creatinine clearance, and serum cobalamin designated as covariates. Similarly, a two-way analysis of covariance was performed to ascertain presence of association between mean methylmalonic acid and the fixed factors, race and SNP genotype, with creatinine clearance and serum cobalamin as covariates. Significant ($p<0.05$) interaction and SNP main effect F tests were explored further using the Tukey and Least Significant Difference procedures, and by assessing interaction graphs. The Bonferroni adjustment was used to correct for alpha inflation arising from multiple testing.

Results

Demographics

Demographic and other baseline health characteristics of the 789 study participants are shown in Table 4. The 591 Caucasian participants in this study represented 75% of the study sample, were slightly older than the 198 African Americans [$t(787)= -2.90$, $p=0.004$], and had more years of education [$t(787)= -7.27$, $p<0.01$]. There were 536 WHAS 1 subjects, who comprised 68% of the study sample. The remaining WHAS 2 subjects (32%) were significantly more educated than their WHAS 1 counterparts [$t(787) = -10.9$, $p<0.01$]. The study population was 71% Caucasian and 29% African American for WHAS 1 participants, and 83% Caucasian and 17% African American for WHAS 2 participants.

The number of chronic diseases did not differ substantially between the African Americans as compared to Caucasians; however, WHAS 1 subjects had more chronic diseases than WHAS 2 subjects due to that cohort's sampling focus on the more disabled portion of the Baltimore population [$t(787)= 9.50$, $p<0.01$]. African American elderly women were more likely to have elevated body mass index values [$t(741)= 5.13$, $p<0.01$], as were WHAS 1 cohort participants [$t(741)= 3.62$, $p<0.01$].

Biochemical Characteristics

Descriptive summary statistics and clinical biochemical profile parameters for the WHAS 1 and 2 participants are presented in Tables 5 and 6. The mean serum cobalamin for African American subjects was 563 pmol/L compared to 461 pmol/L for Caucasian elderly women [$t(770)= 5.24$, $p<0.01$]. Clinically low serum cobalamin was more

prevalent in Caucasian subjects [$t(775)= 2.83$, $p=0.005$] with 3% of Caucasians demonstrating low serum cobalamin concentrations compared to 1% in African Americans. Although mean homocysteine concentrations were similar between African American and Caucasian subjects in both cohort groups, the mean values for WHAS 2 subjects were much lower than WHAS 1 subjects [$t(756)= 4.90$, $p<0.01$]. Caucasian subjects had higher mean methylmalonic acid concentrations at 254 nmol/L compared to 209 nmol/L for African Americans [$t(752)= -3.78$, $p<0.01$], and WHAS 1 Caucasian women had the highest mean methylmalonic acid concentration at 268 nmol/L. Moderate clinical elevations in methylmalonic acid levels [$t(762)= 3.70$, $p<0.01$] were more prevalent in Caucasian elderly women, where 29% of Caucasians experienced moderate to high serum methylmalonic acid concentrations compared to 15.7% in African Americans.

There were no differences between African American and Caucasian women or between participants for WHAS 1 and 2 cohorts in overall mean folate concentration. However, African American women were more likely to have clinically low serum folate, with 44.4% demonstrating concentrations less than 6.8 nmol/L compared to 28.3% for Caucasians [$t(778)= 4.32$, $p=0.00$]. Although the mean serum creatinine concentration for WHAS subjects was in the normal laboratory reference range for renal function assessment, mean values of creatinine clearance estimation for both groups indicated presence of moderately impaired renal status. Estimated glomerular filtration rates of 52 mL/min for Caucasians and 53 mL/min for African Americans corresponded to Stage 3 NKF-K/DOQI criteria. WHAS 2 subjects had better renal function per Cockcroft-Gault creatinine clearance estimation [$t(734)= -2.61$, $p=0.009$]. Both groups were comparable

in their distribution of renal function, and the majority of WHAS subjects experienced mild to moderate renal insufficiency (NKF-KDOQI Stage 2 and 3).

Genetic Data

Of the total 51 SNPs selected for genotyping in the transcobalamin II and transcobalamin II-receptor genes, 29 SNPs were successfully genotyped, resulting in incomplete tagging coverage for both genes. SNPs were dropped if they were monomorphic ($n = 6$) or if they did not meet genotyping quality control thresholds ($n = 16$). Genotype and allele frequencies for the 29 successfully genotyped SNPs are presented in Table 7. For two-way between-groups analysis of covariance statistical association tests, WHAS subjects were divided into one of the three genetic categories (AA, AB, BB) according to their genotypes and stratified by race. Bonferroni adjustment for multiple testing of the 29 SNPs across the three biochemical outcomes in this study yields a corrected alpha significance threshold of 0.0006 (0.05/87). Table 8 provides a summary of the *F* values obtained in this research, including interaction effect, SNP main effects, and race main effects. No result reached the stringent level of significance according to the Bonferroni-adjusted threshold. However, several results were significant at the p=0.01 level and p=0.05 level. These findings may be true associations that fail to survive correction. Due to the exploratory nature of this research and the value of hypothesis generating information, several of these are highlighted below.

Cobalamin

For the two-way analysis of covariance evaluating mean cobalamin concentrations across race and genetic factors adjusted for estimated creatinine clearance, there were two significant SNPs—rs4820888 in the transcobalamin II gene and rs2336573 in the transcobalamin II-receptor gene.

The rs4820888 G|A SNP is located in intron 7 of the transcobalamin II gene, and although there was no significant interaction (race) effect, there was a statistically significant main (genetic) effect [$F(2, 692)= 3.17$, $p=0.04$, partial eta squared = 0.01]. Pairwise comparison of main effects across the genotype groups indicated that mean cobalamin concentrations of GA heterozygotes (M=474 pmol/L) were lower than the AA homozygotes (M=507 pmol/L) at $p=0.018$. Differences between AA homozygotes (M=507 pmol/L) and GG homozygotes (M=470 pmol/L) were not statistically significant at $p=0.06$, as was the difference between GA heterozygotes (M=474 pmol/L) and GG homozygotes (M=470 pmol/L) at $p=0.95$. A significant main race effect was found [$F(1, 692)= 22.37$, $p=<0.01$, partial eta squared=0.032] for African American subjects' higher cobalamin concentrations.

The rs2336573 C|T SNP in the transcobalamin II-receptor gene is a missense polymorphism located in exon 4, resulting in an arginine to glycine amino acid change at codon position 220. The interaction effect between race and rs2336573 genotype category [$F(2, 684)= 0.27$, $p=0.76$] did not reach statistical significance. However, there was a statistically significant main genetic effect with a small effect size [$F(2, 684)= 3.25$, $p=0.04$, partial eta squared 0.01]. Pairwise comparison tests across the main genetic groups indicated that mean serum cobalamin concentrations in the TC group (M=573

pmol/L) were higher than the CC group (*M*=466 pmol/L) at *p*=0.02. Differences between CC homozygotes (*M*=466 pmol/L) and TT (*M*=576 pmol/L) were insignificant at *p*=0.16, as were differences between TT and TC groups significant at *p*=0.96. A significant main race effect was found [*F*(1, 684]= 6.78, *p*=0.009, partial eta squared=0.01] for African Americans' higher cobalamin concentrations.

Homocysteine

The two-way analysis of covariance was performed to evaluate mean homocysteine concentrations on the fixed factors, race and SNP genotype, after designating a singular mean value for WHAS subjects' creatinine clearance, serum folate, and serum cobalamin concentrations. After creatinine clearance, serum folate, and serum cobalamin adjustment, there was no interaction effect [*F*(2, 673)= 2.66, *p*=0.071], and a significant main effect for the transcobalamin II SNP rs9621049 [*F*(2, 673)= 4.97, *p*=0.007, partial eta squared=0.015]. The rs9621049 SNP is a C|T missense polymorphism in exon 7 that results in an amino acid change of a phenylalanine to serine at codon position 348. Simple comparison testing found the mean homocysteine concentration for the TT group (*M*=12.5 μmol/L) was significantly different from the CT group (*M*=10.6 μmol/L) at *p*=0.002. The difference between the TT group (*M*=12.5 μmol/L) was also significantly different from the CC group (*M*=10.5 μmol/L) at *p*=0.003. A significant main race effect was found [*F*(1, 673)= 6.91, *p*=0.009, partial eta squared=0.01] for African Americans' higher homocysteine concentrations.

A second significant result from the analysis of covariance on mean homocysteine concentration included an interaction effect [*F*(2, 666)= 4.58, *p*=0.01, partial eta

squared=0.014] for the transcobalamin II A|G SNP rs4820887 in intron 7 (Figure 3). Simple comparison testing on the interaction effect found that African American subjects with an AA genotype (*M*=17.0 µmol/L) had significantly different mean homocysteine concentrations from both the GA group (*M*=9.1 µmol/L) at *p*=0.001, and the GG group (*M*=11.2 µmol/L) at *p*=0.002. For this SNP, there was also a significant main effect [*F*(2, 666)= 4.73, *p*=0.009, partial eta squared =0.014]. Simple comparison testing on the SNP main effect found that mean homocysteine was different between the AA (*M*=12.9 µmol/L) and GA (*M*=10.5 µmol/L) genotype groups at *p*=0.002, and between the AA and GG (*M*=10.6 µmol/L) genotype groups at *p*=0.003. However, these relationships are not certain nor are the strengths of the significant associations assured; the A allele frequency of 0.09 in both the African Americans and Caucasian WHAS subject groups resulted in a small number of AA homozygotes (*n*=3 for African Americans and *n*=9 for Caucasians) from which wider variability may spuriously be driving significance. For this SNP, there was also a significant main race effect [*F*(1, 666)= 7.94, *p*=0.005, partial eta squared=0.012] for African Americans' higher homocysteine concentrations.

The last SNP found to be associated with mean homocysteine concentration was SNP rs35838082, a C|T missense polymorphism in exon 5 of the transcobalamin II gene resulting in an amino acid change of tryptophan to arginine at codon position 215. There was no interaction effect [*F*(2, 676)= 0.001, *p*=0.98] and a significant SNP main effect [*F*(2, 676)= 3.36, *p*=0.035, partial eta squared=0.01]. Simple comparison testing ascertained higher mean homocysteine concentrations in the CC homozygote group (*M*=10.6 µmol/L) as compared to the TT homozygote group (*M*=8.5 µmol/L) at *p*=0.019. There was no race main effect [*F*(1, 676)= 1.50, *p*=0.22].

Methylmalonic Acid

The two-way analysis of covariance evaluated mean methylmalonic acid concentrations on the fixed factors, race and SNP genotype, after designating a singular mean value for WHAS subjects' creatinine clearance and serum cobalamin. The only significant SNP in this analysis for the outcome parameter methylmalonic acid was rs35838082—a C|T missense polymorphism in exon 5 of the transcobalamin II gene that was also associated with mean homocysteine concentrations. Although a highly significant interaction effect was found [$F(2, 671)= 7.58$, $p=0.004$, partial eta squared=0.011], the low frequency of the T allele in WHAS Caucasians resulted in absent TT and low CT cell sample sizes (TT $n=0$ and CT $n=6$); these small sample numbers increase the risk that the observed significance is spurious. For this SNP, there was also a significant main race effect [$F(1, 671)= 12.62$, $p<0.01$, partial eta squared=0.019] for Caucasians' higher methylmalonic acid concentrations.

Discussion

Reported cobalamin deficiency prevalence indicates evidence of widespread decreases in cobalamin nutritional status for older adults. Understanding which older adults are susceptible to functional cobalamin deficiency could improve healthcare mechanisms and strategies aimed at decreasing onset or speed of disability trajectories. To better understand genetic factors contributing to decreased cobalamin nutrition status in the elderly, this study examined association of variants in the transcobalamin II and transcobalamin II-receptor genes with metabolic parameters in Women's Health and Aging Study 1 and 2 participants. Although no genetic variants reached the stringent

Bonferroni-adjusted significance threshold of p=0.0006, this exploratory analysis did find six variants that were associated with various biochemical parameters ranging from the p=0.004 to p=0.04 level of significance. Concordant with HapMap reference population statistics, two of the six significant variants (rs35838083 and rs4820887) had low allele frequencies that resulted in decreased cell sizes for which to make fully adequate comparisons. The four remaining SNPs are discussed further.

The primary finding from this study was the significant main effect indicating association of a F348S missense SNP in exon 7 (rs9621049) in the transcobalamin II gene with mean homocysteine concentration. After adjusting for estimated creatinine clearance, serum folate, and serum cobalamin, TT homozygotes experienced a 2.0 μmol/L higher mean homocysteine concentration than CC homozygotes, and the difference between mean homocysteine of the TT and CT groups was similar at 1.9 μmol/L. Conformational changes in the transcobalamin II carrier protein as a result of the phenylalanine-to-serine amino acid residue change could result in variable binding efficacy of cobalamin in serum, preventing effective transport to cells, and affecting conversion of homocysteine to methionine.

Elevated homocysteine has been previously reported as a risk factor in the development of cardiovascular disease via a hypothesized connection to atherosclerosis through hypomethylation, generation of reactive oxygen species, and vascular endothelial dysfunction (Di Minno, Tremoli, Coppola, Di Minno, & Lupoli; Lawrence de Koning, Werstuck, Zhou, & Austin, 2003; Zhou & Austin, 2009). However, a recent randomized evaluation of folic acid and vitamin B12 supplementation versus placebo on blood homocysteine concentration did not demonstrate benefit in preventing myocardial

infarction outcomes (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine Collaborative Group, 2010). Presently, it is not known if elevated homocysteine is mediated by interactions between genetic factors such as rs9621049 SNP genotype and an individual's cobalamin nutrition status.

The rs9621049 SNP finding obtained in this study is not consistent with a previous study of transcobalamin II genetic variation on homocysteine concentrations (Lievers et al., 2002). The evaluation by Lievers et al. of transcobalamin II missense SNPs found rs1801198 (P259R) to be significantly associated with elevated homocysteine, whereas rs9621049 was not significant. For the WHAS participants, there was no rs1801198 interaction effect [$F(2, 676)=1.23$, $p=0.29$] or SNP main effect [$F(2, 676)=0.11$, $p=0.90$]. Differences in results obtained may be due to the dissimilarity in patient sampling and demographics as Lievers et al. examined the influence of altered homocysteine levels in younger patients at risk of cardiac disease.

A second key finding from this study was the significant association of two SNPs in intron 7 of the transcobalamin gene with mean serum cobalamin (rs4820888) and homocysteine (rs4820887) concentrations. After adjusting for estimated creatinine clearance, GG rs4820888 homozygotes experienced a 37 pmol/L lower mean cobalamin concentration than AA rs4820888 homozygotes, and the difference between mean serum cobalamin of the GG and GA groups was similar, at 33 pmol/L. After adjusting for estimated creatinine clearance, serum folate, and serum cobalamin, rs4820887's differences in homocysteine concentration mirrored that of rs9621049, where AA homozygotes ($M=12.9$ μmol/L) demonstrated a 2.4 μmol/L higher mean homocysteine concentration compared to AG heterozygotes ($M=10.5$ μmol/L) and GG homozygotes

(*M*=10.5 μmol/L). Chromosomal distance between the rs9621049 in exon 7 and rs4820887 in intron 7 is 3,495 base pairs and the relationship between these two SNPs is not known.

Neither rs4820888, rs4820887, or the chromosomal region (haplotype block) on which they lie, are currently indicated in literature as being associated with biochemical parameters of cobalamin deficiency. Because the genetic distance between both intron 7 SNPs is only 408 base pairs, it is not known if one marker is acting as a surrogate for the other. As this was a candidate gene association study using haplotype tagging to select SNPs for genotyping, it is possible that both SNPs are significant because the real signal is nearby in close linkage disequilibrium to rs4820888 and rs4820887, but was not genotyped in this study.

The final SNP to be highlighted from this study is rs2336573 (A220G), the missense coding SNP in exon 4 of the transcobalamin II-receptor that was associated with mean serum cobalamin concentrations (Figure 4). Regardless of an individual's race, individuals with a CC genotype had significantly lower mean serum cobalamin, demonstrating a 110 pmol/L difference from subjects with a TT genotype. This finding may possibly indicate that the C allele of rs2336573 exerts a functional difference resulting in decreased cobalamin uptake by a cell, reducing intracellular nutrient availability. Because rs2336573 is located in an exon, it is biologically plausible that it could result in functional differences in cobalamin uptake kinetics across a cell's plasma membrane; however, this hypothesis has not been tested in an experimental system. As the transcobalamin II-receptor gene and protein were only just recently identified and purified, little is known about the clinical effects of genetic variation or the relevance it

may hold for patients in cohorts such as WHAS (Quadros et al., 2009). Nonetheless, the rs2336573 results obtained in this study identify an attractive candidate polymorphism for further research.

The work outlined in this study also mirrors recently published WHAS reports on the evaluation of methylmalonic acid with SNPs in the transcobalamin II gene (Matteini, Walston, Bandeen-Roche, Arking, Allen, Fried et al., 2008; Matteini, Walston, Bandeen-Roche, Arking, Allen, Fried et al., 2010). In their candidate gene analysis reports on 326 Caucasian WHAS 1 and 2 subjects, no significant association between methylmalonic acid levels and transcobalamin II SNPs were found.

In addition to genetic association data, this study indicated presence of variability in the mean outcome measures that was not due to genetics. Of the 29 SNPs, there were 18 significant race main effects for the serum cobalamin outcome, and 8 significant race main effects for the homocysteine and methylmalonic acid outcomes. Factors specific to the social contexts of the WHAS participants such as nutrition, socio-economics, quality of life, and mental well-being, may explain a small portion of the variability observed in this study. Additionally, some of these observed findings may be coming from other factors that were not controlled for in this research, including use of medications and presence of comorbid chronic diseases. Although participants' ages were controlled for through the sampling design (WHAS 1 at 65-74, 75-84, >85 and WHAS 2 at 70-74, 75-79), the mean age of WHAS 1 participants at 77.4 years was higher than that of WHAS 2 participants at 73.9 years. Some of the observed race main effects may also be arising from the age differences between the cohorts. Ultimately very little data exists on gene-environment interactions involving social factors and their effects on the transcobalamin

II and the transcobalamin II-receptor genes for which to confirm these findings in the broader literature base.

Strengths of this research include the use of a well-characterized and examined elder adult cohort to ascertain effects of genetic variation on metabolic parameters of cobalamin deficiency. In that there was limited quantity of genomic starting material, this study also demonstrates that use of whole genome amplification to generate genetic data is a sound technique to augment previous clinical and epidemiological research initiatives. Also notable is that this investigation includes examination of genetic variation from a recently characterized gene and protein, the transcobalamin II-receptor.

Limitations of this study are primarily notable for incomplete tagging of the transcobalamin II and transcobalamin II-receptor genes. This stemmed from implementation of stringent quality control criteria and other genotyping challenges associated with using whole genome amplified material of DNA that ranged from 16-18 years old. Another limitation of this research is that serum holotranscobalamin measurements, the amount of cobalamin bound to the transcobalamin II carrier molecule, were not available for WHAS 1 and 2 subjects. Using holotranscobalamin as an outcome variable would have permitted more direct assessment of the relationship between biologically active cobalamin in serum and transcobalamin II-receptor uptake.

Another limitation to this work is that environmental factors known to affect susceptibility to cobalamin deficiency in older adults (such as medications and co-morbidities), were not included in these analyses. Because many genotype frequencies were low in African American and Caucasian subgroups and several covariates were already used, further loss of power from additional covariate inclusion would have

removed ability to detect genetic effects. Had environmental factors been included there may have been less variance in the study's outcomes attributable to genetic influence.

Despite the limitations of this study, the findings obtained suggest that there are additional biologic factors that contribute to an elderly adult's susceptibility to decreased cobalamin nutritional status. Further research in more highly powered and diverse patient cohorts is needed to replicate the transcobalamin II rs9621049 (F348S), rs4820888, rs4820887 findings, and the transcobalamin II-receptor rs2336573 (A220G) finding reported here. The information produced from this report may contribute to identification of valuable cobalamin-related targets for future functional studies.

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Table 4

Selected Sociodemographic and Health Characteristics for WHAS Subjects

Characteristic	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*N*
Age (Years)	WHAS 1**		WHAS 2		Total	
African American	75.7 (7.0)	155	73.0 (2.5)	43	75.1 (6.4)	198*
Caucasian	78.1 (7.8)	381	74.1 (2.7)	210	76.7 (6.8)	591
Education (Years completed)	WHAS 1**		WHAS 2		Total	
African American	8.6 (3.2)	155	11.0 (3.8)	43	9.1 (3.5)	198*
Caucasian	10.3 (3.7)	381	13.2 (3.2)	210	11.3 (3.8)	591
Number of Chronic Diseases[a]	WHAS 1**		WHAS 2		Total	
African American	1.4 (1.6)	155	0.6 (1.1)	43	1.3 (1.5)	198
Caucasian	1.4 (1.4)	381	0.4 (0.9)	210	1.0 (1.3)	591
Body Mass Index (kg/m^2)	WHAS 1**		WHAS 2		Total	
African American	31.5 (16.1)	138	29.3 (6.3)	43	31.0 (14.3)	181*
Caucasian	28.0 (6.1)	352	26.0 (0.9)	210	27.2 (5.7)	562

Note. Summary statistics including mean values, standard deviations (*SD*), and sample size. Significant differences from independent *t*-tests between WHAS 1 and WHAS 2 subjects (**$p<0.01$) and African American and Caucasian subjects (*$p<0.01$) are indicated.

[a]Self-reported diseases include angina, myocardial infarction, coronary artery disease, congestive heart failure, peripheral artery disease, stroke, diabetes mellitus, and cancer.

Table 5

Serum Metabolite and Chemistry Concentration Descriptive Summaries

Variable	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*N*
Cobalamin (pmol/L)	WHAS 1		WHAS 2		Total	
African American	557 (263)	149	579 (267)	43	563 (264)	192*
Caucasian	456 (223)	372	468 (221)	208	461 (222)	580
Homocysteine (μmol/L)	WHAS 1**		WHAS 2		Total	
African American	11.3 (4.3)	149	9.7 (3.2)	39	11.0 (4.1)	188
Caucasian	11.1 (4.3)	369	9.7 (2.6)	201	10.6 (3.8)	570
Methylmalonic acid (nmol/L)	WHAS 1**		WHAS 2		Total	
African American	214 (135)	150	190 (122)	39	209 (133)	189*
Caucasian	268 (135)	364	227 (149)	201	253 (141)	565
Folate (nmol/L)	WHAS 1		WHAS 2		Total	
African American	11.2 (23.0)	152	9.8 (8.3)	43	10.9 (20.1)	195
Caucasian	12.6 (10.5)	375	12.2 (8.1)	210	12.5 (9.7)	585
Creatinine	WHAS 1		WHAS 2		Total	
African American	1.2 (0.9)	152	1.1 (0.2)	42	1.2 (0.8)	194
Caucasian	1.1 (0.4)	366	0.9 (0.2)	209	1.0 (0.3)	575
Creatinine Clearance$_E$ (mL/min)[a]	WHAS 1**		WHAS 2		Total	
African American	52.3 (22.0)	136	57.2 (18.0)	42	53.4 (21.2)	178
Caucasian	50.4 (21.0)	349	54.3 (14.0)	209	51.8 (18.8)	558

Note. Significant differences from independent *t*-tests between WHAS 1 and WHAS 2 subjects (**$p<0.01$) and African American and Caucasian subjects (*$p<0.01$) are indicated.

[a]Estimated creatinine clearance by Cockcroft-Gault formula.

Table 6

Metabolic and Clinical Profiles for WHAS Subjects by Race Category

Clinical Parameter	*N*	%	*N*	%	*N*	%
Cobalamin (pmol/L)	African American*		Caucasian		Total	
Low (<148)	2	1.0	18	3.0	20	2.5
Moderate (149-258)	11	5.6	67	11.3	78	9.9
Normal (>259)	181	91.4	498	84.3	679	86.1
Homocysteine (μmol/L)	African American		Caucasian		Total	
Normal (<14)	149	75.3	486	82.2	635	80.5
Moderate (15-49)	31	15.7	68	11.5	99	12.5
High (>50)	1	0.5	2	0.4	3	0.4
Methylmalonic acid (nmol/L)	African American*		Caucasian		Total	
Normal (<279)	158	79.8	391	66.2	549	69.6
Moderate-High (280-999)	31	15.7	174	29.4	205	26.0
Very High (>1000)	2	1.0	8	1.4	10	1.3
Folate (nmol/L)	African American*		Caucasian		Total	
Low (<6.8)	88	44.4	167	28.3	255	32.3
Normal (>6.9)	107	54.0	418	70.7	525	66.5
Creatinine Clearance$_E$[a]	African American		Caucasian		Total	
Stage 1 (>90)	11	5.6	17	2.9	28	3.5
Stage 2 (60-89)	46	23.2	140	23.7	186	23.6
Stage 3 (30-59)	98	49.5	325	55.0	423	53.6
Stage 4 (15-29)	20	10.1	50	8.5	70	8.9
Stage 5 (<15)	2	1.0	2	0.3	4	0.5

Note. Percentages may not add to 100% due to missing data. Significant differences in means of African American and Caucasian clinical parameter groups from independent *t*-tests (*p<0.01) are indicated.

[a]National Kidney Foundation-KDOQI Clinical Practice Guideline criteria, in mL/min.

Table 7

Genotype and Allele Frequencies for TCNII and TCNII-Receptor SNPs

	African American					Caucasian				
SNP	AA	AB	BB	A[a]	B	AA	AB	BB	A	B
TCNII										
rs16988828	5	39	141	0.13	0.87	10	87	454	0.10	0.90
rs7289549	19	67	103	0.28	0.72	11	92	458	0.10	0.90
rs7286107	12	60	117	0.22	0.78	3	1	561	0.01	0.99
rs9606756	10	48	132	0.18	0.82	28	87	447	0.13	0.87
rs740234	4	29	157	0.10	0.90	35	159	371	0.20	0.80
rs35915865	1	2	188	0.01	0.99	4	19	544	0.02	0.98
rs11703570	14	50	123	0.21	0.79	45	148	367	0.21	0.79
rs35838082	9	54	126	0.19	0.81	0	7	562	0.01	0.99
rs2267163	14	56	116	0.23	0.77	123	225	203	0.43	0.57
rs1801198	16	65	108	0.26	0.74	133	251	183	0.46	0.54
rs4820021	0	6	187	0.02	0.98	14	92	455	0.11	0.89
rs9621049	7	51	134	0.17	0.83	14	101	448	0.11	0.89
rs4820886	4	50	135	0.15	0.85	12	102	450	0.11	0.89
rs4820887	4	27	157	0.09	0.91	9	85	465	0.09	0.91
rs4820888	38	90	62	0.44	0.56	127	235	199	0.44	0.56
rs2301955	17	71	102	0.28	0.72	120	230	220	0.41	0.59
rs2301958	11	59	121	0.21	0.79	32	172	368	0.21	0.79
rs1131603	0	1	195	0.00	1.00	2	49	531	0.05	0.95
rs4820889	5	31	154	0.11	0.89	2	23	547	0.02	0.98
rs2072194	4	44	136	0.14	0.86	115	238	200	0.42	0.58
TCNII-Receptor										
rs173665	7	33	148	0.13	0.88	10	79	466	0.09	0.91
rs250510	3	22	157	0.08	0.92	1	6	539	0.01	0.99
rs2232787	1	3	186	0.01	0.99	1	0	547	0.00	1.00
rs2227288	13	50	125	0.20	0.80	12	98	427	0.11	0.89
rs2336573	21	72	94	0.30	0.70	9	35	512	0.05	0.95
rs2232779	2	18	174	0.06	0.94	5	2	573	0.01	0.99
rs2927707	16	51	118	0.22	0.78	57	202	288	0.29	0.71
rs3760680	29	75	78	0.37	0.63	81	221	242	0.35	0.65
rs8100119	23	65	102	0.29	0.71	5	35	524	0.04	0.96

Note. SNP data in genomic order as occurring on the chromosome.

[a]A denotes the minor allele and B denotes the major allele.

Table 8

Two-Way ANCOVA *F*-Statistics for Cobalamin-Related Clinical Outcomes

	Cobalamin			Homocysteine			MMA[a]		
SNP	Int.	M(S)	M(R)	Int.	M(S)	M(R)	Int.	M(S)	M(R)
TCNII									
rs16988828	0.55	1.31	8.47**	1.13	0.86	0.04	0.39	1.60	1.29
rs7289549	2.36	0.05	14.39**	0.12	0.18	1.67	1.86	0.85	6.87**
rs7286107	0.50	0.46	0.01	0.19	1.30	0.00	0.05	0.10	0.45
rs9606756	0.02	0.01	7.54**	1.41	0.52	3.10	0.09	0.96	1.28
rs740234	0.20	0.14	3.38	0.33	0.46	0.00	1.76	0.58	0.75
rs35915865	0.04	0.57	1.38	0.95	0.58	0.19	0.60	2.18	0.15
rs11703570	0.56	2.73	11.29**	1.61	0.92	0.60	0.39	0.29	1.56
rs35838082	0.03	0.39	3.92*	0.00	3.36*	1.50	8.21**	2.51	12.62**
rs2267163	0.86	1.69	5.90**	1.30	0.16	3.49	0.38	0.06	3.96*
rs1801198	1.25	1.97	6.41*	1.23	0.29	6.21**	0.17	0.01	3.79
rs4820021	0.85	2.11	0.81	0.73	0.01	2.56	0.50	0.04	0.27
rs9621049	2.30	0.77	6.41**	2.66	4.97**	6.91**	0.60	0.01	2.56
rs4820886	2.07	1.24	4.37	1.72	2.89	5.17*	0.69	0.19	3.00
rs4820887	0.75	0.74	5.24*	4.58**	4.73**	7.94**	0.37	0.03	0.76
rs4820888	1.59	3.17*	22.33**	0.80	1.17	5.33*	0.26	0.33	5.58*
rs2301955	1.05	0.98	15.82**	1.17	0.66	1.86	0.57	0.73	2.31
rs2301958	1.11	2.79	10.68**	1.40	0.43	0.07	0.05	0.01	3.70
rs1131603	1.64	1.30	0.15	0.10	0.14	0.02	0.78	0.91	1.81
rs4820889	1.54	1.21	1.52	0.03	0.27	0.83	1.77	1.54	1.22
rs2072194	0.22	0.25	4.72*	1.03	0.58	4.47*	1.13	0.24	3.42
TCNII-Receptor									
rs173665	0.01	1.01	5.55*	0.77	1.04	0.02	0.81	1.23	1.89
rs250510	0.06	2.05	1.87	1.25	1.82	4.38*	0.08	1.35	0.01
rs2232787	0.32	0.98	1.47	0.17	0.03	0.01	3.55	0.32	4.85*
rs2227288	0.27	2.80	5.94*	1.29	0.00	0.85	1.28	0.07	0.09
rs2336573	0.27	3.25*	6.78**	1.86	0.35	0.00	0.29	0.41	2.29
rs2232779	0.39	0.01	3.84*	0.39	0.37	0.06	0.96	0.69	4.10*
rs2927707	2.20	1.59	7.24**	0.76	0.73	5.67*	1.07	0.08	7.75**
rs3760680	1.34	1.95	17.47**	3.51	1.22	1.19	0.40	0.16	5.37*
rs8100119	0.01	2.10	2.72	1.21	0.33	0.17	0.84	0.34	0.55

Note. 2-way ANCOVA *F*-statistics for Interaction Effects (Int.), SNP Genotype Main Effects M(S), and Race Main Effects M(R), after adjustment for covariates. (*) indicates p=0.05 significance and (**) indicates p=0.01 significance. Data shown here are before Bonferroni adjustment.

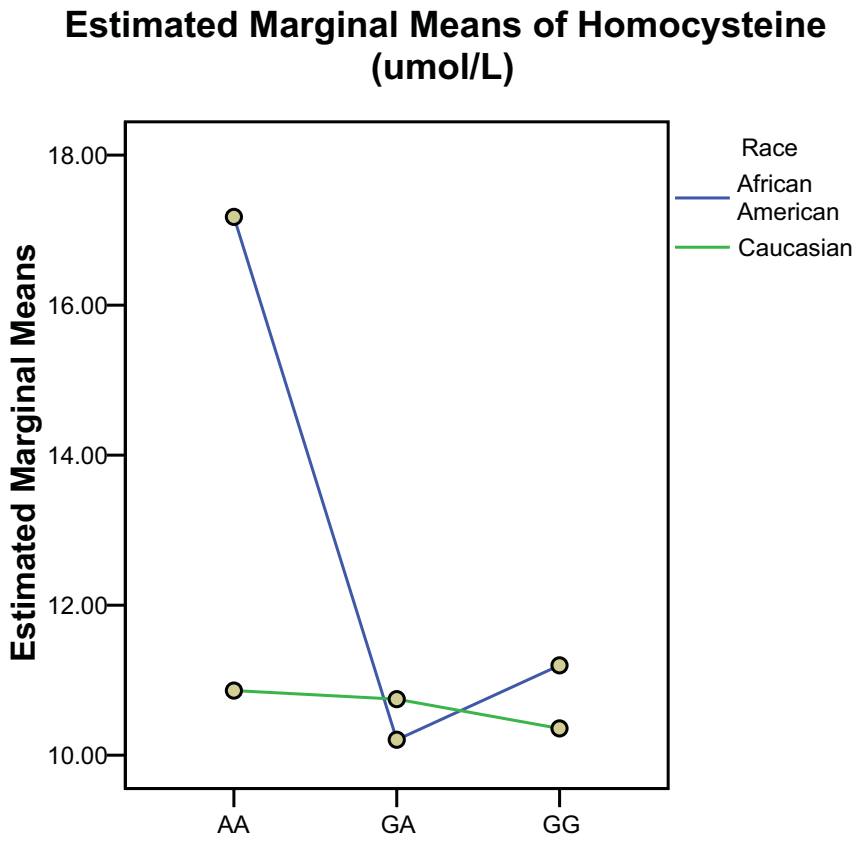

Figure 3. Race by SNP Genotype Interaction for SNP rs4820887 on Mean Homocysteine.

Shown above is the interaction between race and genotype for SNP rs4820887 on mean homocysteine concentrations μmol/L after adjustment for estimated creatinine clearance, serum folate, and serum cobalamin.

