ASSESSMENT OF THE RATE AND DETERMINANTS OF VITAMIN B12 DEFICIENCY IN SOUTH ASIAN AND EUROPEAN WOMEN OF CHILDBEARING AGE IN METRO VANCOUVER

by

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Abstract

Low maternal vitamin B12 (B12) status has been associated with an increased risk for adverse health outcomes in the offspring, including neural tube defects and insulin resistance. A high rate of B12 deficiency has been reported in both South Asians, who comprise one of Canada's largest minority groups, and Canadian women of childbearing age. Comprehensive B12 status assessment should include multiple biomarkers to reduce the risk of misclassification. However, there is no consensus on the appropriate cut-off values to define chronic and marginal B12 deficiency. Our goal was to assess the rate and determinants of B12 deficiency in healthy South Asian and European women aged 19-35 in Metro Vancouver using multiple biomarkers.

We conducted a cross-sectional descriptive study in a convenience sample (n=207) of South Asian and European women (19-35y). Anthropometric measurements and questionnaire data on demographics, lifestyle and diet were collected. Vitamin B12 status was assessed using serum vitamin B12 (SB12), serum holotranscobalamin (holoTC) and plasma methylmalonic acid (MMA) as biomarkers. The association of lifestyle, social, dietary, and genetic variables with B12 status was examined using multiple regression models.

Using conventional SB12 concentration cut-offs, 14% of all participants with biochemical data (n=204) were classified with chronic deficiency (SB12 <148 pmol/L), and 20% with marginal deficiency (SB12 148-220 pmol/L) and there was no difference in rate of deficiency between ethnic groups. The rate of B12 deficiency changed substantially (5-12% decrease) when applying cut-offs for alternate biomarkers (holoTC and MMA) alone or in combination with SB12 to indicate functional deficiency. Vitamin B12 status was influenced positively by dietary B12 intake, and B12 supplement use, and negatively by oral contraceptive use and first generation immigrant status.

We observed a high rate (34%) of B12 inadequacy based on SB12 levels, indicating a necessity for peri-conceptional monitoring of B12 status. Risk factors associated with B12 deficiency should be further assessed to determine whether extra surveillance is required. Use of multiple biomarkers, which reduced our estimates of the rate of inadequacy, should be applied henceforth in B12 status assessment, as SB12 alone may lack accuracy and may not be sensitive for detecting marginal deficiency. As such, further investigation into the appropriate cut-offs for MMA and holoTC is needed.

Preface

This thesis was prepared in partial fulfillment of the requirement for the degree of Master of Science in Human Nutrition. I completed this thesis under the supervision of Dr. Yvonne Lamers (YL) (primary) and Dr. Susan Barr (SB) (interim). YL, SB, and Dr. Angela Devlin (AD), contributed feedback during the preparation of the thesis and Dr. Patricia Janssen contributed feedback on the final draft during the thesis defense. YL developed the study design in collaboration with myself, SB and AD. The research team was comprised of myself, research assistants, undergraduate research volunteers, and other graduate students.

I recruited and enrolled participants, and completed assessments including blood draws, questionnaires, and anthropometric measurements. I performed blood analysis with the help of research assistants at various laboratories (Lamers Lab[1], Devlin Lab[2], Innis Lab[2], Robinson Lab[2]), with the exception of complete blood count (CBC) analysis[3]. The Shatenstein Lab[4] carried out analysis of the food frequency questionnaire. I conducted all laboratory experiments involving biomarker analysis and genotyping, and entry and error checking of all questionnaire data with the help of research assistants. I performed all statistical analyses, in consultation with YL, SB, and Arianne Albert[5].

Preliminary results from the present study were the topic of an oral presentation (Experimental Biology 2013 Conference) and a poster presentation (Canadian Nutrition Society 2013 Conference). Based on the primary objective and exploratory objective 1 of the study, I have prepared a manuscript in collaboration with YL, SB, AD, and Theresa Schroder, which is intended for submission for publication. Other components of this thesis may be the topic of future manuscripts. This study was approved to proceed by the Clinical Research Ethics Board at the University of British Columbia (H11-01216), Vancouver Coastal Health Research Ethics Board (#V11-01216) and Fraser Health Research Ethics Board (FHREB 2013-016). The 'FNH Vitamin Research Fund' funded the project and my student stipend.

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List of Abbreviations

*An 's' added on the end of an acronym implies the plural form of the word or fragment

ASF= animal sourced food
B12 = vitamin B12
BCNS= British Columbia Nutrition Survey
BMI= body mass index
CBC= complete blood count
CCHS= Canadian Community Health Survey
CHMS= Canadian Health Measures Survey
DQ= demographic questionnaire
EAR= estimated average requirement
FFQ= food frequency questionnaire
FUT2= galactoside 2 alpha fucosyl transferase 2
GI= gastrointestinal
Hb= hemoglobin
HC= haptocorrin
Hcy= homocysteine
holoTC= holotranscobalamin
HOMA-IR = homeostatic model assessment of insulin resistance
IF= intrinsic factor
IPAQ= International Physical Activity Questionnaire
LC-MS/MS= liquid chromatography tandem mass spectrometry
LMBRD1= probable lysosomal cobalamin transporter
MCV= mean corpuscular volume
MMA= methylmalonic acid
MTHFR= methylene tetrahydrofolate reductase
MS= methionine synthase
MTRR= methionine synthase reductase
NHANES= National Health and Nutrition Examination Survey
NTD= neural tube defect
OC=oral contraceptives
RBC= red blood cell
RDA = recommended dietary allowance
SA= South Asian or South Asia, *depending on context*
SB12= serum vitamin B12
SD = standard deviation
SES= socioeconomic status
TCN1= transcobalamin 1
TCN2= transcobalamin 2
tHcy=total homocysteine
$\boldsymbol{\chi^2}$= chi square

Acknowledgements

I would like to extend my sincere gratitude to all of the individuals and organizations that made the completion of my thesis possible, beginning with the women whose interest and participation as research subjects in this study was most essential of all.

Foremost, I would like to thank Dr. Yvonne Lamers, my primary supervisor, for inviting me to study at UBC and for her guidance, patience and mentorship. Her generous contributions of time, advice, and resources have been essential to my growth as a researcher and I will forever cherish memories of the enriching experiences I had during my time in her lab.

I would also like to thank the members of my thesis and examining committees, firstly Dr. Susan Barr, for going above and beyond in her duties as a committee member and interim supervisor. Susan, your vast knowledge, thoughtful advice, and constant support and have been thoroughly appreciated and it has been a pleasure and privilege learning from you. Dr. Angela Devlin, for opening up her lab, and lending expertise. Without the help of the Devlin lab the genetic component of my research would not have been possible. Dr. Patti Janssen, for sitting on my examining committee and providing valuable contributions to the final thesis.

Thanks to the Devlin, Innis, Green, and Robinson Labs for contributing laboratory space and resources, and to Dr. Shatenstein's team for providing the dietary assessment tool and training. I would like to acknowledge the laboratory assistance of Tina Li, Dr. Marta Jeruszka-Bielak, Roger Dyer, Janette King, and Dian Sulistyoningrum, the statistical expertise of Arianne Albert, and our undergraduate research volunteers for their help in recruitment and on study days. Deepest gratitude to the community nutritionists at Vancouver Coastal Health and to our colleagues at Fraser Health for providing clinic space and recruitment support. To all the organizations that provided recruitment venues, your role in facilitating uptake of subjects was much appreciated. I would like to acknowledge the support of the Food Nutrition and Health Vitamin Research Fund in funding my research and student stipend.

I would like to thank my fellow HUNU graduate students and all the LFS staff for their support and friendship. In particular, I share the credit of my work with Theresa Schroder, a relentless source of support inside the lab and out. To my family and friends, near and far – I am sincerely grateful for your support (and thesis edits). Finally, Wade, thank you for your love, encouragement and patience over these past few years.

Dedication

I would like to dedicate this thesis to my uncles:

Geoffrey Wasteneys and Michael Savage.

I would never have been successful in this endeavor without their support. Thanks to them for feeding and housing me, and putting up with the perpetual entropy over these past few years.

Chapter 1: Overview

Vitamin B12 (B12) is an essential nutrient that plays crucial roles in the human body ranging from supporting a healthy nervous system to the synthesis of DNA and red blood cells (RBCs). It has been established that impaired maternal B12 status in pregnancy is associated with poor maternal and child health outcomes (1,2).

While mandatory folic acid food fortification by certain countries has effectively reduced neural tube defect (NTD) occurrence (3,4), the well-defined metabolic interface between folate and B12 suggests that B12 may also be an important nutrient in pregnancy, and supports the notion that B12 deficiency may be a nutritional risk factor for NTDs in folate-replete regions such as Canada (5).

Marginal B12 deficiency[6] indicated by B12 biomarker concentrations consistent with B12 depletion has been reported in mothers with a NTD affected pregnancy in multiple countries, including regions without folic acid fortification such as Ireland, establishing B12 deficiency as an independent risk factor for NTDs (6). Ray *et al* determined that 34% of NTDs may be attributable to low maternal B12 status and that one in 20 Canadian women of childbearing age may be B12 deficient during the timeframe dedicated to embryonic neural tube closure (2,5). Therefore, it is critical to ensure B12 adequacy prior to conception.

Maternal B12 deficiency has also been linked to preeclampsia, and various health outcomes for the offspring such as growth inhibition, developmental regression, neurological symptoms, cognitive impairment, behavioral symptoms, and an increased risk for development of chronic disease later in life (7–9). In addition, impaired B12 and folate status can affect *in*

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[6] Contrast with chronic deficiency, which is associated with megaloblastic anemia and neurological outcomes and requires medical attention.

utero methyl nutrient availability. This imbalance has been suggested to affect long-term risk for insulin resistance, type-2 diabetes, and cardiovascular disease. For example, in Pune, India, researchers observed that *in utero* exposure to the combination of high maternal folate and low maternal B12 concentrations was associated with greater adiposity and insulin resistance in children at 6 years, and for mothers post-partum (10,11).

The recommended approach to B12 status assessment involves multiple measurements. Available tools include functional and direct biomarkers[7], and clinical outcomes[8]. Using more than one biomarker allows for a critical evaluation of deficiency and better distinction between the separate chronic and marginal states. Single biomarker assessment drastically increases the chances of misclassification (12). Despite this knowledge, the majority of existing B12 research has only used one biomarker, namely serum vitamin B12 (SB12), and has rarely measured clinical outcomes[9] (13). Consequently, SB12 has been somewhat established as the reference method despite its drawbacks and is the primary outcome of many studies assessing B12 status, even when other biomarkers are measured. It is imperative moving forward that multiple biomarker assessment be implemented in B12 status-related research.