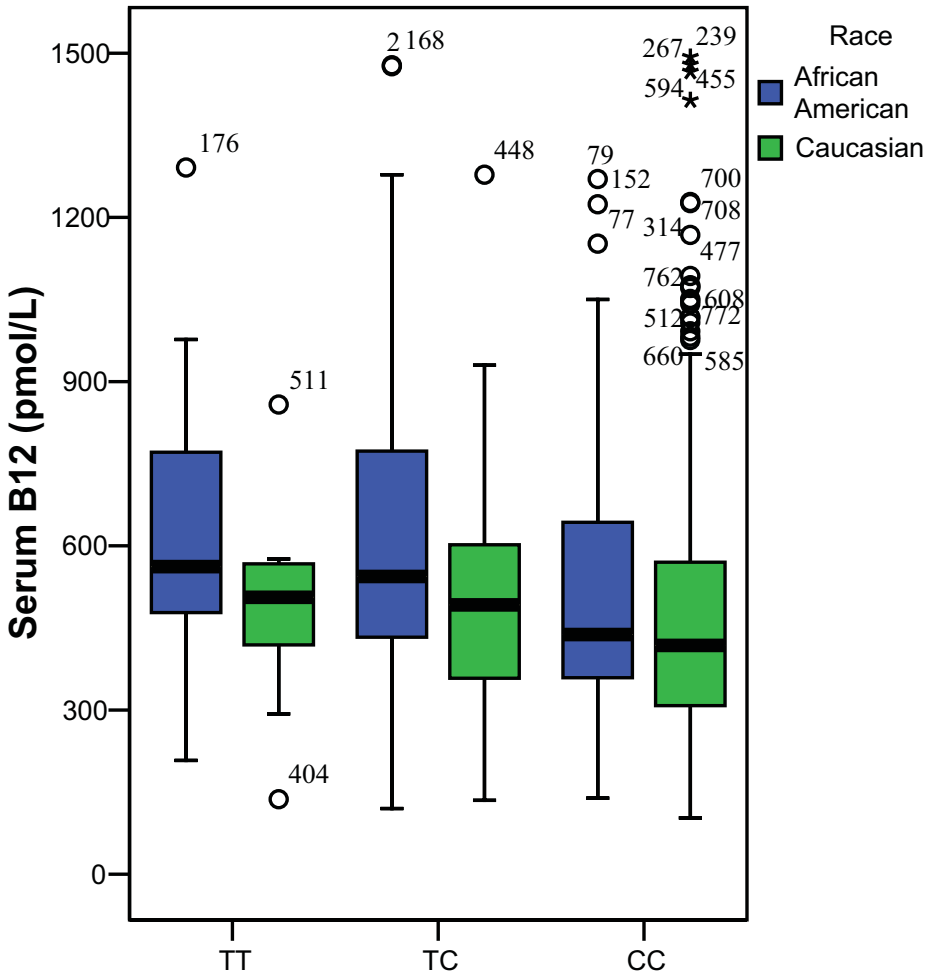

Figure 4. Transcobalamin II-Receptor SNP rs2336573 and Mean Serum Cobalamin. Box and whiskers plot serum cobalamin concentration (pmol/L) in African American and Caucasian WHAS subjects by rs2336573 (A220G) genotype in the transcobalamin II-receptor. The figure also includes the distribution of outlying cases.

CHAPTER 5

ASSOCIATION OF TRANSCOBALAMIN II AND TRANSCOBALAMIN II-RECEPTOR GENETIC VARIATION WITH CLINICAL FEATURES OF VITAMIN B12 DEFICIENCY IN ELDERLY WOMEN

Abstract

Vitamin B12 (cobalamin) deficiency is an insidious and chronically progressive condition that demonstrates increased prevalence in older adult populations. Elder adults are particularly susceptible to its development due to age-related decreases in nutrient absorption, presence of comorbidities and medications that interfere with vitamin B12, and misattribution of vague symptoms to "older age." Confounding accurate detection is clinical heterogeneity in the spectrum of symptom presentation, where clinical presentation spans mild fatigue and weakness to full-blown megaloblastic anemia and permanent neuropathic injury. This study aimed to better understand the heterogeneity observed in clinical cobalamin deficiency by evaluating the role of genetic variation on vitamin B12 deficiency symptom profiles in 795 Women's Health and Aging Study elderly subjects. A candidate gene association study was performed to test for associations between variation in genes involved in vitamin B12 transport and hematologic, neurologic, and functional performance features of cobalamin deficiency. Two genes were studied, the primary cobalamin transport molecule and its receptor

(transcobalamin II and transcobalamin II-receptor). An association was identified between a missense coding SNP (rs11801198) in the transcobalamin II gene and red blood cell mean corpuscular volume. A cluster of SNPs in the promoter region of the transcobalamin II gene were associated with the physical performance parameters, hand grip strength, and walking speed.

Introduction

Vitamin B12, also referred to as cobalamin, is an essential nutrient for survival in human beings. Because vitamin B12 is made only by bacteria, humans are dependent on receiving at least 2-4 μcg per day through dietary intake of animal products, such as meat, poultry, seafood, eggs, dairy, or alternatively, through supplementation practices (National Academy of Sciences, 1998)**.** In the human body, vitamin B12 has two primary forms, methylcobalamin and adenosylcobalamin, and is used as a cofactor for two enzymatic reactions: 1) in the cytoplasm, methylcobalamin overlaps with folate and serves as a methyl donor for the reaction catalyzed by methionine synthase. The products of this reaction provide the molecular precursors for DNA synthesis and cellular methylations, and 2) in the mitochondria, adenosylcobalamin is used as an essential cofactor in the metabolism of odd chain fatty acids and ketogenic amino acids for proper energy balance (Rosenblatt & Fenton, 2001).

Pathophysiologic responses to vitamin B12 deficiency commonly occur in cells that divide rapidly, such as those in the hematopoietic system, and in cells that require methylations for proper neurologic function, such as axons that experience frequent myelin sheath turnover. When methylations and DNA synthesis are disrupted, classical

pathology development includes macrocytic anemia, peripheral neuropathy, and subacute degeneration of the spinal cord (Beck, 2001). However, clinical vitamin B12 deficiency more often presents subtly, spanning a sizeable and heterogeneous preclinical spectrum with nonspecific symptom profiles, including malaise, vertigo, fatigue, mood alterations, sleeping, and gait disturbances (Carmel, 2000). Furthermore, neurologic symptoms may or may not occur before development of macrocytic anemia, and individuals receiving treatment may or may not experience resolution of neurologic symptoms even if using prescribed standardized replacement therapy (Allen, Stabler, Savage, & Lindenbaum, 1990; Lindenbaum, Savage, Stabler, & Allen, 1990).

The challenge of accurately identifying presence of decreased vitamin B12 status and effectively treating it is especially difficult for older adult populations. Nonspecific symptoms of borderline cobalamin status are often erroneously attributed to older age. Presence of common medications, age-related declines in gastric function, and comorbidities in older adults can impair normal vitamin B12 absorption (Baik & Russell, 1999). Estimated vitamin B12 deficiency prevalence in older adults ranges from 6 to as high as over 40% depending on the definition criteria used (Allen, 2009; Baik & Russell, 1999; Johnson et al., 2003; Lindenbaum, Rosenberg, Wilson, Stabler, & Allen, 1994; Pennypacker et al., 1992). If unrecognized, vitamin B12 deficiency could contribute to increased disability trajectories in older adults (Bartali et al., 2006).

Improved understanding of the factors contributing to the heterogeneity of clinical features observed in vitamin B12 deficiency affecting older adults is needed. Recently, research has identified that genetic variation in cobalamin metabolism genes can affect clinical parameters such as serum metabolites (Hazra et al., 2009; Miller, Ramos, Garrod,

Flynn, & Green, 2002; Tanaka et al., 2009). Genes involved in the transport of all biologically active vitamin B12 used by the body are especially attractive candidates in exploring the role of genetic variation in an elder adult's cobalamin status.

To explore this hypothesis, a candidate gene association study was performed to examine if genetic variation in the primary cobalamin carrier molecule (transcobalamin II), and its receptor (transcobalamin II-receptor) were associated with clinical indicators of vitamin B12 deficiency in a population of elderly women. Research subjects were previous participants of the Women's Health and Aging Studies. The clinical phenotypes examined in this study were traits that represented primary manifestations of clinical cobalamin deficiency and spanned hematological, neurologic, and functional performance parameters. Specifically, this study evaluated if genetic variation in the transcobalamin II and transcobalamin II-receptor gene was associated with hemoglobin concentration, mean corpuscular volume, depression scale score, peripheral neuropathy desensitization, hand grip strength, and walking speeds.

Methods

Study Design and Population

The Women's Health and Aging Study (WHAS) 1 and 2 were prospective observational female cohort research initiatives conducted by the National Institute on Aging (NIA) in conjunction with the Johns Hopkins School of Public Health (Fried, Bandeen-Roche, Chaves, & Johnson, 2000; Guralnik, Fried, Simonsick, Bandeen-Roche, & Kasper, 1995). Designed as complementary companion studies, data from WHAS 1 and 2 are often combined for a strengthened analytic approach representative of the

community-dwelling elderly (Bandeen-Roche et al., 2006; Chaves, Ashar, Guralnik, & Fried, 2002; Chaves et al., 2005; Leng, Xue, Tian, Walston, & Fried, 2007; Semba, Garrett, Johnson, Guralnik, & Fried, 2000; Semba et al., 2005; Walston et al., 2005). The WHAS studies represent a broad spectrum of illness and health in the Baltimore, MD community-dwelling female elderly population, as WHAS 1 focused on examination of the one-third most disabled women and WHAS 2 focused on two-thirds of the least disabled individuals. Although outlined briefly here, detailed reports of the sampling, screening, recruitment, and data collection procedures employed in WHAS 1 and 2 are reported elsewhere (Fried et al., 2000; Guralnik et al., 1995). The research protocols used by WHAS 1 and 2 investigators were approved by the Johns Hopkins Medical Institutions institutional review board. The genotyping portion of the WHAS research presented in this report was conducted at the National Human Genome Research Institute, and was approved by the Office of Human Subjects Research at the National Institutes of Health and the institutional review board at the University of Utah.

WHAS 1 (1992-1995)

Using Health Care Financing Administration (HCFA) Medicare enrollees from 12 Baltimore area zip codes, 6,521 elderly women were randomly selected for WHAS 1 screening (Fried, Kasper, Guralnik & Simonsick, 1995). Of the 6,521 age stratified women (65-74, 75-84, and >85) who were selected, 3,841 individuals met screening eligibility criteria and were assessed for physical disability across four domains: 1) mobility and exercise tolerance, 2) upper extremity function, 3) high function tasks (activities of daily living), and 4) basic self care. If participants had trouble performing

tasks in two, three, or four of the domains, and were moderately cognitively impaired as indicated by a score of at least 18 on Mini-Mental State Examination (MMSE), they were invited for WHAS 1 participation (n = 1,409) (Fried, Kasper, Guralnik, & Simonsick, 1995; Guralnik et al., 1995). Following consent, trained interviewers collected data on 1,002 WHAS 1 participants. In subjects' homes, interviewers used standardized assessment protocols to administer health questionnaires, physical examinations, and functional performance measures. A subset of 762 WHAS 1 research subjects consented to phlebotomy. DNA availability from the phlebotomy performed in 1992-1993 included samples for 536 WHAS 1 subjects.

WHAS 2 (1994-1996)

Also randomly selected from the 32,538 Health Care Financing Administration (HCFA) Medicare enrollee records in Baltimore, MD, an age-stratified sample (70-74, 75-79) of 1,630 women was identified for health screening and enrollment. Evaluated across the same four physical disability domains, women with difficulties in none or one of the domains with a Mini-Mental State Examination (MMSE) score of 24 or higher were invited for study participation (n = 880). Subjects assessed over the telephone with an abbreviated MMSE who correctly answered at least 80% of the verbal response items were also eligible. A total of 436 subjects consented and were enrolled in WHAS 2, where they participated in a clinic examination visit conducted by trained study personnel. The same standardized questionnaires, physical examination criteria, and functional performance measures were administered to WHAS 2 subjects as in WHAS 1.

A subset of 405 WHAS 2 research subjects consented to phlebotomy with DNA availability for 253 research subjects.

Blood Assay Measurement

Blood assay measurements used for this study included hemoglobin concentration, mean corpuscular volume (MCV), and serum folate. Nonfasting blood samples for WHAS 1 and 2 subjects were collected using venipuncture into sterile tubes containing ethylenediaminetetraacetic acid as an anticoagulant and processed at the Core Genetics Laboratory in the Johns Hopkins University School of Medicine. Frozen aliquots were sent to Quest Diagnostics (formerly Corning Clinical Laboratories and MetPath) in Teterboro, NJ. Hemoglobin concentrations were measured using the traditional cyanmethemoglobin method. Hemoglobin concentrations under 12.0 g/dL are considered to be low, indicating presence of anemia (World Health Organization, 1968). MCV values were calculated by taking the proportion of blood volume in a subject's sample comprised of erythrocytes (hematocrit) and dividing it by the total red blood cell count number. MCV values are reported in femtoliters (fL) and used to differentiate between various etiologies of anemia: microcytic (<83fL), normocytic (83-103 fL), and macrocytic (>103 fL). For serum folate measurement, frozen aliquots were sent from Johns Hopkins University School of Medicine to Quest Diagnostics (formerly Corning Clinical Laboratories and MetPath) in Teterboro, NJ. At Quest Diagnostics, subjects' serum folate concentrations were determined using a competitive folate-binding protein assay, according to reported methods of the Ciba-Corning Diagnostics Corporation (Medfield, MA). The Quest Diagnostics laboratory's reported normal reference range for

serum folate was 6.8-36.0 nmol/L. The hemoglobin, MCV, and serum folate values available for the WHAS participants included *n* = 752, hemoglobin concentrations, *n* = 744 MCV concentrations, and *n* = 780 folate concentrations.

Neurologic Clinical Measures

Depression

Depressive symptoms in WHAS 1 and 2 subjects were evaluated through use of the Geriatric Depression Scale (GDS), a psychometric depression assessment in elderly adults designed to circumvent somatic depression symptoms and presence of dementia (Yesavage et al., 1982). The GDS is a validated 30-item survey of dichotomous (yes/no) questions requiring approximately 8 minutes to administer. Although the continuous scores were used for analysis in this study, generally accepted clinical scoring cutoffs include no depression (less than or equal to 9), mild depression (scores 10-13), and severe depression (greater than or equal to 14) (Ferrucci, Kittner, Corti, & Guralnik, 1995; Lyness et al., 1997; Norris, Gallagher, Wilson, & Winograd, 1987). Depression scores were available for all 789 WHAS 1 and 2 research subjects in this study.

Peripheral Neuropathy

To assess for presence and severity of peripheral neuropathy, vibration perception testing (VPT) was conducted in both WHAS 1 and 2 cohorts. Unlike other laboratory tests and clinical measurements in WHAS, VPT data collection was not standardized between the two cohorts. WHAS 1 measurement protocols used a vibrometer and WHAS

2 measurement protocols used a tuning fork. To ensure reliability, the genetic analysis performed for this study was restricted to VPT continuous data for WHAS 1 subjects.

VPT testing for the WHAS 1 elderly women was performed on study participants' lower extremities using the Vibratron II (Physitemp Instrument, Inc., Clifton, NJ). Modeled after a diabetic neuropathy protocol, subjects placed their right toe on the Vibratron II's platform and reported whether or not a vibratory stimulus was felt using a two-alternative (yes/no) forced choice procedure (Ferrucci, Kittner, et al., 1995; Maser et al., 1989). Stimulation intensity was progressively decreased in 10% decrements until study subjects could no longer detect vibration. When a study subject provided an erroneous response, the vibration intensity was increased by 10% and the progressive decrements continued until a total of 5 errors were made. Vibration units measured by the Vibratron II were converted to microns, and after identifying the five errors and five lowest correct scores, a participant's mean vibratory threshold was identified by removing the highest and lowest scores and averaging the remaining values. Although the continuous scores were used for analysis in this study, accepted neuropathic functional micron unit cutoffs include normal function (<3.43 units), mild dysfunction (3.44-4.87 units), moderate dysfunction (4.88-6.31 units), and severe dysfunction (>6.31 units) (Resnick, Vinik, Heimovitz, Brancati, & Guralnik, 2001; Volpato, Leveille, Blaum, Fried, & Guralnik, 2005)**.** There were $n = 498$ continuous WHAS 1 vibration scores available for analysis.

Functional Performance Measures

Hand Grip Strength

A JAMAR hand dynamometer was used to measure hand grip strength in WHAS elderly participants (Model #BK-7498; Fred Sammons Inc, Burr Ridge, IL). Testing was performed with subjects in a seated position and elbow flexure at a 90-degree angle (Ferrucci, Guralnik, Bandeen-Roche, Lafferty, Pahor & Fried, 1995). Subjects were asked to grasp the dynamometer and squeeze as hard as possible three times on each hand. The best measure in the stronger hand was recorded and reported in kilograms of force. There were $n = 718$ hand grip strength measurements available for this study's analysis.

Four-Meter Walking Speed

WHAS 1 and 2 participants were asked to walk over a 4-meter course, at their usual speed two times and once, as fast as possible. For some participants, 4 meters was not available in their homes and a distance of 3 meters was used instead. Walking and timing of the walk beginning at a starting line did not start until the command to start was given by the interviewer. Using the faster of the two usual-pace walks, average walking speed was calculated by dividing the length of the walk (in meters) by the time in seconds required to complete it. Subjects were permitted to use a cane, walker, or walking aid, but not assistance from an additional person. There were $n = 767$ walking speed measurements available for this study's analysis.

Genetic Variant Measurement

SNP Selection

Information from publicly available databases (HapMap phase 1 and 2 full dataset, dbSNP build 125, and NCBI build 35) was used to select single nucleotide polymorphisms (SNPs) in the candidate genes, for Caucasians and African Americans. Haploview (4.0) Tagger was used to tag SNPs spanning both candidate genes, including 10kb flanking regions, using an r^2 threshold of 0.9 and a minor allele frequency of 0.05. Other candidates selected included those reported in literature to be associated with clinical vitamin B12 deficiency parameters, and functional variants within exons, promoters, or conserved sequences across species. A total of 51 SNPs were selected including 29 SNPs in the transcobalamin II gene and 22 SNPs in the transcobalamin II-receptor gene.

Whole Genome Amplification

To extract WHAS 1 and 2 subjects' genomic DNA from whole blood samples, study staff at the Core Genetics Laboratory in the Johns Hopkins School of Medicine used the Puregene DNA Purification Kit from GentraSystems, Inc. WHAS subject genomic DNA was plated at Johns Hopkins University and 50ng was provided to the National Human Genome Research Institute for genotyping. Due to limited starting quantities of genetic material, 10 ng of WHAS subject DNA was whole genome amplified using Qiagen's REPLI-g Midi Kit (Product # 150045_100 Rx). This kit is molecularly characterized by use of multiple displacement amplification via a highly processive Φ29 DNA polymerase, which can result in a random amplification of only

one allele. To minimize random allele dropout, two independent rounds of whole genome amplification reactions were performed and pooled for high-throughput genotyping. In pilot analyses, Hardy Weinberg statistics were closely correlated between calculations of whole genome amplified material and those of nonamplified genomic material. All 789 WHAS participant genomic samples were successfully amplified.

SNP Genotyping

Genotyping of the WHAS whole genome amplified material was completed at the National Human Genome Research Institute using the Sequenom MassArray iPLEX platform (San Diego, CA). Sequenom's high-throughput genotyping platform is characterized by a locus-specific PCR reaction producing a 100-base pair segment with the SNP of interest, followed by a locus-specific primer extension reaction producing a mass-modified SNP genotype. Following these serial amplifications and extensions, the mass-modified SNP genotypes were resolved using MALDI-TOF mass spectroscopy.

Statistical Analysis

SPSS (version 12.0) was used to compute descriptive and inferential statistics. Summary data, frequency distributions, normality parameters, and independent *t*-tests were evaluated for WHAS participant demographic characteristics and the study's hematologic, neurologic, and functional performance outcomes. For genetic data, Hardy-Weinberg statistics were calculated for each SNP in the transcobalamin II and transcobalamin II-receptor genes. Allele and genotype frequencies were obtained for African American and Caucasian WHAS subjects. Missing data were evaluated through

SPSS' missing data analysis function, and no nonrandom patterns were observed. WHAS subjects missing genotypes, clinical outcome measurements, or covariate measurement data were removed from analysis. For the outcome and covariate variables used in this study, assessment of linearity, homogeneity of variance, homogeneity of regression slopes, and covariate reliability indicated no violations of statistical assumptions. For the study's laboratory parameters, extreme outliers that were zero (indicating a false laboratory entry) or greater than 3 standard deviations away from the mean were removed from analysis (hemoglobin $n = 0$, MCV $n = 7$, and serum folate $n = 1$).

For hematologic parameters, a two-way analysis of covariance was conducted to ascertain presence of differences in mean hemoglobin concentration, MCV, covaried on serum folate by the independent variables, race and SNP genotype. For neurologic parameters, a two-way analysis of variance was performed to ascertain presence of differences in mean depression score and peripheral vibration sensitivity outcomes by the independent variables, race and SNP genotype. For physical performance parameters, a two-way analysis of variance was performed to assess differences in hand grip strength by race and SNP genotype. An analysis of covariance was conducted to ascertain differences in subjects' 4-meter walking speed by independent variables, race and SNP genotype, covarying on standing height to adjust for the influence of an individual's height on gait length. All analyses of variance and covariance in this study used continuous data. Significant interaction and main effect F tests (p=0.05) were evaluated using the Tukey and Least Significant Difference pairwise comparison test procedures, and by assessing interaction graphs. To correct for alpha inflation arising from multiple testing, the Bonferroni adjustment was used.

Results

Demographics

The demographic and other baseline health characteristics for the WHAS 1 and 2 study participants are summarized in Table 9. Caucasian participants were significantly older than African Americans [$t(787)= -2.90$, $p=0.004$], and WHAS 1 subjects were older than WHAS 2 subjects [$t(787)= 7.01$, $p<0.01$]. Caucasians had more years of education than African Americans [$t(787)= -7.27$, $p<0.01$] and as a cohort, WHAS 2 participants were more highly educated [$t(787)= -10.9$, $p<0.01$]. The number of chronic diseases was higher in WHAS 1 participants due to the cohort's focus on more disabled members of the community [$t(787)= 9.50$, $p<0.01$]. African American participants had elevated body mass index (BMI) parameters compared to the Caucasian participants [$t(741)= 5.13$, $p<0.01$], and WHAS 1 subjects had higher average BMI compared to WHAS 2 subjects [$t(741)= 3.62$, $p<0.01$].

Clinical Characteristics

Summary data for the WHAS subjects' clinical outcome characteristics are shown in Table 10 and frequencies for the clinical diagnoses related to the study's outcome parameters are indicated in Table 11. The mean hemoglobin concentration for African Americans at 12.4 gm/dL was lower than that for Caucasians at 13.3 gm/dL [$t(750)= -8.58$, $p<0.01$]. This difference was also noted for mean corpuscular volume (MCV), where African Americans' mean MCV was significantly lower at 90.3 fL compared to 94.2 fL for Caucasian participants [$t(742)= -8.39$, $p<0.01$]. African Americans subjects had a significantly higher prevalence of anemia compared to Caucasians [$t(750)= 6.04$,

$p<0.01$], and microcytic anemia affected more African Americans than Caucasians [$t(742)= -7.05$, $p<0.01$]. Across the cohorts, WHAS 1 elderly subjects had lower mean hemoglobin concentrations [$t(750)= -3.65$, $p<0.01$]. Although mean depression scale scores were similar between African Americans and Caucasians participants, WHAS 1 elderly women had higher scores than WHAS 2 elderly women [$t(787)= 10.0$, $p<0.01$]. Peripheral neuropathy measurements were only available for WHAS 1 subjects. Mean peripheral sensitivities did not differ between the ethnic groups and at 8.4 microns for African Americans and 8.3 microns for Caucasians, reflected an average of severe neuropathy for the sample. Given the varying and complementary selection criteria between the two different cohorts, these observed differences were expected and mirrored results of other WHAS publications.

Mean hand grip strengths indicate that African American elderly women were physically stronger than Caucasian elderly women [$t(716)= 2.86$, $p=0.004$], and mean 4-meter walking speeds for Caucasian elderly women were significantly faster than the African Americans [$t(765)= -6.25$, $p<0.01$]. WHAS 1 elderly women were less strong [$t(716)= -11.4$, $p<0.01$] and not as fast [$t(765)= -20.3$, $p<0.01$] as WHAS 2 elderly women. Table 12 provides functional performance percentiles for the WHAS elderly subjects on hand grip strength and 4-meter walking speeds. Previous characterization of physical performance measures in WHAS participants ascertained that individuals in the lowest quintile of measurement meet the definition of physical weakness, and physical slowness (Fried et al., 2001).

Genetic Data

Of the 51 SNPs selected for genotyping, only 29 SNPs were genotyped successfully. SNPs that were monomorphic (n = 6) or did not meet genotyping quality control thresholds (n = 16) were dropped. This resulted in incomplete tagging coverage for the transcobalamin II and transcobalamin II-receptor genes. SNPs were not included in the analysis if they were monomorphic or did not meet strict quality-control thresholds. Table 13 shows the genotype (AA, AB, BB) and allele (A, B) frequencies for SNPs that were successfully genotyped in WHAS African American and Caucasian subjects. Bonferroni adjustment for multiple testing of the 29 SNPs across the six clinical outcomes in this study yields a corrected alpha significance threshold of [p=(0.05/174)=0.0003]. Tables 14-16 provide a summary of the F values for interaction effects, SNP main effects, and race main effects obtained from the two-way analysis of variance and analysis of covariance statistical testing, and no results approach the Bonferroni-adjusted threshold level. However, several SNP main effect results were significant at the unadjusted p=0.01 levels for the hematologic parameters, and p=0.05 for the neurologic and functional performance parameters.

Hematologic Parameters

The two-way analysis of covariance of mean hemoglobin concentrations and MCV across race and SNP genotype factors adjusted for serum folate identified four highly significant SNPs, two for hemoglobin concentration and two for MCV (Table 14). Although there were no gross normality violations for the hemoglobin distribution, the MCV distribution demonstrated a bimodal peak that corresponded to differences between

African American's mean MCV of 90.3 fL and Caucasian subjects' mean MCV of 94.2 fL. Levene's test for equality of variances was violated in all analyses of covariance for MCV, resulting in the need for cautious interpretation in SNPs that did not meet more stringent alpha significance levels, such as under 0.025 (Tabachnik & Fidell, 2007).

For the hemoglobin concentration outcome, an A|G SNP in intron 7 of the transcobalamin II gene, rs4820888, demonstrated a significant interaction effect [F(2, 716), p=0.008, partial eta squared = 0.013] between race and genotype. Simple comparison testing type on the interaction effect indicated that African American GG homozygotes (M=12.7 gm/dL) were different from AG heterozygotes (M=12.2 gm/dL) at p=0.04. The SNP main effect of rs4820888 was insignificant [F(2, 716)= 0.694, p=0.5]. Also associated with mean hemoglobin concentration, a significant interaction effect [F(2, 700)= 5.57, p=0.004, partial eta squared = 0.016], and a significant SNP main effect [F(2, 700)= 4.10, p=0.017, partial eta squared = 0.012] was observed for the SNP rs2072194, an A|G polymorphism in intron 8 of the transcobalamin II gene. However, this association was spurious, its strength stemming from too few cases in the African American GG and GA genotype group cells that contained lower hemoglobin concentrations, thus driving the differences detected in the SNP main effect.

For the MCV outcome trait, a missense C|G polymorphism in exon 6 of the transcobalamin II gene, rs1801198 (P259R), demonstrated a significant interaction effect [F(2, 709)= 5.37, p=0.005, partial eta squared = 0.015]. Simple pairwise comparison tests were performed and identified statistically significant differences between the GG and CG groups (p=0.002) and the GG and CC groups (p=0.029) of African American elderly subjects. African American GG homozygotes had lower MCV (M=86.1 fL) compared to

CG heterozygotes (*M*=91.4 fL) and CC homozygotes (*M*=89.7 fL). There was no graphical interaction observed between African Americans and Caucasians for the MCV trait by rs1801198 genotype. Pairwise comparison tests across genotype groups in Caucasian elderly subjects were insignificant. Although there was a significant SNP main effect for rs1801198 [$F(2, 709)= 3.73$, $p=0.025$, partial eta squared = 0.011], it was likely driven by the strength of the association observed in African Americans. A second SNP associated with MCV was rs2232787, a synonymous SNP (S280S) in exon 5 of the transcobalamin II-receptor gene with a significant interaction effect [$F(2, 690)= 4.874$, $p=0.028$, partial eta squared = 0.007]. However, this SNP was monomorphic in both African American and Caucasian elderly subjects; discrepancies in MCV of genotype group cell sizes with zero, one, or two research subjects were driving this signal. In summary, the key finding identified in the analysis of hematologic parameters that was not attributable to low genotype frequencies was that of rs1801198 on MCV in African American subjects.

Neurologic Parameters

The two-way analysis of variance evaluating geriatric depression score and peripheral neuropathy measurements across race and SNP genotype factors identified no SNPs significantly associated with neurologic vitamin B12 deficiency parameters at the $p=0.01$ level, and three SNPs at the $p=0.05$ level (Table 15). The association closest to the 0.01 alpha threshold was a significant interaction effect [$F(2, 738)= 5.64$, $p=0.018$, partial eta squared 0.008] observed for the rs2232787 synonymous SNP (S280S) in exon 5 of the transcobalamin II-receptor gene; however, this was the same monomorphic SNP

from the MCV results. Inadequate cell sizes for the AA homozygotes (*n*=1 for African Americans, *n*=1 for Caucasians) and AG heterozygotes (*n*=3 for African Americans, *n*=0 for Caucasians) were driving wide variability in mean depression scores, resulting in false association.

Demonstrating weaker significance were three SNPs associated with peripheral neuropathy micron measurements. The first SNP, rs4820886, a G|T polymorphism in intron 7 of the transcobalamin II gene, demonstrated a significant SNP main effect [*F*(2, 470)= 3.67, *p*=0.026, partial eta squared 0.016] between genotype groups. The Tukey post-hoc analysis test ascertained significant differences between the mean micron measurement levels of GT heterozygotes (*M*=7.3 microns) and TT homozygotes (*M*=8.6 microns) at *p*= 0.04. The second SNP, rs2227288, a C|G polymorphism in intron 4 of the transcobalamin II-receptor gene on peripheral neuropathy micron measurement, demonstrated a significant interaction effect [*F*(2, 451)= 3.21, *p*=.041, partial eta squared = 0.014]. The Tukey post-hoc comparison test identified significant differences of mean micron measurements across genotype groups in African Americans and Caucasians. For Caucasian elderly women, mean micron measurements in GC heterozygotes (*M*=9.8) were significantly different from GG homozygotes (*M*=8.0) at *p*=0.024, and also significantly different from CC homozygotes (*M*=5.4) at *p*=0.006. However, the minor C allele comprised small cell sizes, at *n*=10 for African Americans and *n*=6 for Caucasians. The last SNP reaching significance with mean micron measurement was rs2927707, a T|C polymorphism located in intron 1 of the transcobalamin II-receptor gene, which demonstrated a weakly significant interaction effect [*F*(2, 448)= 3.02, *p*=0.05, partial eta squared = 0.013]. In African American elderly women, Tukey post-hoc testing

ascertained that African American CC homozygotes (*M*=10.9 microns) were significantly different from TT homozygotes (*M*=8.4 microns) at *p*=0.048.

In summary, four significant SNPs were found to be associated with neurologic parameters, one for depression (rs2232787) that was spurious due to low genotype frequencies from a monomorphic SNP, and three for peripheral neuropathy (rs4820886, rs2227288, and rs2927707). There was a significant main effect between genotype groups for rs4820886 independent of race. For rs227288, small genotype frequencies in the minor allele group drove false association. For rs2927707, differences in peripheral neuropathy measurements across genotype groups were identified in African Americans.

Functional Performance Parameters

At the *p*=0.05 level, the two-way analysis of variance for hand grip strength identified four significant SNPs, and the two-way analysis of covariance for walking speed, adjusted for standing height, identified three significant SNPs (Table 16). The majority of these observed findings were clustered in the five prime region of the transcobalamin II gene, including rs16988828 in intron 1, rs7289549 in intron 1, rs7286107 in intron 1, rs9606756 in exon 2, and rs11703570 in intron 3. The strongest of these significant associations is rs7286107, a C|T polymorphism in intron 1, demonstrating a SNP main effect in genotype groups across mean walking speeds [*F*(2, 708)= 4.18, *p*=0.016, partial eta squared = 0.012]. Simple comparison testing to ascertain which genotype groups were significantly different from each other showed that CT heterozygotes (*M*=0.54 m/s) had lower mean walking speeds than TT homozygotes (*M*=0.78 m/s) at *p*=0.018.

The final SNP in this analysis to be significantly associated with a functional performance parameter was rs2232779 in the transcobalamin II-receptor gene, an A|G polymorphism in intron 1 that had a significant interaction effect [$F(2, 726)= 3.47$, $p=0.032$, partial eta squared = 0.010] on walking speed. Pairwise comparison testing identified differences in mean walking speeds for Caucasians between TT homozygotes ($M=0.67$ m/s) and TC heterozygotes ($M=1.3$ m/s) at $p=0.006$, and between TT and CC ($M=0.80$ m/s) homozygotes at $p=0.023$, but inadequate cell frequency numbers were responsible for this effect (TT $n = 4$ and CT $n =2$). In summary, differences in functional performance not attributable to low cell frequencies were identified in a small cluster of six significant SNPs in the five prime region of the transcobalamin II gene.

Discussion

A candidate gene association study was performed to ascertain if genetic variation in the transcobalamin II and transcobalamin II-receptor genes was associated with clinical phenotype parameters of B12 deficiency in a cohort of older adult women. Using banked samples and previously catalogued clinical measurements, genotypes were generated and analyzed for their association with clinical traits using a two-way analysis of variance and covariance. Although no genetic variant association reached the level of statistical significance required from the Bonferroni adjustment ($p=0.0003$), this exploratory analysis did find a transcobalamin II variant that was significant with the MCV trait at $p=0.008$, and a cluster of lower-level significant SNPs associating with physical performance parameters in a region of the transcobalamin II gene.

The first, and primary, finding from this study was a significant interaction effect between the transcobalamin II rs11801198 (P259R) SNP and the clinical parameter MCV in African American elderly women (Figure 5). African American subjects with a GG genotype had significantly lower MCV values compared to other genotype groups. Currently, there are no published reports that identify effects of rs11801198 on the MCV trait. However, this SNP is suspected to exert a functional biologic effect, most notably resulting in altered cobalamin-dependent metabolite levels in serum (Afman, Lievers, van der Put, Trijbels, & Blom, 2002; Lievers et al., 2002).