It is possible that some ethnic groups, particularly populations of South Asian (SA) descent, may be at increased risk for B12 deficiency (14). Prevalent deficiency in SAs has been reported in rural, urban, affluent, and low-income populations (15,16), and among vegetarians and non-vegetarians (15,17,18), worldwide. Increased susceptibility to B12 deficiency may be at least partially attributable to dietary and lifestyle practices, and genetics. Reduced animal-sourced food (ASF) intake and variants in genes involved in B12 metabolism and gut health have

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[7] Serum vitamin B12, holotrascobalamin, total homocysteine, and methylmalonic acid
[8] To assess symptoms of megaloblastic anemia and the neurological consequences of vitamin B12 deficiency
[9] For this reason our primary rate assessments will be based on serum B12 and other biomarkers will be considered only in exploratory analysis

been associated with poor B12 status in SA populations. These observations are relevant because SAs comprise one of the largest minority groups in Metro Vancouver (19). Trends towards the maintenance of religious, cultural and dietary integrity and reduced use of healthcare services, especially by women, may position SA women as uniquely vulnerable to B12 deficiency (20,21). Notwithstanding, B12 deficiency rates and determinants in SA women have not been assessed in Canada.

My thesis project developed from the questions: **[1]**[10] are SA women at greater risk for B12 deficiency than European women, and if so **[2]** what are potential dietary, genetic, and lifestyle related risk factors that could explain an increased susceptibility to B12 deficiency, and **[3]** does the choice of multiple biomarkers and cut-off values implemented for B12 status assessment influence the rate of deficiency? To tackle these questions, YL developed an original study protocol that SB, AD and I further contributed to.

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[10] Note that **[Boxed Brackets]** represent a list, whereas **(Parentheses)** represent in text references.

Chapter 2: General Literature Review

This chapter will provide a brief introduction to B12 function and metabolism, followed by background on: **[1]** dietary sources of vitamin B12, **[2]** dietary vitamin B12 recommendations; **[3]** the suggested approaches for assessing B12 status and classifying B12 deficiency; **[4]** health risks associated with maternal B12 deficiency; **[4]** the prevalence of deficiency in populations of women; **[5]** background on SA populations; and **[6]** known risk factors for deficiency relevant to women of childbearing age.

2.1   Vitamin B12 Synthesis, Absorption, and Metabolism

Vitamin B12, or cobalamin, was discovered as a red crystalline liver isolate that had a curative effect on the autoimmune disorder pernicious anemia (22). It is a large heterocyclic structure with a corrin nucleus and four reduced pyrrole rings linked by a cobalt molecule. Specific B12 isomers are distinguished by a functional R group attached to the cobalt that determines the co-factor form and function. Only two of these isomers, methylcobalamin, and 5'-deoxyadenosylcobalamin **(Table 2-1)** appear to be active in human metabolism.

Table 2-1 Common Vitamin B12 Isomers

Vitamer	R Group	Role in Human Metabolism	Primary Source
Methylcobalamin	-CH$_3$	One-carbon metabolism	Animal sourced foods
5'-Deoxyadenosylcobalamin	-5'-Deoxyadenosyl	Odd chain fatty acid metabolism	Animal sourced foods
Cyanocobalamin	-CN	Proposed minor role in cyanide transport (23)	Supplements
Hydroxycobalamin	-OH	N/A	Supplements

2.2   Dietary Sources of Vitamin B12

The presence of B12 in the human food supply depends on a 30-step enzymatic process carried out exclusively by select microorganisms (24). This process does not occur in plants, thus plant foods do not represent a viable dietary source of B12. There is a common misconception that select plant foods (e.g. spirulina) are good dietary sources of B12 but they actually contain

metabolically inactive B12 analogues (25). Plant products that have been fermented, contaminated with feces, or grown in bio-enriched soil may contain small trace amounts of B12 but these sources are insufficient to meet dietary requirements at normal intakes (26). Furthermore, although *de novo* synthesis by microorganisms occurs in the human gut, the ileal absorption site occurs prior to the location of synthesis. Consequently, humans must rely on ASFs, supplements and/or fortified foods to maintain B12 adequacy. Common dietary sources of B12 are summarized in **Table 2-2** (27–29).

Table 2-2 Vitamin B12 Content of Common Foods

Food	Portion	Vitamin B12 content (raw) µg per portion	Bioavailability
Clams (30)	100g	49.4	72%
Beef Liver	100g	59.3	N/A
Beef (Standard Grade) (31)	100 g	1.4	N/A
Chicken (25)	100g	0.4	61-65%
Pork	100g	0.8	N/A
Lamb (25)	100g	2.5	56-89%
Fish (Sockeye Salmon) (25)	100g	5.8	42%
Milk (32)	100g		8-10%
2% Partly Skimmed		0.5	
3.5% Homogenized		0.5	N/A
1% Skimmed		0.5	N/A
Cheese (Cheddar)	100g	0.8	N/A
Cottage Cheese (2%)	100g	0.5	N/A
Whole Egg (25)	35g (1 small egg)	1.8	<9%
Breakfast Cereal[a]	100g	0.0	N/A
Fortified Soy Beverage	100g	0.4	N/A
Fortified Soy Meat Analogues (e.g. Meatless Meatball)	100g	1.5	N/A
Nutritional Yeast	12g	26.6	N/A
Tempe	100g	0.1	N/A

Data derived from (33) unless otherwise specified
[a]For example, Post Shredded Wheat ® or General Mills Cheerios ®
N/A = no information available

The absolute amount of B12 available from ASFs depends on **[1]** light exposure and **[2]** losses due to cooking, which vary depending on food source, duration, temperature, and cooking methods (25,31,34–38). For example, after microwave treatment, the B12 content of milk was reduced by 50%, whereas boiling only caused a 30% loss (25,34).

General bioavailability of B12 in food is reported to be 50% but this assumed value ranges significantly by source (35). Meat and seafood/shellfish products have higher bioavailability than non-meat ASFs such as dairy products and eggs (25,36,37) (**Table 2-2**). Based on this assumption, overall absorption of equivalent intakes of B12 would be lower for vegetarians than for omnivores (38).

Cost, availability, dietary preference, and culturally and religiously motivated food choices are all factors that affect ASF consumption and impose challenges for achieving adequate B12 intake. For populations unlikely to maintain B12 adequacy through regular dietary means, supplements or fortified foods are recommended but not universally accessible due to expense and availability. In Canada, B12 fortification guidelines follow the 'principle of nutritional equivalence' and foods mimicking animal products, such as simulated meats and plant-based milk replacements (e.g. soy milk), must contain equal B12 to their reference foods (39). In addition to these products, one particularly good non-ASF source of dietary B12 is fortified brewer's (or nutritional) yeast.

Many industrialized countries, including Canada, enforce mandatory fortification of flour and ready-to-eat cereals with folic acid (40). Fortification with B12 has been proposed by several researchers as a positive addition but it is not practiced in Canada (41–43). Selective fortification of flour products with B12 is practiced in several regions including the United States, and mandatory fortification is practiced in Cameroon, Cuba, Benin, Ghana, Cote d'Ivoire, Jordan Kenya, Mali, Mauritania, Niger, Rwanda, Senegal, Tanzania, Republic of Togo, Uganda, Burkina Faso, Uruguay, and Palestine (Flour Fortification Initiative, Atlanta, GA, *unpublished data*).

2.3 Absorption, Tissue Uptake and Metabolism of Vitamin B12

Vitamin B12 must overcome a complex absorption pathway before it can assume its metabolic role (**Figure 2-1**). This process depends on a variety of molecules that cleave, bind, chaperone, and transport B12 (44,45)

Figure 2-1 Absorption Mechanism of Vitamin B12

Stomach

1. B12 binds HC following release from food protein by HCl + pepsin

2. IF is secreted by parietal cells

3. B12-HC and IF travel to duodenum

LEGEND
- Vitamin B12 (B12)
- Intrinsic Factor (IF)
- Transcobalamin II (TCN2)
- Haptocorrin (HC)
- Receptor (RC)

Small Intestine (Ileum)

4. B12 released from HC by pancreatic proteases

5. B12 binds IF

6A. B12 crosses the ileal enterocyte via CUBAM Rc

6B. B12 crosses ileal enterocyte via passive diffusion

CUBAM Rc

Luminal Membrane

7. B12 released from IF in lysosome

8. B12 exits lysosome via LMBRD1 Rc

MRP1 Rc   Megalin Rc   LMBRD1 Rc   Lysosome   Ileal enterocyte

Blood stream

Basolateral Membrane

9. B12 enters the bloodstream via the MRP1 or Megalin Rc

10B. B12 binds to HC (80%) - this portion stays in the bloodstream or is stored in the liver. It is secreted into bile and recycled by enterohepatic circulation and reabsorbed. The small amount that isn't reabsorbed is excreted.

10A. B12 binds to TCN2 (~20%) - forming the complex holotranscobalamin

11. Holotranscobalamin enters the cell via the CD320 Rc

CD320 Rc

Tissue (cell)

Cytoplasmic Membrane

LMBRD1 Rc

12. B12 released from holotranscobalamin in the lysosome

Lysosome

13. B12 exits lysosome via LMBRD1 Rc

14B. B12 trafficked to mitochondria to function as a co-factor for L-methylmalonyl-CoA mutase in odd-chain-fatty acid metabolism

14B. B12 trafficked to function as a co-factor for methione synthase in 1-carbon metabolism

L-methylmalonyl-CoA

Methyl-THF   Homocysteine

L-methylmalonyl-CoA mutase

Methionine synthase

Succinyl-CoA

THF   Methionine

Mitochondrial Compartment

Cytoplasmic Compartment

This figure depicts the absorption mechanism of vitamin B12. In the small intestine two pathways are shown – 6A. transport by the CUBAM receptor, and 6B. passive diffusion across the luminal membrane. **Abbreviations:** HCl= Hydrochloric Acid, Rc= Receptor, LMBRD1 Rc= Probable lysosomal cobalamin transporter, MRP1 RC=Multidrug resistance protein 1, CD320 Rc= Transcobalamin 2 receptor, THF= Tetrahydrofolate. Adapted from (46,47). Refer to text (page 9 and 10 for more detail)

Following ingestion of food, gastric hydrochloric acid facilitates the conversion of pepsinogen to pepsin, which acts to liberate B12 from the food protein matrix. Free B12 binds to the protein haptocorrin (HC), which protects it from denaturation in the stomach and allows passage into the small intestine. Coincidentally, intrinsic factor (IF), a highly glycosylated transport protein, is secreted from parietal cells in the stomach. In the duodenum, HC is digested by pancreatic proteases (i.e. trypsin), allowing IF to bind B12. The IF-B12 complex is transported into the ileal enterocyte by receptor-mediated endocytosis facilitated by the IF-B12 receptor, cubam, located in the distal ileum. The cubam receptor is comprised of two proteins: cubilin which binds IF-B12, and amnionless which internalizes cubilin-IF-B12. Approximately one to three percent of free B12 can be taken up by passive diffusion which allows absorption of an adequate portion from supplemental mega doses of B12 given to individuals with malabsorption (46,47). After entering the enterocyte, IF-B12 is degraded in the lysosome and free B12 transverses the lysosomal membrane (via the probable lysosomal cobalamin transporter (LMBRD1)) to enter the enterocyte cytoplasm (25). Entry of B12 into the bloodstream from the basolateral side of the cell occurs by transmembrane transport of the vitamin, in free form, with the assistance of the ABC transporter multidrug resistance protein 1 (50). In the bloodstream ~20% of B12 is immediately bound to transcobalamin 2 (TCN2) to form holotranscobalamin (holoTC) which represents the active fraction of B12 available to the tissue (51). The other ~80% binds to HC and this fraction is metabolically inactive. B12 bound to HC is eventually transferred into the portal circulation for clearance by hepatocytes or for entry into systemic circulation.

Vitamin B12 bound as holoTC enters hepatocytes and other cells by the calcium-dependent endocytic receptor transmembrane CD320 protein (or the TCN2 receptor) (28). The

Megalin receptor can also perform this function, especially in the kidney where it is responsible for facilitating reabsorption of TCN2 bound B12. HoloTC is degraded in the lysosome and enters the cytoplasm via the LMBRD1 receptor. In the cytoplasm, free B12 is trafficked to its site of activity and converted to its active co-factor form by various cobalamin-assisting proteins.

Vitamin B12 co-enzymes are required for two metabolic reactions **(Figure 2-2)**. Methylcobalamin acts as a co-factor for methionine synthase (MS) as an intermediate methyl group carrier. Methylcobalamin donates its methyl group to homocysteine (Hcy) during Hcy remethylation, which occurs in tandem with the donation of a methyl group from 5-methyltetrahydrofolate. After approximately two thousand reactions, the cobalamin cofactor of MS becomes oxidized and requires regeneration by a reaction controlled by methionine synthase reductase (MTRR) in which S-adenosylmethionine donates a methyl group (52). If Hcy remethylation is inhibited, Hcy concentrations rise and 5-methyltetrahydrofolate accumulates in response. The second co-enzyme, 5-deoxyadenosylcobalamin facilitates the isomerization of L-methylmalonyl CoA into succinyl CoA, a key intermediate in the Krebs cycle. This step is a critical continuation following the degradation of branched chain amino acids, odd chain fatty acid, and cholesterol carbon skeletons into propionyl-CoA (53,54). If this pathway is inhibited, L-methylmalonyl CoA is converted into methylmalonic acid (MMA), which accumulates in B12 deficiency.

Figure 2-2 Roles of Vitamin B12 in Human Metabolism
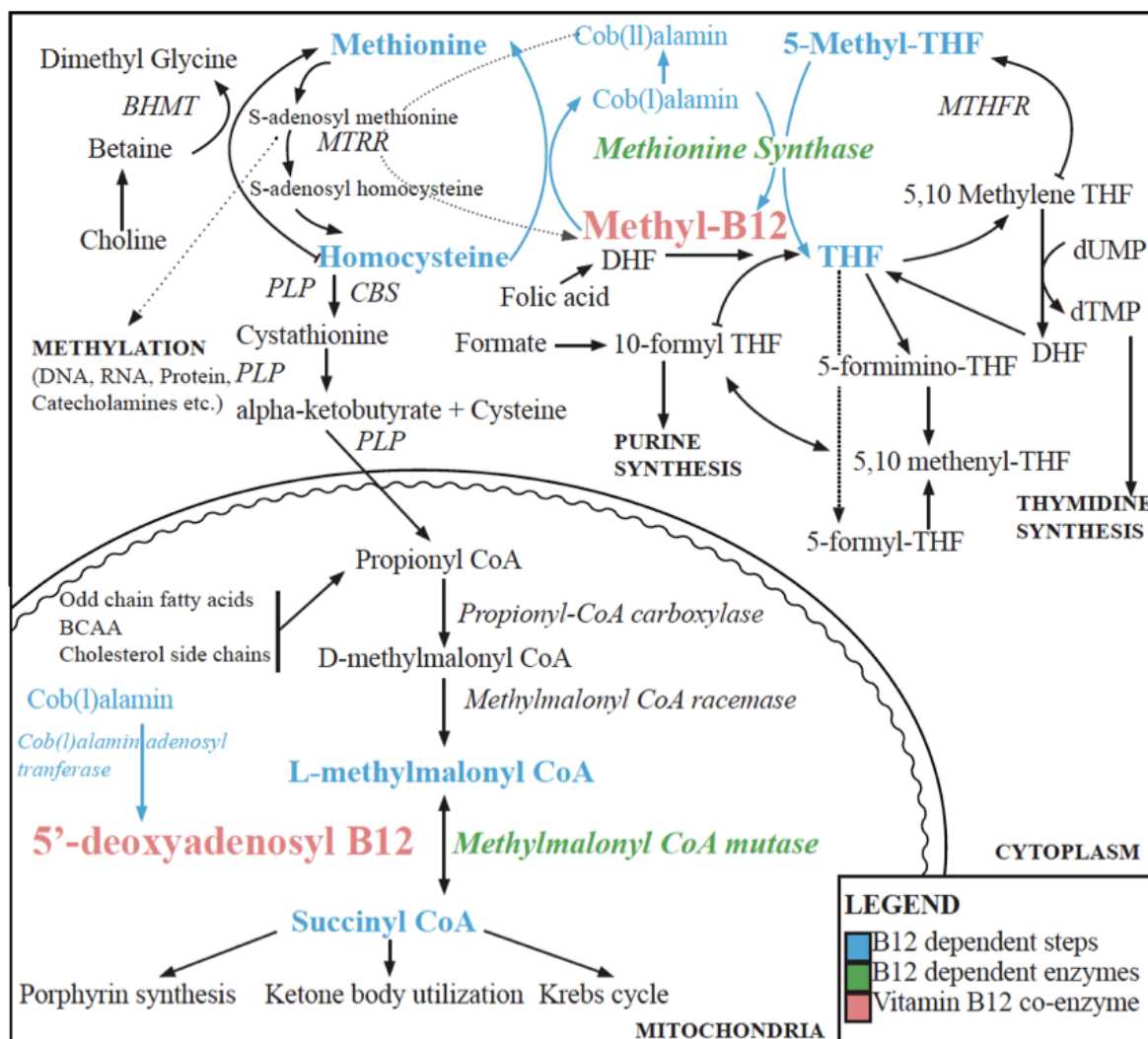
This figure depicts the involvement of vitamin B12 in two metabolic pathways: 1-carbon-metabolism, depicted in the cytoplasm, and Odd-chain-fatty acid-metabolism, depicted in the Mitochondria. *Enzymes* are depicted in italics. **Abbreviations:** B12=vitamin B12, THF= Tetrahydrofolate, MTHFR= methylene-tetrahydrofolate-reductase, dUMP= deoxyuridine monophosphate, dTMP=deoxythymine monophosphate, DHF=dihydrofolate, PLP= pyridoxal phosphate, BCAA= Branched chain amino acids, CBS= Cystathionine beta synthase, BHMT=Betaine homocysteine S-methyltransferase, MTRR= methionine synthase reductase. Adapted from (55,56)

Unabsorbed B12 is degraded in the small intestine (57) and circulating B12 that is not utilized is either stored (primarily in the liver) or secreted into bile (approx. 5-10 µg/day). The majority (60-75%) of biliary B12 is subsequently reabsorbed (3-5 µg) via enterohepatic circulation. Vitamin B12 is also conserved by reuptake in the kidney. A small amount is excreted

(0.1-0.2% total body content) in urine and feces. Individuals with malabsorption of B12 have proportionally greater losses (58). The timeframe before deficiency appears varies depending on total B12 reserves (1-4 mg in healthy individuals) and the level of dietary restriction and malabsorption (59). Starting with sufficient stores, it may take two to five years for deficiency to develop once absorption is completely disrupted. Individuals with intact absorption only incur very small daily losses from the total B12 pool so even if intakes are restricted deficiency may take years to develop.

2.4 Dietary Vitamin B12 Recommendations

The current recommended dietary allowance (RDA) for B12 (**2.4 µg/day** for adults) was estimated based on the amount of B12 needed to maintain **[1]** normal hematological status (mean corpuscular volume (MCV), hemoglobin (Hb), and reticulocyte count) and **[2]** adequate SB12 concentrations (>150 pmol/L) (35).

The evidence supporting the current requirements comes primarily from studies monitoring patients with pernicious anemia in remission, and vegetarians with insufficient intake. Darby *et al* determined an average requirement of 1.5 µg/day, which was used in the algorithm to determine B12 requirements (**Table 2-3)** (60). A coefficient of variation of 10% was applied in the absence of data on the standard deviation (SD) of B12 requirements to determine the RDA. The current RDA is based on the sum of the estimated average requirement (EAR) and twice the coefficient of variation to cover the needs of 97-98% of the population.

Table 2-3 Determination of Adult Estimated Average Requirements for Vitamin B12 Intake

Step 1: 1.5 µg/day intramuscular dose required to maintain normal hematological status and SB12 in PA patients
Step 2: Subtract 0.5 µg/day to account for biliary losses
Step 3: 1.0 µg/day required not accounting for bioavailability
Step 4: Apply a value of 50% average absorption = **2ug/day EAR**
Assumptions: (1) 0.5 µg/day lost in bile without biliary reabsorption, (2) average absorption from food is 50%, (3) normal serum vitamin B12 is 150 pmol/L, (4) normal hematological status relies on mean corpuscular volume, hemoglobin, and reticulocyte response. Abbreviations: SB12= serum vitamin B12, PA= pernicious anemia, EAR=estimated average requirement

Current dietary B12 recommendations for different life stages are summarized in **Table 2-4** and special considerations in **Table 2-5**. Recommended intakes for individuals one to 18 years of age are extrapolated from adult requirements. Adequate intake for infants zero to six months old is based on the intake of infants fed exclusively human breast milk; adequate intake for infants seven to 12 months old is adjusted for weight based on adequate intake of newborns. In pregnancy and lactation, recommendations take into consideration fetal deposition and mammary secretions of B12, respectively.