Current population-based studies identify that hematologic parameters including hemoglobin and MCV percentiles in African American individuals are slightly lower than in Caucasian individuals (Cheng, Chan, Cembrowski, & van Assendelft, 2004). Although it has been proposed that differences in hemoglobin and MCV in African Americans may be attributable to socio-economic differences between the two populations, recent studies have identified alternative causes, such as genetic factors (Beutler & West, 2005). Ultimately, reasons for the rs11801198 finding in this study are unknown, and further research would be needed to validate this association in independent cohorts and ascertain its magnitude in a more adequately powered sample of elderly African American women.

A second finding from this work is the cluster of SNPs in the five prime region of the transcobalamin II gene associated with the cobalamin functional performance parameters. These SNPs span a genomic distance of approximately 5kb, extending across intron 1, exon 2, and intron 3, indicating that they are likely travelling together as part of a haplotype block. In addition to protein coding regions, this block is likely to include

sequences that regulate the expression of transcobalamin II messenger RNA, including enhancers and silencers. SNPs associated with decreased physical performance could act by influencing vitamin B12 availability and increase risk of functional vitamin B12 deficiency in elderly individuals.

Two recent reports of transcobalamin II candidate gene associations with frailty parameters in Caucasian Women's Health and Aging Study 1 and 2 subjects were published (Matteini, Walston, Bandeen-Roche, Arking, Allen, Fried et al., 2008; Matteini, Walston, Bandeen-Roche, Arking, Allen, Fried et al., 2010). These efforts identified significant associations between the frailty syndrome and transcobalamin II SNPs, specifically rs2267163 (and rs11801198 in linkage disequilibrium), which are located in the genomic region of intron 5 and exon 6. In comparison, results reported in this paper were slightly different in that the location of the significant SNPs was in intron 1 through intron 3.

Reasons for this difference may be in the varying analytic approaches used. Matteini and colleagues collapsed five continuous measurements of functional performance traits (walking speed, hand grip strength, energy level, body mass, and physical activity) into dichotomous outcomes according to widely accepted frailty parameters. Research subjects with at least three of the five frailty traits were categorized as being frail and entered into multivariate logistic regression models with SNP genotypes. In comparison, the analysis of covariance and analysis of variance approach outlined in this work used continuous data and conducted analysis of African American subjects in addition to Caucasian subjects. As both efforts identified findings in the transcobalamin II gene as being significant to functional performance in older adults, the

transcobalamin II gene remains an attractive biologic candidate for further replication and validation with a larger sample of older adults.

This study also assessed presence of variability in the mean outcome measures that was not due to genetics. For hemoglobin, MCV, and walking speed outcomes, the majority of SNPs had significant race main effects. There was one significant race main effect for depression score and peripheral neuropathy, 14 significant race main effects for hand grip strength, and 23 for walking speed. The variability in outcome measures in the race main effects was arising from factors related to the social contexts of the WHAS subjects, including nutrition, comorbidities, socio-economic status, or access to health care. For the traits hemoglobin and MCV, it is understood that social factors in addition to biologic differences account for clinical variability between African Americans and Caucasians (Beutler & West, 2005). However, there is no data on gene-environment interactions involving social mechanisms that is known to affect transcobalamin II and the transcobalamin II-receptor gene function in the broader literature base. Although this study indicates that social factors are present and impacting the outcome measures, this research does not ascertain which factors from an individual's environment are generating the observed association.

The strengths of this research include the use of a well-characterized elder adult research cohort such as WHAS 1 and 2 to investigate effects of genetic variation on clinical parameters of cobalamin deficiency. Additionally, because this study made use of very small amounts of genomic DNA, it demonstrates that use of whole genome amplification is a sound methodological technique to complement previous epidemiological investigations, such as WHAS 1 and 2.

Limitations of this work include the inability to fully tag both genes, due to the genotyping challenges associated with using whole genome amplified material from WHAS subject DNA that was between 16 and 18 years old. Because this is a candidate gene association study, it is important to note that although the findings identify new transcobalamin II and transcobalamin II-receptor genetic associations, knowledge of these associations cannot provide information as to their role in biologic causation of clinical vitamin B12 deficiency.

Another limitation to this research is that environmental factors known to affect cobalamin status in older adults, such as medication use and comorbidities, were not included in the analyses. Since frequencies were low in many African American and Caucasian SNP genotype subgroups, greater loss of statistical power through additional covariate inclusion would have impaired ability to detect genetic effects. However, if data on medications and comorbidities was incorporated, there may have been less variance in the study's outcomes attributable to SNP genotype.

The MCV and physical performance genetic associations identified in this research support improved understanding of clinical heterogeneity of cobalamin deficiency parameters in older adult women. Additional research is required to replicate rs1801198 SNP (P259R) and the SNP cluster associations identified in the transcobalamin II gene, and to ascertain their biologic significance in the development and progression of vitamin B12 deficiency. The results obtained in this study may contribute to new analytic targets for future functional analyses of vitamin B12 related research.

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Table 9

Selected Demographic and Health Characteristics for WHAS Subjects

Characteristic	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*N*
Age (Years)	WHAS 1**		WHAS 2		Total	
African American	75.7 (7.0)	155	73.0 (2.5)	43	75.1 (6.4)	198*
Caucasian	78.1 (7.8)	381	74.1 (2.7)	210	76.7 (6.8)	591
Education (Years completed)	WHAS 1**		WHAS 2		Total	
African American	8.6 (3.2)	155	11.0 (3.8)	43	9.1 (3.5)	198*
Caucasian	10.3 (3.7)	381	13.2 (3.2)	210	11.3 (3.8)	591
Number of Chronic Diseases[a]	WHAS 1**		WHAS 2		Total	
African American	1.4 (1.6)	155	0.6 (1.1)	43	1.3 (1.5)	198
Caucasian	1.4 (1.4)	381	0.4 (0.9)	210	1.0 (1.3)	591
Body Mass Index (kg/m^2)	WHAS 1**		WHAS 2		Total	
African American	31.5 (16.1)	138	29.3 (6.3)	43	31.0 (14.3)	181*
Caucasian	28.0 (6.1)	352	26.0 (0.9)	210	27.2 (5.7)	562

Note. Summary statistics including mean values, standard deviations (*SD*), and sample size. Significant differences on independent *t*-tests between WHAS 1 and WHAS 2 subjects (**$p<0.01$) and African American and Caucasian subjects (*$p<0.01$) are indicated.

[a]Self-reported diseases include angina, myocardial infarction, coronary artery disease, congestive heart failure, peripheral artery disease, stroke, diabetes mellitus, and cancer.

Table 10

Descriptive Summary of Cobalamin-Related Clinical Outcome Variables

Clinical Outcome[a]	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*N*
Hemoglobin (gm/dL)	WHAS 1**		WHAS 2		Total	
African American	12.3 (1.6)	144	12.6 (1.0)	41	12.4 (1.5)	185*
Caucasian	13.2 (1.3)	367	13.4 (1.1)	200	13.3 (1.2)	580
Mean Corpuscular Volume (fL)	WHAS 1		WHAS 2		Total	
African American	90.3 (6.3)	143	90.4 (6.9)	41	90.3 (6.4)	184*
Caucasian	95.1 (5.2)	361	92.7 (4.8)	199	94.2 (5.2)	560
Geriatric Depression Score	WHAS 1**		WHAS 2			
African American	7.2 (5.1)	155	4.2 (4.1)	43	6.6 (5.0)	198
Caucasian	8.2 (5.9)	381	3.9 (3.8)	210	6.7 (5.6)	591
Peripheral Neuropathy[a] (microns)	WHAS 1					
African American	8.4 (4.6)	144				
Caucasian	8.3 (4.4)	354				
Hand Grip Strength (kg)	WHAS 1**		WHAS 2		Total	
African American	21.9 (5.9)	129	27.1 (5.6)	43	23.2 (6.3)	172*
Caucasian	19.8 (5.4)	341	24.8 (4.7)	205	21.7 (5.7)	546
4-Meter Walking Speed (m/s)	WHAS 1**		WHAS 2		Total	
African American	0.5 (0.2)	145	0.9 (0.3)	43	0.6 (0.3)	188*
Caucasian	0.6 (0.3)	372	1.1 (0.3)	207	0.8 (0.4)	579

Note. Significant differences in means from independent *t*-tests between WHAS 1 and WHAS 2 subjects (**$p<0.01$), and African American and Caucasian subjects (*$p<0.01$) are indicated.

[a]Vibratron II peripheral neuropathy measurements were collected only on WHAS 1 participants.

Table 11

Clinical Profile Descriptive Summaries for WHAS Subjects by Race Category

Clinical Parameter	*N*	%	*N*	%	*N*	%
Hemoglobin (gm/dL)	African American*		Caucasian		Total	
Anemia (<12.0)	60	30.3	75	12.7	135	17.1
Normal (>12.0)	125	63.1	492	83.2	617	78.2
Mean Corpuscular Volume (fL)	African American*		Caucasian		Total	
Microcytic (<83)	30	15.2	9	1.5	39	4.9
Normal (83-103)	151	76.3	529	89.5	680	86.2
Macrocytic (>103)	3	1.5	22	3.7	25	3.2
Geriatric Depression Score	African American		Caucasian		Total	
None (0-9)	148	74.7	445	75.3	593	75.2
Mild (10-13)	23	11.6	66	11.2	89	11.3
Moderate/Severe (>13)	27	13.6	80	13.5	107	13.6
Peripheral Neuropathy	African American		Caucasian		Total	
None (<3.44)	14	7.1	41	6.9	55	7.0
Mild (3.44-4.87)	21	10.6	42	7.1	63	8.0
Moderate (4.88-6.31)	24	12.1	52	8.8	76	9.6
Severe (>6.31)	85	42.9	219	37.1	304	38.5

Note. Percentages may not add to 100% due to missing data. Significant differences in means of African American and Caucasian clinical parameter groups from independent *t*-tests (*$p<0.01$) are indicated.

Table 12

Functional Performance Percentiles in WHAS Elderly Subjects

Clinical Parameter	African American	Caucasian	Total
Hand Grip Strength (kg)	*N*=188	*N*=546	*N*=718
20th percentile	18.00	17.00	17.00
40th percentile	22.00	20.00	20.00
60th percentile	24.00	23.00	24.00
80th percentile	28.00	26.00	27.00
4-Meter Walking Speed (m/s)	*N*=172	*N*=579	*N*=767
20th percentile	0.34	0.48	0.43
40th percentile	0.51	0.67	0.63
60th percentile	0.64	0.83	0.78
80th percentile	0.85	1.08	1.03

Table 13

Genotype and Allele Frequencies for TCNII and TCNII-Receptor SNPs

	African American					Caucasian				
SNP	AA	AB	BB	A[a]	B	AA	AB	BB	A	B
TCNII										
rs16988828	5	39	141	0.13	0.87	10	87	454	0.10	0.90
rs7289549	19	67	103	0.28	0.72	11	92	458	0.10	0.90
rs7286107	12	60	117	0.22	0.78	3	1	561	0.01	0.99
rs9606756	10	48	132	0.18	0.82	28	87	447	0.13	0.87
rs740234	4	29	157	0.10	0.90	35	159	371	0.20	0.80
rs35915865	1	2	188	0.01	0.99	4	19	544	0.02	0.98
rs11703570	14	50	123	0.21	0.79	45	148	367	0.21	0.79
rs35838082	9	54	126	0.19	0.81	0	7	562	0.01	0.99
rs2267163	14	56	116	0.23	0.77	123	225	203	0.43	0.57
rs1801198	16	65	108	0.26	0.74	133	251	183	0.46	0.54
rs4820021	0	6	187	0.02	0.98	14	92	455	0.11	0.89
rs9621049	7	51	134	0.17	0.83	14	101	448	0.11	0.89
rs4820886	4	50	135	0.15	0.85	12	102	450	0.11	0.89
rs4820887	4	27	157	0.09	0.91	9	85	465	0.09	0.91
rs4820888	38	90	62	0.44	0.56	127	235	199	0.44	0.56
rs2301955	17	71	102	0.28	0.72	120	230	220	0.41	0.59
rs2301958	11	59	121	0.21	0.79	32	172	368	0.21	0.79
rs1131603	0	1	195	0.00	1.00	2	49	531	0.05	0.95
rs4820889	5	31	154	0.11	0.89	2	23	547	0.02	0.98
rs2072194	4	44	136	0.14	0.86	115	238	200	0.42	0.58
TCNII-Receptor										
rs173665	7	33	148	0.13	0.88	10	79	466	0.09	0.91
rs250510	3	22	157	0.08	0.92	1	6	539	0.01	0.99
rs2232787	1	3	186	0.01	0.99	1	0	547	0.00	1.00
rs2227288	13	50	125	0.20	0.80	12	98	427	0.11	0.89
rs2336573	21	72	94	0.30	0.70	9	35	512	0.05	0.95
rs2232779	2	18	174	0.06	0.94	5	2	573	0.01	0.99
rs2927707	16	51	118	0.22	0.78	57	202	288	0.29	0.71
rs3760680	29	75	78	0.37	0.63	81	221	242	0.35	0.65
rs8100119	23	65	102	0.29	0.71	5	35	524	0.04	0.96

Note. SNP data in genomic order as occurring on the chromosome.

[a]A denotes the minor allele and B the major allele.

Table 14

Two-Way ANCOVA *F*-Statistics for Hematologic Parameters

	Hemoglobin			Mean Corpuscular Volume		
SNP	Interaction	Main(S)	Main(R)	Interaction	Main(S)	Main(R)
TCNII						
rs16988828	0.25	0.06	8.73**	0.75	1.30	8.70**
rs7289549	0.02	0.10	26.40**	2.63	0.59	12.61**
rs7286107	0.22	1.06	1.58	0.49	1.58	5.23*
rs9606756	2.38	1.13	45.94**	0.60	0.33	17.42**
rs740234	1.48	0.86	4.74*	0.20	0.24	13.83**
rs35915865	2.41	1.97	0.09	0.56	1.06	4.10*
rs11703570	2.11	0.13	18.60**	1.13	0.02	37.87**
rs35838082	0.57	1.64	19.13**	0.26	2.65	17.46**
rs2267163	1.74	0.91	65.10**	3.67*	2.98	59.91**
rs1801198	0.84	0.28	56.92**	5.37**	3.73*	66.29**
rs4820021	0.07	0.11	14.11**	1.44	0.79	19.97**
rs9621049	1.46	0.30	19.60**	0.26	0.01	20.86**
rs4820886	1.33	0.50	19.88**	0.25	0.01	9.24**
rs4820887	1.73	0.77	19.20**	1.86	0.65	5.03*
rs4820888	4.81**	0.69	57.50**	2.92	0.86	77.88**
rs2301955	2.73	0.27	33.28**	1.21	0.02	50.60**
rs2301958	1.01	0.36	19.17**	0.53	0.66	33.81**
rs1131603	1.81	1.11	0.03**	0.40	2.29	0.59
rs4820889	0.64	0.55	6.66**	0.35	0.49	10.24**
rs2072194	5.57**	4.10**	50.29**	0.22	0.69	23.31**
TCNII-Receptor						
rs173665	0.01	0.67	18.70**	0.37	0.26	20.61**
rs250510	1.01	0.77	0.94	0.44	0.15	6.92**
rs2232787	0.14	0.67	0.50	4.87**	0.19	9.89**
rs2227288	2.70	0.78	25.75**	1.43	1.62	14.69**
rs2336573	0.45	1.07	37.68**	1.52	0.73	26.95**
rs2232779	0.27	0.04	0.04	0.81	1.22	7.80**
rs2927707	0.20	0.46	48.90**	0.10	0.33	38.26**
rs3760680	0.01	0.19	67.00**	0.74	0.43	49.36**
rs8100119	0.32	0.76	14.82**	0.28	0.94	16.87**

Note. ANCOVA *F*-statistics covaried on serum folate, with Interaction Effect, SNP Main Effect (S), and Race Main Effect (R) values shown. (*) indicates p=0.05 significance and (**) indicates p=0.01 significance. Data shown are before Bonferroni adjustment.

Table 15

Two-Way ANOVA *F*-Statistics for Neurologic Parameters

	Geriatric Depression Score			Peripheral Neuropathy		
SNP	Interaction	Main (S)	Main (R)	Interaction	Main (S)	Main (R)
TCNII						
rs16988828	2.08	0.41	2.41	2.49	0.94	2.29
rs7289549	0.63	1.36	0.59	0.13	0.53	0.05
rs7286107	2.90	1.16	0.73	2.90	2.40	4.51*
rs9606756	0.05	1.95	0.00	0.13	0.94	0.24
rs740234	0.85	0.12	1.28	0.34	0.34	0.05
rs35915865	1.60	0.29	1.95	2.48	0.84	3.57
rs11703570	0.06	0.15	0.00	0.85	0.51	0.14
rs35838082	0.49	1.63	0.72	0.12	0.12	0.15
rs2267163	0.20	0.97	0.24	0.98	1.02	0.81
rs1801198	0.02	0.85	0.32	1.42	1.69	1.58
rs4820021	0.93	0.63	0.31	0.01	0.08	0.00
rs9621049	0.55	0.16	0.20	0.78	2.57	0.14
rs4820886	0.06	0.36	0.09	1.29	3.67*	1.10
rs4820887	0.09	0.25	0.06	0.47	0.86	0.37
rs4820888	0.10	0.21	0.50	1.72	1.26	0.00
rs2301955	0.21	0.75	0.03	2.10	1.09	1.02
rs2301958	1.45	0.49	0.83	1.98	0.90	0.75
rs1131603	1.40	0.73	1.46	--	0.11	0.13
rs4820889	1.12	0.33	0.65	1.63	0.50	1.77
rs2072194	1.32	0.72	1.22	0.89	2.87	0.18
TCNII-Receptor						
rs173665	1.20	0.16	0.32	1.26	0.03	0.71
rs250510	0.79	1.82	1.37	1.84	0.40	1.51
rs2232787	5.64*	2.10	5.50*	0.01	0.35	0.01
rs2227288	2.58	0.27	1.40	3.21*	2.32	0.08
rs2336573	0.14	1.13	1.13	0.30	0.40	0.43
rs2232779	0.39	4.51	0.05	0.13	2.34	0.01
rs2927707	0.49	0.80	0.04	3.02*	0.61	3.05
rs3760680	0.20	0.63	0.22	2.32	0.06	1.22
rs8100119	0.14	0.03	0.09	0.65	1.61	0.45

Note. F-statistics with Interaction Effect, SNP Main Effect (S), and Race Main Effect (R) values. (*) indicates p=0.05 and (**) indicates p=0.01 significance. Data shown here are before Bonferroni adjustment.

Table 16

Two-Way ANOVA and ANCOVA *F*-Statistics for Functional Performance Parameters

	Hand Grip Strength			4-Meter Walking Speed		
SNP	Interaction	Main (S)	Main (R)	Interaction	Main (S)	Main (R)
TCNII						
rs16988828	3.64*	1.25	7.44**	0.23	0.28	5.44*
rs7289549	3.69*	2.29	16.22**	3.84*	0.80	29.49**
rs7286107	1.56	0.54	5.11*	1.52	4.18*	0.54
rs9606756	0.86	3.74*	0.68	0.19	1.04	8.60**
rs740234	0.74	1.34	4.49*	0.33	0.77	3.81*
rs35915865	0.03	0.73	0.29	0.07	0.11	1.05
rs11703570	3.08*	1.69	10.65**	0.39	0.13	11.67**
rs35838082	0.26	0.19	0.62	0.65	0.12	7.48**
rs2267163	1.23	0.44	3.18	0.07	1.59	14.29**
rs1801198	0.23	1.22	5.23*	0.07	2.77	18.10**
rs4820021	2.15	0.27	0.00	0.42	0.14	8.49**
rs9621049	0.04	0.07	2.53	0.39	0.12	6.68**
rs4820886	0.14	0.10	0.89	0.71	0.17	4.12*
rs4820887	1.42	1.75	0.48	0.41	0.05	3.81*
rs4820888	0.03	0.21	7.62**	0.99	0.11	26.95**
rs2301955	0.26	1.06	8.25**	1.47	0.28	14.82**
rs2301958	0.55	0.56	6.37**	1.85	0.74	5.47*
rs1131603	1.77	1.59	3.38	0.49	0.11	0.10
rs4820889	0.26	1.91	0.94	0.66	1.53	8.25**
rs2072194	1.66	1.77	0.13	0.84	1.30	11.72**
TCNII-Receptor						
rs173665	0.38	0.47	4.13*	0.68	2.33	3.22
rs250510	0.01	1.70	0.24	0.23	1.37	0.48
rs2232787	--	--	--	1.33	0.35	3.48
rs2227288	0.69	0.15	4.66*	1.50	1.52	14.80**
rs2336573	0.72	0.64	1.98	1.43	0.22	5.01*
rs2232779	1.42	1.69	4.01*	3.47*	2.03	5.12*
rs2927707	0.19	0.12	6.16**	0.78	0.69	16.73**
rs3760680	1.89	1.06	4.91*	0.42	0.43	22.33**
rs8100119	0.10	1.81	2.26	0.01	0.72	6.69**

Note. *F*-statistics with Interaction, SNP (S), and Race (R) main effect values. (*) indicates p=0.05 and (**) p=0.01 significance, before Bonferroni adjustment.

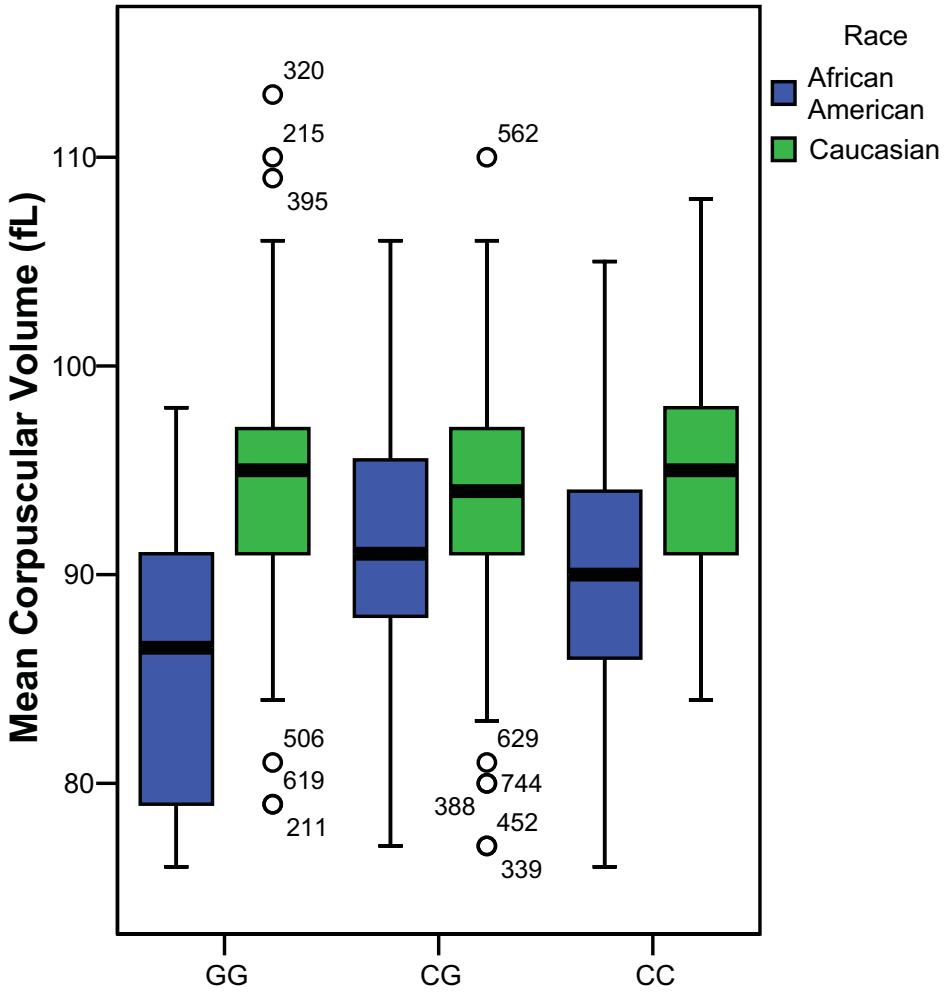

Figure 5. Transcobalamin II SNP rs1801198 and Mean Corpuscular Volume.

Box and whiskers plot mean MCV for African American and Caucasian WHAS subjects by genotype of rs1801198 (P259R) in the transcobalamin II gene. The distribution of outlying cases is included.

CHAPTER 6

TRANSLATION OF GENETICS AND GENOMICS FOR NURSING—PERSONALIZED MEDICINE

Abstract

Scientific advances in genetics and genomics will be incorporated into healthcare soon. The tailoring of treatment to an individual's genetic make up has been termed Personalized Medicine. These advances are promising and are receiving significant attention; however, many nurses are caught in the gap between technologic advances and clinical diffusion and uptake. Four elements of Personalized Medicine are described in this paper, which include 1) discovery of novel biology that guides clinical translation mechanisms; 2) genetic risk assessment; 3) molecular diagnostic technology; and 4) pharmacogenetics and pharmacogenomics. Opportunities for engagement of the nursing profession that are presented by Personalized Medicine are addressed. Successful design and implementation of Personalized Medicine will hinge on the roles of nurses conducting or participating in collaborative initiatives that are furthering genetic/genomic applications.

Introduction

Formally completed in 2003, The Human Genome Project provided a reference map of human genome DNA sequence (The International Human Genome Sequencing Consortium, 2001, 2004). Similarly, the International Haplotype Map Project (HapMap, www.hapmap.org), completed in 2005, provided a map of variation in human DNA and how this variation was arranged in populations (The International HapMap Consortium, 2005). Fruits of these research efforts are revolutionizing biology, medicine, and ultimately, healthcare delivery (Collins, Green, Guttmacher, & Guyer, 2003; Collins & McKusick, 2001). Clinical translation of these landmark scientific achievements will contribute to Personalized Medicine, where an individual's DNA can be used to finely tailor healthcare practices. Health advances include personalized risk assessments that predict disease development years before clinical appearance, use of precisely tuned molecular screening tests and clinical diagnostics, and safer and more effective pharmaceutical agents prescribed to match individuals' genetic constitution. Further developing and fueling these innovative applications is the continued discovery of novel biology in high-tech laboratory settings across the country and throughout the world.

Scientific achievement beyond The Human Genome Project and the HapMap is being fueled by unprecedented technological advancement. Cost of sequencing an individual's genome in 2002 was 1 billion dollars, but through Illumina, Inc., Personal Genome Sequencing Service can now be commercially performed for $48,000 (Illumina, 2009). Furthermore, it is estimated that this price will drop to under $1000 per genome in the next 2-3 years, a magnitude and rate of cost reduction that surpasses the gains made by the microprocessor industry. Because the human genome is approximately 6 billion

data points, the sheer volume of scientific data is overwhelming, and data flowing from studies examining the associations between genetic sequences and disease has been likened to 'drinking water from a fire hose' (Hunter & Kraft, 2007). A PubMed search using the terms "genetics" and "genomics" comparing 1989-1999 and 1999-2009 demonstrates this rapid increase: 576,170 and 7,412 results, respectively, for 1989-1999, compared to 1,169,310 and 47,297 for the past decade (http://www.ncbi.nlm.nih.gov/sites/entrez/ queried on 30 November 2009).

Intense mainstream media coverage of scientific progress in genetics and genomics has resulted in public expectation that these advances are already incorporated into clinical practice. Capitalizing on this environment, several companies have launched direct-to-consumer marketing campaigns for genomic profiling tests. Some testing companies provide test results and interpretations that are not supported by scientific data (Janssens et al., 2008). Others may include information that is difficult for the consumer to interpret in the absence of knowledgeable practitioner guidance (Gollust, Wilfond, & Hull, 2003; Kutz, 2006). Current examples include services offered by *23andMe, DeCode,* and *Navigenics*, where for $300-$1500, a consumer can submit a sample of their DNA and obtain personalized analyses of their genetic code. Information contained in these companies' reports includes a person's lifetime risk for development of health conditions such as osteoporosis, cancer, diabetes, and heart disease.

Previous scholarly papers in nursing have discussed genetics, and genomics, but none outline Personalized Medicine while identifying implications and opportunities for nurses. In this paper, I outline four conceptual Personalized Medicine elements, describe their healthcare implications and the current translation obstacles they face, and outline

implications for nurses in research, education, and practice settings. Although the biologic advances fueling Personalized Medicine offer sound proof of technologic possibility, it will likely be one or two decades until there is enough evidence concerning its application before nurses can use the entirety of an individual's genome to guide personalized patient healthcare. During this long incubation period, health care patterns and practices will begin to incorporate elements of genetics. The field of nursing should anticipate and contribute to these developments and play an integral role in their translation and application.

Clinical Application of Genetics and Genomics:

Personalized Medicine

Personalized Medicine, in lay definition, is "using information about a person's genetic makeup to tailor strategies for the *detection*, *treatment*, or *prevention of disease*" (Collins, 2005). This new paradigm of healthcare rests on four key elements, the first driving the latter three, which contain direct clinical relevance to the nursing community (Figure 6): (1) discovery of novel biologic processes, which serve as the foundation for all clinical translation; (2) personalized risk assessments; (3) enhanced diagnostic accuracy through molecular profiling; and (4) pharmacogenetics and pharmacogenomics. Throughout the paper, refer to Table 17 for a concept glossary and Table 18 for relevant reference genetics and genomics websites.

The Foundation of Personalized Medicine—

New Biology, New Drugs

Novel biologic knowledge arising from the Human Genome Project and HapMap has great implications for advances in future healthcare practices. Validated Genome Wide Association Study (GWAS) associations identify 100 new loci for over 40 common health and disease traits, and are publicly searchable in an online catalogue maintained by the National Human Genome Research Institute at www.genome.gov/26525384 (Hindorff et al., 2009; Manolio, Brooks, & Collins, 2008). This knowledge translates to understandings of biologic networks not previously known as involved with common diseases (Figure 7). Through these new pathway connections, opportunities for improved clinical measurement and therapeutic manipulation will arise. A relevant example is illustrated by the case of age-related macular degeneration (Figure 8), one of the first conditions to be analyzed with the GWAS approach.

While gene/disease associations like those for age-related macular degeneration arose from the Human Genome Project and HapMap, several additional ongoing scientific initiatives are expected to yield similar biomedical impact. These include ENCylopedia Of DNA Elements (ENCODE), 1000 Genomes Project, Human Microbiome Project, and the Cancer Genome Atlas. As nurses play integral roles in generation of new knowledge and clinical realization of novel technologies, understanding significance of these efforts can help drive their clinical translation and application.

ENCODE

ENCODE is a public research consortium for identification of all parts of the human genome (within each gene) that regulate normal physiologic and biologic functions (National Human Genome Research Institute, 2008). Findings from ENCODE have shown that regions of the human genome previously thought to have no obvious or direct protein coding function are extremely important in gene regulation; expansive areas of intronic 'junk' DNA actually regulate protein production in distant genes and chromosomes (The ENCODE Project Consortium, 2007). Demonstrating enormous complexity, new structural categories are realized through ENCODE; instead of clear translation boundaries between exons and introns, there are alternative start and stop sites that have temporal and spatial uniqueness. When completed, ENCODE will demonstrate how an individual's biologic make-up may be linked to a person's responses in health, illness, and injury states.

1000 Genomes Project

Through HapMap and GWAS efforts, common genetic variants have been identified that contribute to complex chronic diseases. But the effects of those identified variants account for very little, in terms of risk, of developing a particular trait or phenotype. For example, obesity GWAS research identifies two common variants in the FTO and MC4R (melanocortin-4 receptor) genes carried by ~20% of those of European-descent. These variants exert physiologic effects on Body Mass Index but account for only 2% of adult variability of the trait (Frayling et al., 2007; Loos et al., 2008). Rare and difficult to find genetic variants may explain more of the variability in BMI, but are not

currently known. Sequencing the genomes of more individuals will allow for identification of rare variants with greater and more sizeable genetic effects (The 1000 Genomes Project Analysis Group and Steering Committee, 2008). In September 2008, an international consortium launched the 1000 Genomes Project to generate an extensive catalogue of human genetic variation capable of increasing discoveries of new biology from a population perspective.

Human Microbiome Project

Understanding how bacterial colonies interact in our bodies is increasingly important (Hsiao & Fraser-Liggett, 2009). Bacteria and the colonies with which they live interact elegantly and intricately in contexts of greater host micro-environments. Recently, presence of specific types of bacteria in gut flora were found to predict occurrence of Type 1 Diabetes in experimental mouse models (Wen et al., 2008). Although symbiotic bacteria outnumber human body cells by 10:1, the species living upon and within us have yet to be cataloged and information on how they communicate with each other and their human hosts is unknown (Turnbaugh et al., 2007). Using genomic sequencing, analyses of microbes from intestinal epithelial tissue in animals and individuals with obesity, intestinal disease, and cancer identified unique microbial profiles (Chu et al., 2004; Eckburg & Relman; Turnbaugh et al., 2006). To ascertain the extent that microbial communities participate in health and disease, the NIH Roadmap for Medical Research established the Human Microbiome Project (HMP) (The Human Microbiome Project, 2007). A global consortium, the HMP will provide genome sequence for floral communities across various human epithelial tissues. Initial data

identify great diversity within and across individuals dependent on tissue sampling site (Grice et al., 2008).

Cancer Genome Atlas

In 2007, the National Cancer Institute and the National Human Genome Research Institute began large-scale genome sequencing of human tumor cells from cancer cohorts to provide a comprehensive catalogue of malignancy abnormalities (Collins & Barker, 2007; The Cancer Genome Atlas, 2008). Characterization of glioblastoma, a common brain malignancy in adults noted for poor survival outcomes, yielded molecular profiles that may be used to reclassify and target clinical treatments (The Cancer Genome Atlas Research Network, 2008). Continued progress with other common tumor types such as ovarian and pancreatic cancer are revolutionizing how molecular derangements in malignancies are understood, and are expanding diagnostic, prognostic, and treatment options.

Genetic Risk Assessment

Human genetic variation holds great promise in predicting the development of costly chronic and preventable diseases. For example, carriers of Single Nucleotide Polymorphism (SNP) risk alleles in the Transcription Factor 7-like 2 (TCF7L2) gene have significantly increased lifetime risk of developing diabetes (Helgason et al., 2007; Prokunina-Olsson et al., 2009). Using the information contained in a person's genetic constitution for these purposes is increasingly attractive given that recent Genome-Wide Association Studies (GWAS) delineate numerous novel and quantifiable genetic risks for

common health conditions including heart disease, depression, colorectal cancer, and osteoporosis (Figure 7) (McPherson et al., 2007; Scott et al., 2007; Tomlinson et al., 2007; Wellcome Trust Case Control Consortium, 2007).