Table 2-4 Daily Recommended Intakes for Vitamin B12

Life stage	AI	EAR	RDA	UL
0-6 months	0.4 µg/day			
7-12 months	0.5 µg/day			
1-3 years		0.7 µg/day	0.9 µg/day	
4-8 years		1.0 µg/day	1.2 µg/day	
9-13 years		1.5 µg/day	1.8 µg/day	
14-18 years		2.0 µg/day	2.4 µg/day	
19-50 years		2.0 µg/day	**2.4 µg/day[a]**	N/A no UL set
50-70+ years		2.0 µg/day	2.4 µg/day primarily from fortified foods and supplements	
Pregnancy (18-50 years)		2.2 µg/day	2.6 µg/day	
Lactation (18-50 years)		2.4 µg/day	2.8 µg/day	

[a]Recommendation relevant to the sample population
AI = adequate intake, EAR= estimated average requirements, RDA= recommended daily allowance, UL= tolerable upper intake level
Adapted from: (35)

Table 2-5 Populations with Special Considerations Related to Dietary Vitamin B12 Intake

Population	Considerations
Adults >50	Consume B12 from supplements or fortified foods to address prevalence of atrophic gastritis (10-30% of individuals >50 y) and food bound cobalamin malabsorption
Infants of deficient mothers or infants on a low ASF diet	Supplement from birth to address insufficient liver stores, depleted breast milk stores, or reduced dietary intake.
Individuals with severe malabsorption syndromes or gastrointestinal bypass surgery	Consult physician for treatment.
Individuals with chronic diarrhea	May require increased oral or parenteral doses to replace losses.
Individuals with conditions affecting bioavailability (i.e. acid reducing medication, atrophic gastritis, genetic disorders)	Consume B12 from supplements or fortified foods to address decreased bioavailability from food

A recent systematic review conducted by Dutch researchers suggested that B12 requirements vary significantly by the individual and are potentially higher than the current recommendations for healthy populations (38). From pooled data, they estimated that daily losses are likely in the range of 1.4 to 5.1 µg/day suggesting that requirements may be as high as 3.8-20.7 µg/day for adults, far exceeding the current recommendations. The data included in this systematic review was taken from older studies (1958 – 1991) that used non-specific radioisotope methods, which can potentially overestimate B12 concentrations and cannot be repeated for ethical reasons. As such, their suggestions cannot be substantiated without further research. In the future, B12 requirements should be re-evaluated using higher order analytical methods and multiple biomarker assessment to assess absorption, storage, excretion and the relationship between blood concentrations and long-term clinical outcomes.

2.5   Biomarkers of Vitamin B12 Status

B12 status can be assessed by quantifying direct and functional biomarkers **(Figure 2-3)**.

Figure 2-3 Direct and Functional Biomarkers used to Assess Vitamin B12 Status
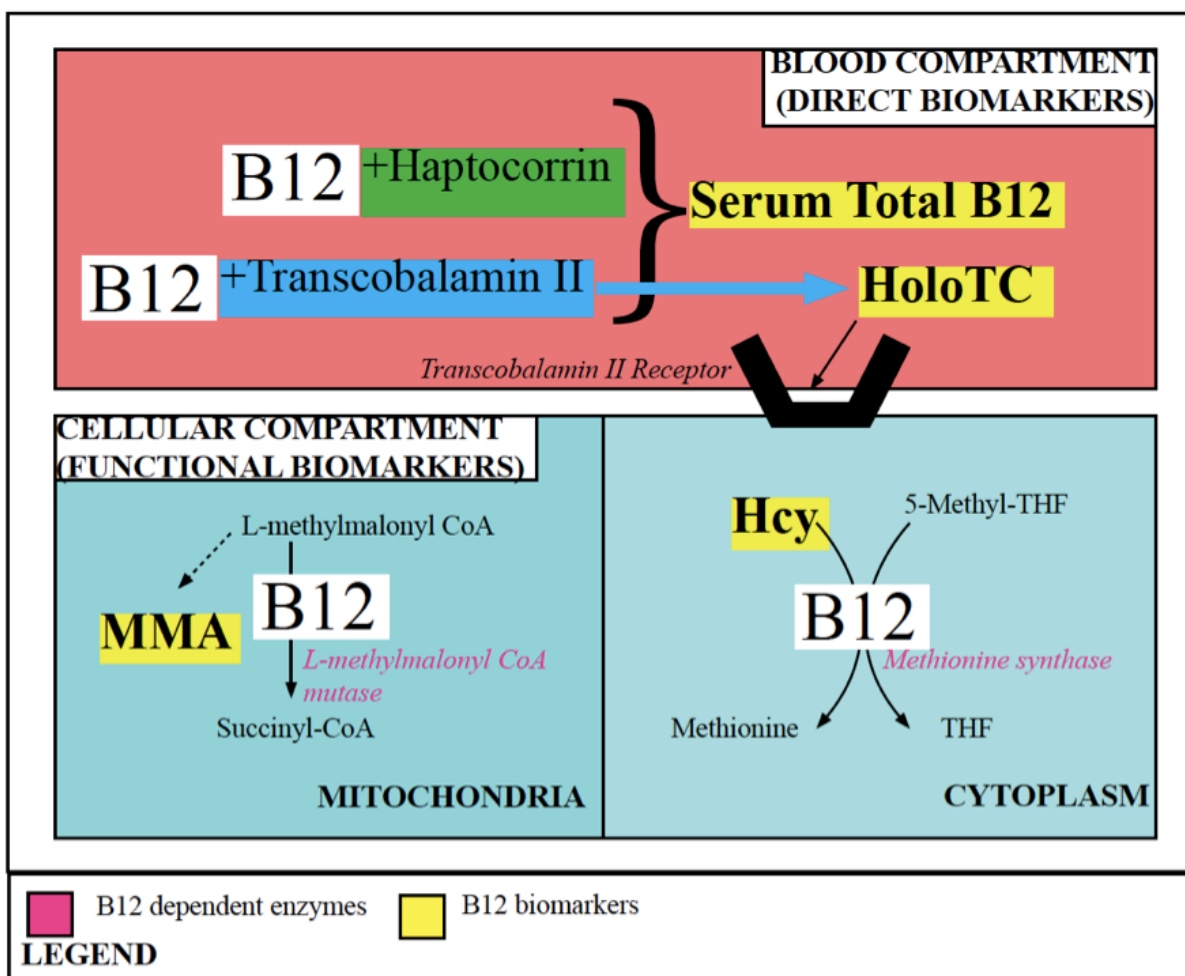
Vitamin B12 biomarkers are depicted in their respective compartments. This figure illustrates that direct biomarkers are representative of the concentration of B12 in the blood compartment, whereas functional biomarkers represent the concentration of metabolites generated by B12 dependent metabolic functions in the cellular compartment.
Abbreviations: B12= Vitamin B12, MMA=methylmalonic acid, Hcy= total homocysteine, THF= tetrahydrofolate, TCN2= transcobalamin 2. Adapted from Quay and Lamers 2012. (61)

2.5.1   Direct Biomarkers of Vitamin B12 Status

Two direct biomarkers, SB12 and holoTC, exist for B12 status assessment and reflect a broad range of B12 status and intakes (62).

Total B12[11] can be assessed in serum or plasma and measures B12 as well as B12 analogues bound to all B12 transport proteins. In this respect it is a non-specific indicator of B12 status because B12 analogues and B12 not bound to TCN2 are unavailable to the tissue. Under some conditions SB12 might not accurately reflect metabolic adequacy (63). For instance, blood levels of SB12 could be normal or elevated but the tissue might be deficient in individuals with excessive analogue binding, and in several disease states (64). Conversely in those with mild to severe HC deficiency (or transcobalamin 1 (TCN1) deficiency) SB12 may be falsely low despite sufficient B12 bound as holoTC and sufficient tissue concentrations. Despite these limitations most experts argue that SB12 is the preferred direct biomarker pending validation and standardization of commercial holoTC assays (12,65,66). It has been suggested that a single highly specific reference method measuring B12 by higher order techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS) would be preferable to the current quantification methods as it would allow for improved distinction between true B12 and B12 analogues (12). Assays for assessing B12 concentrations in milk using LC-MS/MS have been previously described but there are no validated methods for biological samples (67).

Holotranscobalamin, or "active-B12" is the fraction of circulating B12 that is taken up by human tissue via the TCN2 receptor (66). It can be measured in serum or plasma and offers several advantages over SB12 and the functional biomarkers. For one, it appears to be a better biomarker of B12 status during pregnancy compared to SB12 because concentrations remain

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[11] The term serum vitamin B12 (SB12) will be used throughout this thesis

stable and are not influenced to the same extent by hormonal changes and hemodilution (68). It is also less sensitive to repeated mega doses of B12 and has been shown in pregnant women to gradually elevate over three days and then plateau, whereas SB12 continues to increase over time and may overestimate adequacy (68,69). The majority of studies comparing SB12 and holoTC suggest that holoTC is superior as it changes sooner than any of the other B12 biomarkers during the development of deficiency and correlates better with tHcy and MMA than SB12 (70). It has been reported to have two to six % higher sensitivity than SB12 for detecting cases of marginal deficiency (65). Transcobalamin 2 has a reduced affinity for B12 analogues compared to other B12 binding proteins, so B12 analogues have a lesser impact on holoTC concentrations. Despite these advantages, the cost and availability of assays (especially clinical settings) deter the use of holoTC, especially in clinical practice (71,72). Holotranscobalamin concentrations can be affected by liver disease, impaired renal function, and conditions involving macrophage elevation (66). In addition, cut-off values for holoTC used to reflect B12 status are not as well established as for the other B12 biomarkers. Reported cut-offs values range from 11 to 65 pmol/L in the literature but the most commonly referenced cut-off is 35 pmol/L (73). Investigation of the appropriate cut-offs for holoTC should be pursued, especially in regards to defining a cut-off to represent marginal deficiency (65).

2.5.2   Functional Biomarkers of Vitamin B12 Status

Functional biomarkers that reflect intracellular B12 status include plasma total Hcy (tHcy) and MMA concentrations. These biomarkers reflect early changes in B12 status. Several studies have observed that both indices increase at SB12 concentrations above the accepted cut-off for chronic B12 deficiency of 148 pmol/L and may therefore act as an early alert for the eventual development of B12 deficiency (65,74,75).

Low vitamin B12 status can cause elevated plasma Hcy concentrations by reducing the flux of Hcy remethylation when the one-carbon metabolism pathway is limited by reduced activity of B12-dependent MS. Although B12 is the major determinant of Hcy concentrations in folic acid-fortified regions, Hcy has very poor specificity as a biomarker as it is also influenced by the concentration of other B vitamins, namely folate, vitamin B6, and riboflavin. In addition to B vitamin deficiency, renal failure, diabetes, and CVD may also result in elevated Hcy, although it should be noted that elevations in Hcy have not shown a causal relationship with adverse clinical outcomes of these conditions, and B vitamin therapy does not improve clinical outcomes despite lowering Hcy concentration (76). Overall, Hcy has limited utility as a biomarker for B12 assessment.

Methylmalonic acid accumulates due to the impaired conversion of L-methylmalonyl-CoA to succinyl CoA. The sensitivity of MMA for detecting chronic deficiency is >95%. MMA has improved specificity over Hcy as it is not influenced by the status of other B vitamins. The proposed cut-off values for this biomarker range from 210 to 480 nmol/L (77–79), though the most commonly used is 270 nmol/L (65). Cut-offs have been established based on the inflection point at which MMA accumulates as SB12 decreases. A recent two-stage statistical model based on data from the United States National Health and Nutrition Examination Survey (NHANES) showed that the median MMA concentration for individuals divided by two distinct inflection points was 120 nmol/L in the group with highest B12 (adequate), 148 nmol/L in the group with intermediate B12 (marginal deficiency), and 281 nmol/L in the group with lowest SB12, suggesting that higher cut-offs used in the literature (e.g. >370 nmol/L) may be too high (80).

In addition to the limitations stated above, genetic variation, disease, medications, pregnancy (hemodilution), as well as procedural and technical errors can affect B12 status assessment. **Table 2-6** summarizes cut-offs and limitations.

Table 2-6 Proposed Biomarker Cut-offs and Limitations for Assessment of Vitamin B12 Status

Biomarker	Proposed cut-off		Techniques	Limitations
	Chronic Deficiency	Marginal Deficiency		
Direct				
SB12	<148 pmol/L <126 pmol/L[a]	148-221 pmol/L 126-287 pmol/L[a]	Radioisotopic assay, microbiological assay, microparticle enzyme immunoassay, chemiluminescent immunoassay	Not sensitive for diagnosing marginal deficiency. Possible neurological consequences at normal concentrations. Significant rates of false positive and false negative results (i.e. falsely elevated in several disease states and transcobalamin I deficiency) (67) . Female sex hormones and hemodilution in pregnancy may reduce concentrations. Not appropriate for monitoring response to treatment in chronic deficiency (186).
HoloTC	<35 pmol/L	N/A	Microparticle enzyme immunoassay	Not superior for determining chronic deficiency. Sensitive to acute dietary changes. High cost. Sensitive to renal function.
Functional				
MMA	>260-370 nmol/L	>210-260 nmol/L	LC-MS/MS, GC-MS	Affected by renal function, bacterial overgrowth. Requires mass spectrometry for analysis. High cost.
tHcy	>13-15 µmol/L	N/A	Liquid chromatography + fluorescence detection, colorimetric enzyme assay, GC-MS, LC-MS/MS	Low specificity. Affected by renal function, liver disease, other B vitamin status (folate, vitamin B6), genetic polymorphisms (*MTHFR* C677T).

Abbreviations: SB12=Serum total vitamin B12, HoloTC=holotranscobalamin, MMA=methylmalonic acid, LC-MS/MS= liquid chromatography tandem mass spectrometry, GC-MS = gas chromatography tandem mass spectrometry, tHcy=total homocysteine, *MTHFR*= methylenetetrahydrofolate reductase
[a] 'Bailey' cut-offs (79,83)

2.5.3   Classification of Vitamin B12 Deficiency

Current classification of B12 inadequacy acknowledges **[1]** chronic B12 deficiency and **[2]** marginal B12 deficiency (98). Chronic deficiency is rare because depletion of liver stores is a very slow process, whereas population based data indicates that marginal deficiency may occur in a sizable proportion of the general population worldwide (82). Marginal deficiency is prevalent in North America, affecting between 10 and 25% of the general population depending on the region. In contrast, estimated rates for chronic deficiency are 1-2% in the United States and 4.6% in Canada (1,83). These distinct conditions have unique determinants and consequences, which are summarized below. Chronic deficiency[12] may also be referred to as clinical, outright, or severe deficiency. Megaloblastic anemia and progressive neurological and cognitive impairment, as well as several gastrointestinal (GI) symptoms, can result as symptoms of chronic B12 deficiency (84).

Mechanistically, megaloblastic anemia occurs due to the functional folate deficiency that results from restriction of MS activity. This reduction in activity leads to a reduced availability of THF and thus of 5,10-methylene THF required for de-novo purine and pyrimidine synthesis, and thereby to impaired red blood cell (RBC) maturation in the bone marrow (85). Physical and behavioural symptoms of megaloblastic anemia include fatigue, dyspnea, pallor, listlessness, and glossitis, which can be confirmed by clinical evidence of macrocytosis, reduced RBC population and RBC longevity, immature RBC nuclei, and hypersegmentation in granulocytes. It is diagnosed by an increased MCV (normal range 80-100 fL) or by assessment of a blood smear. Less than ten percent of individuals with SB12 <148 pmol/L (i.e. chronic B12 deficiency) present with true megaloblastic anemia (12).

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[12] *This term will be used for the duration of this thesis*

The neurological and cognitive consequences of B12 depletion are related to the essential role of B12 in the development and function of the central nervous system (86). Inherited disorders that affect B-vitamin status have been linked to mental retardation, psychiatric disorders, seizures, and myelopathy (84). Neurological consequences specific to B12 deficiency include progressive sub-acute combined degeneration of the spinal cord, loss of proprioception, weakness of lower limbs, peripheral neuropathy, cerebral demyelination, optic atrophy and progressive cognitive decline (87,88). These symptoms may respond to treatment depending on their duration and severity. B12 deficiency has also been associated with various cognitive and neuropsychiatric disorders such as dementia, Alzheimer's, affective disorder, depression, bipolar disorder, panic disorder, brain atrophy and psychosis (89,90).

Lastly, gastrointestinal symptoms such as sore tongue (glossitis), appetite loss, flatulence and constipation often accompany B12 deficiency. It is unclear whether these symptoms are direct causes of deficiency or whether they are solely attributable to underlying causes of B12 deficiency related to GI dysfunction.

Hematological symptoms of B12 deficiency can be attenuated if sufficient folate is provided as folic acid to generate THF, which provides the precursors for folate forms needed for nucleotide synthesis. Provision of folate thereby allows for RBC maturation. However, despite the correction of the hematological signs of B12 deficiency by folate, it does not correct the accumulation of MMA and can lead to masking of B12 deficiency.

Several researchers have observed that the metabolic profile of high folate and low B12, which may be common in folic acid fortified regions, may exacerbate the clinical outcomes associated with B12 deficiency. Synchronous high folate and low B12 status has been associated with anemia and cognitive impairment in the elderly (91) and obesity and insulin resistance in

the offspring of mothers with this metabolic profile (11). Miller *et al* determined that functional evidence of B12 deficiency, including elevated tHcy, MMA, and low holoTC was more pronounced in older adults with elevated plasma folate (92). Morris *et al* reported relatively higher odds for incidence of anemia (OR: 3.1; 95% confidence interval (CI): 1.5, 6.6) and cognitive impairment (OR: 2.6; 95% CI: 1.1, 6.1) in the group with low B12 status (defined as SB12 concentrations <148 pmol/L or MMA concentrations >210 nmol/L) and high serum folate concentrations (defined as concentrations >59 nmol/L), compared to the group with low B12 status and normal serum folate concentrations in the elderly NHANES population (>60 years of age) (93). This observational data suggested a possible reduction in B12 dependent enzyme activity under circumstances of high folate and low B12 status. Selhub *et al* observed that a combination of low plasma B12 concentrations (<148 pmol/L) and/or high plasma MMA concentrations (>210 pmol/L) and elevated folate concentrations (>59 nmol/L) was associated an increased incidence of anemia, macrocytosis, and cognitive impairment (91). It has yet to be determined whether supraphysiological folate concentrations have a direct negative impact on B12 related health outcomes in individuals with B12 deficiency. This question is pertinent in folic acid fortified regions, such as Canada, where a large proportion (40%) of the population have higher than adequate folate status (>1360 nmol/L), and chronic B12 deficiency affects 4.6% of the general population (1,94).

Marginal B12 deficiency[13], otherwise referred to as subclinical deficiency, is most often characterized by moderate B12 depletion and changes in concentrations of functional B12 biomarkers (MMA and Hcy), without the presence of clinical deficiency symptoms. Carmel has proposed four possible trajectories of the disease. By his suggestion, marginal deficiency can

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[13] This term will be used for the duration of this thesis

remit completely without treatment, eventually progress to clinical symptomatic deficiency, rapidly accelerate towards chronic deficiency (e.g. as is observed when gastritis converts to pernicious anemia), or indefinitely fluctuate between chronic and marginal states (65,95). Beyond changes in functional metabolites, observational studies suggest that marginal B12 deficiency is associated with an increased risk for chronic diseases associated with ocular (96), bone (97,98), cognitive and neurological (99), and vascular (100) health, and poor pregnancy outcomes (5). There is currently debate over the ideal approach to monitoring and managing marginal deficiency. Experts believe that the condition warrants further investigation given the abundance of associated health risks (101,102).

2.5.4   Proposed Cut-off Values for Defining Chronic and Marginal Vitamin B12 Deficiency

Proposed cut-offs for chronic and marginal B12 deficiency are summarized in **Table 2-6.** It should be noted that there is a lack of consensus on the appropriate clinical cut-offs, in general, and for special population groups such as women of childbearing age. The 'conventional' cut-off for chronic deficiency is meant to reflect the potential for the appearance of clinical symptoms including megaloblastic anemia and neurological outcomes at SB12 concentrations below 148 pmol/L. This cut-off was established by calculating 3 SD from reference range mean of a SB12 adequate population (35). Validation of cut-off values will require investigation into the relationship between blood biomarker concentrations clinical outcomes using reliable measurement procedures to accurately reflect health risks or adverse clinical outcomes, which are currently not established for marginal deficiency. This could prove difficult given the discordance between appearance of neurological and hematological outcomes and the potential for inconsistency in biomarker concentration changes.

Building on the 'conventional' cut-off approach, researchers in the United States have devised a two-step statistical model to represent the relationship between MMA and SB12 (80). Based on this model they identified two clear inflection points at which MMA concentrations rise at a significantly greater rate in relation to decreasing SB12 concentrations. From these observations they proposed two cut-offs, <128 pmol/L to represent chronic deficiency, and 128-287 pmol/L to represent marginal deficiency. Their models were based on a large sample size and took into account various confounders such as age and excluded individuals with renal failure. While the authors put forth some obvious limitations including the choice of mathematical model, which they have noted influences the cut-point, their method improves upon the method implemented for the conventional cut-offs, and their suggested cut-offs could represent more appropriate division points at which to separate marginal and chronic cases of deficiency. Both sets of cut-offs will be considered for assessment of the present study population and will be referred to throughout this thesis.

2.5.5   Suggested Ideal Approach and Alternative Methods for Vitamin B12 Status Assessment

Paradoxically, 20% of individuals with chronic deficiency defined by SB12 concentrations alone do not display clinical or metabolic signs of deficiency, and in contrast ~50% of patients with metabolic evidence of deficiency have SB12 concentrations >148 pmol/L (65,103). Therefore, the preferred approach, to account for sensitivity and specificity issues and other limitations including the lack of appropriate cut-offs, is to use at least two biomarkers to measure B12 status. The experts involved in the NHANES roundtable on biomarkers of B12 status recommend a pairing of SB12 and MMA to address specificity issues with Hcy and the need for further investigation of the utility of holoTC (12). Despite these recommendations, functional assessment of deficiency is not always completed. This is often due to the requirement

for sophisticated technology, cost and training of personnel. Of 127 systematically reviewed studies that measured B12 deficiency only 13% assessed MMA in addition to a direct biomarker (13). Suggestions have been also been made to include measurements of cognitive impairment and malabsorption (e.g. IF antibody tests) so that distinction between degree of deficiency and the clinical implications of deficiency can be discerned. This could also address the problem of some individuals with clinically proven deficiency only displaying elevations in one functional biomarker (either MMA or Hcy) (12,103) but may not be feasible for routine clinical monitoring and large population-based studies. In clinical settings, comprehensive B12 status assessment may be foregone and suspected deficiency may be addressed with prophylactic B12 treatment, especially in strict vegetarians and patients who have undergone gastric surgery (81).