Of particular interest is if the SNPs identified in GWAS reports can be used in preventive medicine, to predict illnesses. Sponsored by the Agency for Healthcare Research and Quality (AHRQ), the U.S. Preventive Services Task Force (USPSTF) evaluates health screening mechanisms for efficacy, cost, and accuracy, and includes well known examples of screening standards (Atkins, Fink, & Slutsky, 2005; Harris et al., 2001; U.S. Preventive Services Task Force, 2002a, 2002b, 2003a, 2003b, 2004, 2005). Presently, there is not enough evidence to guide a USPSTF review of using genomic SNP sequence for preventive screening (Burke & Psaty, 2007; Gwinn & Khoury, 2006; Khoury, Yang, Gwinn, Little, & Dana Flanders, 2004). For example, many gene-gene and gene-environment interactive effects are unknown, attributable risk over one's lifetime is small to modest for many SNPs, and population SNP allele frequencies are not yet fully established (Janssens et al., 2007; Khoury, Little, Gwinn, & Ioannidis, 2007). Further contributing to delays in clinical translation are inadequate experimental designs, biased or erroneous interpretation of scientific data, and public dissemination of results from less rigorous research (Little et al., 2002; Moonesinghe, Khoury, & Janssens, 2007). In order to properly inform a USPSTF investigation, novel genetic variant associations must be consistently replicated across various settings (Burke & Psaty, 2007).

Knowledge gaps aside, the most heated debates involving SNP genetic testing concern provision of maximal benefit while limiting harm to patients (Khoury, Gwinn, Burke, Bowen, & Zimmern, 2007). Possible adverse unintended consequences of SNP

genomic profiling include increased anxiety stemming from a test that does not impact health outcomes and excessive financial cost for little clinical advantage (Burke & Zimmern, 2004). Equally important is need for scientific data demonstrating how patients will use personalized genetic risk information once they receive it (McBride & Brody, 2007; Thompson, 2007).

As mentioned previously, several companies market and sell genomic "risk" profiles directly to consumers (Burke & Press, 2006; Janssens et al., 2008). Proposed advantages of direct-to-consumer genomic profiling are increased availability, privacy, convenience, and enhanced market translation of molecular research advances (Goddard et al., 2007). Test results are interpreted and communicated to customers via a report that is mailed to them or accessed via the Internet. Detailed test interpretations and lifestyle recommendations may also be provided. Regulatory protection against false health claims for consumers using these services is a key deficiency as genomic technologies flood healthcare markets (Katsanis, Javitt, & Hudson, 2008; The Secretary's Advisory Committee on Genetics Health and Society, 2008).

To enhance accurate clinical translation of SNP associations, numerous resources are available to guide how biologic information can be used (Table 18). The Centers for Disease Control has developed and maintained *HuGENet* (Human Genome Epidemiology Network), a research database similar to *The Cochrane Collaboration Reviews*, where results of population-based gene-environment associations can be searched and obtained (Higgins et al., 2007; Lin et al., 2006; Seminara et al., 2007). Launched in 2004, The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) is an independent and multidisciplinary panel that critically evaluates evidence

supporting use of genomic tests in clinical practice through assessment of analytic validity, clinical validity, and clinical utility (Teutsch et al., 2009). Genetic test recommendations are available for a variety of conditions, including venous thromboembolism, breast cancer, and Lynch syndrome (HNPCC). Building on the foundations of EGAPP, The Genomic Applications in Practice and Prevention Network (GAPPNet) brings together more collaborative stakeholders in order to better outline and disseminate current knowledge; develop an evidence-based recommendation process for review of newly released genetics/genomics technologies; translate research into real-world dissemination; and develop comprehensive education, outreach, and surveillance programs (Khoury, Feero, et al., 2009). Professional organizations are also assuming positions of leadership by clarifying a clinician's role when managing patient inquiries about genomic profiling. For example, The American Society for Human Genetics issued a position statement outlining scope of clinical services that can be safely and legitimately provided to consumers (Hudson, Javitt, Burke, & Byers, 2007).

Diagnostics

Nurses can expect that technology-driven increases in diagnostic capacity will yield dramatic advancements for Personalized Medicine. Efficient diagnosis is presently a benchmark standard for healthcare quality and patient safety, and improved specificity and sensitivity for clinical decision-making is a key feature of many programs of research (Bissonnette & Bergeron, 2006; Dietel & Sers, 2006; Institute of Medicine, 2001; Snyderman & Langheier, 2006). The ability to generate and analyze enormous amounts

of unique biologic data is fueling three fields of clinical application: infectious disease, cancer, and biomarker discovery.

Infectious Disease

In prescribing antimicrobial agents, the inability to reach a definitive clinical diagnosis is a significant challenge. Diagnostic certainty in treating infectious disease decreases patient inflammatory responses, transmission risks, and harmful exposure to clinicians (Bissonnette & Bergeron, 2006; Diekema et al., 2004; McGowan & Tenover, 2004; Raoult, Fournier, & Drancourt, 2004; Tenover, 2006; The Alliance for the Prudent Use of Antibiotics, 2005). However, culturing of patient specimens typically requires 1-2 days for results, and until they are obtained, incorrect use of broad-spectrum prescriptive agents yields high pharmaceutical costs and the formation of antibiotic-resistant bacterial strains.

Genomic science is revealing why it is so challenging to effectively identify organisms resulting in a patient's clinical deterioration. Microbial genome sequence comparison shows evidence of horizontal gene transfer, resulting in enormous differences across and between singular pathogenic strains, shattering standard dogma of bacterial classification and clinical treatment (Fraser & Rappuoli, 2005). Applying these findings to clinical settings, it is hoped that ultra-fast sequencing of microbial genomes from infectious specimens can provide agent identification in 1-2 hours, versus the traditional culturing standard of 24-48 hours (Bissonnette & Bergeron, 2006).

Cancer

As a professional discipline, oncology currently experiences the most clinical progress in working towards Personalized Medicine, where molecular tumor profiles can be used to correlate targeted treatment regiments. Classic examples include breast cancers expressing human epidermal growth factor receptor-2 protein (HER2/neu) and chronic myelogenous leukaemia with positive Philadelphia chromosome status. Molecular understanding of both conditions has yielded extraordinarily successful response rates to drug therapy with the monoclonal antibody Herceptin, and tyrosine kinase inhibitor Gleevec. These therapeutic agents target specific and clinically measurable genetic changes (Fischer, Streit, Hart, & Ullrich, 2003). Further expansion of molecular cancer profiling beyond these examples is being driven by the Cancer Genome Atlas Project (see above) and high-throughput screening technologies (Ludwig & Weinstein, 2005; Srivastava, 2006).

As with other areas of genomic scientific advancement, translation into clinical treatment and screening mechanisms is proving challenging. Presently, a formidable challenge preventing Personalized Medicine from being realized in cancer care is reconciling the unexpected difference between meaningful clinical diagnostic standards with personalized genomic tumor profiling (Ludwig & Weinstein, 2005). For example, genetic and genomic molecular profiles challenge current oncologic survival and treatment curves, necessitating redefined scoring criteria for the Tumor, Nodes, and Metastasis (TNM) staging system, the widely used clinical grading system for cancer diagnosis stratification (Lam, Shvarts, Leppert, Figlin, & Belldegrun, 2005; Leong, 2006; Nguyen & Schrump, 2006; Piccaluga et al., 2008). Also contributing to the translation

gap is the need for improved clinical research standards when validating positive scientific findings or performing cross-comparison analyses across different study populations. Spanning both research and clinical settings—differences in tumor specimen collection, laboratory processing and testing procedures, and clinical phenotype documentation are proving to be a significant source of confounding variability when trying to validate high-throughput genomic screening signals (Compton, 2007; Srivastava, 2006).

Biomarker Discovery

Nurses are widely familiar with biomarkers, or singular proteins and molecules in body fluids associated with diseases, as a way to facilitate personalized approaches to patient health. The linking of biomarkers to clinical outcome measurements presently represents a fruitful area of biologic research, and efforts can be classified into genetic, proteomic, antigen and auto-antibody classes (Srivastava, 2006). Powerful molecular approaches, such as microarray platforms, are fueling biomarker discovery because of technological ability to simultaneously evaluate thousands of molecules within and across numerous samples and patients (He, 2006). In the coming years, biomarkers will contribute greatly to Personalized Medicine by providing increasingly precise mechanisms of disease detection, prognostication, and therapeutic monitoring (Rai, 2007). Presently, the technology-driven increases in data volume and precision have yet to replace less accurate and widely used screening tests, such as the prostate-specific antigen, and ovarian cancer antigen-125 tests. A chief reason for biomarker adoption delay is the vast amount of time and work that is required to appropriately screen and

validate initial findings before reliable reproducibility is obtained (Ransohoff, 2004). Development chronology occurs over many years with five distinct basic and clinical research development phases: (1) preclinical discovery, (2) assay analytical validation, (3) case/control cohort investigation, (4) longitudinal observation, and lastly, (5) prospective case/control investigation (Pepe et al., 2001). Stage progression occurs in a "funnel" format, where initial screens of many molecules are examined and eliminated in order to meet rigorous specificity and sensitivity criteria, while also demonstrating potential for predictive capacity (Srivastava, 2006). Poor reproducibility early on prohibits further application and progression to clinical translation.

Pharmacogenomics

Pharmacogenomics evolved from pharmacogenetics, a field established 50 years ago when it was demonstrated that a person's genetic inheritance could effect drug metabolism (Alving, Carson, Flanagan, & Ickes, 1956; Evans & Relling, 2004; Meyer, 2004). Building on the pharmacogenetic paradigm of one-gene and one-drug, pharmacogenomics studies how numerous genes interact with each other and the environment (Evans & McLeod, 2003; Goldstein, Tate, & Sisodiya, 2003; R. Weinshilboum, 2003; R. M. Weinshilboum & Wang, 2006). Presently, pharmacogenomic science understands and predicts adverse reactions to medical therapeutics based upon an individual's DNA sequence (Ginsburg, Konstance, Allsbrook, & Schulman, 2005; A. D. Roses, 2004). Pharmacogenomics is projected to have broad clinical utility in multifactorial diseases in order to reduce adverse drug reactions (Phillips, Veenstra, Oren, Lee, & Sadee, 2001).

A recent illustrative case study for Personalized Medicine is warfarin (Coumadin), a frequently prescribed anticoagulant. Warfarin demonstrates costly challenges such as limited therapeutic windows, large dosage differences across individuals, risk for serious bleeding sequelae, inability to estimate patient drug-responses from medical and physical criteria, and need for frequent International Normalized Ratio (INR) monitoring via phlebotomy (Daly & King, 2003). Traditional dosing factors incorporated include diet, age, gender, dietary intake, body weight, and use of other medications (Wadelius et al., 2007). More recently, variation in genes encoding the hepatic microsomal enzyme cytochrome P450 2C9 (CYP2 C9) and the vitamin K epoxide reductase complex 1 (VKORC1) demonstrated influence respectively on warfarin pharmacokinetics and pharmacodynamics (Higashi et al., 2002; Rieder et al., 2005). Single Nucleotide Polymorphisms (SNPs) in CYP29 and VKORC1 genes explained 35-50% of dosage difference variability among patients—leading to development of a clinical genetic test for alleles resulting in altered dose requirements (Aquilante et al., 2006; Gage et al., 2004; Geisen et al., 2005). On August 16, 2007, the Federal Drug Administration (FDA) updated the warfarin label to support combined CYP 2C9 and VKORC1 genetic testing, with up to 35% of individuals benefiting from lower starting doses (FDA Press Conference on Warfarin Transcript, 2007).

Though the FDA's position incorporating genomic information prior to dosing is indicative of movement towards pharmacogenomics in Personalized Medicine, it is not a formal recommendation to conduct CYP2 C9/VKORC1 genetic testing prior to anticoagulation. Furthermore, warfarin is the first mainstream drug for which the FDA provides genomic recommendations and is not yet successful from a combination

perspective of uptake, utility, and cost perspectives. Recent warfarin genetic testing studies show that CYP2 C9/VKORC1 is predictive of INR level but not bleeding or thrombotic sequelae, and that cost effectiveness remains a key concern (Eckman, Rosand, Greenberg, & Gage, 2009; Millican et al., 2007; Rieder et al., 2005). As a result, Centers for Medicare and Medicaid Services (CMS) could not conclude from available evidence that warfarin pharmacogenetic testing was better than existing care coverage, and denied reimbursement for CYP2/C9 testing on May 4, 2009 (Jensen et al., 2009). Opening the door for decision reversal if clinical trials demonstrate significant and cost-effective clinical utility, CMS issued the decision "pursuant to Coverage with Evidence Development (CED)." For widespread clinical uptake in the U.S., use of the CYP2C9/VKORC1 genetic test (currently priced at $300-$500) in current clinical trials will need to demonstrate ability to affordably improve current standards of care through prevention of adverse bleeding and thrombotic events for CMS coverage to be issued (Figure 9).

Implications and Opportunities for Nursing in Personalized Medicine

Personalized Medicine, or healthcare practices tailored to a person's genetic make-up, is poised to enter healthcare stemming from unprecedented technology advances. Implementation and uptake of Personalized Medicine is presently hampered by lack of clinical application evidence and could benefit greatly from nursing's patient-focused approach. Across the four domains outlined in this paper, nurses are uniquely positioned to reconcile the competing perspectives of scientific innovation with practical

matters of clinical adoption and dissemination. The expertise of nurses across all research, education, and practice roles could be harnessed to address the global nature of the clinical translation challenges outlined thus far.

Research

Nurse scientists and scholars presently have limitless possibilities for which to become involved in the translation of Personalized Medicine from research settings to clinical application mechanisms. For example, academic nursing leaders can evaluate their current research programs and integrate any number of genetics/genomics themes into institutional and individual grant submissions to fill recently identified priorities of evidentiary need (Khoury et al., 2008; Khoury, McBride et al., 2009; NIH Consensus Development Program, 2009). Individually, interested nurse scientists can develop expertise to incorporate genetics/genomics into their current programs of research (Barr et al.; Ersig, Williams, Hadley, & Koehly, 2009; Meilleur et al., 2009; Voss et al., 2008). This can be done by collaborating with knowledgeable colleagues in multidisciplinary settings or by obtaining supplemental training to add genetic questions, outcomes, or markers to current scholarly efforts. Because many nurse scientists are currently most familiar with research incorporating the latter three Personalized Medicine domains (genetic risk assessment, diagnostics, and pharmacogenetics), this section will highlight nursing research opportunities presented by recent biologic initiatives.

The ENCylopedia Of DNA Elements (ENCODE), 1000 Genomes Project, Human Microbiome Project, and the Cancer Genome Atlas represent key opportunities for nurse scientists to ask clinically relevant scientific questions. As nurses have long appreciated

the uniqueness of a patient's biologic make-up, they can empower the scientific community to pair clinically observed phenotypes with generated sequence data. For example, nurses can link newly identified genetic elements (silencers, enhancers, and promoters) from ENCODE with knowledge of a patient's environment and clinical status to better understand, predict, or manipulate functional health outcomes in various diseases. From the 1000 Genomes Project, nurses can more finely search genetic data to select and study effects of rare genetic variants that may be responsible for biologic traits in their patient populations. Nursing scientists can ensure the Human Microbiome Project findings are translated into clinically useful tools by cross-comparing microbial sequence from a wide range of patients (varying health and disease states, age groups), aligning clinical infection measurements with microbial sequence results, developing standards for clinical specimen sampling protocols, identifying what quality control mechanisms are needed, and developing affordable models of health care access for clinical use (Bryant, Venter, Robins-Browne, & Curtis, 2004; Burnett, Henchal, Schmaljohn, & Bavari, 2005; Call, 2005; Loy & Bodrossy, 2006; Simon, 2003; Tenover, 2007). Cancer Genome Atlas sequence will become most useful if nurse researchers can help oncologic scientists to create genomic profiles that correlate meaningfully with clinical phenotype characteristics, such as symptom presentation and disease progression, for each tumor type.

Education

Because genetics and genomics science is accelerating and its impact on health is growing in significance, nursing educators can prepare for Personalized Medicine by

nurturing development of genetic/genomic expertise at their institutions and in their students. Nurses have been at the fore-front of genetic education initiatives for many years, and efforts in designing basic genetic/genomic professional competencies, curriculum guidelines, and nursing faculty resources are well noted (Calzone et al., 2009; Greco & Salveson, 2009; Jenkins & Calzone, 2007; Lewis, Calzone, & Jenkins, 2006; Prows, Glass, Nicol, Skirton, & Williams, 2005; Read, Dylis, Mott, & Fairchild, 2004; Seibert, Edwards, & Maradiegue, 2007). Cumulatively, these efforts have been so effective that for 2009 credentialing, the American Association of Colleges of Nursing has integrated the genetic competencies into three of the nine Essentials of Baccalaureate Education: liberal education for baccalaureate generalist nursing practice (I), clinical prevention and population health (VII), and baccalaureate generalist nursing practice (IX) (American Association of Colleges of Nursing, 2008). Despite these tremendous advances, a key challenge presented by Personalized Medicine will be how to best prepare professional nurses to move beyond single-gene concepts to that of genomics, where patients will need extensive help in understanding complex and probabilistic health information spanning multiple genes and the environment (Guttmacher, Porteous, & McInerney, 2007). The difficulty of this transition faces not just nursing but all healthcare professionals, for which the Secretary's Advisory Committee in Genetics Health and Society is preparing a detailed report for the Health and Human Services Secretary in its 2010 Genetics Education and Training Task Force report (Secretary's Advisory Committee on Genetics Health and Society, 2009).

Practice

No other group of professional nurses will be as heavily impacted by the science of Personalized Medicine as clinically practicing registered nurses and advanced practice registered nurses. Because health promotion interventions and lifestyle management needs rely on genetic components of disease development, nursing clinicians are expected to translate genomic risk factor information into practical language for patients, families, and communities. Pursuant, the Essential Nursing Competencies for Genetics and Genomics were established in 2005 with 50 professional nursing stakeholder organizations endorsing minimal genetic/genomic services every practicing nurse should be capable of providing (Lewis et al., 2006). A similar consensus effort is currently underway for advanced practice nursing competencies (Seibert, Greco, & Tinley, 2009). When applying these competencies to recent scientific findings such as the Human Microbiome Project, practicing nurses can appreciate that despite their patients being enrolled in nutritional counseling or adhering to recommended guidelines, gene-environment effects of microbiotic floral communities may proffer great challenges to best clinical management efforts.

However, the most powerful translational promoter of Personalized Medicine will be the collaborations between nursing scientists/researchers and practicing nurses. For example, upon combining symptom profiles with genomic data, collaborating nursing teams can submit findings to scientific initiatives such as The Pharmacogenomics Knowledge Base (Pharmacogenomics Knowledge Base, 2009)**;** contributing to these efforts will ensure understanding how a patient's unique biology predicts response to medication or adherence to prescriptive requirements (Sangkuhl, Berlin, Altman, &

Klein, 2008). Similarly, advanced practice nurses or registered nurses who collaborate with molecular scientists in tertiary care settings can help guide adaptation and feasibility trials of molecular diagnostic tools like the 1-2 hour ultra-fast microbial sequencing platforms. As such, baccalaureate-prepared nurses who are interested in research, genetics, and the scientific process can seek clinical genetic/genomic research studies that are ongoing in their practice settings. For example, a motivated intensive care nurse in an academic research setting can quickly and powerfully organize and promote the sampling, recruitment, and enrollment of patients in a genomic investigation trial. The benefit to science from practicing nurses who work with senior scientific mentors in these ways is untold, and largely an untapped resource at present.

Concluding Remarks

This paper outlines how personalization of healthcare practices will be affected through continued waves of scientific knowledge about how an individual's DNA guides tailored healthcare interventions. Despite the presence of these opportunities and the great potential for improved health across the four domains, scientific initiatives outlined in this paper will fall short of desired outcomes without the commitment and global participation of the nursing community. As these technologic efforts expand and accelerate, it is hoped that professional nurses of all backgrounds heed this call to increase their practical involvement in the realization of Personalized Medicine. The country's 2.9 million Registered Nurses represent a valuable and untapped resource at the forefront of Personalized Medicine to translate biologic findings into practical clinical applications. Because nurses "hold the keys" to accurate patient observation and

healthcare practice dissemination, they are the foremost health discipline capable of bringing full investment in scientific advancement to fruition—yielding advanced health for patients, families, and communities.

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Table 17

Personalized Medicine Working Glossary

Term/Acronym	Definition*
Allele	Varying (or variant) forms of a gene at a specific location in the genome.
Analytic validity	Accuracy and reliability a genetic/genomic test detects a particular genetic characteristic.
Biomarkers	Biological molecules in blood or other body fluids whose parameters can be associated with disease presence and severity. Can be detected and measured by many different methods such as laboratory assays and imaging technology.
CNV	Copy Number Variation; where combinations of 2-3 nucleotides are continually repeated in non-coding portions of the genome, i.e.: CACACACACACACA.
Clinical utility	Degree with which a genetic or genomic test will provide benefit to patients after accounting for potential harms.
Clinical validity	Ability of a genetic/genomic test to detect or predict a clinical condition or outcome.
Deletion	A mutation caused by the removal of DNA from the chromosome. Deletions can be of any length, from one base pair to a large chromosomal segment (millions of base pairs).
Enhancer	Short stretch of regulatory DNA sequence that signals where transcription factors should bind. Enhancers modulate rate of transcription and can be found great distances away from the gene it regulates.
Epigenetics	Study of heritable differences in gene function which occur without changes in DNA sequence (i.e.: methylation patterns).
Exon	Region of a gene encoding for a particular portion of the complete protein.
Gene	The functional and physical unit of heredity passed from parent to offspring. Genes contain information for making a specific protein.

* Adapted from publicly available Department of Health and Human Services glossaries.

Table 17 Continued

Term/Acronym	Definition
Genetic Testing	Generic term for an array of techniques that analyze DNA, RNA or proteins for general health or medical identification purposes. Currently over 1200 tests are clinically available.
Genome	The entirety of an individual's genetic code, approximately 6 billion nucleotides comprising 23,500 genes.
Genomics	Scientific study of a genome—including any or all combinations of genes, their functions, and their interactions with each other and the surrounding environment.
Genotype	An individual's 2 alleles at a specific loci.
Haplotype	Combinations of SNP alleles located close to one another on a chromosome. If close together, haplotypes can be inherited as units or blocks.
HapMap	The Haplotype Map: a map of all inherited genetic variation (haplotypes) in the human genome.
Heterozygous	Having two different forms of a particular gene (AB).
Homozygous	Having two identical forms of a particular gene (AA).
Indel	An insertion/deletion polymorphism where AA, AB, BB yield: insertion/insertion, insertion/deletion, deletion/deletion.
Insertion	A mutation caused by the insertion of DNA from the chromosome. Insertions can also be of variable length (one to many base pairs).
Intron	Non-coding sequences of DNA that are removed from the RNA transcript prior to exportation from the nucleus.
Locus	The physical location of a gene or gene segment on a chromosome.
Loci	The plural of locus.
Methylation	Chemical reactions that place a methyl group (3 hydrogen atoms and 1 carbon atom) on the DNA nucleotide cytosine (C); presence of methylation silences genetic expression.

Table 17 Continued

Term/Acronym	Definition
Multifactorial Dx	Diseases caused by interactions of numerous genes with environmental factors. Examples include obesity, diabetes, heart disease and cancer.
Mutation	Permanent and structural alteration in DNA. Most cause little, if any harm. If in a critical location, such as the DNA repair genes in BRCA 1 and 2, can cause severe disease such as early onset cancer.
Negative Predictive Value	Probability that patients with a negative genetic/genomic test result will not get a specific disease or condition.
Phenotype	A patient's observable clinical and physiologic characteristics as a result of inherited genotype interacting with their environment.
Polymorphism	The existence of multiple genotypes in a population, at one locus. Variants are not due to mutations in DNA because they occur at a frequency greater than can occur by evolutionary (slow) means. Polymorphisms may take several forms, including SNPs, CNVs, and insertion/deletion (indel's).
Positive Predictive Value	Probability that patients with a positive genetic/genomic test result will get a specific disease or condition.
Promoter	Short stretch of regulatory DNA sequence that signals where transcription should start in a gene (for the RNA polymerase).
Sensitivity (clinical)	Percent of patients with positive genetic/genomic test result that are correctly identified as having the defined clinical trait.
Silencer	Short stretch of regulatory DNA sequence that signals where chromatin should become condensed. This blocks other enzymes from accessing the DNA strands to prevent transcription.
Specificity (clinical)	Percent of patients with a negative genetic/genomic test result that are correctly identified as *not* having the defined clinical trait.

Table 17 Continued

Term/Acronym	Definition
SNPs	Single Nucleotide Polymorphism(s). The difference of a single base pair at a specific position in the genome between 2 different individuals in a population. Most are inconsequential, but if in a coding region, may cause changes in gene efficiency and/or function.
Variant	Another word for polymorphism. There are different types of variants, such as SNPs, CNVs, insertion/deletion (indels), and RFLPs.

Table 18

Genetics and Genomics Scientific and Clinical Translation Resources

Resource Title	Reference Websites Locations
GENERAL INFORMATION	
Centers for Disease Control	www.cdc.gov
Current Drug Trials	www.clinicaltrials.gov/
Database of Genotype and Phenotype	www.ncbi.nlm.nih.gov/sites/entrez?Db=gap
Food and Drug Administration	www.fda.gov/
Genomic Careers for Students	http://www.genome.gov/27538514
Gene Tests	www.genetests.org/
Human Genome Epidemiology Network	www.cdc.gov/genomics/hugenet/
Illumina, Personalized Genome Service	http://www.everygenome.com/
NCHPEG	www.nchpeg.org/
NCI's Early Detection Research Network	edrn.nci.nih.gov/
NIH	www.nih.gov
NHGRI	www.genome.gov/
National Office of Public Health Genomics	www.cdc.gov/genomics/
Pharmacogenomics Knowledge Base	www.pharmgkb.org/
CLINICAL TRANSLATION	
EGAPP	www.egappreveiws.org
GAPPNet	www.cdc.gov/genomics/GAPPNet/index.htm
HuGENet	www.cdc.gov/genomics/hugenet/default.htm
PROFESSIONAL SOCIETY RECOMMENDATIONS*	
American Society for Human Genetics	www.ashg.org/pdf.dtc_statement.pdf
American College of Medical Genetics	http://www.acmg.net/AM/Template.cfm?Section=Policy_Statements&Template=/CM/HTMLDisplay.cfm&ContentID=4157
International Society of Nurses in Genetics	http://www.isong.org/about/ps_consumer_marketing.cfm
National Society of Genetic Counselors	http://www.nsgc.org/about/position.cfm#DTC

*Direct-to-Consumer Testing and Complex Disease Management

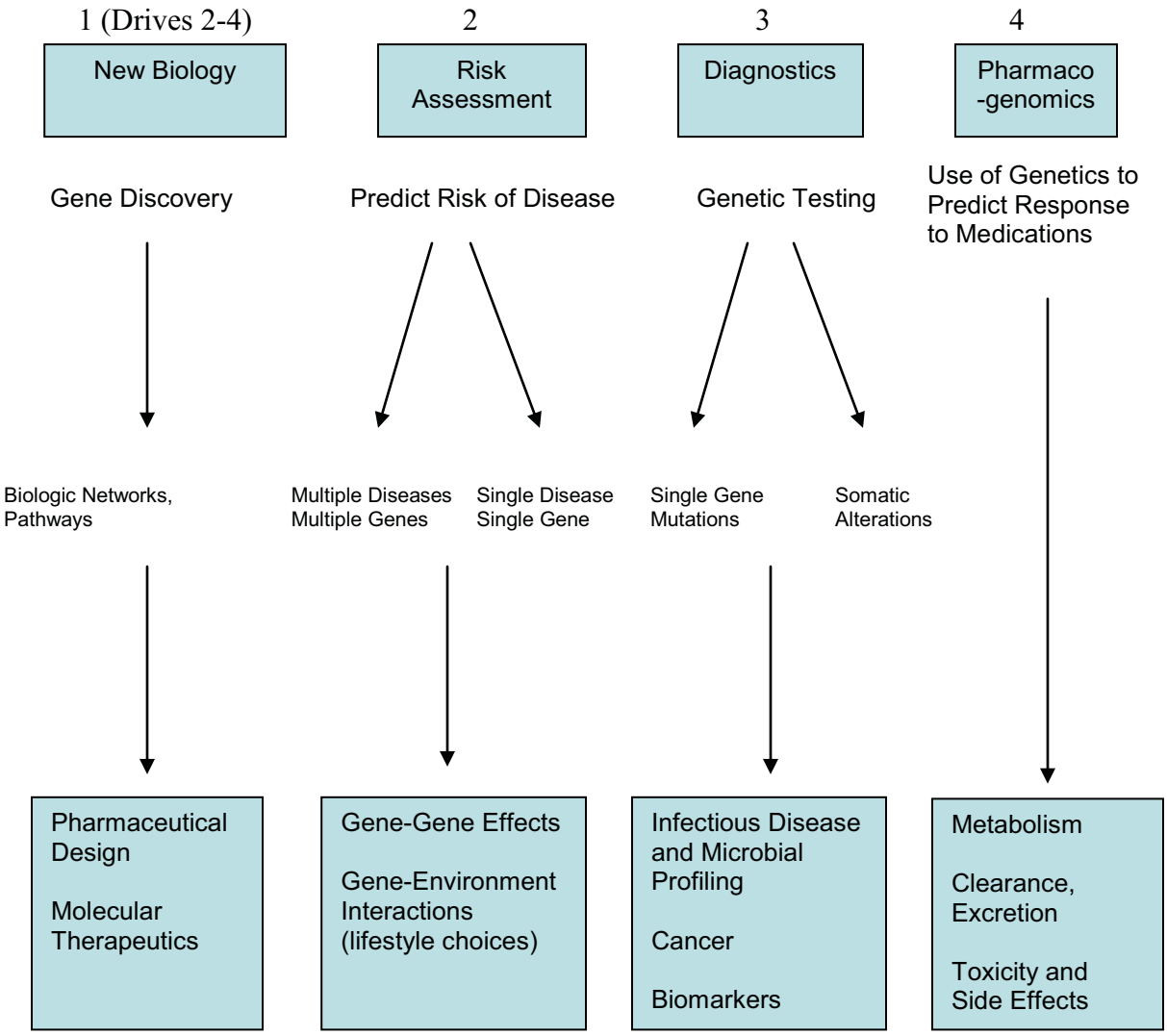

Figure 6. The Four Elements of Personalized Medicine.

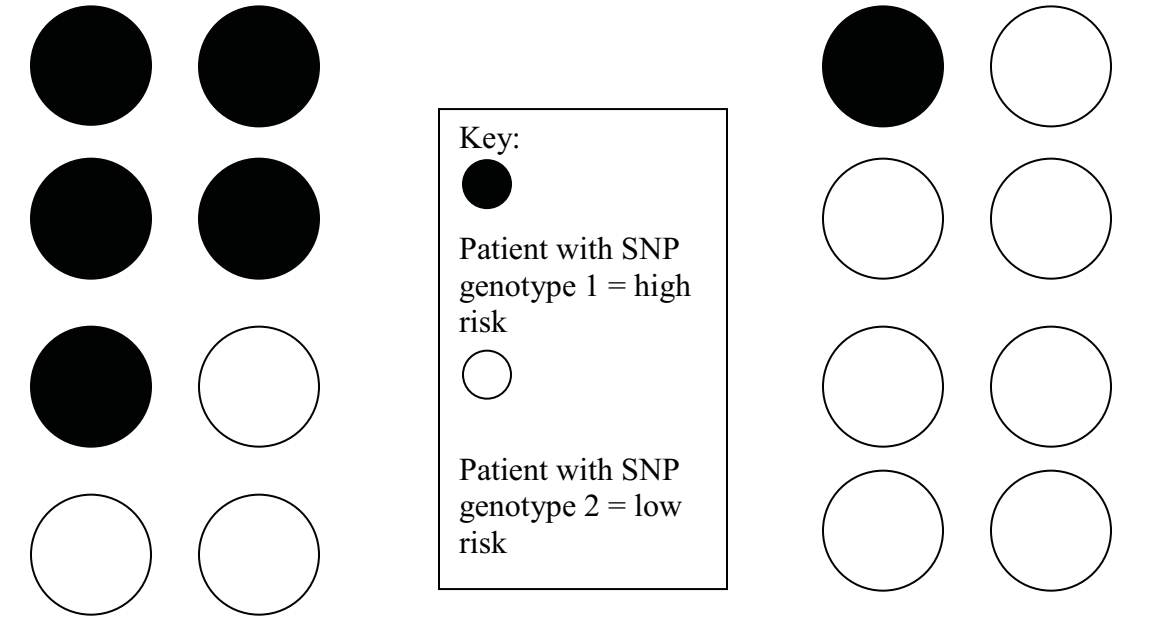

Figure 7. Basic Foundation of Genome Wide Association Studies.

Shown above is the basic model for Genome Wide Association Studies, where DNA from diseased patients is compared to healthy individuals to detect differences. Patient DNA is placed on a chip and millions of SNPs can be tested at once. This approach permits identification of genes that cannot be predicted by selecting candidates from known disease pathways, allowing for discovery of novel genes contributing to complex diseases.

Age-related Macular Degeneration (AMD) is a complex and multi-factorial disease, is the leading cause of blindness with 1.75 million affected Americans, and is projected to affect 1 in 4 individuals >75 years old (R. Klein, Peto, Bird, & Vannewkirk, 2004; Ting, Lee, & MacDonald, 2009). In 2004-2005, researchers using HapMap identified a genetic variant responsible for 50% increased risk of developing AMD in the Complement Factor H gene (Edwards, et al., 2005; Haines, et al., 2005; R. J. Klein, et al., 2005). Complement Factor H is part of the inflammatory complement cascade, and helped lend credibility to concurrent investigations on inflammatory pathways as causative mechanisms of AMD, such as those involving C-Reactive Protein (Seddon, Gensler, Milton, Klein, & Rifai, 2004; Seddon, George, Rosner, & Rifai, 2005). These research efforts permitted development of prospective assessments of the role of the contributory SNP in patients; findings validated the role of genetics and inflammation in AMD (Schaumberg, et al., 2006). Follow-up investigation ascertained interactive effects of the Complement Factor H variant with modifiable environmental risk factors, including Body Mass Index, smoking, regular aspirin intake, and dietary habits (Schaumberg, Hankinson, Guo, Rimm, & Hunter, 2007). This culminated in the formation of a large, randomized, controlled clinical trial enrolling 4,757 patients from 11 medical centers (M. L. Klein, et al., 2008). The multi-disciplinary collaborative effort, "The Age Related Eye Disease Study", ascertained pharmacogenetic effects of treatment with zinc and antioxidant supplements according to SNP genotypes, one of which being in the Complement Factor H gene.

Figure 8. Age-Related Macular Degeneration: From HapMap to Clinical Trial in <5 Years.