In regions where laborious assays are not feasible due to high costs and/or lacking infrastructure, dried blood spot analysis can be an economical and field friendly alternative assessment tool for studies with large sample sizes. Advantages of dried blood spot analysis include less invasive blood sampling, elimination of blood preparation steps, and ability to store and ship large numbers of samples at room temperature. Schroder *et al* have described a highly sensitive method for MMA quantitation in dried blood spots using LC-MS/MS that allows for the measurement of a functional biomarker without a derivatization step (104). There is also a novel B12 breath test available that measures $^{13}CO_2$ generated by the metabolism of an oral dose of labelled 1-^{13}C-propionate. It measures the activity of B12 dependent L-methylmalonyl-CoA mutase and has been validated to assess B12 status (105).

Even with the use of multiple cut-offs and multiple biomarkers there is still a chance of non-deficient individuals being classified as deficient, and vice-versa. Bailey *et al* have proposed four combined metabolic profiles to define individuals assessed by SB12 and MMA (106). They

are as follows: **[1]** low SB12 and elevated MMA, **[2]** low SB12 and normal MMA, **[3]** normal SB12 and elevated MMA, and **[4]** normal SB12 and normal MMA. The researchers proposed that based on various demographic, biochemical, clinical, and lifestyle characteristics, profile 1 and 3 were indicative of a true deficiency, whereas, profile 2 and 4 were more likely to represent sufficient individuals. Profile 3 individuals were thought to have a milder deficiency than profile 1 individuals, as well as potentially falsely elevated MMA concentrations, whereas profile 2 individuals were assumed to have probable falsely low SB12 concentrations. While this analysis was conducted in a large sample and assessed the differences between profiles using extensive indicators, the analysis did not include holotranscobalamin assessment, which may provide further insight into the likelihood of falsely low SB12 concentrations vs. true depletion.

2.6   Health Risks Associated With Impaired Maternal Vitamin B12 Status

Vitamin B12 is an important nutrient during pregnancy not only for normal physical and cognitive development but also for ensuring adequate B12 status of the infant in the early stages of life. Maternal B12 deficiency has been associated with increased risk for preeclampsia (107) low birth weight (108), intrauterine growth retardation (109), neurological symptoms in the infant (110–112), and impaired cognitive function in infants, children, and adolescents (113,114). Increased risk for these conditions has primarily been observed in relation to severe maternal B12 depletion. However, increased risk for several health outcomes, namely NTDs and insulin resistance, has been associated with marginal maternal B12 depletion.

2.6.1   Neural Tube Defects

Neural tube defects occur at a rate of 0.58 per thousand pregnancies in Canada (4). This spectrum of conditions, which includes spina bifida, anencephaly, and encephalocele, results from the failed closure of the neural tube during the third or fourth week of gestation. Neural tube defects are associated with debilitating developmental, neurocognitive and physical

disabilities (115). The exact mechanism of NTD development is not fully understood and is proposed to be a highly individualized and multi-factorial process. Maternal obesity, valproic acid, and nutritional status have been identified as potential risk factors for NTDs (116).

It was estimated that achieving optimal folate status could prevent 50-60% of all NTDs (117) and maternal folic acid supplementation has been shown to reduce the incidence of NTDs substantially (118). Following universal fortification of processed cereal grains in 1998, there was a 46% reduction in the rate of NTDs in Canada (3). In addition, folate deficiency has become virtually nonexistent in the general Canadian population, though 22% of women of childbearing age are estimated to be below the cut-off deemed optimal for NTD protection (RBC folate >906 nmol/L) (94,119). In consideration of the large proportion of unplanned pregnancies (~50%), the Society of Obstetricians and Gynecologists of Canada suggests peri-conceptional folic acid supplements be used by all women of childbearing age regardless of intent to bear children, to support the prevention of NTDs (120).

The benefits of folic acid are hypothesized to be partially attributable to the provision of methyl groups required for DNA methylation (121). A reduction in the methyl group availability is associated with a widening of the anterior neuropore in rodent models (122), and a reduction in maternal methionine to Hcy ratio and DNA methylation has been observed in NTD affected cases in humans (123). This suggests that other methyl-nutrients, particularly B12, could also be risk factors for NTDs. This is especially relevant in Canada given that one in 20 Canadian women of childbearing age may be B12 deficient during the timeframe dedicated to embryonic NT closure (2).

Low levels of B12 have been measured in the amniotic fluid and blood of mothers with NTD affected pregnancies pre and post-fortification (6,124–126). In addition, low maternal

SB12 concentrations have been associated with an increased risk of NTDs in regions without folic acid fortification such as Ireland, establishing B12 as an independent risk factor for NTD risk and as the main nutritional determinant of NTD risk in folic acid fortified regions (6). Ray *et al* conducted a case-control study of women (n=89) in Ontario with a NTD (myelomeningocele or anencephaly) affected pregnancy compared to controls (n=422) with a healthy pregnancy and observed increased odds (OR: 2.9; 95% CI: 1.2, 6.9) of NTDs for individuals in the lowest quartile of holoTC concentrations. They estimated that up to 34% of remaining NTD cases in Canada could be attributed to B12 deficiency (5). A recent meta-analysis that included data from nine case-control studies (compiling a total of n=567 cases, n=1566 controls) reported an overall increased odds (OR: 2.41; 95% CI: 1.90, 3.06) of fetal NTD for offspring born to mothers with low B12 status (defined as SB12 <150 pmol/L 'or' holoTC <25%ile 'or' MMA >75%ile) (127). Thompson *et al* have suggested the need for a randomized controlled trial to assess the benefits of B12 and folic acid compared to folic acid alone in a population with low B12 status and a high prevalence of NTDs such as India (115). Overall, evidence suggests that B12 is likely an important nutrient in pregnancy in regards to reducing the risk for NTD.

2.6.2 Adiposity and Glucose Homeostasis

Evidence is accumulating to suggest that maternal nutrition status during the pre and post-natal periods may contribute, through epigenetic programming, to cardiovascular and metabolic health later in life. Maternal B12 deficiency has been associated with an increased risk for insulin resistance postpartum and in the offspring, which could potentially increase long-term risk for the development of diabetes and metabolic syndrome (10,11,128). In the Pune Maternal Nutrition Study, which was a longitudinal investigation conducted in a population of 700 women living in Indian villages, maternal B12 deficiency (<150 pmol/L, observed in 60-71% of the women depending on week of gestation) was associated with higher homeostatic model

assessment of insulin resistance (HOMA-IR) scores in six year old offspring. This measure is an indicator of insulin resistance calculated from fasting plasma glucose and insulin concentrations. Concurrent elevated folate and low B12 concentrations in the women during pregnancy were associated with the highest HOMA-IR scores in the offspring (11). By 12 years of age this relationship had disappeared (129) but further investigation is necessary to determine whether this observation could be confounded by the developmental rise in insulin resistance observed during puberty (130). Maternal B12 deficiency was also associated with greater maternal adiposity and HOMA-IR scores, and increased odds of developing diabetes (OR=5.2) at five years post-partum (10). In another longitudinal study that investigated the effect of various micronutrient supplements during pregnancy, maternal B12 deficiency (<148 pmol/L) at baseline was associated with increased HOMA-IR scores in the offspring at six to eight years independent of exposure to micronutrient supplementation and maternal folate status (131). In summary, maternal B12 deficiency may be associated with adverse impacts on the metabolic health of the mother and offspring but further research is needed to clarify this relationship and to understand the determining mechanisms.

2.7   Prevalence of Vitamin B12 Deficiency in Women of Childbearing Age

Based on data from the Canadian Health Measures Survey (CHMS) (Cycle 1 2007-2009), 4.6% of all Canadians have SB12 concentrations <148 pmol/L indicative of chronic deficiency, and 18.5% have concentrations between 148 and 220 pmol/L indicative of marginal deficiency. The subgroup of women aged 20-45 y had 6.1% and 19.9% percent chronic deficiency and marginal deficiency, respectively (1). Due to suppressed data the prevalence of B12 deficiency could not be determined for sub-regions and for women of different ethnicities. This data was not intended to be extractable by sub-group and is not representative of specific regions or subpopulations (1). The most relevant data on Canadian women of childbearing age is from a

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retrospective cross-sectional analysis of n=10622 women of childbearing age from Ontario (15-46 y). The authors reported B12 deficiency (defined as SB12 <125 pmol/L) in 6.9% of non-pregnant women, 5.2% of pregnant women at less than four gestational weeks and 10.1% of pregnant women at greater than four gestational weeks (2). Vitamin B12 status assessment did not include multiple biomarkers in this survey. Several other studies have observed moderate to substantial rates of B12 deficiency in pregnant and non-pregnant women in several geographic regions (**Table 2-7**). Rates of potential chronic deficiency (highest cut-off <150 pmol/L) between 5.2 and 60% have been observed. Pregnant women have a higher prevalence of deficiency than non-pregnant women primarily due to hemodilution and hormonal changes in pregnancy (132).

Table 2-7 Vitamin B12 Deficiency Rates in Women of Childbearing Age

Population	Location	Study Design	Subjects	Percent Deficient	Cut-offs [a]
Non-pregnant Women	Germany(133)	Cross sectional survey	n-1266; 18-40 y	15%	148 pmol/L
	Canada (Ontario)(5)	Case control	n=6888; 15-46y	6.9%	125 pmol/L
	Vietnam (134)	Cross sectional survey	n=1526; 15-49 y	12%	148 pmol/L
	Denmark (Copenhagen Capital Region *formerly Copenhagen Country)(135)*	Cross sectional survey	n=3173; 30-60 y	8.4%	148 pmol/L
	New Zealand (Auckland)(136)	Cross sectional survey	n=135; ≥20 y	12.9%	150 pmol/L
Pregnant Women	Canada (Newfoundland)(137)	Cross sectional survey	(n=1424; 15-47 y)	25%	130 pmol/L
	Nepal (District of Sarlahi(138)	Baseline data from randomized controlled trial	(n=1158; 15-45 y)	28%	150 pmol/L
	Venezuela (Gran Caracas)(139)	Cross sectional survey	(n=1283; ≥14 y)	61%	150 pmol/L
	Canada (Ontario)(2)	Case control	<28 days gestation (n=1244; 15-46 y)	5.2%	125 pmol/L
			>28 days gestation (n=2490; 15-46 y)	10%	
	India (Pune)(11)	Longitudinal survey	18 weeks gestation (n=700; 19-23 y)	60%	150 pmol/L
	India (Bangalore)(140)	Cross sectional survey	>14 weeks gestation (n=366; 18-40 y)	51.1%	150 pmol/L
	Auckland, New Zealand(141)	Case control study	28-31 weeks gestation (n=166, mean age 25-26 y)	29-33%	200 pg/mL

[a]Cut-offs are for serum B12 concentrations. All cut-offs are expressed in pmol/L for continuity – they may have been reported as pg/mL in the original article and were converted to pmol/L as 1.35 pg/mL = 1 pmol/L

2.8 South Asian Populations: Risk for Chronic Disease and Health Inequities in Canada

South Asian ethnicity includes individuals identifying as Bangladeshi, Bengali, East Indian, Goan, Gujarati, Hindu, Ismaili, Kashmiri, Nepali, Pakistani, Punjabi, Sikh, Sinhalese, Sri Lankan or Tamil ethnicity, but does not include individuals of other ethnic descent who practice SA religions. At a population of over 1.5 million (4.8% of the Canadian population), SAs are the largest minority group in Canada (142). In Metro Vancouver, 11% of the population is of SA descent, making up 16.1% of the total SA population in Canada (142). This proportion is higher

in suburban cities in Metro Vancouver such as Surrey (19.1%) and Abbotsford (27.5%) (143).