Despite strong scientific genetic evidence of efficacy, warfarin classically demonstrates the systematic, structural, and clinical evidentiary challenges awaiting pharmacogenomics in Personalized Medicine (Phillips & Van Bebber, 2006). Because tests must demonstrate high predictive value for adverse events, metabolically accurate dosing ranges and treatment effects will need to be established according to allele prevalence in populations (Smits et al., 2005). Study designs specific to population genetics do not reflect current standards for FDA drug evaluation and approval, where criteria are dependent upon 20th century infrastructure and measurements (Califf, 2004; Haga, Thummel, & Burke, 2006; Woodcock, Witter, & Dionne, 2007). Moving forward, a key concern is that randomized clinical trials cannot account for genomic variability across study participants, or clearly delineate patient groups per genetic responses to pharmaceutical agents. Common approaches to address the issue involve patient stratification according to race or ethnicity, but question of appropriateness is necessary as genetics research identifies serious flaws in these categories (Bamshad, Wooding, Salisbury, & Stephens, 2004; Doyle, 2006; Foster & Sharp, 2002; Lee, 2007). To better account for biotechnologic advancement, many proposals call for overhauled federal regulatory networks allowing improved focus on pharmacogenomic clinical research including: synchronized payment systems; genetic/genomic education for patients and healthcare providers; and clinical care focusing on an individual's dynamic probability for disease development (Califf, 2004). What may achieve this most quickly is integration across government networks to form clinically accurate, large-scale, prospective observational cohorts (A. Roses, 2007).

A barrier to large-scale clinical genetic research efforts is reluctance in wholly embracing research efforts, stemming from public fears of genetic discrimination. To better address these concerns, the Genetic Information Non-discrimination Act was passed in 2008. This legislation guarantees basic protection for individuals receiving genetic testing, so that they are protected against employer and health insurance discrimination practices (Slaughter, 2008). Limitations of the legislation are that it does not protect individuals from discrimination in the military or for long term care coverage determinations, in addition to considerations that the legislative language is not specific enough regarding what is considered a "genetic test" (Baruch & Hudson, 2008; McGuire & Majumder, 2009).

Figure 9. The Pharmacogenomic and Policy Implications of CYP 2C9 and VKORC1 Genetic Testing.

CHAPTER 7

CONCLUSIONS

Summary of Dissertation Research and Its Scientific Context

The overarching intent of the work performed in this dissertation research was to "bridge" seemingly disparate disciplines and worlds. As exemplar to this inspired path, the problem of clinical heterogeneity observed in cobalamin deficiency of older adults was investigated using genetic technology through a behavioral lifespan lens—a common perspective in nursing research. The theoretical underpinnings of vitamin B12 as a health concern extend back to the early 1800s from simple observations of confounding clinical trajectories that resulted in tragic loss of life. Three Nobel prizes were issued to individuals able to solve intricately complex puzzles of nature, permanently improving morbidity and mortality for all humankind. Collectively, as science continued its forward movement, the age of molecular genetics produced ability to clone cobalamin-related genes and manipulate model organisms for more sophisticated awareness of why cobalamin is an essential nutritional requirement.

Despite these tremendous and storied advancements, there is still not a clear understanding as to why certain individuals are severely affected by small changes in cobalamin status, while others remain completely unaffected. Not all "low" serum cobalamin and metabolite test results paint the clinical picture of deficiency, and not all

"normal" test results indicate that metabolic sufficiency is present (Carmel 2000; Carmel et al., 1999). Clinical signs alone cannot be the only metric, as severe metabolic crises can occur in their absence and may be reversible with supplemental therapy (Carmel et al., 1995; Carmel, Sinow, & Karnaze, 1987).

Study Context Within Broader Literature Base

The overarching hypothesis of this dissertation research was that there exists a genetic basis to the phenomenon of clinical heterogeneity observed in cobalamin deficiency affecting older adults. The use of a well-characterized and measured phenotype is instrumental in exploring genetic associations; otherwise, carefully generated genetic data are at risk of being spurious or meaningless upon combination with clinical data. Use of the Women's Health and Aging Study Cohorts (WHAS) permitted examination of a sophisticated combination of clinical traits that either would not have been possible, or at the very least, quite costly to prospectively collect the amount and type of data used in this study.

Although the WHAS 1 and 2 publications on physical disability trajectories are widely known and number into the several hundreds, linking the original WHAS data to a genetics focus is not nearly as common. For genetic researchers that have realized that carefully catalogued phenotypes are contained within these extraordinary epidemiologic research initiatives, genetic-focused analyses have been performed. Walston and colleagues examined the role of genetic variation in the interleukin-6 gene and its relationship with circulating inflammatory biomarkers, and muscle, weakness, and frailty measurements in WHAS 2 subjects (Walston et al., 2005). Seibert and colleagues

examined polymorphic variation in the human myostatin (GDF-8) gene and its relationship with various strength measures in WHAS 1 and WHAS 2 participants to ascertain a genetic basis of skeletal mass decline (Seibert, Xue, Fried, & Walston, 2001). And recently, genome-wide association studies were undertaken to evaluate the effect of genetic variation in iron metabolism genes on serum iron concentrations in WHAS 1 and 2 subjects (Tanaka et al., 2010).

However, the most closely relevant effort to the dissertation research has been the recent work by Matteini et al. in evaluating the WHAS 1 and 2 cohorts for susceptibility to frailty through study of genetic variation in one-carbon metabolism genes (Matteini et al., 2008; Matteini et al., 2010). In the 2008 and 2010 reports, SNPs from vitamin B12 candidate genes were selected, genotyped, and analyzed for their relationship with serum methylmalonic acid and frailty syndrome. Frailty syndrome was characterized as WHAS participants having at least 3 of the 5 following indicators: slow walking speed, weak hand grip strength, decreased energy level, decreased body mass, and low physical activity. Although the dissertation results corresponded to Matteini's findings of no SNPs being associated with methylmalonic acid, data related to the physical performance of the WHAS 1 and 2 participants was somewhat divergent. For example, rs2267163 (in intron 5) was found to have significant association with frailty syndrome according to Matteini and colleagues. In the dissertation study, neither rs2267163 nor the SNPs in close proximity to it were significantly associated with the continuous functional performance outcomes, hand grip strength and walking speed. Instead, a small cluster of several SNPs in the five prime region of the gene (intron 1-intron 3) were identified as being significant.

Reasons for these differing results may lie in the varying analytic approaches used and the population sample differences between the research efforts. Whereas this dissertation study directly analyzed continuous traits of WHAS participants in analyses of variance and covariance, Matteini and colleagues collapsed five different continuous physical performance measurements (walking speed, hand grip strength, energy level, body mass, and physical activity) into dichotomous outcomes (slow, weak, decreased energy, decreased muscle density, low activity) to create a 'frail' profile before entering them into multivariate logistic regression models.

Additionally, because Matteini's genotyping used the Illumnia BeadArray genotyping technology, analysis was restricted to WHAS 1 and 2 subjects with ample remaining amounts of native genomic DNA for chip typing requirements (i.e., at least 100 ng). As a result, the final sample size available for analysis was $n = 326$ Caucasian and $n = 90$ African American subjects, representing 35.6% of the total 1,167 WHAS 1 and 2 subjects who consented to phlebotomy. Furthermore, results are only reported for Caucasian subjects since African Americans were too underpowered to detect any effects, yielding a final sample size that was 27.9% of the initial WHAS 1 and 2 subject pool who had blood samples drawn. Because the dissertation used whole genome amplification to augment remaining genomic material, many more WHAS 1 and 2 subjects' DNA were able to be analyzed than in Matteini's reports. Furthermore, use of two-way analysis of variance and covariance in the dissertation study permitted evaluation of SNP effects across all 789 African Americans and Caucasian WHAS subjects collectively, which provided greater statistical power in assessing presence and impact of genetic differences.

Study Rationale

The broader goals of WHAS 1 and 2 research initiatives are to identify which functional determinants may (or may not) contribute to physical disability, the frailty syndrome, and the development of comorbidities in older adults (Fried, Ferrucci, Darer, Williamson, & Anderson, 2004). Availability of genetic data greatly enhances the biologic understanding of these processes. Thus, the dissertation aims of evaluating genetic variation in the transcobalamin II and transcobalamin II-receptor genes on cobalamin deficiency parameters in WHAS subjects were selected to better understand factors leading to poorer health status in aging individuals.

Ascertaining biologic correlates to functional determinant outcome measures has been a fruitful area of WHAS research. For example, several WHAS 1 and WHAS 2 studies outline a variety of biologic, socio-demographic, and psychologic factors that affect the dissertation's functional performance outcomes, hand grip strength and walking speed. Presence of reactive oxygen species in skeletal muscle, as measured by serum protein carbonyls, was independently associated with poor hand grip strength (Howard et al., 2007). Hand grip strength measurements were shown to be significant predictors of cardiovascular disease, respiratory disease, and total mortality for up to 5 years (Rantanen et al., 2003). Decreased serum insulin-like growth factor-I and elevated serum interleukin-6 concentrations were associated with slower walking speeds (Cappola et al., 2003). Tobacco smoking and cognitive decline were found to affect physical decline trajectories, including walking speeds, independent of potential confounders (Atkinson et al., 2005). And lastly, although presence of pain did not predict walking difficulties for WHAS subjects, report of widespread musculoskeletal pain was found to be associated

with onset and deterioration of walking capacity (Leveille, Bean, Ngo, McMullen, & Guralnik, 2007). Similar to these published efforts, the dissertation study was designed to better identify those biologic factors contributing to functional impairment and decline of health status across cobalamin-related outcomes in WHAS 1 and 2 subjects.

The Dissertation's Candidate Genes

Transcobalamin II and the transcobalamin II-receptor were candidate genes selected from a careful evaluation of cobalamin pathophysiology in humans. In selecting both, they represented the system of serum transport and target cell uptake for all biologically active cobalamin in the body, and thus the "least common denominator" that could provide the most information about an individual's clinical deficiency from a genetic perspective. The transcobalamin II-receptor gene was only recently identified, the protein purified and structure elucidated (Quadros, Nakayama, & Sequeira, 2009). Until this time, very little information on the transcobalamin II-receptor was available in public databases and published reports. At the time of dissertation writing, no information existed about the contribution of genetic variation in the transcobalamin II-receptor gene to clinical conditions affecting humans. Thus, the dissertation research is the first examination of association between transcobalamin II-receptor genetic variation and clinical outcomes in individuals. Knowledge obtained from the dissertation contributes to a better understanding of the importance of this candidate gene to human health, while also generating more focused targets for functional laboratory analyses. Understanding which part of the transcobalamin II-receptor gene yields increased cobalamin uptake for

certain individuals as compared to others may enhance clinical care treatments and help prevent deficiency from occurring.

There were also other interesting genes considered for evaluation, had the dissertation study been adequately powered to investigate more genes. Other possible candidates included intrinsic factor (the molecule pairing with dietary cobalamin in the duodenum) and its receptor on ileal enterocytes. Representing a 'class' of targets are the cobalamin-cofactor synthesis groups, nicknamed "cbl." After the transcobalamin II-receptor endocytoses cobalamin into a lysosome, the cobalamin is shuttled through a series of cytoplasmic cbl compartments where chemical reactions prepare cobalamin for use in the mitochondria or cytoplasm (Coelho et al., 2008). There are eight cbl complementation groups (cblA, cblB, cblC, cblD, cblE, cblE, cblF, cblG, cblH), and candidate selection of cbl groups responsible for intracellular trafficking to the cytoplasm (cblF, cblC, or cblD) or mitochondria (cblD, cblA, or cblB) would also have been meaningful candidates for investigation.

The Dissertation's Cobalamin-Deficiency Phenotype Traits

Selection of the phenotype characteristics representative of cobalamin deficiency was undertaken with the intent to strike a balance between the classic "hallmarks" and the chronic, subtle "preclinical" symptoms. Hemoglobin concentrations, mean corpuscular volumes (MCV), and the biochemical assays including serum cobalamin, methylmalonic acid, and total homocysteine are all regularly utilized components in deriving a clinical diagnosis of cobalamin deficiency (Beck, 2001). However, subtle signs of cobalamin deficiency, such as mood and sleep alterations, gait disturbances, peripheral insensitivity,

and fatigue, also accompany the subclinical metabolic definitions that are yielding higher prevalence rates identified in older adult populations (Carmel, 2000). It was important for the dissertation research to include assessment of quantitative traits that included these subclinical aspects of the cobalamin deficiency trajectory, including depression, peripheral insensitivity, physical strength, and walking capacity. That being noted, these outcomes were objective measurements of biological and physical traits. It also would have been interesting to explore the genetic basis of subjective phenomena that is associated with cobalamin deficiency, such as fatigue symptoms and perceived quality of life.

Biochemical Metabolites

The first data-based paper in this dissertation ascertained if genetic variation in the transcobalamin II and transcobalamin II-receptor gene was associated with any of the biochemical parameters traditionally used in making a clinical diagnosis of cobalamin deficiency. Results identified two significant genetic variants that were very promising for future research, because they were both located in genetic positions responsible for amino acid changes. The first coding variant, rs2336573, in exon 4 of the transcobalamin II-receptor gene, encodes an arginine to glycine switch (R220G) and was associated with serum cobalamin after adjusting for creatinine clearance. The second coding variant, rs9621049, in exon 7 of the transcobalamin II gene (F348S), was associated with homocysteine concentrations after adjusting for creatinine clearance, serum folate, and serum cobalamin.

In evaluating the transcobalamin II-receptor genotype group differences of rs2336573, even though none of the mean serum cobalamin values were "low," there was a 112-115 pmol/L difference between the CC genotype and the other genotype groups. However, measurement of serum cobalamin reflects not just the biologically active B12 in the circulation that is bound to transcobalamin II, but also includes the longer circulating bound forms, transcobalamin I and III (20-30 days). Since approximately 80% of serum cobalamin measures the cobalamin attached to transcobalamin I and III, it is not clear how much different forms of the transcobalamin II-receptor on cell surfaces would impact serum cobalamin levels. Additional research efforts should analyze the association of rs2336573 with measures of holotranscobalamin, the fraction of B12 that is only bound to transcobalamin II. In doing so, there would be a more direct biologic link between SNP influence, substrate availability in serum, and uptake capacity by the receptor on the cell surface.

The second key biochemical finding was the association between rs9621049 in the transcobalamin II gene with homocysteine concentration. There were significant differences between the three genotype groups independent of race. Three percent of elderly women belonging to the TT genotype group had the highest mean homocysteine concentration (12.5 μmol/L), which approaches the clinically significant threshold of 'elevated' at 15.0 μmol/L. Homocysteine elevations, even in the absence of metabolic cobalamin deficiency in tissues, may be a risk factor for development of cardiovascular diseases and occurrence of thrombotic events (Arnesen et al., 1995; Boushey, Beresford, Omenn, & Motulsky, 1995; Selhub et al., 1995). However, this link is not well understood, since recent randomized evaluation of folic acid and vitamin B12

supplementation versus placebo on blood homocysteine concentration did not have any effect on preventing myocardial infarction outcomes (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine Collaborative Group, 2010).

The broader significance of the associations observed for these two genetic variants is not clear. The measured partial eta effect sizes, at 0.01 for rs2336573 and at 0.015 for rs9621049, are very small—indicating that the effect on biochemical parameter means approaches 1%. This effect is typical compared to other phenotype characterizations of complex traits demonstrating both environmental and genetic components, and illustrates the difficulty of ascertaining meaning from genetic variability observed across individuals.

Clinical Parameters

The second data-based paper in this dissertation ascertained if genetic variation in the transcobalamin II and transcobalamin II-receptor gene was associated with any of the hematologic, neurologic, and functional performance parameters that comprise some of the clinical assessments and symptoms indicative of cobalamin deficiency. Results identified one significant coding variant in the transcobalamin II gene associated with mean corpuscular volume (MCV), and second, a genomic region of interest in the transcobalamin II gene associated with hand grip strength and walking speed.

The first finding identified was differences in MCV by rs1801198 SNP allele. This SNP is a coding polymorphism encoding a proline to arginine switch at position 259 (P259R) in exon 6 of the transcobalamin II gene and demonstrated a significant interaction effect after adjusting for folate status. In African American WHAS elderly

women, GG homozygotes had the lowest mean MCV of 86.1 fL, compared to CG (91.4 fL) and CC (90.1 fL) genotype groups. The reason for this result is not clear; there is no published research on the effect of transcobalamin II genetic variation on hematologic traits such as MCV. Although it is widely known that there is a race effect for the hematological traits, MCV and hemoglobin, the reasons for this difference cannot currently be explained by socio-economic differences, and genetic reasons for this possible difference are just beginning to be explored. While values for MCV are at physiologically lower levels for African American as compared to their Caucasian counterparts, the large difference found in this dissertation study suggests a biologic influence from rs1801198 occurring within the African American group only.

The second finding was the identification of a cluster of significant SNPs in the five prime (front end) of the transcobalamin II gene with the physical performance measures, hand grip strength and walking speed. Singularly, none of these SNPs on their own were highly significant, but that they were clustered within a specific region of one gene and associating only with the strength-related outcome measures is intriguing. Because of the high assay dropout stemming from working with whole genome amplified material (of genomic DNA that was 16-18 years old) the analysis for this research conducted a series of independent association tests, and the significance obtained from each SNP was relatively muted. However, reperforming the analyses in blocks of SNPs that are inherited together could likely produce a signal for this region that is more intense. If the signal obtained in the dissertation research is found to be true after further validation, this would mean that elderly patients with specific transcobalamin II allelic

combinations may be predisposed to experience physical weakness and slowness compared to others.

Dissertation Research Limitations and Future Research

The most significant shortcoming of this research is the inability to completely "tag" both the transcobalamin II and transcobalamin II-receptor genes with full genetic coverage. Due to the difficulty of working with whole genome amplified material from DNA that was 16-18 years old, there was a tremendous amount of assay loss that resulted in an inability to analyze SNPs in heredity blocks as they occur on the chromosome, also called "haplotype blocks."

Although the counter-measure to this was to independently analyze SNPs singularly, great loss of power to detect significant loci in WHAS subjects was the cost of running many tests. But as the clustered findings of the functional performance parameters indicate, the analyses are not independent, making the Bonferroni adjustment an especially restrictive and overly stringent corrective mechanism. Furthermore, the fact that many singular tests were run in linked areas of the genome, it is likely that existing signals found in this dissertation research would be greatly amplified upon performance of a haploblock analysis. Because of poorly performing assays, regenotyping WHAS whole genome amplified material will not be able to fill existing holes in blocks of the two candidate genes to produce the required coverage. Other alternatives to validating the signals obtained in this dissertation include 1) imputation of population-specific reference genotypes from HapMap, and 2) transitioning efforts to another adult cohort with similar

clinical measurements but with better genomic DNA quality, and genotype selected transcobalamin II, transcobalamin II-receptor SNPs.

As with any candidate gene association study, positive findings (even if validated in independent cohorts) do not yield information about molecular mechanism or causality. Although knowledge of association can provide increased understanding of how genetics may be involved with a clinical phenotype, it cannot answer the question as to whether a SNP with functional effects results in a disease or health status changes. Additional studies involving different methodologic and laboratory approaches would be required to address the issue of causation. Despite the genotyping shortcomings and the inferential methodologic limitations, this dissertation research provides several hypothesis-generating genetic variants to evaluate in further cohorts of elderly adults. If validated, these variants may become meaningful targets for laboratory research to better understand their functional consequences and lay the foundations for future translation into clinical interventions.

A second limitation to this study is that analyses were conducted without adjusting for all of the environmental factors known to impact B12 nutrition in older adults. For example, Chapter 2 indicated numerous medications (antiepileptic agents, proton pump inhibitors, histamine receptor antagonists, the antidiabetic drug metformin, antibiotics, and cholestyramine) and comorbid conditions (intestinal diseases, gastric or ileal resections, alcohol intake, smoking, diabetes mellitus, and lymphoma) known to affect cobalamin nutritional status and deficiency parameters (Wolters, Strohle, & Hahn, 2004).

The overarching reason that many of these factors were not incorporated into the dissertation analysis is that accounting for them as covariates would have consumed further statistical power. As frequencies in African American and Caucasian genotype subgroups were low for many SNPs, the genetic effects being studied accounted for a small percentage of variance in each outcome trait, and many outcome variables were already being analyzed with two and three covariates—including additional medications and comorbidities would have further decreased statistical power to detect changes in study outcomes due to genetics. Because the research advantages of their inclusion could not offset the decrease in statistical power that would have occurred, they were not taken into account. Further validation efforts should aim for a sample size that is large enough to fully account for environmental factors such as medication use and comorbid conditions.

A third concern regarding this work is that the use of WHAS, despite all the advantages and strengths it carries in performing genetic research, could possibly be limiting. WHAS and similar aging research initiatives from the National Institute on Aging constitute the "state of the science," and many citations in this dissertation look to the precedent that was set by these landmark efforts. Unfortunately, this carries a risk of sampling error being built into the knowledge base, in that previous WHAS findings are driving further research conducted in the WHAS cohorts.

However, the issue of clinical heterogeneity in cobalamin deficiency of older adults, as reported by multiple non-WHAS sources in Chapter 2, is a distinctly separate and significant problem. As little genetic research has been published regarding the WHAS participants and genetic contributors to vitamin B12 deficiency were gaining in

their health significance for older adults, this dissertation represented a unique research opportunity and a valuable learning experience.

The final limitation of this research is that it was a secondary data analysis of pre-existing studies (WHAS 1, 2). Although it added new information to the field through the generation of candidate-gene SNP genotypes, the analysis of selected outcome measurements and covariates in this study was limited to what data was available. Already mentioned is that use of serum cobalamin is not a direct measurement of biologically active cobalamin, the fraction of vitamin B12 that is bound to the transcobalamin II molecule. Serum "holotranscobalamin" measurements, cobalamin bound to transcobalamin II, were unavailable for WHAS subjects. This limitation made inference about how a genetic variant may be exerting a biologic effect (i.e., on serum metabolites) more difficult.

Implications for Leadership and Policy

Using the knowledge of biologic diversity provided through resources such as the Human Genome Project and the Haplotype Map, genomics research is unraveling complexities of chronic diseases in humans (Hindorff et al., 2009; Manolio, Brooks, & Collins, 2008). Without the ability to translate these findings into clinically relevant and meaningful tools that can be practically applied to patient care settings, many of these technologic breakthroughs will remain limited to the halls of science where they were conceived. The dissertation research was undertaken with an overarching interest in bridging these seemingly disconnected worlds. However, simply performing the research,

without effort of broader education or outreach, would not help facilitate true understanding between the worlds of nursing research and genomic science.

Chapter 6, the final manuscript of this dissertation, reflects an exercise in academic nursing leadership. The effort was undertaken to highlight the opportunities in genetics/genomics for nurses to advance human health through the adoption and utilization of Personalized Medicine. Policy, research, education, and practice implications were addressed in order to provide a bridge between the genetic technologies used in Chapters 4 and 5's research, and what nurses from varying settings may expect upon seeing the same technologies in their local healthcare settings. For nurses who are able to incorporate and implement these incredible tools, significant groundwork will be laid for sound health policy derivation and widespread health benefit for patients, families, and communities.

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APPENDIX A

WHOLE GENOME AMPLIFICATION FOR DISSERTATION

Scientific Protocol for Qiagen REPLI-g Midi Kit (Product #150045_100)

Reagents:

Microcentrifuge tubes	Pipetes and pipet tubes
Water bath or heating block	Ice
Microcentrifuge	Nuclease-free water
Vortexer	

Before Starting:

Prepare Buffer DLB by adding 500 ul nuclease-free water to the tube. Mix thoroughly and centrifuge briefly. Reconstituted buffer DLB can be stored for 6 months at -20 degrees C. Buffer DLB is pH-labile. Avoid neutralization with CO_2. All buffers and reagents should be vortexed before use to ensure thorough mixing. Set heating block to 30 degrees C.

Protocol:

1. Prepare a sufficient amount of Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer) for the total number of whole genome amplification reactions.

   Preparation of Buffer D1:

Reconstituted Buffer DLB	5.0 ul
Nuclease-free water	35.0 ul
Total	40.0 ul (for 7 reactions)

   Preparation of Buffer N1:

Solution B (stop solution)	8.0 ul
Nuclease-free water	72.0 ul
Total	80.0 ul (for 7 reactions)

2. Place 5.0 ul of template DNA into a microcentrifuge tube.
3. Add 5.0 ul of Buffer D1 to the DNA. Mix by vortexing and centrifuge briefly.
4. Incubate the samples at room temperature for 3 minutes.

Scientific Protocol for Qiagen REPLI-g Midi Kit (Product #150045_100)

1. Add 10.0 ul of Buffer N1 to the samples. Mix by vortexing and centrifuge briefly.
2. Thaw REPLI-g DNA polymerase on ice. Thaw all other components at room temperature; vortex then centrifuge briefly.
7. Prepare a master mix on ice. Mix and centrifuge briefly.

   Preparation of Master Mix:

   REPLI-g Mini Reaction Buffer 29.0 ul
   REPLI-g Mini DNA Polymerase 1.0 ul (for 1 reaction)
8. Add 30 ul of master mix to 20 ul of denatured DNA.
9. Incubate at 30 degrees C for 10-16 hours. The maximum DNA yield is achieved using an incubation time of 16 hours.
10. Inactivate REPLI-g DNA Polymerase by heating the sample for 3 minutes at 65 degrees C.
11. Store amplified DNA at 4 degrees C for short-term storage or -20 degrees C for long-term storage.

APPENDIX B

MANUAL GENOTYPE CALLING OF WHOLE GENOME AMPLIFIED MATERIAL

Parameters for Manual Calling in Typer 4.0

1. No signals under log/height arc threshold of .25 are called.
2. No use of "lasso" function to manually change multiple calls simultaneously; spectra of each call must be individually inspected for accuracy. If inaccurate, change to No Call, save file, and issue recall.
3. Spectral peaks of all "aggressive" calls at bleed-border edge of heterozygote/homozygote cluster overlap regions are inspected to ensure they are accurately heterozygous or homozygous.
4. Low-probability calls can be made manually if they are not located in red or yellow wells.
5. Peaks in yellow and red wells can be called as long as using parameter guidelines.
6. Inspect spectral peak and log/height scatter plot to assess the following:
    a. On the spectra, where is the Unexpected Primer (UEP) in relation to the expected SNP allele?
        i. If UEP difficult to visualize and separate from allele peak = No Call
        ii. If UEP is distinctly separate and visible = Call
    b. On the spectra, where does the midline of the call's peak lie in relation to the expected allele mass?
        i. If midline of peak is not present at expected allele mass = No Call
        ii. If midline of peak is present at expected allele mass = Call
    c. On the spectra, what is the amplitude of a candidate peak in relation to the underlying baseline?
        i. If difficult or impossible to distinguish from baseline = No Call
        ii. If candidate peak can be is noticeably higher or distinguishable from baseline = Call

Parameters for Manual Calling in Typer 4.0

a. On the log/height plot, how far away is the candidate call from the closest cluster of calls?
    i. If far away from cluster = No Call; or conservatively follow parameter 6a through c.
    ii. If within or close to existing cluster = Call
b. On the log/height plot, is the candidate call within a region of cluster bleed border overlap?
    i. If yes = No Call; or conservatively follow parameter 6a through c.
    ii. If no = Call according to 6d.

APPENDIX C

HUMAN SUBJECTS CLASSIFICATION FOR

DISSERTATION RESEARCH

Electronic Mail Correspondence Regarding IRB Approval for Dissertation Research

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IRB_00031719

PI: Emma Kurnat-Thoma

Title: Impact of Genetic Variation on Vitamin B12 Metabolism: A Retrospective Analysis Of the Women's Health and Aging Study

Thank you for submitting your request for approval of this project. The IRB has administratively reviewed your application and has determined on 12/4/2008 that your project does NOT meet the definitions of Human Subjects Research according to Federal regulations. Therefore, IRB oversight is not required or necessary for your project.

This determination of non-human subjects research only applies to the project as submitted to the IRB. Since this determination is not an approval, it does not expire or need renewal. Remember that all research involving human subjects must be approved or exempted by the IRB before the research is conducted.

If you have questions about this, please contact our office at 581-3655 and we will be happy to assist you. Thank you again for submitting your proposal.

Click IRB_00031719 to view the application.

APPENDIX D

DISSERTATION DATA

Table 19

Hemoglobin

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	12.60	0.84	4
	GA	12.34	1.26	35
	AA	12.33	1.52	132
	Total	12.34	1.45	171
Caucasian	GG	13.05	1.60	10
	GA	13.22	1.27	84
	AA	13.32	1.24	431
	Total	13.30	1.25	525
Total	GG	12.92	1.41	14
	GA	12.96	1.33	119
	AA	13.09	1.37	563
	Total	13.06	1.36	696

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	12.29	1.39	18
	CG	12.27	1.66	60
	GG	12.35	1.33	98
	Total	12.32	1.45	176
Caucasian	CC	13.19	1.44	11
	CG	13.28	1.15	88
	GG	13.30	1.26	436
	Total	13.29	1.25	535
Total	CC	12.63	1.46	29
	CG	12.87	1.46	148
	GG	13.12	1.32	534
	Total	13.05	1.36	711

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	12.85	1.07	11
	CT	12.10	1.64	54
	TT	12.37	1.35	110
	Total	12.32	1.44	175
Caucasian	CC	13.73	0.93	3
	CT	12.10	.	1
	TT	13.30	1.25	535
	Total	13.30	1.24	539
Total	CC	13.04	1.08	14
	CT	12.10	1.62	55
	TT	13.14	1.31	645
	Total	13.06	1.36	714

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	12.22	0.97	9
	GA	12.32	1.20	44
	AA	12.34	1.55	123
	Total	12.33	1.44	176
Caucasian	GG	13.87	1.21	26
	GA	13.58	1.05	80
	AA	13.19	1.26	430
	Total	13.28	1.25	536
Total	GG	13.45	1.36	35
	GA	13.13	1.26	124
	AA	13.00	1.38	553
	Total	13.05	1.36	712

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	13.17	0.67	3
	TC	12.59	1.18	29
	TT	12.25	1.50	144
	Total	12.32	1.45	176
Caucasian	CC	13.15	1.17	34
	TC	13.28	1.27	150
	TT	13.31	1.26	355
	Total	13.30	1.25	539
Total	CC	13.15	1.13	37
	TC	13.17	1.27	179
	TT	13.01	1.42	499
	Total	13.06	1.37	715

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	13.80	.	1
	CT	14.15	1.20	2
	TT	12.31	1.44	174
	Total	12.34	1.45	177
Caucasian	CC	13.02	0.56	4
	CT	13.28	0.98	19
	TT	13.29	1.26	518
	Total	13.29	1.25	541
Total	CC	13.18	0.60	5
	CT	13.36	1.00	21
	TT	13.04	1.38	692
	Total	13.05	1.36	718

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	12.77	1.02	12
	AT	12.40	1.26	46
	TT	12.29	1.55	115
	Total	12.35	1.45	173
Caucasian	AA	12.98	1.51	42
	AT	13.16	1.21	143
	TT	13.37	1.22	349
	Total	13.28	1.25	534
Total	AA	12.93	1.41	54
	AT	12.98	1.27	189
	TT	13.10	1.39	464
	Total	13.05	1.36	707

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	13.08	1.21	9
	CT	12.18	1.71	51
	CC	12.34	1.33	115
	Total	12.33	1.45	175
Caucasian	CT	13.60	0.81	7
	CC	13.28	1.25	536
	Total	13.29	1.25	543
Total	TT	13.08	1.21	9
	CT	12.35	1.69	58
	CC	13.12	1.32	651
	Total	13.05	1.36	718

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	11.77	1.92	13
	TC	12.32	1.69	55
	CC	12.37	1.23	104
	Total	12.31	1.45	172
Caucasian	TT	13.39	1.08	120
	TC	13.32	1.29	211
	CC	13.28	1.22	194
	Total	13.32	1.22	525
Total	TT	13.23	1.27	133
	TC	13.11	1.44	266
	CC	12.96	1.30	298
	Total	13.07	1.35	697

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	12.02	1.90	15
	CG	12.30	1.65	63
	CC	12.39	1.23	97
	Total	12.33	1.45	175
Caucasian	GG	13.36	1.09	130
	CG	13.28	1.32	236
	CC	13.26	1.26	176
	Total	13.29	1.25	542
Total	GG	13.22	1.26	145
	CG	13.07	1.45	299
	CC	12.95	1.31	273
	Total	13.06	1.36	717

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	12.32	1.56	6
	TT	12.33	1.44	173
	Total	12.33	1.44	179
Caucasian	AA	13.50	1.33	14
	AT	13.40	1.32	87
	TT	13.28	1.22	434
	Total	13.31	1.24	535
Total	AA	13.50	1.33	14
	AT	13.33	1.35	93
	TT	13.01	1.35	607
	Total	13.06	1.36	714

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	12.38	0.90	6
	CT	12.23	1.27	44
	CC	12.36	1.52	128
	Total	12.32	1.44	178
Caucasian	TT	13.14	1.81	14
	CT	13.58	1.12	92
	CC	13.25	1.26	431
	Total	13.30	1.26	537
Total	TT	12.91	1.61	20
	CT	13.14	1.32	136
	CC	13.04	1.37	559
	Total	13.06	1.37	715

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	12.12	1.00	4
	GT	12.25	1.29	43
	TT	12.33	1.51	129
	Total	12.31	1.44	176
Caucasian	GG	13.38	0.83	12
	GT	13.59	1.25	93
	TT	13.23	1.24	433
	Total	13.30	1.24	538
Total	GG	13.07	1.01	16
	GT	13.16	1.41	136
	TT	13.02	1.36	562
	Total	13.05	1.36	714

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	11.10	0.00	2
	GA	12.33	1.08	23
	GG	12.33	1.51	149
	Total	12.32	1.45	174
Caucasian	AA	13.67	1.28	9
	GA	13.57	1.23	78
	GG	13.23	1.24	446
	Total	13.29	1.25	533
Total	AA	13.20	1.55	11
	GA	13.29	1.30	101
	GG	13.00	1.37	595
	Total	13.05	1.36	707

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	12.71	1.12	37
	AG	12.15	1.57	83
	AA	12.30	1.40	57
	Total	12.32	1.44	177
Caucasian	GG	13.02	1.30	123
	AG	13.32	1.27	222
	AA	13.44	1.17	191
	Total	13.29	1.25	536
Total	GG	12.95	1.26	160
	AG	13.00	1.46	305
	AA	13.18	1.31	248
	Total	13.05	1.37	713

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	12.81	1.17	15
	CT	12.30	1.27	67
	CC	12.26	1.60	94
	Total	12.32	1.45	176
Caucasian	TT	13.04	1.30	117
	CT	13.30	1.28	215
	CC	13.43	1.16	212
	Total	13.29	1.24	544
Total	TT	13.01	1.28	132
	CT	13.06	1.34	282
	CC	13.07	1.42	306
	Total	13.06	1.36	720

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	12.71	1.03	9
	CG	12.29	1.29	55
	GG	12.30	1.54	114
	Total	12.32	1.44	178
Caucasian	CC	13.02	1.64	31
	CG	13.22	1.25	164
	GG	13.37	1.20	351
	Total	13.31	1.25	546
Total	CC	12.95	1.52	40
	CG	12.98	1.32	219
	GG	13.11	1.37	465
	Total	13.06	1.36	724

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	14.20	.	1
	TT	12.32	1.42	181
	Total	12.33	1.43	182
Caucasian	CC	13.60	0.57	2
	TC	13.36	1.14	47
	TT	13.29	1.26	507
	Total	13.29	1.25	556
Total	CC	13.60	0.57	2
	TC	13.38	1.14	48
	TT	13.03	1.37	688
	Total	13.06	1.36	738

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	13.14	0.58	5
	GA	12.05	2.13	30
	GG	12.34	1.27	141
	Total	12.31	1.44	176
Caucasian	AA	13.40	0.14	2
	GA	13.52	1.01	22
	GG	13.30	1.26	522
	Total	13.31	1.25	546
Total	AA	13.21	0.49	7
	GA	12.67	1.88	52
	GG	13.09	1.32	663
	Total	13.06	1.37	722

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	10.30	1.83	4
	GA	12.36	1.29	43
	AA	12.32	1.45	123
	Total	12.28	1.44	170
Caucasian	GG	13.40	1.09	110
	GA	13.28	1.32	224
	AA	13.22	1.25	193
	Total	13.29	1.25	527
Total	GG	13.29	1.25	114
	GA	13.14	1.36	267
	AA	12.87	1.40	316
	Total	13.04	1.37	697

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	12.53	0.84	7
	CT	12.08	1.82	31
	CC	12.35	1.38	136
	Total	12.31	1.45	174
Caucasian	TT	13.51	0.80	10
	CT	13.16	1.22	77
	CC	13.33	1.26	442
	Total	13.30	1.24	529
Total	TT	13.11	0.93	17
	CT	12.85	1.49	108
	CC	13.10	1.35	578
	Total	13.06	1.37	703

CD320_rs250510		*M*	*SD*	*N*
African American	TT	14.17	1.03	3
	CT	11.96	1.53	21
	CC	12.34	1.43	144
	Total	12.32	1.46	168
Caucasian	TT	13.30	.	1
	CT	13.38	1.14	5
	CC	13.29	1.23	514
	Total	13.29	1.23	520
Total	TT	13.95	0.94	4
	CT	12.23	1.55	26
	CC	13.08	1.34	658
	Total	13.05	1.35	688

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	12.50	.	1
	AG	13.70	0.42	2
	GG	12.32	1.46	173
	Total	12.34	1.45	176
Caucasian	AA	12.80	.	1
	GG	13.29	1.24	521
	Total	13.29	1.24	522
Total	AA	12.65	0.21	2
	AG	13.70	0.42	2
	GG	13.05	1.36	694
	Total	13.05	1.36	698

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	12.13	0.86	10
	GC	12.00	1.82	47
	GG	12.47	1.29	118
	Total	12.32	1.44	175
Caucasian	CC	13.01	1.49	11
	GC	13.49	1.15	94
	GG	13.28	1.26	407
	Total	13.31	1.25	512
Total	CC	12.59	1.28	21
	GC	12.99	1.57	141
	GG	13.10	1.31	525
	Total	13.06	1.37	687

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	12.25	1.36	21
	TC	12.45	1.41	68
	CC	12.25	1.52	84
	Total	12.33	1.46	173
Caucasian	TT	13.73	1.71	9
	TC	13.54	1.33	35
	CC	13.29	1.22	486
	Total	13.31	1.24	530
Total	TT	12.70	1.60	30
	TC	12.82	1.47	103
	CC	13.13	1.32	570
	Total	13.07	1.36	703

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	12.55	0.49	2
	CT	12.31	1.10	18
	CC	12.33	1.48	160
	Total	12.33	1.43	180
Caucasian	TT	12.84	0.51	5
	CT	13.00	0.28	2
	CC	13.32	1.25	547
	Total	13.31	1.25	554
Total	TT	12.76	0.49	7
	CT	12.37	1.06	20
	CC	13.09	1.37	707
	Total	13.07	1.36	734

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	12.27	1.24	16
	CT	12.19	1.82	47
	TT	12.38	1.32	108
	Total	12.32	1.46	171
Caucasian	CC	13.30	1.09	56
	CT	13.27	1.19	195
	TT	13.33	1.31	271
	Total	13.31	1.24	522
Total	CC	13.07	1.20	72
	CT	13.06	1.40	242
	TT	13.06	1.38	379
	Total	13.06	1.37	693

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	12.32	1.36	29
	CT	12.37	1.35	69
	CC	12.26	1.50	71
	Total	12.32	1.41	169
Caucasian	TT	13.26	1.37	79
	CT	13.37	1.27	205
	CC	13.30	1.15	234
	Total	13.32	1.23	518
Total	TT	13.01	1.42	108
	CT	13.12	1.36	274
	CC	13.06	1.31	305
	Total	13.07	1.35	687

Table 19 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	12.17	1.39	23
	CT	12.32	1.40	59
	TT	12.36	1.47	96
	Total	12.32	1.43	178
Caucasian	CC	12.82	2.65	5
	CT	13.46	1.24	35
	TT	13.28	1.23	498
	Total	13.29	1.25	538
Total	CC	12.29	1.64	28
	CT	12.74	1.44	94
	TT	13.14	1.32	594
	Total	13.05	1.36	716

Table 20

Mean Corpuscular Volume

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	93.00	7.70	4
	GA	90.51	5.29	35
	AA	89.91	6.71	131
	Total	90.11	6.45	170
Caucasian	GG	96.40	4.97	10
	GA	93.37	5.01	81
	AA	94.29	5.28	427
	Total	94.18	5.24	518
Total	GG	95.43	5.77	14
	GA	92.51	5.24	116
	AA	93.26	5.94	558
	Total	93.18	5.83	688

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	92.83	5.80	18
	CG	89.30	6.72	60
	GG	90.25	6.23	97
	Total	90.19	6.41	175
Caucasian	CC	92.55	5.15	11
	CG	94.27	5.23	86
	GG	94.14	5.23	431
	Total	94.13	5.23	528
Total	CC	92.72	5.47	29
	CG	92.23	6.36	146
	GG	93.43	5.63	528
	Total	93.15	5.80	703

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	93.82	6.62	11
	CT	89.83	6.51	54
	TT	90.19	6.31	109
	Total	90.31	6.42	174
Caucasian	CC	96.33	5.51	3
	CT	99.00	.	1
	TT	94.13	5.22	528
	Total	94.15	5.22	532
Total	CC	94.36	6.28	14
	CT	90.00	6.57	55
	TT	93.45	5.61	637
	Total	93.20	5.77	706

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	90.67	7.86	9
	GA	90.18	6.38	44
	AA	90.16	6.28	122
	Total	90.19	6.35	175
Caucasian	GG	93.65	4.11	26
	GA	93.38	5.22	80
	AA	94.41	5.29	423
	Total	94.22	5.23	529
Total	GG	92.89	5.36	35
	GA	92.24	5.84	124
	AA	93.46	5.80	545
	Total	93.22	5.79	704

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	90.33	7.51	3
	TC	90.24	6.24	29
	TT	90.17	6.51	143
	Total	90.19	6.44	175
Caucasian	CC	96.15	3.58	34
	TC	93.93	5.22	148
	TT	94.12	5.34	350
	Total	94.20	5.23	532
Total	CC	95.68	4.18	37
	TC	93.33	5.55	177
	TT	92.98	5.97	493
	Total	93.21	5.81	707

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	90.00	.	1
	CT	85.00	2.83	2
	TT	90.35	6.47	173
	Total	90.29	6.45	176
Caucasian	CC	93.25	5.62	4
	CT	93.37	3.98	19
	TT	94.23	5.27	511
	Total	94.19	5.23	534
Total	CC	92.60	5.08	5
	CT	92.57	4.58	21
	TT	93.25	5.84	684
	Total	93.22	5.80	710

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	88.83	5.06	12
	AT	90.57	5.07	46
	TT	90.32	7.10	114
	Total	90.28	6.47	172
Caucasian	AA	95.23	4.79	40
	AT	93.74	5.25	141
	TT	94.20	5.26	346
	Total	94.16	5.22	527
Total	AA	93.75	5.52	52
	AT	92.96	5.37	187
	TT	93.24	6.00	460
	Total	93.20	5.80	699

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	94.00	3.74	9
	CT	90.33	6.36	51
	CC	89.84	6.56	114
	Total	90.20	6.42	174
Caucasian	CT	95.57	3.26	7
	CC	94.12	5.21	529
	Total	94.14	5.19	536
Total	TT	94.00	3.74	9
	CT	90.97	6.29	58
	CC	93.37	5.71	643
	Total	93.18	5.77	710

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	86.08	6.36	12
	TC	90.84	6.87	55
	CC	90.10	6.10	104
	Total	90.05	6.44	171
Caucasian	TT	94.18	5.21	119
	TC	93.74	5.39	209
	CC	94.42	4.72	191
	Total	94.09	5.11	519
Total	TT	93.44	5.79	131
	TC	93.14	5.84	264
	CC	92.89	5.63	295
	Total	93.09	5.74	690

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	86.21	7.28	14
	CG	91.44	6.37	63
	CC	89.70	6.16	97
	Total	90.05	6.44	174
Caucasian	GG	94.31	5.61	129
	CG	93.97	5.28	233
	CC	94.48	4.84	173
	Total	94.22	5.22	535
Total	GG	93.52	6.25	143
	CG	93.44	5.62	296
	CC	92.76	5.81	270
	Total	93.20	5.82	709

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	87.33	9.24	6
	TT	90.26	6.35	172
	Total	90.16	6.46	178
Caucasian	AA	93.71	5.51	14
	AT	94.16	6.09	86
	TT	94.19	4.96	428
	Total	94.17	5.16	528
Total	AA	93.71	5.51	14
	AT	93.72	6.49	92
	TT	93.06	5.68	600
	Total	93.16	5.78	706

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	89.33	4.46	6
	CT	90.30	7.93	44
	CC	90.07	5.96	127
	Total	90.10	6.43	177
Caucasian	TT	95.07	4.63	14
	CT	93.98	5.19	91
	CC	94.21	5.23	425
	Total	94.20	5.20	530
Total	TT	93.35	5.21	20
	CT	92.78	6.42	135
	CC	93.26	5.68	552
	Total	93.17	5.81	707

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	91.25	4.72	4
	GT	89.95	7.95	43
	TT	90.03	5.94	128
	Total	90.04	6.44	175
Caucasian	GG	93.17	5.25	12
	GT	94.27	4.87	92
	TT	94.18	5.27	427
	Total	94.18	5.20	531
Total	GG	92.69	5.04	16
	GT	92.90	6.32	135
	TT	93.23	5.70	555
	Total	93.15	5.81	706

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	89.00	12.73	2
	GA	91.96	7.68	23
	GG	89.96	6.21	148
	Total	90.21	6.48	173
Caucasian	AA	93.11	4.20	9
	GA	93.62	5.19	78
	GG	94.27	5.23	439
	Total	94.16	5.21	526
Total	AA	92.36	5.75	11
	GA	93.24	5.85	101
	GG	93.19	5.80	587
	Total	93.18	5.80	699

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	88.46	5.80	37
	AG	90.82	6.31	82
	AA	90.09	6.95	57
	Total	90.09	6.45	176
Caucasian	GG	94.60	4.91	121
	AG	93.88	5.10	219
	AA	94.32	5.43	189
	Total	94.20	5.18	529
Total	GG	93.16	5.74	158
	AG	93.05	5.62	301
	AA	93.34	6.07	246
	Total	93.17	5.80	705

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	89.07	4.76	15
	CT	90.54	5.41	67
	CC	90.17	7.34	93
	Total	90.22	6.45	175
Caucasian	TT	95.02	4.94	115
	CT	93.84	5.24	212
	CC	94.16	5.25	210
	Total	94.22	5.19	537
Total	TT	94.33	5.26	130
	CT	93.05	5.46	279
	CC	92.93	6.24	303
	Total	93.23	5.78	712

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	88.44	5.59	9
	CG	89.96	4.83	55
	GG	90.50	7.08	113
	Total	90.23	6.38	177
Caucasian	CC	94.67	4.57	30
	CG	93.79	5.51	161
	GG	94.39	5.17	348
	Total	94.23	5.24	539
Total	CC	93.23	5.44	39
	CG	92.81	5.59	216
	GG	93.44	5.93	461
	Total	93.24	5.80	716

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	94.00	.	1
	TT	90.20	6.46	180
	Total	90.22	6.45	181
Caucasian	CC	102.50	2.12	2
	TC	94.55	5.06	47
	TT	94.17	5.21	500
	Total	94.24	5.21	549
Total	CC	102.50	2.12	2
	TC	94.54	5.01	48
	TT	93.12	5.83	680
	Total	93.24	5.80	730

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	85.80	8.44	5
	GA	90.48	7.56	29
	GG	90.09	6.12	141
	Total	90.03	6.44	175
Caucasian	AA	94.50	6.36	2
	GA	94.09	4.29	22
	GG	94.23	5.23	515
	Total	94.22	5.19	539
Total	AA	88.29	8.50	7
	GA	92.04	6.55	51
	GG	93.34	5.69	656
	Total	93.19	5.81	714

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	88.00	6.48	4
	GA	89.60	6.24	42
	AA	90.22	6.52	123
	Total	90.01	6.43	169
Caucasian	GG	94.27	5.37	109
	GA	93.95	5.24	220
	AA	94.48	5.08	191
	Total	94.21	5.20	520
Total	GG	94.04	5.50	113
	GA	93.25	5.63	262
	AA	92.81	6.05	314
	Total	93.18	5.81	689

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	90.29	2.21	7
	CT	89.94	7.77	31
	CC	90.24	6.29	135
	Total	90.19	6.44	173
Caucasian	TT	95.80	5.85	10
	CT	94.40	4.43	77
	CC	94.08	5.35	435
	Total	94.16	5.23	522
Total	TT	93.53	5.37	17
	CT	93.12	5.92	108
	CC	93.17	5.82	570
	Total	93.17	5.81	695

CD320_rs250510		*M*	*SD*	*N*
African American	TT	87.00	9.64	3
	CT	90.67	5.35	21
	CC	90.15	6.59	143
	Total	90.16	6.48	167
Caucasian	TT	97.00	.	1
	CT	95.20	5.36	5
	CC	94.16	5.21	507
	Total	94.17	5.21	513
Total	TT	89.50	9.33	4
	CT	91.54	5.55	26
	CC	93.28	5.79	650
	Total	93.19	5.81	680

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	80.00	.	1
	AG	88.00	9.90	2
	GG	90.44	6.42	172
	Total	90.35	6.46	175
Caucasian	AA	101.00	.	1
	GG	94.15	5.21	514
	Total	94.16	5.21	515
Total	AA	90.50	14.85	2
	AG	88.00	9.90	2
	GG	93.22	5.76	686
	Total	93.19	5.79	690

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	89.10	4.28	10
	GC	91.72	6.69	46
	GG	89.78	6.31	118
	Total	90.25	6.35	174
Caucasian	CC	92.50	4.90	10
	GC	94.13	5.25	94
	GG	94.28	5.17	402
	Total	94.22	5.17	506
Total	CC	90.80	4.81	20
	GC	93.34	5.85	140
	GG	93.26	5.76	520
	Total	93.20	5.76	680

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	89.52	4.57	21
	TC	89.53	6.44	68
	CC	91.30	6.69	83
	Total	90.38	6.40	172
Caucasian	TT	95.56	8.22	9
	TC	94.20	6.18	35
	CC	94.14	5.10	479
	Total	94.17	5.23	523
Total	TT	91.33	6.40	30
	TC	91.12	6.70	103
	CC	93.72	5.45	562
	Total	93.23	5.77	695

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	93.00	2.83	2
	CT	89.78	6.16	18
	CC	90.32	6.50	159
	Total	90.30	6.43	179
Caucasian	TT	97.80	3.70	5
	CT	98.50	3.54	2
	CC	94.16	5.21	540
	Total	94.21	5.21	547
Total	TT	96.43	3.99	7
	CT	90.65	6.47	20
	CC	93.29	5.76	699
	Total	93.25	5.78	726

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	90.06	7.33	16
	CT	89.91	6.58	47
	TT	90.55	6.34	107
	Total	90.33	6.47	170
Caucasian	CC	94.48	4.29	56
	CT	93.92	5.28	194
	TT	94.17	5.30	266
	Total	94.11	5.18	516
Total	CC	93.50	5.39	72
	CT	93.14	5.76	241
	TT	93.13	5.84	373
	Total	93.17	5.76	686

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	90.03	6.10	29
	CT	89.57	6.06	68
	CC	90.86	6.99	71
	Total	90.20	6.47	168
Caucasian	TT	93.90	5.59	77
	CT	94.17	5.17	202
	CC	94.02	5.22	232
	Total	94.06	5.24	511
Total	TT	92.84	5.96	106
	CT	93.01	5.75	270
	CC	93.28	5.83	303
	Total	93.10	5.81	679

Table 20 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	87.91	5.74	23
	CT	90.32	6.07	59
	TT	90.78	6.83	95
	Total	90.25	6.48	177
Caucasian	CC	93.20	9.78	5
	CT	94.14	6.35	35
	TT	94.21	5.08	491
	Total	94.20	5.21	531
Total	CC	88.86	6.73	28
	CT	91.74	6.41	94
	TT	93.65	5.54	586
	Total	93.21	5.81	708

Table 21

Cobalamin (B12)

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	484.60	126.53	5
	GA	579.40	239.84	35
	AA	555.97	270.99	123
	Total	558.81	260.84	163
Caucasian	GG	306.38	100.81	8
	GA	437.71	242.41	83
	AA	468.33	224.95	427
	Total	460.92	227.27	518
Total	GG	374.92	139.32	13
	GA	479.74	249.25	118
	AA	487.93	238.58	550
	Total	484.35	239.22	681

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	620.33	284.44	18
	CG	534.57	244.33	58
	GG	557.00	261.93	90
	Total	556.03	258.03	166
Caucasian	CC	381.91	171.09	11
	CG	489.36	234.80	86
	GG	458.51	226.39	429
	Total	461.95	227.01	526
Total	CC	529.90	270.99	29
	CG	507.57	238.87	144
	GG	475.59	235.64	519
	Total	484.52	238.05	692

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	555.73	183.24	11
	CT	554.02	283.46	54
	TT	556.63	255.21	100
	Total	555.72	259.48	165
Caucasian	CC	485.00	378.37	3
	CT	701.00	.	1
	TT	463.58	225.44	524
	Total	464.15	226.03	528
Total	CC	540.57	220.82	14
	CT	556.69	281.52	55
	TT	478.49	232.77	624
	Total	485.95	237.46	693

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	561.88	255.22	8
	GA	554.90	266.91	40
	AA	560.92	263.38	118
	Total	559.52	262.29	166
Caucasian	GG	460.25	174.68	24
	GA	464.98	237.81	82
	AA	460.47	223.90	419
	Total	461.17	223.78	525
Total	GG	485.66	198.36	32
	GA	494.46	250.24	122
	AA	482.55	236.58	537
	Total	484.79	237.17	691

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	543.67	389.96	3
	TC	533.78	256.15	27
	TT	562.95	260.07	136
	Total	557.86	260.08	166
Caucasian	CC	474.71	269.94	31
	TC	463.35	217.17	153
	TT	458.97	224.78	344
	Total	461.16	225.05	528
Total	CC	480.79	275.42	34
	TC	473.91	224.09	180
	TT	488.43	239.67	480
	Total	484.29	237.33	694

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	525.00	.	1
	CT	484.50	37.48	2
	TT	562.90	264.81	165
	Total	561.74	262.59	168
Caucasian	CC	331.75	199.99	4
	CT	387.78	168.02	18
	TT	465.94	227.71	509
	Total	462.28	226.19	531
Total	CC	370.40	193.56	5
	CT	397.45	161.92	20
	TT	489.67	240.77	674
	Total	486.18	239.07	699

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	609.38	243.72	13
	AT	497.04	204.63	45
	TT	566.47	269.92	106
	Total	550.82	252.67	164
Caucasian	AA	473.15	242.48	39
	AT	440.14	224.87	142
	TT	469.44	222.83	342
	Total	461.76	224.83	523
Total	AA	507.21	247.65	52
	AT	453.83	220.98	187
	TT	492.40	238.11	448
	Total	483.02	234.69	687

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	494.78	199.66	9
	CT	576.69	286.86	48
	CC	554.89	254.04	108
	Total	557.95	260.70	165
Caucasian	CT	455.83	101.33	6
	CC	463.30	226.43	526
	Total	463.21	225.37	532
Total	TT	494.78	199.66	9
	CT	563.26	274.61	54
	CC	478.90	233.71	634
	Total	485.64	237.46	697

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	484.42	263.59	12
	TC	524.19	256.74	47
	CC	579.78	262.13	104
	Total	556.73	261.07	163
Caucasian	TT	462.66	222.67	117
	TC	453.32	219.07	211
	CC	471.80	237.27	189
	Total	462.19	226.40	517
Total	TT	464.68	225.71	129
	TC	466.23	227.52	258
	CC	510.13	251.32	293
	Total	484.85	238.42	680

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	468.00	259.22	13
	CG	530.34	245.31	56
	CC	584.21	268.51	96
	Total	556.77	261.09	165
Caucasian	GG	462.60	222.09	125
	CG	450.63	225.47	235
	CC	468.70	226.64	172
	Total	459.28	224.78	532
Total	GG	463.11	224.79	138
	CG	465.97	231.14	291
	CC	510.08	248.25	268
	Total	482.36	237.34	697

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	417.17	130.62	6
	TT	561.85	260.90	163
	Total	556.71	258.58	169
Caucasian	AA	399.43	163.02	14
	AT	424.10	163.23	92
	TT	472.14	237.89	418
	Total	461.76	225.52	524
Total	AA	399.43	163.02	14
	AT	423.67	160.86	98
	TT	497.31	247.64	581
	Total	484.92	237.35	693

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	546.29	325.18	7
	CT	617.10	273.01	42
	CC	536.39	249.37	119
	Total	556.98	259.33	168
Caucasian	TT	520.29	258.95	14
	CT	441.10	203.09	93
	CC	462.59	230.68	419
	Total	460.33	226.77	526
Total	TT	528.95	274.71	21
	CT	495.85	240.44	135
	CC	478.91	236.70	538
	Total	483.72	238.49	694

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	614.50	423.22	4
	GT	612.05	271.73	43
	TT	536.56	248.99	120
	Total	557.86	259.86	167
Caucasian	GG	570.42	257.14	12
	GT	440.13	201.08	95
	TT	460.21	231.33	420
	Total	459.10	227.12	527
Total	GG	581.44	291.03	16
	GT	493.70	238.25	138
	TT	477.18	237.27	540
	Total	482.87	238.98	694

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	654.67	540.11	3
	GA	604.24	290.92	21
	GG	552.49	250.85	140
	Total	560.99	260.94	164
Caucasian	AA	532.33	223.98	9
	GA	447.39	206.04	77
	GG	459.70	229.37	437
	Total	459.13	225.85	523
Total	AA	562.92	304.28	12
	GA	481.00	234.30	98
	GG	482.21	237.90	577
	Total	483.45	238.49	687

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	539.57	266.12	35
	AG	522.48	219.77	79
	AA	622.68	300.67	53
	Total	557.86	259.86	167
Caucasian	GG	448.88	245.76	116
	AG	457.21	217.68	223
	AA	473.66	227.02	186
	Total	461.20	227.20	525
Total	GG	469.90	252.65	151
	AG	474.28	219.75	302
	AA	506.70	252.31	239
	Total	484.52	238.92	692

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	572.00	266.71	16
	CT	525.84	228.89	64
	CC	590.85	285.81	86
	Total	563.97	263.63	166
Caucasian	TT	458.68	230.73	107
	CT	463.38	229.29	220
	CC	464.62	222.78	206
	Total	462.92	226.67	533
Total	TT	473.42	237.63	123
	CT	477.45	230.29	284
	CC	501.80	249.29	292
	Total	486.91	239.67	699

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	613.40	282.57	10
	CG	503.04	213.01	53
	GG	583.82	275.02	105
	Total	560.10	259.02	168
Caucasian	CC	477.79	256.12	28
	CG	448.36	228.39	164
	GG	466.01	221.28	343
	Total	461.22	225.12	535
Total	CC	513.47	266.37	38
	CG	461.71	225.48	217
	GG	493.62	239.91	448
	Total	484.85	237.26	703

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	268.00	.	1
	TT	564.16	260.02	171
	Total	562.44	260.24	172
Caucasian	CC	645.50	275.06	2
	TC	465.38	209.65	48
	TT	460.34	226.55	494
	Total	461.47	225.10	544
Total	CC	645.50	275.06	2
	TC	461.35	209.36	49
	TT	487.04	239.75	665
	Total	485.72	237.79	716

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	513.25	182.90	4
	GA	474.39	234.16	28
	GG	581.38	266.69	135
	Total	561.81	261.91	167
Caucasian	AA	364.00	59.40	2
	GA	468.18	293.26	22
	GG	461.67	222.28	509
	Total	461.58	224.98	533
Total	AA	463.50	163.45	6
	GA	471.66	259.00	50
	GG	486.77	237.14	644
	Total	485.49	238.00	700

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	604.67	119.06	3
	GA	534.31	260.46	39
	AA	566.49	266.05	121
	Total	559.49	262.20	163
Caucasian	GG	457.84	225.35	109
	GA	460.29	213.15	222
	AA	463.80	236.92	184
	Total	461.03	224.04	515
Total	GG	461.78	224.13	112
	GA	471.35	221.88	261
	AA	504.54	253.50	305
	Total	484.70	237.35	678

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	569.50	261.49	6
	CT	597.00	350.38	27
	CC	555.38	243.27	133
	Total	562.66	262.67	166
Caucasian	TT	466.25	260.76	8
	CT	490.08	220.57	76
	CC	451.87	225.55	435
	Total	457.69	225.32	519
Total	TT	510.50	256.37	14
	CT	518.11	263.25	103
	CC	476.11	233.75	568
	Total	483.13	238.99	685

CD320_rs250510		*M*	*SD*	*N*
African American	TT	357.00	83.61	3
	CT	606.38	231.30	21
	CC	558.32	262.26	136
	Total	560.85	257.45	160
Caucasian	TT	199.00	.	1
	CT	508.17	208.90	6
	CC	458.51	224.14	503
	Total	458.58	223.91	510
Total	TT	317.50	104.41	4
	CT	584.56	226.45	27
	CC	479.75	236.12	639
	Total	483.00	236.23	670

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	567.00	.	1
	AG	368.00	65.05	2
	GG	568.59	265.04	165
	Total	566.19	263.60	168
Caucasian	AA	243.00	.	1
	GG	461.57	226.32	511
	Total	461.14	226.31	512
Total	AA	405.00	229.10	2
	AG	368.00	65.05	2
	GG	487.69	240.59	676
	Total	487.10	240.19	680

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	423.60	110.39	10
	GC	561.29	232.97	41
	GG	582.24	281.93	114
	Total	567.42	264.76	165
Caucasian	CC	387.50	253.56	12
	GC	441.90	193.61	93
	GG	469.21	233.66	398
	Total	462.21	227.38	503
Total	CC	403.91	198.08	22
	GC	478.43	212.84	134
	GG	494.38	249.42	512
	Total	488.20	241.27	668

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	616.71	261.70	21
	TC	602.05	292.60	65
	CC	520.58	227.57	78
	Total	565.18	261.38	164
Caucasian	TT	480.78	199.96	9
	TC	512.63	229.28	32
	CC	456.96	225.97	479
	Total	460.80	225.77	520
Total	TT	575.93	249.55	30
	TC	572.55	275.39	97
	CC	465.87	227.07	557
	Total	485.83	238.80	684

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	647.50	267.99	2
	CT	617.80	300.16	15
	CC	554.80	255.06	153
	Total	561.45	258.47	170
Caucasian	TT	421.00	170.37	5
	CT	442.50	282.14	2
	CC	460.82	225.53	535
	Total	460.38	224.90	542
Total	TT	485.71	208.65	7
	CT	597.18	295.30	17
	CC	481.72	235.49	688
	Total	484.51	237.12	712

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	450.93	224.32	15
	CT	600.09	265.55	44
	TT	560.71	260.22	103
	Total	561.24	260.11	162
Caucasian	CC	468.53	212.15	53
	CT	458.31	222.49	192
	TT	462.90	234.77	269
	Total	461.76	227.59	514
Total	CC	464.65	213.31	68
	CT	484.75	237.06	236
	TT	489.98	245.67	372
	Total	485.60	239.39	676

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	578.36	273.01	28
	CT	593.21	282.72	66
	CC	515.81	236.59	69
	Total	557.90	263.29	163
Caucasian	TT	484.87	264.93	75
	CT	460.71	230.92	206
	CC	458.11	207.42	230
	Total	463.09	225.88	511
Total	TT	510.28	269.07	103
	CT	492.86	250.50	272
	CC	471.43	215.47	299
	Total	486.02	238.75	674

Table 21 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	555.38	255.89	21
	CT	596.02	291.37	59
	TT	541.30	243.85	88
	Total	562.27	262.57	168
Caucasian	CC	508.75	71.25	4
	CT	516.09	252.72	33
	TT	457.58	224.64	491
	Total	461.63	225.93	528
Total	CC	547.92	235.59	25
	CT	567.35	279.38	92
	TT	470.31	229.42	579
	Total	485.92	239.02	696

Table 22

Homocysteine

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	8.80	1.64	5
	GA	10.91	4.15	34
	AA	11.04	4.18	117
	Total	10.94	4.12	156
Caucasian	GG	10.10	2.51	8
	GA	10.98	3.76	81
	AA	10.37	3.73	415
	Total	10.46	3.72	504
Total	GG	9.60	2.24	13
	GA	10.96	3.86	115
	AA	10.52	3.84	532
	Total	10.58	3.82	660

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	10.27	3.52	18
	CG	11.49	3.86	54
	GG	10.82	4.38	87
	Total	10.99	4.11	159
Caucasian	CC	10.95	4.12	11
	CG	9.84	2.92	85
	GG	10.63	3.85	416
	Total	10.50	3.72	512
Total	CC	10.53	3.70	29
	CG	10.48	3.40	139
	GG	10.66	3.94	503
	Total	10.62	3.82	671

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	9.00	2.46	11
	CT	11.30	3.86	52
	TT	11.05	4.37	95
	Total	10.99	4.12	158
Caucasian	CC	9.13	1.91	3
	CT	10.90	.	1
	TT	10.47	3.72	510
	Total	10.46	3.71	514
Total	CC	9.03	2.28	14
	CT	11.29	3.82	53
	TT	10.56	3.83	605
	Total	10.59	3.81	672

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	13.05	7.12	8
	GA	10.35	3.52	38
	AA	10.99	4.06	113
	Total	10.94	4.14	159
Caucasian	GG	11.05	2.57	24
	GA	10.59	3.89	80
	AA	10.41	3.73	407
	Total	10.47	3.70	511
Total	GG	11.55	4.14	32
	GA	10.52	3.76	118
	AA	10.54	3.81	520
	Total	10.58	3.82	670

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	5.80	0.57	2
	TC	11.10	4.03	25
	TT	11.01	4.15	132
	Total	10.96	4.14	159
Caucasian	CC	10.63	4.34	30
	TC	10.26	3.73	146
	TT	10.58	3.64	338
	Total	10.49	3.70	514
Total	CC	10.33	4.37	32
	TC	10.39	3.77	171
	TT	10.70	3.79	470
	Total	10.60	3.81	673

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	11.38	3.17	13
	AT	10.58	4.08	44
	TT	11.10	4.32	100
	Total	10.98	4.15	157
Caucasian	AA	11.32	3.54	38
	AT	10.98	4.21	139
	TT	10.20	3.49	332
	Total	10.50	3.72	509
Total	AA	11.34	3.42	51
	AT	10.89	4.17	183
	TT	10.41	3.71	432
	Total	10.61	3.83	666

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	10.40	.	1
	CT	14.90	.	1
	TT	10.91	4.15	159
	Total	10.93	4.13	161
Caucasian	CC	11.53	4.00	4
	CT	10.07	3.69	18
	TT	10.52	3.72	495
	Total	10.51	3.72	517
Total	CC	11.30	3.50	5
	CT	10.33	3.76	19
	TT	10.61	3.83	654
	Total	10.61	3.82	678

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	8.48	3.24	9
	CT	10.93	3.56	48
	CC	11.16	4.42	101
	Total	10.94	4.14	158
Caucasian	CT	9.70	1.51	6
	CC	10.52	3.75	512
	Total	10.51	3.73	518
Total	TT	8.48	3.24	9
	CT	10.80	3.40	54
	CC	10.62	3.87	613
	Total	10.61	3.83	676

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	11.50	3.87	9
	TC	11.52	4.62	47
	CC	10.72	3.96	100
	Total	11.01	4.16	156
Caucasian	TT	9.87	3.03	114
	TC	10.38	3.77	208
	CC	11.00	3.97	181
	Total	10.49	3.71	503
Total	TT	9.99	3.11	123
	TC	10.59	3.96	255
	CC	10.90	3.96	281
	Total	10.61	3.82	659

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	11.52	4.10	6
	TT	10.95	4.12	156
	Total	10.97	4.11	162
Caucasian	AA	10.38	3.49	13
	AT	9.97	3.53	92
	TT	10.57	3.66	405
	Total	10.46	3.63	510
Total	AA	10.38	3.49	13
	AT	10.06	3.57	98
	TT	10.68	3.79	561
	Total	10.58	3.76	672

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	12.63	5.11	10
	CG	10.92	4.15	55
	CC	10.88	4.05	93
	Total	11.00	4.15	158
Caucasian	GG	9.90	3.05	121
	CG	10.40	3.81	230
	CC	10.95	3.88	167
	Total	10.46	3.68	518
Total	GG	10.11	3.31	131
	CG	10.50	3.87	285
	CC	10.92	3.93	260
	Total	10.59	3.80	676

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	13.77	6.70	7
	CT	10.08	3.85	40
	CC	11.11	3.97	114
	Total	10.97	4.12	161
Caucasian	TT	11.81	2.94	14
	CT	10.78	3.84	90
	CC	10.36	3.69	408
	Total	10.48	3.71	512
Total	TT	12.47	4.47	21
	CT	10.56	3.84	130
	CC	10.53	3.77	522
	Total	10.59	3.81	673