Many SA individuals in Canada choose to settle in large culturally and religiously resilient multi-generational communities (54). This phenomenon contributes to the persistence of traditional food practices and influences the approach to and overall quality of the healthcare they receive.

Female SA populations in Western countries experience cultural barriers to optimal health including racial discrimination, class inequities, poverty, and social isolation, resulting in inadequate and inequitable access to health care (144). South Asian women are less likely than women of other ethnicities to make use of pre and peri-natal support services and prefer to interact with female and/or SA healthcare practitioners (145,146). They have expressed desire for targeted health services and clinics to address their unique needs (146).

South Asians have, on average, lower income (23% < low income cut-offs) than the general Canadian population (16% < low income cut-offs) and the average income of SA women is only 62% of their male counterparts (19). Therefore, cost for health services such as nutritional supplements, which are not covered by Medicare, could be prohibitive for some women. These unique barriers reinforce the need for additional healthcare services that take ethnic background and cultural traditions into consideration. Without these measures SA women may be at a disadvantage when it comes to maternal healthcare and might not receive the appropriate nutritional counseling necessary for optimal pregnancy outcomes.

Furthermore, SA populations currently have the highest rate and risk for chronic disease of all ethnic groups in Canada (147,148). As B12 deficiency is hypothesized to be involved in the pathophysiology of many chronic conditions, it is plausible that the development of B12 deficiency and chronic disease could be mechanistically intertwined. Collectively, these social and biological factors may contribute to an increased risk for B12 deficiency in SA populations.

2.9 Risk Factors for B12 Deficiency

General risk factors for B12 deficiency are summarized in Table **2-8**.

Table 2-8 General Risk Factors for Vitamin B12 Deficiency

Factor	Examples
Advanced age	N/A
Genetic factors	Single nucleotide polymorphisms in B12 related genes, deficiencies in B12 receptors and transporters, inborn errors of metabolism, genetic predisposition to disease, Immerslund Grasbeck syndrome
Social factors	Religion, socioeconomic status, inhabitation of rural regions, food insecurity, poor diet diversity
Lifestyle factors	Obesity, chronic alcohol abuse, tobacco use
Infections and parasites	*Diphyllobothrium latum, Helicobacter pylori, Giardia lamblia,* Tropical sprue
Gastrointestinal conditions	Pernicious anemia, gastroesophageal reflux disease, peptic ulcers, Zollinger-Ellison syndrome, atrophic gastritis, inflammatory bowel disease, irritable bowel syndrome, celiac disease, pancreatic insufficiency
Disease states	Type 1 and 2 diabetes, autoimmune hypothyroidism, human immunodeficiency virus, acquired immunodeficiency syndrome
Pharmaceuticals	Proton pump inhibitors, metformin, H_2 receptor antagonists, oral contraceptives, nitric oxide anesthesia
Dietary factors	Animal sourced food restricted diets, impaired mastication and swallowing abilities

Adapted from: (61)

These factors are associated with a range of physiological, metabolic, and behavioral changes, which depending on the risk factor can include the development of autoantibodies against B12 transport proteins and parietal cells, reduced function and concentration of B12 transport proteins and receptors, reduced gastric acid and pancreatic secretion resulting in impaired B12 absorption, removal or scarring of the distal ileum – the site of B12 absorption, malabsorption and chronic diarrhea leading to increased B12 losses, and insufficient B12 intakes.

These changes can result in: **[1]** complete malabsorption of B12 from the GI tract to the bloodstream and from re-circulated bile; **[2]** partial malabsorption of B12 from the GI tract to the blood stream and from re-circulated bile; **[3]** insufficient replacement of excretory B12 losses; **[4]** incomplete transport of B12 from the bloodstream to metabolically active tissues, or **[5]** partial malabsorption of B12 from food.

These effects can then lead to either the progressive or rapid depletion of B12 stores, which initially results in the state of marginal deficiency. Marginal deficiency may require moderate supplementation or increased dietary intake and can, but does not absolutely progress, to chronic deficiency. This transition can be very slow or rapid depending on the cause of deficiency. Chronic deficiency requires immediate correction of the disease state through increased dietary B12 intake, B12 supplementation, or pharmacological doses of B12, and repletion therapy in severe cases of malabsorption.

The following subchapter summarizes the determinants of deficiency with the potential to affect the B12 status of healthy women of childbearing age. Factors that will be covered include **[1]** ethnicity, **[2]** inadequate dietary intake, **[3]** socioeconomic status (SES), **[4]** obesity, **[5]** genetics, and **[6]** OCs.

2.9.1   Ethnicity

Certain ethnic groups have been observed to have a high prevalence of B12 deficiency. Regions of interest include SA, Latin America (where ~40% of children and adults had evidence of B12 inadequacy), and Africa (where 70% of Kenyan school children displayed inadequacy) (149–151). In North America groups of European and SA descent appear to be at greater risk than other ethnic groups. The SA population is of particular interest due to common B12 restrictive dietary practices and evidence from Canadian immigration studies and a Canadian based survey of vulnerability to B12 deficiency (14,152).

In Canada, the largest ethnic groups include individuals of European, Asian and SA descent (153). North American studies have suggested that SA and European ethnic groups may be at greater risk of deficiency than other ethnic groups based on comparisons that have been made with those of African and Asian descent. In the United States, lower SB12 concentrations have been observed in Caucasian and Asian Indian groups compared to Asian and African

American groups. For example, a study of 725 elderly (>60 years) multiethnic subjects found the highest rates of deficiency in Caucasian American men and the least in African American and Asian American women (154). Another study in elderly (≥65 years), physically disabled women from the Women's Health and Aging Study in the United States reported higher mean MMA concentrations (p=0.0001) and lower SB12 concentrations (p<0.0003) in white women compared to African American women (155). Serum B12 concentrations were also significantly lower in two studies comparing Asian Indians to other ethnic groups (**Table 2-9**).

Table 2-9 Vitamin B12 Deficiency Rates in Migrant South Asian Populations

Location	Subjects	Mean SB12	% Deficient	Deficiency Cut-offs[a]
United Kingdom (156)	138 Hindu Men	270 pmol/L	69%	N/A
	European Men	357 pmol/L	N/A	
United States (157)	**Multiethnic Adults**			180 pmol/L
	Indian	182 pmol/L	46.7%	
	Other Ethnic Groups	306 pmol/L	10.5%	
United States (158)	**Multiethnic Adults**			
	Asian Indians	204 pmol/L	N/A	N/A
	Caucasians	320 pmol/L		
[a]Based on serum B12 concentrations				

In contrast to Europeans and SAs, people of Asian descent may be at lower risk for B12 deficiency (159). Reasons for this include a diet rich in sources of B12 such as seafood and pork, and a persistent increase in meat consumption (the major source of dietary B12) over time. Furthermore, the lowest rates of pernicious anemia, an autoimmune disorder that results in the destruction of parietal cells and IF and the major cause of severe B12 malabsorption, is observed in Asian populations (159,160).

There is a wealth of evidence to support the hypothesis that SA populations, including SA women of childbearing age, are at high risk for B12 deficiency **(Table 2-10)**.

Table 2-10 Vitamin B12 Deficiency Rates in South Asian Populations

Population	Location	Subjects	Mean Serum Vitamin B12 (pmol/L)	Percent Deficient	Deficiency Cut-offs[a]
Adults	Pune, India (15)	Adults (27-55 y)	124-161 pmol/L	47%	<150 pmol/L
	Pune, India (16)	Men	119 pmol/L	67%	
	Northern India (161)	Adults			
	Bangladesh (162)	Adults	N/A	8%	151 pmol/L
			N/A	13%	
	Gujarat, India (163)	Adults (n=48 men, n=52 women)			211 pg/mL
		Men	453.2 pmol/L	38%	
		Women	414.8 pmol/L	38%	
	Bangalore, India (164)	Healthy elderly urban subjects (n=175, >60 yr.)	N/A	16%	150 pmol/L
	Pune, India(165)	Adults (25-92 y, n=300) with coronary artery disease	167.4 ± 297.7	86.7%	<147.6 pmol/L
Children	North India (166)	**Children aged 6-30 months**			<150 pmol/L
		Breastfed	183 pmol/L	36%	
		Non Breastfed	334 pmol/L	9%	

[a]Based on serum B12 concentrations

The observed risk is often underlined by the common practice of lacto-vegetarian diets in SA populations. Vegetarianism in SA countries typically begins at birth in contrast to the adoption of vegetarian diets in adolescence or adulthood in western countries. This predisposes individuals to B12 inadequacy, especially if the mother is also deficient, as there is no opportunity to build up stores (15). Deficiency is also prevalent in omnivorous SAs, which suggests that other risk factors, such as biological factors or infection, may also contribute to risk. In addition to high rates of deficiency, very high rates of health outcomes associated with low B12 status have been reported in SA populations including NTD rates as high as 6-11.4/1000 births in India (167). Studies in populations living in SA countries have consistently reported very low SB12 concentrations across many demographics (**Table 2-10)**.

Immigrant SA populations may also be at risk of inadequate B12 status. In 1985, a report on 138 Hindu vegetarians residing in the United Kingdom with displays of chronic B12 deficiency provided evidence that deficiency might not be reversed after immigration to an industrialized country. Indians living abroad have been observed to have relatively lower SB12 concentrations than other ethnic groups (**Table 2-9,** page 36).

Vitamin B12 deficiency rates have not been adequately assessed in SA Canadians. The only relatively recent data on the B12 status of SA Canadians comes from a randomly selected population of SA patients (n=988, aged 18-84) attending a Toronto doctor's office who had their charts retrospectively reviewed. A rate of 22% deficiency (<132 pmol/L) was observed in all patients assessed (n=988) and 46% deficiency was observed in patients with at least one available B12 measurement (n=222) (14).

2.9.2 Inadequate Dietary Vitamin B12 Intake

Inadequate dietary B12 intake occurs as a result of the dietary intake patterns associated with **[1]** vegetarian or vegan diets, **[2]** poverty-related reductions in ASF consumption, and **[3]** religiously or culturally motivated avoidance of ASFs. The common theme in all of these scenarios is reduction or absence of ASFs from the diet.