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	14.88	8.73	4
	GT	10.26	3.89	41
	TT	11.13	3.95	115
	Total	11.00	4.12	160
Caucasian	GG	11.68	3.04	12
	GT	10.90	3.89	92
	TT	10.36	3.70	409
	Total	10.49	3.72	513
Total	GG	12.48	4.90	16
	GT	10.70	3.89	133
	TT	10.53	3.76	524
	Total	10.61	3.82	673

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	11.30	4.58	35
	AG	10.78	3.88	73
	AA	11.11	4.19	52
	Total	11.00	4.12	160
Caucasian	GG	11.02	4.04	115
	AG	10.37	3.78	213
	AA	10.33	3.43	183
	Total	10.50	3.72	511
Total	GG	11.09	4.16	150
	AG	10.47	3.80	286
	AA	10.50	3.62	235
	Total	10.62	3.83	671

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	17.03	8.94	3
	GA	9.09	3.18	20
	GG	11.13	3.97	134
	Total	10.99	4.11	157
Caucasian	AA	11.54	3.92	9
	GA	10.96	3.97	76
	GG	10.38	3.67	424
	Total	10.48	3.72	509
Total	AA	12.92	5.65	12
	GA	10.57	3.88	96
	GG	10.56	3.76	558
	Total	10.60	3.82	666

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	10.71	3.91	16
	CT	10.63	3.95	62
	CC	11.28	4.37	81
	Total	10.97	4.15	159
Caucasian	TT	11.24	4.13	106
	CT	10.27	3.75	211
	CC	10.32	3.38	202
	Total	10.49	3.70	519
Total	TT	11.17	4.09	122
	CT	10.35	3.79	273
	CC	10.59	3.71	283
	Total	10.60	3.82	678

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	10.33	3.23	10
	CG	10.97	3.99	52
	GG	10.89	4.13	99
	Total	10.88	4.02	161
Caucasian	CC	11.21	3.34	28
	CG	11.01	4.12	160
	GG	10.18	3.45	333
	Total	10.49	3.68	521
Total	CC	10.98	3.29	38
	CG	11.00	4.08	212
	GG	10.34	3.63	432
	Total	10.58	3.77	682

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	11.93	7.52	4
	GA	11.70	4.50	25
	GG	10.86	3.93	131
	Total	11.02	4.11	160
Caucasian	AA	7.50	2.55	2
	GA	10.22	3.21	21
	GG	10.50	3.71	496
	Total	10.48	3.68	519
Total	AA	10.45	6.36	6
	GA	11.03	3.99	46
	GG	10.57	3.75	627
	Total	10.60	3.79	679

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	12.30	.	1
	TT	10.94	4.09	164
	Total	10.95	4.08	165
Caucasian	CC	8.45	1.20	2
	TC	11.38	4.61	48
	TT	10.43	3.60	480
	Total	10.51	3.71	530
Total	CC	8.45	1.20	2
	TC	11.39	4.56	49
	TT	10.56	3.74	644
	Total	10.61	3.80	695

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	14.43	4.29	3
	GA	10.89	3.46	36
	AA	10.89	4.32	117
	Total	10.96	4.14	156
Caucasian	GG	10.15	3.21	106
	GA	10.32	3.64	215
	AA	10.82	4.09	180
	Total	10.47	3.73	501
Total	GG	10.27	3.30	109
	GA	10.41	3.62	251
	AA	10.85	4.18	297
	Total	10.58	3.83	657

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	9.87	2.50	6
	CT	11.19	4.57	25
	CC	11.01	4.09	128
	Total	11.00	4.11	159
Caucasian	TT	11.75	4.32	8
	CT	10.50	4.31	73
	CC	10.47	3.61	424
	Total	10.49	3.72	505
Total	TT	10.94	3.66	14
	CT	10.68	4.36	98
	CC	10.59	3.73	552
	Total	10.61	3.82	664

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	13.80	.	1
	AG	9.35	3.32	2
	GG	10.96	4.14	158
	Total	10.95	4.12	161
Caucasian	AA	13.40	.	1
	GG	10.47	3.72	497
	Total	10.48	3.72	498
Total	AA	13.60	0.28	2
	AG	9.35	3.32	2
	GG	10.59	3.83	655
	Total	10.59	3.82	659

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs250510		*M*	*SD*	*N*
African American	TT	17.20	5.81	3
	CT	9.99	2.89	21
	CC	10.95	4.18	129
	Total	10.94	4.14	153
Caucasian	TT	13.80	.	1
	CT	8.87	2.35	6
	CC	10.44	3.72	489
	Total	10.43	3.70	496
Total	TT	16.35	5.04	4
	CT	9.74	2.78	27
	CC	10.55	3.82	618
	Total	10.55	3.82	649

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	11.02	3.13	10
	GC	11.54	4.44	39
	GG	10.64	4.07	109
	Total	10.89	4.11	158
Caucasian	CC	11.70	4.04	12
	GC	10.27	3.45	92
	GG	10.52	3.79	387
	Total	10.51	3.74	491
Total	CC	11.39	3.58	22
	GC	10.65	3.80	131
	GG	10.55	3.85	496
	Total	10.60	3.83	649

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	10.20	3.54	20
	TC	10.82	4.20	62
	CC	11.35	4.23	75
	Total	10.99	4.13	157
Caucasian	TT	10.68	3.79	9
	TC	11.05	3.03	31
	CC	10.39	3.72	466
	Total	10.43	3.68	506
Total	TT	10.34	3.56	29
	TC	10.90	3.84	93
	CC	10.52	3.81	541
	Total	10.57	3.80	663

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	12.37	4.67	15
	CT	11.17	4.78	42
	TT	10.76	3.73	98
	Total	11.03	4.13	155
Caucasian	CC	10.64	3.76	51
	CT	10.40	3.57	188
	TT	10.57	3.84	262
	Total	10.51	3.73	501
Total	CC	11.04	4.02	66
	CT	10.54	3.82	230
	TT	10.62	3.80	360
	Total	10.63	3.83	656

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	10.90	2.12	2
	CT	10.24	2.71	14
	CC	11.02	4.22	147
	Total	10.95	4.09	163
Caucasian	TT	11.20	3.05	5
	CT	11.30	2.97	2
	CC	10.46	3.68	521
	Total	10.47	3.67	528
Total	TT	11.11	2.64	7
	CT	10.38	2.66	16
	CC	10.58	3.81	668
	Total	10.58	3.77	691

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	10.21	3.82	27
	CT	10.47	4.19	62
	CC	11.72	4.16	67
	Total	10.96	4.14	156
Caucasian	TT	11.26	4.95	75
	CT	10.29	3.15	198
	CC	10.33	3.67	224
	Total	10.46	3.71	497
Total	TT	10.98	4.68	102
	CT	10.33	3.42	260
	CC	10.65	3.83	291
	Total	10.58	3.82	653

Table 22 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	10.91	3.70	21
	CT	10.55	3.95	55
	TT	11.20	4.30	85
	Total	10.94	4.10	161
Caucasian	CC	12.08	4.86	4
	CT	11.23	3.33	32
	TT	10.44	3.75	478
	Total	10.50	3.74	514
Total	CC	11.10	3.81	25
	CT	10.80	3.73	87
	TT	10.55	3.85	563
	Total	10.60	3.83	675

Table 23

Methylmalonic Acid

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
	GG	180.40	105.05	5
African American	GA	211.37	128.10	35
	AA	197.64	117.83	116
	Total	200.17	119.29	156
Caucasian	GG	248.38	124.04	8
	GA	275.65	156.66	80
	AA	246.89	136.24	411
	Total	251.53	139.65	499
Total	GG	222.23	117.64	13
	GA	256.09	150.94	115
	AA	236.05	133.87	527
	Total	239.29	136.76	655

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	178.11	80.15	18
	CG	186.89	89.11	54
	GG	214.38	139.49	87
	Total	200.94	119.05	159
Caucasian	CC	302.27	176.86	11
	CG	241.29	159.25	83
	GG	253.25	135.81	413
	Total	252.36	140.79	507
Total	CC	225.21	137.23	29
	CG	219.85	138.19	137
	GG	246.49	137.12	500
	Total	240.08	137.59	666

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	182.55	78.81	11
	CT	188.67	93.00	52
	TT	209.81	135.30	95
	Total	200.96	119.53	158
Caucasian	CC	229.00	19.52	3
	CT	232.00	.	1
	TT	249.97	139.72	505
	Total	249.81	139.18	509
Total	CC	192.50	72.30	14
	CT	189.49	92.29	53
	TT	243.61	139.69	600
	Total	238.24	136.30	667

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	197.38	110.07	8
	GA	193.58	123.51	38
	AA	203.67	120.48	113
	Total	200.94	120.07	159
Caucasian	GG	228.96	78.96	24
	GA	236.43	117.66	80
	AA	255.34	147.00	402
	Total	251.09	140.29	506
Total	GG	221.06	86.92	32
	GA	222.63	120.73	118
	AA	244.00	143.11	515
	Total	239.10	137.32	665

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	101.50	23.33	2
	TC	246.64	164.84	25
	TT	193.79	107.34	132
	Total	200.94	119.09	159
Caucasian	CC	266.00	147.75	30
	TC	243.63	134.00	145
	TT	253.16	142.28	334
	Total	251.20	140.14	509
Total	CC	255.72	148.58	32
	TC	244.08	138.42	170
	TT	236.34	135.87	466
	Total	239.24	137.02	668

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	204.00	.	1
	CT	401.00	.	1
	TT	198.53	118.44	159
	Total	199.82	118.78	161
Caucasian	CC	325.50	232.75	4
	CT	326.22	164.41	18
	TT	248.14	138.48	490
	Total	251.49	140.78	512
Total	CC	301.20	208.76	5
	CT	330.16	160.70	19
	TT	235.98	135.45	649
	Total	239.13	137.53	673

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	222.46	140.88	13
	AT	208.57	152.66	44
	TT	198.10	103.63	100
	Total	203.05	121.74	157
Caucasian	AA	242.82	136.85	38
	AT	258.52	134.93	138
	TT	247.60	140.09	328
	Total	250.23	138.28	504
Total	AA	237.63	136.76	51
	AT	246.45	140.63	182
	TT	236.03	134.01	428
	Total	239.02	135.94	661

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	170.00	37.20	9
	CT	189.35	102.30	48
	CC	208.79	131.31	101
	Total	200.68	119.67	158
Caucasian	CT	373.00	231.24	6
	CC	250.10	138.62	507
	Total	251.54	140.31	513
Total	TT	170.00	37.20	9
	CT	209.76	133.11	54
	CC	243.24	138.19	608
	Total	239.56	137.36	671

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	202.56	119.31	9
	TC	217.85	108.44	46
	CC	192.81	126.00	101
	Total	200.76	120.50	156
Caucasian	TT	263.14	159.04	112
	TC	245.82	139.28	206
	CC	251.36	129.63	180
	Total	251.72	140.52	498
Total	TT	258.64	156.85	121
	TC	240.71	134.42	252
	CC	230.32	131.17	281
	Total	239.56	137.65	654

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	207.60	113.61	10
	CG	207.62	106.37	55
	CC	199.57	131.97	93
	Total	202.88	121.87	158
Caucasian	GG	257.73	155.72	119
	CG	246.82	137.09	228
	CC	249.57	132.17	166
	Total	250.24	139.89	513
Total	GG	253.84	153.11	129
	CG	239.20	132.42	283
	CC	231.61	134.01	259
	Total	239.09	137.25	671

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	245.83	133.08	6
	TT	200.23	120.18	156
	Total	201.92	120.54	162
Caucasian	AA	254.46	100.31	13
	AT	249.01	149.36	90
	TT	252.89	139.69	402
	Total	252.24	140.38	505
Total	AA	254.46	100.31	13
	AT	248.81	147.76	96
	TT	238.17	136.48	558
	Total	240.02	137.45	667

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	160.43	60.93	7
	CT	203.90	130.03	40
	CC	203.95	120.64	114
	Total	202.04	120.91	161
Caucasian	TT	271.38	90.48	13
	CT	248.44	120.76	90
	CC	251.57	145.93	404
	Total	251.52	140.47	507
Total	TT	232.55	96.39	20
	CT	234.74	124.89	130
	CC	241.09	142.02	518
	Total	239.60	137.56	668

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	152.50	44.81	4
	GT	203.07	128.43	41
	TT	203.48	120.78	115
	Total	202.10	121.28	160
Caucasian	GG	260.92	74.03	12
	GT	252.22	122.26	91
	TT	250.36	146.10	405
	Total	250.94	140.66	508
Total	GG	233.81	82.29	16
	GT	236.95	125.80	132
	TT	239.99	142.12	520
	Total	239.24	137.77	668

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	185.00	27.73	3
	GA	190.65	132.14	20
	GG	203.00	122.93	134
	Total	201.08	122.65	157
Caucasian	AA	251.78	78.94	9
	GA	246.49	121.19	76
	GG	252.36	144.73	419
	Total	251.46	140.36	504
Total	AA	235.08	74.73	12
	GA	234.85	124.93	96
	GG	240.40	141.25	553
	Total	239.49	137.95	661

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	219.46	147.54	35
	AG	209.18	128.71	73
	AA	180.48	84.88	52
	Total	202.10	121.28	160
Caucasian	GG	262.19	143.47	115
	AG	247.00	138.36	210
	AA	251.90	142.16	181
	Total	252.21	140.74	506
Total	GG	252.22	145.06	150
	AG	237.24	136.72	283
	AA	235.96	134.73	233
	Total	240.17	137.90	666

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	231.94	194.23	16
	CT	202.92	120.61	62
	CC	195.42	104.05	81
	Total	202.02	121.62	159
Caucasian	TT	261.31	143.84	106
	CT	240.71	129.08	208
	CC	259.97	152.17	200
	Total	252.45	141.54	514
Total	TT	257.46	150.76	122
	CT	232.03	127.96	270
	CC	241.36	142.86	281
	Total	240.54	138.68	673

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	179.80	149.64	10
	CG	211.23	139.11	52
	GG	197.85	108.26	99
	Total	201.05	121.14	161
Caucasian	CC	255.86	151.94	28
	CG	256.08	139.48	158
	GG	250.11	141.53	330
	Total	252.25	141.23	516
Total	CC	235.84	153.12	38
	CG	244.98	140.40	210
	GG	238.05	136.27	429
	Total	240.07	138.37	677

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	131.00	.	1
	TT	202.27	120.50	164
	Total	201.84	120.26	165
Caucasian	CC	162.00	18.38	2
	TC	238.02	124.02	48
	TT	253.35	141.56	475
	Total	251.60	139.84	525
Total	CC	162.00	18.38	2
	TC	235.84	123.67	49
	TT	240.24	138.19	639
	Total	239.70	136.99	690

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	164.00	77.90	4
	GA	213.80	100.31	25
	GG	202.34	126.73	131
	Total	203.18	121.74	160
Caucasian	AA	191.50	21.92	2
	GA	295.43	163.56	21
	GG	249.34	139.59	491
	Total	250.99	140.55	514
Total	AA	173.17	62.76	6
	GA	251.07	137.65	46
	GG	239.44	138.22	622
	Total	239.64	137.74	674

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	159.33	23.25	3
	GA	215.64	130.69	36
	AA	197.58	120.10	117
	Total	201.01	121.45	156
Caucasian	GG	262.49	161.43	104
	GA	239.00	128.91	213
	AA	254.87	132.73	179
	Total	249.65	137.73	496
Total	GG	259.60	160.08	107
	GA	235.63	129.16	249
	AA	232.22	130.73	296
	Total	238.02	135.52	652

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	182.67	90.88	6
	CT	229.36	170.45	25
	CC	196.76	108.92	128
	Total	201.35	119.85	159
Caucasian	TT	291.50	69.03	8
	CT	249.11	143.52	73
	CC	250.30	141.69	419
	Total	250.78	141.01	500
Total	TT	244.86	94.16	14
	CT	244.07	150.18	98
	CC	237.77	136.54	547
	Total	238.86	137.75	659

CD320_rs250510		*M*	*SD*	*N*
African American	TT	175.67	90.59	3
	CT	155.50	35.84	20
	CC	207.31	128.48	130
	Total	199.92	120.81	153
Caucasian	TT	234.00	.	1
	CT	200.67	100.82	6
	CC	251.82	142.59	484
	Total	251.16	142.05	491
Total	TT	190.25	79.51	4
	CT	165.92	58.19	26
	CC	242.40	140.80	614
	Total	238.99	138.93	644

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	133.00	.	1
	AG	166.50	98.29	2
	GG	199.71	119.80	158
	Total	198.88	119.10	161
Caucasian	AA	497.00	.	1
	GG	251.34	141.37	492
	Total	251.84	141.66	493
Total	AA	315.00	257.39	2
	AG	166.50	98.29	2
	GG	238.79	138.14	650
	Total	238.80	138.26	654

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	232.10	160.90	10
	GC	218.66	105.04	38
	GG	191.87	119.57	110
	Total	200.86	119.19	158
Caucasian	CC	230.50	103.92	12
	GC	235.51	130.80	90
	GG	252.52	142.77	384
	Total	248.83	139.76	486
Total	CC	231.23	129.44	22
	GC	230.51	123.54	128
	GG	239.02	140.12	494
	Total	237.06	136.49	644

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	193.57	129.12	21
	TC	196.29	121.07	62
	CC	204.65	118.61	74
	Total	199.87	120.30	157
Caucasian	TT	245.44	90.14	9
	TC	233.61	107.26	31
	CC	252.40	143.65	461
	Total	251.11	140.80	501
Total	TT	209.13	119.68	30
	TC	208.73	117.40	93
	CC	245.79	141.32	535
	Total	238.88	137.85	658

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	132.50	38.89	2
	CT	200.00	172.49	14
	CC	200.20	112.97	147
	Total	199.35	118.13	163
Caucasian	TT	368.40	232.81	5
	CT	326.00	241.83	2
	CC	251.44	139.44	516
	Total	252.84	140.93	523
Total	TT	301.00	222.79	7
	CT	215.75	177.59	16
	CC	240.08	135.62	663
	Total	240.13	137.67	686

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	211.93	100.98	15
	CT	186.12	101.27	41
	TT	205.04	130.91	99
	Total	200.70	120.73	155
Caucasian	CC	277.08	179.43	50
	CT	254.82	149.22	187
	TT	243.87	124.35	259
	Total	251.35	140.41	496
Total	CC	262.05	166.27	65
	CT	242.46	144.06	228
	TT	233.13	127.21	358
	Total	239.29	137.60	651

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	193.32	134.51	28
	CT	197.02	122.50	62
	CC	213.52	118.48	66
	Total	203.33	122.56	156
Caucasian	TT	263.72	143.46	74
	CT	249.48	145.11	198
	CC	242.20	133.13	220
	Total	248.37	139.52	492
Total	TT	244.39	143.90	102
	CT	236.97	141.61	260
	CC	235.58	130.26	286
	Total	237.52	136.91	648

Table 23 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	204.95	170.95	21
	CT	175.02	77.00	56
	TT	220.58	129.28	84
	Total	202.70	121.65	161
Caucasian	CC	232.75	58.20	4
	CT	249.44	108.20	32
	TT	250.86	142.45	473
	Total	250.62	139.97	509
Total	CC	209.40	157.75	25
	CT	202.08	96.00	88
	TT	246.29	140.85	557
	Total	239.11	137.24	670

Table 24

Geriatric Depression Scale Score

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	8.40	4.88	5
	GA	6.54	4.77	39
	AA	6.30	5.11	141
	Total	6.41	5.02	185
Caucasian	GG	2.60	2.72	10
	GA	7.06	5.76	87
	AA	6.59	5.60	454
	Total	6.60	5.61	551
Total	GG	4.53	4.42	15
	GA	6.90	5.46	126
	AA	6.52	5.48	595
	Total	6.55	5.46	736

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	6.79	5.57	19
	CG	5.81	4.48	67
	GG	6.84	5.24	103
	Total	6.47	5.01	189
Caucasian	CC	4.73	2.90	11
	CG	6.22	5.22	92
	GG	6.73	5.66	458
	Total	6.61	5.55	561
Total	CC	6.03	4.82	30
	CG	6.04	4.91	159
	GG	6.75	5.58	561
	Total	6.57	5.42	750

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	8.17	5.44	12
	CT	5.25	3.77	60
	TT	7.03	5.34	117
	Total	6.53	4.96	189
Caucasian	CC	3.33	2.08	3
	CT	16.00	.	1
	TT	6.64	5.56	561
	Total	6.64	5.56	565
Total	CC	7.20	5.28	15
	CT	5.43	3.99	61
	TT	6.71	5.52	678
	Total	6.62	5.42	754

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	8.40	6.65	10
	GA	6.83	5.46	48
	AA	6.33	4.83	132
	Total	6.56	5.09	190
Caucasian	GG	8.43	6.01	28
	GA	6.62	5.51	87
	AA	6.48	5.50	447
	Total	6.60	5.53	562
Total	GG	8.42	6.09	38
	GA	6.70	5.47	135
	AA	6.44	5.35	579
	Total	6.59	5.42	752

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	5.50	5.26	4
	TC	6.10	5.09	29
	TT	6.55	5.02	157
	Total	6.46	5.01	190
Caucasian	CC	8.71	6.46	35
	TC	6.64	5.60	159
	TT	6.32	5.43	371
	Total	6.56	5.57	565
Total	CC	8.38	6.36	39
	TC	6.55	5.52	188
	TT	6.39	5.31	528
	Total	6.54	5.43	755

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	5.00	.	1
	CT	1.50	2.12	2
	TT	6.48	5.01	188
	Total	6.42	5.00	191
Caucasian	CC	8.25	6.18	4
	CT	8.42	8.64	19
	TT	6.49	5.41	544
	Total	6.56	5.55	567
Total	CC	7.60	5.55	5
	CT	7.76	8.47	21
	TT	6.48	5.30	732
	Total	6.53	5.41	758

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	7.07	5.41	14
	AT	6.42	5.12	50
	TT	6.30	5.01	123
	Total	6.39	5.04	187
Caucasian	AA	6.73	5.63	45
	AT	6.51	5.12	148
	TT	6.54	5.71	367
	Total	6.55	5.54	560
Total	AA	6.81	5.54	59
	AT	6.49	5.11	198
	TT	6.48	5.54	490
	Total	6.51	5.42	747

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	6.33	5.12	9
	CT	5.59	4.14	54
	CC	6.83	5.35	126
	Total	6.46	5.03	189
Caucasian	CT	3.86	4.74	7
	CC	6.67	5.56	562
	Total	6.63	5.56	569
Total	TT	6.33	5.12	9
	CT	5.39	4.21	61
	CC	6.70	5.52	688
	Total	6.59	5.43	758

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	6.07	5.06	14
	TC	6.29	5.03	56
	CC	6.62	5.03	116
	Total	6.48	5.01	186
Caucasian	TT	6.37	5.77	123
	TC	6.26	5.60	225
	CC	7.25	5.55	203
	Total	6.65	5.63	551
Total	TT	6.34	5.68	137
	TC	6.27	5.48	281
	CC	7.02	5.37	319
	Total	6.61	5.48	737

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	5.94	4.85	16
	CG	6.11	5.05	65
	CC	6.76	5.06	108
	Total	6.47	5.03	189
Caucasian	GG	6.33	5.68	133
	CG	6.49	5.69	251
	CC	6.98	5.25	183
	Total	6.61	5.55	567
Total	GG	6.29	5.58	149
	CG	6.41	5.56	316
	CC	6.90	5.18	291
	Total	6.57	5.42	756

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	5.17	3.43	6
	TT	6.47	5.03	187
	Total	6.42	4.98	193
Caucasian	AA	5.36	4.13	14
	AT	6.17	5.88	92
	TT	6.76	5.56	455
	Total	6.63	5.58	561
Total	AA	5.36	4.13	14
	AT	6.11	5.75	98
	TT	6.67	5.41	642
	Total	6.58	5.43	754

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	6.86	4.98	7
	CT	6.25	5.04	51
	CC	6.49	5.01	134
	Total	6.44	4.99	192
Caucasian	TT	4.86	3.01	14
	CT	6.96	5.84	101
	CC	6.57	5.57	448
	Total	6.60	5.57	563
Total	TT	5.52	3.78	21
	CT	6.72	5.58	152
	CC	6.55	5.44	582
	Total	6.56	5.43	755

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	5.25	4.03	4
	GT	6.12	5.02	50
	TT	6.52	5.05	135
	Total	6.39	5.01	189
Caucasian	GG	5.50	3.42	12
	GT	6.67	5.77	102
	TT	6.70	5.59	450
	Total	6.67	5.58	564
Total	GG	5.44	3.44	16
	GT	6.49	5.53	152
	TT	6.66	5.47	585
	Total	6.60	5.44	753

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	5.25	3.86	4
	GA	6.33	5.14	27
	GG	6.55	5.01	157
	Total	6.49	4.99	188
Caucasian	AA	5.56	3.13	9
	GA	6.89	5.83	85
	GG	6.56	5.55	465
	Total	6.59	5.56	559
Total	AA	5.46	3.20	13
	GA	6.76	5.65	112
	GG	6.56	5.41	622
	Total	6.57	5.42	747

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	6.24	5.06	38
	AG	6.52	4.95	90
	AA	6.31	5.09	62
	Total	6.39	5.00	190
Caucasian	GG	6.87	5.28	127
	AG	6.83	5.89	235
	AA	6.38	5.46	199
	Total	6.68	5.60	561
Total	GG	6.73	5.22	165
	AG	6.75	5.64	325
	AA	6.36	5.37	261
	Total	6.61	5.45	751

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	7.47	4.94	17
	CT	6.55	5.09	71
	CC	6.13	5.00	102
	Total	6.41	5.02	190
Caucasian	TT	7.01	5.36	120
	CT	6.37	5.49	230
	CC	6.46	5.72	220
	Total	6.54	5.55	570
Total	TT	7.07	5.30	137
	CT	6.41	5.39	301
	CC	6.35	5.49	322
	Total	6.50	5.42	760

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	8.91	4.70	11
	CG	6.17	4.93	59
	GG	6.37	5.03	121
	Total	6.46	5.00	191
Caucasian	CC	6.03	5.45	32
	CG	6.78	5.22	172
	GG	6.67	5.77	368
	Total	6.67	5.58	572
Total	CC	6.77	5.37	43
	CG	6.63	5.15	231
	GG	6.60	5.59	489
	Total	6.62	5.44	763

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	0.00	.	1
	TT	6.54	5.00	195
	Total	6.51	5.01	196
Caucasian	CC	6.00	1.41	2
	TC	6.57	5.80	49
	TT	6.62	5.54	531
	Total	6.62	5.55	582
Total	CC	6.00	1.41	2
	TC	6.44	5.81	50
	TT	6.60	5.40	726
	Total	6.59	5.42	778

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	4.20	3.56	5
	GA	5.23	4.16	31
	GG	6.73	5.19	154
	Total	6.42	5.03	190
Caucasian	AA	6.00	4.24	2
	GA	7.43	8.19	23
	GG	6.64	5.50	547
	Total	6.67	5.61	572
Total	AA	4.71	3.50	7
	GA	6.17	6.23	54
	GG	6.66	5.43	701
	Total	6.61	5.47	762

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	10.25	9.78	4
	GA	6.43	5.38	44
	AA	6.26	4.74	136
	Total	6.39	5.02	184
Caucasian	GG	6.21	5.27	115
	GA	6.63	5.83	238
	AA	6.82	5.46	200
	Total	6.61	5.58	553
Total	GG	6.34	5.46	119
	GA	6.60	5.75	282
	AA	6.59	5.18	336
	Total	6.55	5.44	737

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	7.14	7.01	7
	CT	5.48	3.27	33
	CC	6.65	5.23	148
	Total	6.46	5.01	188
Caucasian	TT	7.20	5.03	10
	CT	7.25	5.32	79
	CC	6.50	5.65	466
	Total	6.62	5.59	555
Total	TT	7.18	5.71	17
	CT	6.73	4.86	112
	CC	6.53	5.55	614
	Total	6.58	5.45	743

CD320_rs250510		*M*	*SD*	*N*
African American	TT	10.67	7.57	3
	CT	4.32	3.87	22
	CC	6.52	4.91	157
	Total	6.32	4.90	182
Caucasian	TT	3.00	.	1
	CT	4.00	3.03	6
	CC	6.63	5.59	539
	Total	6.59	5.57	546
Total	TT	8.75	7.27	4
	CT	4.25	3.66	28
	CC	6.60	5.44	696
	Total	6.52	5.41	728

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	21.00	.	1
	AG	5.67	1.53	3
	GG	6.41	4.95	186
	Total	6.47	5.01	190
Caucasian	AA	3.00	.	1
	GG	6.52	5.53	547
	Total	6.51	5.53	548
Total	AA	12.00	12.73	2
	AG	5.67	1.53	3
	GG	6.49	5.39	733
	Total	6.50	5.40	738

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	9.38	5.80	13
	GC	5.90	4.25	50
	GG	6.46	5.06	125
	Total	6.52	4.96	188
Caucasian	CC	5.25	5.22	12
	GC	7.09	6.13	98
	GG	6.53	5.45	427
	Total	6.61	5.58	537
Total	CC	7.40	5.81	25
	GC	6.69	5.58	148
	GG	6.52	5.36	552
	Total	6.58	5.42	725

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	7.05	6.11	21
	TC	5.78	5.16	72
	CC	6.71	4.40	94
	Total	6.39	4.91	187
Caucasian	TT	8.00	6.40	9
	TC	6.03	6.13	35
	CC	6.59	5.55	512
	Total	6.58	5.59	556
Total	TT	7.33	6.10	30
	TC	5.86	5.47	107
	CC	6.61	5.38	606
	Total	6.53	5.43	743

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	15.00	1.41	2
	CT	6.61	3.93	18
	CC	6.46	5.08	174
	Total	6.56	5.02	194
Caucasian	TT	11.60	6.27	5
	CT	8.50	7.78	2
	CC	6.58	5.57	573
	Total	6.63	5.59	580
Total	TT	12.57	5.41	7
	CT	6.80	4.16	20
	CC	6.55	5.46	747
	Total	6.61	5.45	774

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	6.88	4.95	16
	CT	5.61	4.57	51
	TT	6.70	5.02	118
	Total	6.42	4.90	185
Caucasian	CC	6.60	4.46	57
	CT	6.42	5.68	202
	TT	6.55	5.52	288
	Total	6.50	5.47	547
Total	CC	6.66	4.54	73
	CT	6.25	5.48	253
	TT	6.59	5.37	406
	Total	6.48	5.33	732

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	5.83	5.71	29
	CT	6.35	5.19	75
	CC	6.23	4.28	78
	Total	6.21	4.89	182
Caucasian	TT	5.77	4.68	81
	CT	6.48	5.64	221
	CC	6.89	5.79	242
	Total	6.55	5.58	544
Total	TT	5.78	4.94	110
	CT	6.44	5.52	296
	CC	6.73	5.47	320
	Total	6.47	5.41	726

Table 24 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	6.87	5.78	23
	CT	6.51	4.97	65
	TT	6.47	4.90	102
	Total	6.53	5.01	190
Caucasian	CC	5.60	6.19	5
	CT	6.71	6.91	35
	TT	6.65	5.53	524
	Total	6.65	5.62	564
Total	CC	6.64	5.76	28
	CT	6.58	5.69	100
	TT	6.62	5.43	626
	Total	6.62	5.47	754

Table 25

Peripheral Neuropathy

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	4.19	1.56	5
	GA	8.80	4.54	31
	AA	8.43	4.48	97
	Total	8.35	4.48	133
Caucasian	GG	9.23	4.72	8
	GA	7.85	4.32	57
	AA	8.49	4.40	258
	Total	8.40	4.39	323
Total	GG	7.29	4.51	13
	GA	8.19	4.39	88
	AA	8.47	4.42	355
	Total	8.39	4.41	456

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	8.15	5.46	18
	CG	8.13	4.44	47
	GG	8.91	4.45	71
	Total	8.54	4.57	136
Caucasian	CC	8.17	3.82	6
	CG	8.11	4.68	54
	GG	8.38	4.36	269
	Total	8.33	4.39	329
Total	CC	8.15	5.02	24
	CG	8.12	4.54	101
	GG	8.49	4.37	340
	Total	8.39	4.44	465

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	8.23	5.72	10
	CT	8.20	4.62	46
	TT	8.62	4.42	80
	Total	8.45	4.55	136
Caucasian	CC	9.39	6.49	3
	CT	18.78	.	1
	TT	8.40	4.41	331
	Total	8.44	4.45	335
Total	CC	8.50	5.64	13
	CT	8.43	4.82	47
	TT	8.44	4.40	411
	Total	8.44	4.47	471

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	8.61	4.98	9
	GA	7.93	3.90	28
	AA	8.55	4.75	99
	Total	8.42	4.58	136
Caucasian	GG	7.81	4.15	15
	GA	7.59	4.23	47
	AA	8.59	4.48	271
	Total	8.41	4.44	333
Total	GG	8.11	4.39	24
	GA	7.72	4.09	75
	AA	8.58	4.55	370
	Total	8.41	4.47	469

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	8.81	6.20	4
	TC	8.41	4.41	16
	TT	8.56	4.55	117
	Total	8.55	4.55	137
Caucasian	CC	9.58	5.07	24
	TC	8.77	4.44	87
	TT	8.05	4.30	223
	Total	8.35	4.41	334
Total	CC	9.47	5.12	28
	TC	8.72	4.42	103
	TT	8.22	4.39	340
	Total	8.41	4.44	471

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	8.90	.	1
	CT	16.25	.	1
	TT	8.43	4.53	137
	Total	8.49	4.55	139
Caucasian	CC	5.79	3.38	3
	CT	6.28	3.06	11
	TT	8.45	4.43	321
	Total	8.35	4.40	335
Total	CC	6.57	3.17	4
	CT	7.11	4.10	12
	TT	8.44	4.46	458
	Total	8.39	4.44	474

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	7.64	5.56	10
	AT	9.21	4.35	37
	TT	8.35	4.45	87
	Total	8.53	4.50	134
Caucasian	AA	7.81	4.40	33
	AT	8.09	4.33	86
	TT	8.58	4.49	212
	Total	8.38	4.43	331
Total	AA	7.77	4.63	43
	AT	8.43	4.35	123
	TT	8.51	4.47	299
	Total	8.42	4.45	465

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	8.30	6.07	9
	CT	8.27	4.37	39
	CC	8.45	4.36	87
	Total	8.39	4.46	135
Caucasian	CT	7.26	1.85	3
	CC	8.40	4.43	331
	Total	8.39	4.41	334
Total	TT	8.30	6.07	9
	CT	8.20	4.24	42
	CC	8.41	4.41	418
	Total	8.39	4.42	469

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	9.43	5.13	10
	TC	9.24	3.85	38
	CC	7.96	4.66	86
	Total	8.43	4.49	134
Caucasian	TT	8.17	4.13	72
	TC	8.46	4.47	119
	CC	8.37	4.45	131
	Total	8.36	4.38	322
Total	TT	8.32	4.25	82
	TC	8.65	4.33	157
	CC	8.21	4.53	217
	Total	8.38	4.41	456

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	10.22	5.22	12
	CG	9.10	3.93	41
	CC	7.86	4.68	83
	Total	8.44	4.55	136
Caucasian	GG	8.33	4.37	80
	CG	8.40	4.39	138
	CC	8.29	4.55	119
	Total	8.35	4.43	337
Total	GG	8.58	4.50	92
	CG	8.56	4.29	179
	CC	8.11	4.60	202
	Total	8.37	4.46	473

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	8.62	4.15	5
	TT	8.49	4.56	134
	Total	8.50	4.54	139
Caucasian	AA	9.06	3.79	8
	AT	8.60	4.19	49
	TT	8.38	4.49	272
	Total	8.43	4.43	329
Total	AA	9.06	3.79	8
	AT	8.60	4.15	54
	TT	8.41	4.51	406
	Total	8.45	4.45	468

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	7.96	0.70	5
	CT	7.12	4.06	34
	CC	9.00	4.74	99
	Total	8.50	4.55	138
Caucasian	TT	8.97	6.14	8
	CT	7.74	4.00	52
	CC	8.38	4.41	271
	Total	8.29	4.39	331
Total	TT	8.58	4.74	13
	CT	7.50	4.01	86
	CC	8.54	4.51	370
	Total	8.35	4.44	469

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	6.53	2.45	3
	GT	6.93	3.54	34
	TT	9.04	4.73	99
	Total	8.45	4.50	136
Caucasian	GG	9.77	5.08	7
	GT	7.63	4.12	54
	TT	8.49	4.47	273
	Total	8.38	4.43	334
Total	GG	8.80	4.58	10
	GT	7.36	3.90	88
	TT	8.64	4.54	372
	Total	8.40	4.45	470

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	7.86	0.90	3
	GA	7.24	4.48	15
	GG	8.72	4.61	117
	Total	8.53	4.55	135
Caucasian	AA	9.59	6.01	5
	GA	8.00	4.45	42
	GG	8.38	4.39	282
	Total	8.35	4.41	329
Total	AA	8.94	4.65	8
	GA	7.80	4.43	57
	GG	8.48	4.45	399
	Total	8.41	4.45	464

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	8.35	4.07	26
	AG	9.28	4.69	64
	AA	7.59	4.53	47
	Total	8.52	4.56	137
Caucasian	GG	8.75	4.90	85
	AG	8.20	4.27	131
	AA	8.29	4.23	118
	Total	8.37	4.42	334
Total	GG	8.66	4.70	111
	AG	8.55	4.43	195
	AA	8.09	4.31	165
	Total	8.42	4.45	471

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	6.03	3.40	9
	CT	9.01	4.73	55
	CC	8.28	4.52	73
	Total	8.43	4.57	137
Caucasian	TT	8.66	4.70	87
	CT	8.18	4.34	122
	CC	8.32	4.19	130
	Total	8.36	4.37	339
Total	TT	8.41	4.64	96
	CT	8.44	4.47	177
	CC	8.31	4.30	203
	Total	8.38	4.43	476

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	5.61	1.96	5
	CG	9.27	4.82	46
	GG	8.34	4.57	87
	Total	8.55	4.62	138
Caucasian	CC	8.72	4.61	23
	CG	8.13	4.56	101
	GG	8.45	4.30	217
	Total	8.37	4.39	341
Total	CC	8.17	4.40	28
	CG	8.49	4.66	147
	GG	8.42	4.37	304
	Total	8.42	4.45	479

TCN2_rs1131603		*M*	*SD*	*N*
African American	TT	8.51	4.59	142
	Total	8.51	4.59	142
Caucasian	CC	6.84	3.47	2
	TC	8.38	4.53	24
	TT	8.34	4.42	320
	Total	8.33	4.41	346
Total	CC	6.84	3.47	2
	TC	8.38	4.53	24
	TT	8.39	4.47	462
	Total	8.38	4.46	488

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	8.31	2.57	4
	GA	9.36	4.58	20
	GG	8.20	4.56	112
	Total	8.38	4.51	136
Caucasian	AA	3.36	.	1
	GA	7.10	3.04	13
	GG	8.44	4.45	328
	Total	8.38	4.41	342
Total	AA	7.32	3.14	5
	GA	8.47	4.14	33
	GG	8.38	4.48	440
	Total	8.38	4.44	478

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	7.91	5.27	4
	GA	9.96	4.00	28
	AA	7.96	4.47	102
	Total	8.38	4.44	134
Caucasian	GG	8.14	4.12	71
	GA	8.61	4.41	132
	AA	8.03	4.51	126
	Total	8.28	4.38	329
Total	GG	8.12	4.15	75
	GA	8.84	4.36	160
	AA	8.00	4.48	228
	Total	8.31	4.40	463

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	7.55	6.08	5
	CT	8.04	4.36	21
	CC	8.79	4.66	110
	Total	8.63	4.64	136
Caucasian	TT	9.31	4.64	7
	CT	9.31	4.64	44
	CC	8.24	4.40	274
	Total	8.41	4.44	325
Total	TT	8.57	5.10	12
	CT	8.90	4.55	65
	CC	8.40	4.48	384
	Total	8.48	4.50	461

CD320_rs250510		*M*	*SD*	*N*
African American	TT	9.74	4.77	3
	CT	11.51	5.13	12
	CC	8.01	4.29	115
	Total	8.38	4.46	130
Caucasian	TT	6.70	.	1
	CT	7.16	2.69	4
	CC	8.37	4.45	314
	Total	8.34	4.42	319
Total	TT	8.98	4.18	4
	CT	10.43	4.95	16
	CC	8.27	4.40	429
	Total	8.35	4.43	449

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	7.04	.	1
	AG	10.86	4.49	2
	GG	8.46	4.57	135
	Total	8.49	4.54	138
Caucasian	AA	6.44	.	1
	GG	8.40	4.43	320
	Total	8.39	4.42	321
Total	AA	6.74	0.42	2
	AG	10.86	4.49	2
	GG	8.41	4.46	455
	Total	8.42	4.45	459

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	7.00	3.05	10
	GC	8.01	4.60	36
	GG	8.91	4.70	90
	Total	8.53	4.59	136
Caucasian	CC	5.45	3.55	6
	GC	9.77	4.93	59
	GG	7.99	4.25	250
	Total	8.27	4.43	315
Total	CC	6.42	3.22	16
	GC	9.10	4.86	95
	GG	8.23	4.39	340
	Total	8.35	4.48	451

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	9.24	3.17	16
	TC	7.99	4.37	52
	CC	8.98	4.98	65
	Total	8.62	4.56	133
Caucasian	TT	8.07	3.57	8
	TC	8.24	4.30	21
	CC	8.36	4.46	293
	Total	8.35	4.41	322
Total	TT	8.85	3.28	24
	TC	8.06	4.33	73
	CC	8.47	4.55	358
	Total	8.43	4.46	455

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	5.10	1.26	2
	CT	6.15	3.14	12
	CC	8.82	4.65	127
	Total	8.54	4.58	141
Caucasian	TT	6.52	2.47	5
	CT	5.68	1.08	2
	CC	8.37	4.38	338
	Total	8.33	4.35	345
Total	TT	6.11	2.20	7
	CT	6.08	2.91	14
	CC	8.50	4.46	465
	Total	8.39	4.42	486

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	10.96	5.37	13
	CT	8.40	4.21	32
	TT	8.34	4.53	88
	Total	8.61	4.57	133
Caucasian	CC	7.43	3.56	40
	CT	8.77	4.54	107
	TT	8.38	4.52	168
	Total	8.39	4.42	315
Total	CC	8.29	4.30	53
	CT	8.69	4.45	139
	TT	8.37	4.52	256
	Total	8.46	4.46	448

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	9.51	4.64	21
	CT	8.23	4.56	55
	CC	8.44	4.70	55
	Total	8.52	4.62	131
Caucasian	TT	7.22	3.98	52
	CT	8.87	4.69	121
	CC	8.37	4.29	144
	Total	8.38	4.42	317
Total	TT	7.88	4.28	73
	CT	8.67	4.65	176
	CC	8.39	4.40	199
	Total	8.42	4.48	448

Table 25 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	8.67	3.46	15
	CT	7.78	4.73	48
	TT	8.76	4.78	75
	Total	8.41	4.63	138
Caucasian	CC	10.99	5.18	5
	CT	7.65	3.56	23
	TT	8.36	4.47	305
	Total	8.35	4.43	333
Total	CC	9.25	3.94	20
	CT	7.74	4.36	71
	TT	8.43	4.53	380
	Total	8.36	4.48	471

Table 26

Hand Grip Strength

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	28.75	5.85	4
	GA	21.61	6.65	31
	AA	23.61	6.05	127
	Total	23.36	6.24	162
Caucasian	GG	19.89	7.62	9
	GA	22.08	4.86	80
	AA	21.74	5.84	420
	Total	21.76	5.73	509
Total	GG	22.62	8.09	13
	GA	21.95	5.40	111
	AA	22.17	5.94	547
	Total	22.14	5.89	671

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	23.94	6.58	18
	CG	22.98	6.14	57
	GG	23.33	6.24	90
	Total	23.28	6.21	165
Caucasian	CC	16.20	7.98	10
	CG	21.75	5.41	85
	GG	21.91	5.64	423
	Total	21.77	5.70	518
Total	CC	21.18	7.92	28
	CG	22.25	5.72	142
	GG	22.16	5.77	513
	Total	22.13	5.86	683

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	25.80	6.16	10
	CT	22.94	5.91	52
	TT	23.25	6.37	103
	Total	23.31	6.21	165
Caucasian	CC	18.33	2.52	3
	CT	16.00	.	1
	TT	21.72	5.72	518
	Total	21.69	5.71	522
Total	CC	24.08	6.34	13
	CT	22.81	5.93	53
	TT	21.97	5.85	621
	Total	22.08	5.87	687

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	18.63	2.67	8
	GA	23.60	6.57	42
	AA	23.53	6.17	116
	Total	23.31	6.22	166
Caucasian	GG	20.04	5.89	24
	GA	21.65	5.66	79
	AA	21.84	5.74	417
	Total	21.73	5.74	520
Total	GG	19.69	5.27	32
	GA	22.32	6.04	121
	AA	22.21	5.87	533
	Total	22.11	5.89	686

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	23.75	4.11	4
	TC	24.79	7.40	28
	TT	22.96	5.96	134
	Total	23.29	6.19	166
Caucasian	CC	20.61	4.13	33
	TC	22.06	5.43	146
	TT	21.71	5.93	343
	Total	21.74	5.69	522
Total	CC	20.95	4.19	37
	TC	22.50	5.85	174
	TT	22.06	5.96	477
	Total	22.11	5.85	688

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	27.00	.	1
	CT	24.00	2.83	2
	TT	23.16	6.30	164
	Total	23.20	6.26	167
Caucasian	CC	24.75	4.57	4
	CT	23.50	6.95	18
	TT	21.68	5.64	502
	Total	21.77	5.69	524
Total	CC	25.20	4.09	5
	CT	23.55	6.61	20
	TT	22.05	5.84	666
	Total	22.11	5.86	691

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	26.92	5.26	12
	AT	22.21	6.55	42
	TT	23.22	6.08	110
	Total	23.23	6.22	164
Caucasian	AA	21.26	5.66	39
	AT	21.96	5.74	137
	TT	21.72	5.72	341
	Total	21.75	5.71	517
Total	AA	22.59	6.02	51
	AT	22.02	5.92	179
	TT	22.08	5.84	451
	Total	22.11	5.86	681

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	24.22	6.69	9
	CT	22.91	5.92	46
	CC	23.26	6.27	110
	Total	23.22	6.17	165
Caucasian	CT	22.57	7.48	7
	CC	21.68	5.70	518
	Total	21.70	5.72	525
Total	TT	24.22	6.69	9
	CT	22.87	6.07	53
	CC	21.96	5.83	628
	Total	22.06	5.86	690

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	22.14	7.26	14
	TC	22.98	6.71	46
	CC	23.55	5.88	103
	Total	23.27	6.22	163
Caucasian	TT	21.47	6.06	115
	TC	22.38	5.43	209
	CC	21.24	5.47	184
	Total	21.76	5.61	508
Total	TT	21.54	6.17	129
	TC	22.49	5.67	255
	CC	22.07	5.72	287
	Total	22.13	5.79	671

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	22.25	6.78	16
	CG	24.02	6.82	55
	CC	23.15	5.65	94
	Total	23.35	6.16	165
Caucasian	GG	21.48	6.23	124
	CG	22.06	5.59	233
	CC	21.47	5.47	167
	Total	21.73	5.71	524
Total	GG	21.57	6.27	140
	CG	22.43	5.88	288
	CC	22.07	5.58	261
	Total	22.12	5.85	689

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	20.67	7.74	6
	TT	23.39	6.10	162
	Total	23.29	6.15	168
Caucasian	AA	22.43	8.27	14
	AT	22.50	6.00	88
	TT	21.54	5.52	417
	Total	21.73	5.69	519
Total	AA	22.43	8.27	14
	AT	22.38	6.09	94
	TT	22.06	5.74	579
	Total	22.11	5.84	687

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	22.86	6.36	7
	CT	23.55	5.21	44
	CC	23.22	6.51	117
	Total	23.29	6.15	168
Caucasian	TT	21.42	5.33	12
	CT	21.71	5.52	92
	CC	21.70	5.72	417
	Total	21.70	5.67	521
Total	TT	21.95	5.60	19
	CT	22.30	5.47	136
	CC	22.04	5.93	534
	Total	22.09	5.82	689

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	21.75	2.63	4
	GT	23.49	5.39	43
	TT	23.27	6.58	118
	Total	23.29	6.21	165
Caucasian	GG	21.70	5.70	10
	GT	21.60	5.60	93
	TT	21.74	5.73	419
	Total	21.72	5.70	522
Total	GG	21.71	4.91	14
	GT	22.20	5.59	136
	TT	22.08	5.96	537
	Total	22.10	5.86	687

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	19.00	4.58	3
	GA	25.17	4.91	23
	GG	23.08	6.39	139
	Total	23.30	6.22	165
Caucasian	AA	20.86	6.62	7
	GA	21.68	5.77	77
	GG	21.75	5.69	433
	Total	21.73	5.70	517
Total	AA	20.30	5.89	10
	GA	22.48	5.75	100
	GG	22.07	5.89	572
	Total	22.11	5.86	682

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	23.35	5.73	34
	AG	23.44	6.82	78
	AA	23.02	5.58	54
	Total	23.28	6.19	166
Caucasian	GG	21.98	5.93	117
	AG	21.77	5.47	216
	AA	21.52	5.79	184
	Total	21.73	5.68	517
Total	GG	22.29	5.89	151
	AG	22.21	5.89	294
	AA	21.86	5.77	238
	Total	22.11	5.84	683

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	24.13	5.57	15
	CT	23.75	6.37	60
	CC	22.86	6.27	91
	Total	23.30	6.23	166
Caucasian	TT	21.49	5.32	111
	CT	22.16	5.67	212
	CC	21.47	5.89	204
	Total	21.75	5.69	527
Total	TT	21.80	5.40	126
	CT	22.51	5.86	272
	CC	21.90	6.04	295
	Total	22.12	5.85	693

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	25.22	5.38	9
	CG	23.18	6.39	50
	GG	23.08	6.22	108
	Total	23.23	6.21	167
Caucasian	CC	21.59	5.77	29
	CG	22.08	5.70	159
	GG	21.50	5.71	341
	Total	21.68	5.70	529
Total	CC	22.45	5.82	38
	CG	22.34	5.87	209
	GG	21.88	5.87	449
	Total	22.05	5.86	696

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	32.00	.	1
	TT	23.15	6.16	169
	Total	23.20	6.18	170
Caucasian	CC	19.50	6.36	2
	TC	22.66	5.64	44
	TT	21.65	5.70	491
	Total	21.72	5.69	537
Total	CC	19.50	6.36	2
	TC	22.87	5.75	45
	TT	22.03	5.85	660
	Total	22.08	5.84	707

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	23.60	4.39	5
	GA	24.21	7.26	28
	GG	23.07	6.04	133
	Total	23.28	6.20	166
Caucasian	AA	20.50	9.19	2
	GA	23.82	6.28	22
	GG	21.57	5.63	506
	Total	21.66	5.67	530
Total	AA	22.71	5.41	7
	GA	24.04	6.79	50
	GG	21.88	5.74	639
	Total	22.04	5.84	696

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	17.33	3.79	3
	GA	22.79	7.07	38
	AA	23.55	5.84	121
	Total	23.25	6.15	162
Caucasian	GG	21.38	6.23	106
	GA	21.92	5.40	220
	AA	21.67	5.78	184
	Total	21.72	5.71	510
Total	GG	21.27	6.20	109
	GA	22.05	5.67	258
	AA	22.41	5.87	305
	Total	22.09	5.85	672

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	25.00	9.47	6
	CT	23.93	6.75	28
	CC	22.98	5.98	132
	Total	23.22	6.23	166
Caucasian	TT	21.25	5.04	8
	CT	21.96	5.34	72
	CC	21.68	5.73	432
	Total	21.71	5.65	512
Total	TT	22.86	7.20	14
	CT	22.51	5.80	100
	CC	21.99	5.81	564
	Total	22.08	5.83	678

CD320_rs250510		*M*	*SD*	*N*
African American	TT	19.00	5.00	3
	CT	25.14	5.11	22
	CC	23.02	6.45	135
	Total	23.24	6.30	160
Caucasian	TT	18.00	.	1
	CT	23.80	4.49	5
	CC	21.80	5.64	500
	Total	21.81	5.62	506
Total	TT	18.75	4.11	4
	CT	24.89	4.95	27
	CC	22.06	5.83	635
	Total	22.15	5.82	666

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AG	26.00	5.66	2
	GG	23.13	6.31	165
	Total	23.16	6.29	167
Caucasian	AA	22.00	.	1
	GG	21.79	5.67	507
	Total	21.79	5.67	508
Total	AA	22.00	.	1
	AG	26.00	5.66	2
	GG	22.12	5.86	672
	Total	22.13	5.85	675

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	23.83	6.95	12
	GC	23.37	5.68	41
	GG	22.63	6.32	111
	Total	22.90	6.19	164
Caucasian	CC	20.42	5.92	12
	GC	21.75	5.62	91
	GG	21.82	5.76	394
	Total	21.77	5.73	497
Total	CC	22.13	6.55	24
	GC	22.25	5.67	132
	GG	22.00	5.89	505
	Total	22.05	5.86	661

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	22.59	4.06	17
	TC	22.47	6.34	62
	CC	23.99	6.53	85
	Total	23.27	6.26	164
Caucasian	TT	21.44	7.26	9
	TC	21.91	5.42	34
	CC	21.77	5.65	472
	Total	21.78	5.66	515
Total	TT	22.19	5.27	26
	TC	22.27	6.01	96
	CC	22.11	5.84	557
	Total	22.14	5.84	679

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	31.00	.	1
	CT	25.00	5.90	15
	CC	23.06	6.21	153
	Total	23.28	6.21	169
Caucasian	TT	24.80	3.96	5
	CT	17.00	7.07	2
	CC	21.73	5.71	529
	Total	21.74	5.70	536
Total	TT	25.83	4.36	6
	CT	24.06	6.38	17
	CC	22.03	5.85	682
	Total	22.11	5.86	705

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	23.50	7.88	16
	CT	23.43	6.37	47
	TT	23.10	6.02	100
	Total	23.23	6.28	163
Caucasian	CC	21.11	4.54	54
	CT	21.99	5.98	186
	TT	21.84	5.78	266
	Total	21.82	5.73	506
Total	CC	21.66	5.51	70
	CT	22.28	6.07	233
	TT	22.18	5.86	366
	Total	22.16	5.90	669

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	21.96	4.89	27
	CT	22.92	6.00	64
	CC	24.30	6.83	70
	Total	23.36	6.24	161
Caucasian	TT	21.68	6.46	74
	CT	22.05	5.85	207
	CC	21.55	5.44	222
	Total	21.78	5.76	503
Total	TT	21.75	6.06	101
	CT	22.26	5.89	271
	CC	22.21	5.91	292
	Total	22.16	5.92	664

Table 26 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	21.90	6.74	20
	CT	23.96	6.27	54
	TT	22.98	6.02	91
	Total	23.17	6.19	165
Caucasian	CC	19.40	9.91	5
	CT	22.79	4.53	33
	TT	21.77	5.72	481
	Total	21.81	5.70	519
Total	CC	21.40	7.31	25
	CT	23.52	5.68	87
	TT	21.96	5.78	572
	Total	22.14	5.84	684

Table 27

Walking Speed

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs16988828		*M*	*SD*	*N*
African American	GG	0.68	0.22	5
	GA	0.61	0.26	34
	AA	0.63	0.31	132
	Total	0.62	0.29	171
Caucasian	GG	0.77	0.37	10
	GA	0.76	0.32	83
	AA	0.81	0.35	430
	Total	0.80	0.35	523
Total	GG	0.74	0.32	15
	GA	0.72	0.31	117
	AA	0.77	0.35	562
	Total	0.76	0.34	694

TCN2_rs7289549		*M*	*SD*	*N*
African American	CC	0.54	0.22	19
	CG	0.56	0.31	62
	GG	0.67	0.28	93
	Total	0.62	0.29	174
Caucasian	CC	0.92	0.30	10
	CG	0.83	0.34	86
	GG	0.79	0.35	437
	Total	0.80	0.35	533
Total	CC	0.67	0.31	29
	CG	0.71	0.35	148
	GG	0.77	0.34	530
	Total	0.75	0.34	707

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs7286107		*M*	*SD*	*N*
African American	CC	0.55	0.26	10
	CT	0.54	0.30	57
	TT	0.67	0.28	105
	Total	0.62	0.29	172
Caucasian	CC	0.56	0.23	3
	CT	0.33	.	1
	TT	0.80	0.35	532
	Total	0.80	0.35	536
Total	CC	0.55	0.24	13
	CT	0.54	0.30	58
	TT	0.78	0.34	637
	Total	0.76	0.34	708

TCN2_rs9606756		*M*	*SD*	*N*
African American	GG	0.49	0.22	8
	GA	0.67	0.34	43
	AA	0.62	0.27	123
	Total	0.62	0.29	174
Caucasian	GG	0.72	0.37	26
	GA	0.81	0.36	83
	AA	0.80	0.34	422
	Total	0.80	0.34	531
Total	GG	0.66	0.35	34
	GA	0.76	0.36	126
	AA	0.76	0.33	545
	Total	0.75	0.34	705

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs740234		*M*	*SD*	*N*
African American	CC	0.70	0.33	4
	TC	0.67	0.28	29
	TT	0.61	0.30	140
	Total	0.62	0.29	173
Caucasian	CC	0.74	0.28	32
	TC	0.83	0.37	153
	TT	0.79	0.34	351
	Total	0.80	0.35	536
Total	CC	0.73	0.28	36
	TC	0.80	0.36	182
	TT	0.74	0.34	491
	Total	0.75	0.34	709

TCN2_rs35915865		*M*	*SD*	*N*
African American	CC	0.74	.	1
	CT	0.70	0.12	2
	TT	0.62	0.30	172
	Total	0.62	0.29	175
Caucasian	CC	0.81	0.29	4
	CT	0.88	0.45	18
	TT	0.80	0.34	516
	Total	0.80	0.34	538
Total	CC	0.80	0.25	5
	CT	0.87	0.43	20
	TT	0.75	0.34	688
	Total	0.75	0.34	713

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs11703570		*M*	*SD*	*N*
African American	AA	0.65	0.21	13
	AT	0.61	0.27	45
	TT	0.62	0.32	113
	Total	0.62	0.30	171
Caucasian	AA	0.76	0.34	43
	AT	0.78	0.33	139
	TT	0.81	0.35	348
	Total	0.80	0.35	530
Total	AA	0.73	0.31	56
	AT	0.74	0.32	184
	TT	0.77	0.35	461
	Total	0.76	0.34	701

TCN2_rs35838082		*M*	*SD*	*N*
African American	TT	0.61	0.23	9
	CT	0.56	0.32	51
	CC	0.65	0.29	113
	Total	0.62	0.30	173
Caucasian	CT	0.94	0.29	6
	CC	0.80	0.35	532
	Total	0.80	0.35	538
Total	TT	0.61	0.23	9
	CT	0.60	0.33	57
	CC	0.77	0.34	645
	Total	0.76	0.34	711

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2267163		*M*	*SD*	*N*
African American	TT	0.67	0.22	13
	TC	0.65	0.36	48
	CC	0.60	0.27	109
	Total	0.62	0.29	170
Caucasian	TT	0.79	0.35	118
	TC	0.82	0.37	209
	CC	0.77	0.32	195
	Total	0.80	0.35	522
Total	TT	0.78	0.34	131
	TC	0.79	0.37	257
	CC	0.71	0.31	304
	Total	0.75	0.34	692

TCN2_rs1801198		*M*	*SD*	*N*
African American	GG	0.63	0.23	15
	CG	0.67	0.34	57
	CC	0.59	0.28	101
	Total	0.62	0.29	173
Caucasian	GG	0.80	0.35	128
	CG	0.82	0.37	232
	CC	0.76	0.31	178
	Total	0.80	0.35	538
Total	GG	0.78	0.35	143
	CG	0.79	0.36	289
	CC	0.70	0.31	279
	Total	0.76	0.34	711

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820021		*M*	*SD*	*N*
African American	AT	0.57	0.19	6
	TT	0.62	0.30	170
	Total	0.62	0.29	176
Caucasian	AA	0.79	0.37	14
	AT	0.83	0.36	90
	TT	0.79	0.35	429
	Total	0.80	0.35	533
Total	AA	0.79	0.37	14
	AT	0.81	0.36	96
	TT	0.74	0.34	599
	Total	0.75	0.34	709

TCN2_rs9621049		*M*	*SD*	*N*
African American	TT	0.62	0.20	7
	CT	0.66	0.34	46
	CC	0.60	0.28	123
	Total	0.62	0.29	176
Caucasian	TT	0.78	0.34	14
	CT	0.80	0.39	98
	CC	0.80	0.34	421
	Total	0.80	0.35	533
Total	TT	0.73	0.31	21
	CT	0.76	0.38	144
	CC	0.75	0.33	544
	Total	0.75	0.34	709

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820886		*M*	*SD*	*N*
African American	GG	0.58	0.15	4
	GT	0.67	0.34	46
	TT	0.60	0.28	124
	Total	0.62	0.29	174
Caucasian	GG	0.80	0.36	12
	GT	0.79	0.38	98
	TT	0.80	0.34	424
	Total	0.80	0.34	534
Total	GG	0.75	0.33	16
	GT	0.75	0.37	144
	TT	0.76	0.33	548
	Total	0.75	0.34	708

TCN2_rs4820887		*M*	*SD*	*N*
African American	AA	0.56	0.13	3
	GA	0.70	0.31	24
	GG	0.61	0.29	145
	Total	0.62	0.29	172
Caucasian	AA	0.84	0.39	9
	GA	0.79	0.39	81
	GG	0.80	0.34	439
	Total	0.80	0.35	529
Total	AA	0.77	0.36	12
	GA	0.77	0.37	105
	GG	0.75	0.34	584
	Total	0.76	0.34	701

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820888		*M*	*SD*	*N*
African American	GG	0.66	0.27	36
	AG	0.61	0.28	81
	AA	0.60	0.32	58
	Total	0.62	0.29	175
Caucasian	GG	0.77	0.30	122
	AG	0.80	0.36	220
	AA	0.80	0.35	190
	Total	0.79	0.34	532
Total	GG	0.74	0.30	158
	AG	0.75	0.35	301
	AA	0.75	0.36	248
	Total	0.75	0.34	707

TCN2_rs2301955		*M*	*SD*	*N*
African American	TT	0.69	0.23	16
	CT	0.62	0.29	64
	CC	0.61	0.31	94
	Total	0.62	0.30	174
Caucasian	TT	0.76	0.31	114
	CT	0.82	0.35	218
	CC	0.80	0.36	208
	Total	0.80	0.35	540
Total	TT	0.75	0.30	130
	CT	0.77	0.35	282
	CC	0.74	0.36	302
	Total	0.76	0.34	714

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs2301958		*M*	*SD*	*N*
African American	CC	0.72	0.21	10
	CG	0.59	0.26	52
	GG	0.63	0.31	114
	Total	0.62	0.29	176
Caucasian	CC	0.72	0.32	32
	CG	0.79	0.33	160
	GG	0.81	0.36	349
	Total	0.80	0.35	541
Total	CC	0.72	0.30	42
	CG	0.74	0.33	212
	GG	0.77	0.36	463
	Total	0.76	0.34	717

TCN2_rs1131603		*M*	*SD*	*N*
African American	TC	0.85	.	1
	TT	0.62	0.29	178
	Total	0.62	0.29	179
Caucasian	CC	0.73	0.20	2
	TC	0.79	0.34	47
	TT	0.80	0.35	501
	Total	0.80	0.35	550
Total	CC	0.73	0.20	2
	TC	0.79	0.34	48
	TT	0.75	0.34	679
	Total	0.75	0.34	729

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

TCN2_rs4820889		*M*	*SD*	*N*
African American	AA	0.64	0.25	5
	GA	0.66	0.30	30
	GG	0.61	0.30	139
	Total	0.62	0.29	174
Caucasian	AA	1.11	0.39	2
	GA	0.86	0.41	22
	GG	0.80	0.34	517
	Total	0.80	0.35	541
Total	AA	0.77	0.34	7
	GA	0.75	0.36	52
	GG	0.76	0.34	656
	Total	0.76	0.34	715

TCN2_rs2072194		*M*	*SD*	*N*
African American	GG	0.38	0.48	3
	GA	0.65	0.33	38
	AA	0.62	0.28	128
	Total	0.62	0.30	169
Caucasian	GG	0.79	0.34	110
	GA	0.81	0.36	222
	AA	0.79	0.34	192
	Total	0.80	0.35	524
Total	GG	0.78	0.35	113
	GA	0.79	0.36	260
	AA	0.72	0.33	320
	Total	0.76	0.34	693

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs173665		*M*	*SD*	*N*
African American	TT	0.56	0.21	6
	CT	0.60	0.25	29
	CC	0.63	0.31	138
	Total	0.62	0.29	173
Caucasian	TT	0.55	0.27	8
	CT	0.78	0.33	77
	CC	0.81	0.35	440
	Total	0.80	0.35	525
Total	TT	0.56	0.23	14
	CT	0.73	0.32	106
	CC	0.76	0.35	578
	Total	0.75	0.34	698

CD320_rs250510		*M*	*SD*	*N*
African American	TT	0.55	0.18	3
	CT	0.70	0.31	21
	CC	0.62	0.29	144
	Total	0.63	0.29	168
Caucasian	TT	0.47	.	1
	CT	0.91	0.36	6
	CC	0.80	0.35	509
	Total	0.80	0.35	516
Total	TT	0.53	0.16	4
	CT	0.75	0.33	27
	CC	0.76	0.34	653
	Total	0.76	0.34	684

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2232787		*M*	*SD*	*N*
African American	AA	0.06	.	1
	AG	0.77	0.19	2
	GG	0.62	0.29	172
	Total	0.62	0.29	175
Caucasian	AA	1.05	.	1
	GG	0.80	0.35	518
	Total	0.80	0.35	519
Total	AA	0.56	0.70	2
	AG	0.77	0.19	2
	GG	0.75	0.34	690
	Total	0.75	0.34	694

CD320_rs2227288		*M*	*SD*	*N*
African American	CC	0.64	0.23	12
	GC	0.70	0.31	42
	GG	0.60	0.28	118
	Total	0.63	0.29	172
Caucasian	CC	0.94	0.41	11
	GC	0.80	0.37	94
	GG	0.80	0.34	403
	Total	0.80	0.35	508
Total	CC	0.78	0.36	23
	GC	0.77	0.35	136
	GG	0.75	0.34	521
	Total	0.76	0.34	680

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2336573		*M*	*SD*	*N*
African American	TT	0.68	0.36	20
	TC	0.62	0.33	68
	CC	0.62	0.23	83
	Total	0.62	0.29	171
Caucasian	TT	0.66	0.15	8
	TC	0.83	0.26	32
	CC	0.80	0.35	487
	Total	0.80	0.35	527
Total	TT	0.67	0.32	28
	TC	0.68	0.33	100
	CC	0.78	0.35	570
	Total	0.76	0.34	698

CD320_rs2232779		*M*	*SD*	*N*
African American	TT	0.59	0.04	2
	CT	0.57	0.19	16
	CC	0.63	0.30	159
	Total	0.62	0.29	177
Caucasian	TT	0.67	0.24	4
	CT	1.28	0.32	2
	CC	0.80	0.35	543
	Total	0.80	0.35	549
Total	TT	0.64	0.19	6
	CT	0.65	0.30	18
	CC	0.76	0.34	702
	Total	0.76	0.34	726

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs2927707		*M*	*SD*	*N*
African American	CC	0.63	0.25	14
	CT	0.64	0.29	47
	TT	0.61	0.30	109
	Total	0.62	0.29	170
Caucasian	CC	0.78	0.32	55
	CT	0.81	0.37	191
	TT	0.81	0.34	273
	Total	0.80	0.35	519
Total	CC	0.75	0.31	69
	CT	0.77	0.36	238
	TT	0.75	0.34	382
	Total	0.76	0.34	689

CD320_rs3760680		*M*	*SD*	*N*
African American	TT	0.63	0.35	28
	CT	0.63	0.32	71
	CC	0.62	0.23	70
	Total	0.62	0.29	169
Caucasian	TT	0.78	0.27	75
	CT	0.83	0.38	209
	CC	0.79	0.34	232
	Total	0.80	0.35	516
Total	TT	0.74	0.30	103
	CT	0.78	0.38	280
	CC	0.75	0.32	302
	Total	0.76	0.34	685

Table 27 Continued

Means, Standard Deviations, and Sample Sizes by Race and SNP

CD320_rs8100119		*M*	*SD*	*N*
African American	CC	0.58	0.37	20
	CT	0.65	0.30	61
	TT	0.61	0.27	93
	Total	0.62	0.29	174
Caucasian	CC	0.68	0.24	5
	CT	0.81	0.27	30
	TT	0.80	0.35	499
	Total	0.80	0.35	534
Total	CC	0.60	0.34	25
	CT	0.70	0.30	91
	TT	0.77	0.35	592
	Total	0.75	0.34	708