Under circumstances of inadequate B12 intake, daily losses from liver stores may not be adequately replenished and deficiency symptoms can appear as early as two to six years after B12 intake is restricted (168). Well planned vegetarian diets are touted as protective against poor cardiovascular health, cancer, obesity, and other chronic degenerative diseases but poorly planned vegetarian diets may lack essential nutrients such as protein, omega 3 fatty acids, vitamin D, calcium, iron, zinc, iodine and B12 (169–171). Additionally, vegetarian diets are often rich in folate, which may contribute to masking of the hematological symptoms of B12

inadequacy (172). Vegetarians are also prone to iron deficiency which can result in damage to the gastric mucosa and lead to atrophic gastritis and decreased B12 absorption (173). Compared to omnivores, individuals eating on the vegetarian spectrum, which ranges from complete ASF restriction ("vegan"), to modest consumption of ASFs such as milk and eggs ("lacto-ovo" vegetarian), to inclusion of some fish and shellfish ("pescatarian"), have an increased risk of B12 deficiency and elevated Hcy (174). This outcome has been reported by many observational studies in populations including men (175) pregnant women (176) and the elderly (177). Further, a cross-sectional study in Germany reported that 0% of omnivores had elevated serum MMA compared to 5% of low meat consumers, 32% of lacto-ovo vegetarians, and 43% of vegans (169). Lacto-vegetarianism is common in regions of SA, particularly Northern India where the staple diet includes foods such as cereals, millets, vegetables, milk and milk products (178). Non-vegetarians in this region add occasional eggs, poultry, and small amounts of meat to their diet but net animal protein intake is relatively lower than the omnivorous western diet (178). In a study on pregnant women in India, low consumption of fish and yoghurt increased risk for B12 deficiency (140). Refsum *et al* observed that only 38% of female and male adult SA participants consumed animal products more than once a month (15). The National Family Health Survey in India reported that one third of women ate no meat products, and one tenth ate no dairy products (179). Given the propensity for ASF restriction in SA populations, differences in B12 intake may exist between ethnic groups and dietary intake may be a strong determinant of B12 status in SAs.

There is a common misconception that it takes decades for B12 deficiency to develop in individuals with adequate stores and without malabsorption related disorders. Vegetarian diets maintained for a duration of as little as two years have been associated with the presence and

development of deficiency. Donaldson *et al* observed 76% marginal deficiency (SB12 <221 pmol/L) in a sample of individuals who had been vegetarians for two to four years (180). Hermann *et al* observed 66% deficiency in individuals who had adhered to vegetarian intake practices for at least two years (181). Pre-existing deficiency was not assessed in either of these studies. While restrictive and long-term vegetarian diets increase the magnitude and rate of development of deficiency, deficiency develops eventually in vegetarians of all persuasions who do not supplement their diet or regularly consume adequate amounts of ASFs to satisfy B12 requirements as is possible with well planned lacto-ovo, pescatarian, and pollotarian (no red meat) diets, and for vegetarians with occasional meat intake. In British Columbia, 5.8% of the general population and 14% of adult females self-identified as vegetarians (182). This group estimate includes individuals who reported at least occasional consumption of fish (74.9%), poultry (57.6%), red meat (22.4%), and dairy products and eggs (97.3% and 92.3%, respectively).

Dietitians of Canada recommend at least three daily servings of B12 rich foods for adults. Vitamin B12 rich foods highlighted within their educational literature include Red Star™ nutritional yeast, fortified dairy and non-dairy milk, and fortified meat analogs, but meat is the main contributor to total B12 intake in the general North American population (183).

Canadian Community Health Survey (CCHS) data from 2004 estimates that females over 19 in Canada have an estimated mean B12 intake of 3.5 μg/day, and that ≤5-8% of this adult female population (aged 19-50 y) do not meet the EAR for dietary B12 intake depending on whether they consume B12 containing supplements (184,185). Median B12 intake in pregnant women in India was 1.5 μg/day (IQR: 0.86, 1.96), which is significantly lower than recommended daily intakes. There is no data on ethnic specific B12 intakes in Canada. Rates of

dietary B12 inadequacy for Canadian women are reported in **Table 2-11.** Likelihood of

inadequacy from diet alone was similar for supplement and non-supplement users but

supplement use was associated with lower rates of inadequacy.

Table 2-11 Prevalence of Dietary B12 Inadequacy in Canadian Women

Group (Females)	Diet only (supplement users)	Diet only (non supplement users)	Diet + supplements in supplement users
		Values presented as (%) SE	
14-18 y	15(3)	20(9)	<5
19-50 y	12(3)	8(7)	<5
>51 y	9(3)	14(4)	<5
Adapted from (185)			

2.9.3 Socioeconomic Status

Individuals inhabiting developing countries are subject to poverty, below average

sanitation, low education, poor diet, and suboptimal healthcare (13). Low income and education

have been observed to impact micronutrient intake and status, contributing to prevalent B12

deficiency in developing regions worldwide (186,187). Despite these consistent observations,

some researchers have failed to observe this trend, particularly in SA populations. In Northern

India 76.1% of urban slum dwellers vs. 88.4% of urban non-slum dwellers reported B12 intakes

lower than the RDA (161). Lower intakes were more common in vegetarians but interestingly,

slum dwellers were more likely to report sufficient intake of ASF suggesting that poverty might

not determine B12 status in SA populations, or that ASF consumption is not determined by

income. Data is not available on the impact of socioeconomic status on B12 intake of Canadians.

2.9.4 Obesity

Obesity, defined by the World Health Organization as a body mass index (BMI) ≥ 30

kg/m^2, and B12 deficiency may have a bi-directional relationship. Mechanistically, it has been

proposed that MMA accumulation may result in impaired mitochondrial respiration and could

reduce substrate (e.g. fatty acid) oxidation leading to increased retention of adipose stores;

impaired mitochondrial respiration has been observed in neural tissue in rodents (140,188,189). In addition, reduced Hcy re-methylation could reduce protein synthesis and lean tissue deposition by limiting methionine availability. However, in the Hordaland Homocysteine Study, tHcy concentrations were not associated with lean tissue mass or BMI after controlling for total cysteine concentrations (190). Obesity may in turn be accompanied by changes in the absorption, excretion or metabolism of B12. In Israel, obese children (BMI >95%ile) and adolescents were at risk of lower SB12 concentrations compared to non-obese controls (OR=4.33(1.54-12.2)) (191), and B12 concentrations were inversely correlated with BMI (r =-0.221, p=0.001) in a case control study comparing obese and overweight adults to non-obese adults in Turkey (192). Evidence suggests that concentrations of B12 biomarkers are impacted in obese individuals, but it is not clear whether this is a true depletion or rather a redistribution of the vitamin without consequences for cellular depletion.

2.9.5   Genetics

Genetic variants in B vitamin metabolism related genes have been associated with low B12 biomarker concentrations as well as increased risk for B12 related health outcomes, such as NTDs, diabetes and preeclampsia (122,193,194).

With the exception of inborn errors of metabolism in B12 related genes, no individual gene variant has emerged as a major determinant of B12 status (195). However, multiple gene variants may have collective effects on B12 metabolism that are more significant than the effect of a single variant on its own (196). For instance, the coincident occurrence of *MTHFR* C677T and cystathionine beta synthase T833C (rs5742905) variants has been shown to collectively increase NTD risk above that of the individual variants (197). It is possible that some cases of unexplained marginal deficiency could be influenced by genetic variation. Specific variants in the genes encoding MTHFR, MS, MTRR, the transcobalamin receptor (TCN2 receptor),

transcobalamin II (TCN2), the cubam receptor, and galactoside 2 alpha L-fucosyl transferase 2, otherwise referred to as fucosyl transferase 2 (FUT2) have been investigated. As this thesis has focused on four gene variants, they will be discussed in further detail below.

2.9.5.1 *MTHFR* C677T (rs 1801133)

The *MTHFR* C677T variant is one of the most thoroughly studied in relation to B vitamin metabolism and health outcomes related to B vitamin depletion. This variant results in an amino acid substitution of an alanine to valine at codon 222, which generates an enzyme product with reduced thermal stability.

The TT genotype has been associated with elevated Hcy (198), particularly in subjects with low folate status (199), reduced total folate (200)and a shift towards accumulation of folate vitamers involved in purine and pyrimidine synthesis at the expense of Hcy remethylation (201). As well, the inverse relationship between both folate and B12 status, and Hcy concentration, is more pronounced in those with the TT genotype (202).

Greater susceptibility to B12 deficiency (SB12 <148 pmol/L) has been reported in individuals homozygous for the T allele of the *MTHFR* C677T variant (OR: 1.78; 95% CI: 1.25, 2.54) (135). Some studies have reported an increased risk for NTDs associated with the TT genotype of the *MTHFR* 677TT variant but the results of multiple trials have been conflicting (203).

The prevalence of the CT and TT genotypes for this variant are significantly lower in SAs compared to Europeans (204). In SA populations this variant is not associated with NTD risk (OR: 0.97; 95% CI: 0.71, 1.28), or altered Hcy levels (204). It is possible that this gene variant could account for some variation in B12 status and that the effects could be ethnic specific.

2.9.5.2   *TCN2* Variants (rs 1801198, 9606756)

Variants in the gene encoding TCN2 have been associated with impaired B12 status.

The *TCN2* C776G variant is defined by an arginine to proline substitution at codon 259 that causes a reduction in the transcription of TCN2 (205). Lower holoTC concentrations have been observed in individuals with the GG genotype of the *TCN2* C776G variant compared to individuals with the CC genotype (206). Despite the effect on holoTC, the concentration of other B12 biomarkers remains the same in a B12 adequate environment (207). This variant has also been associated with an increased risk for NTD in the offspring (OR: 1.34; 95% CI: 1.08, 1.64) (122), though not all studies have observed this effect (208).

Reductions in holoTC concentrations have also been associated with the *TCN2* A67G variant. Norwegian adults (50-64 y) with the GG genotype and AG genotype had 30% and 15% lower holoTC concentrations than individuals with the AA genotype, respectively (209). The authors of this study suggested that the G allele of both the *TCN2* C776G variant and the *TCN2* A67G variant contribute to a reduction in holoTC concentrations but that the *TCN2* A67G variant has the greater effect (209).

Given that TCN2 gene variants have only been shown to affect holoTC concentrations, it is not clear whether these variants have a clinically significant influence on other B12 biomarkers or clinical outcomes of B12 depletion.

2.9.5.3   *FUT2* A893G variant (rs602662)

The fucosyl transferase (*FUT2*) gene encodes galactoside 2-α-L-fucosyl transferase 2, the enzyme responsible for synthesis of H antigen, the precursor of the ABO histo-blood group antigens in body fluids and throughout the intestinal mucosa. Individuals homozygous for nonfunctional *FUT2* alleles fail to present ABO antigens and are referred to as 'non-secretors'.

Individuals with functional enzymes, or 'secretors' have a greater risk of infection from pathogens that can attach to α-1,2-fucosylated glycans on epithelial cells (210). Consequently, secretors are observed to have higher susceptibility to *H. pylori* infection and gastritis, and impaired B12 status (211,212).

One variant with significant evidence for an effect on B12 biomarker concentrations is the *FUT2* A893G variant (rs 602662). Homozygosity for the A allele corresponds to inactive *FUT2* and non-secretor status. Tanawar *et al* investigated the association of this variant with SB12 concentrations in 1146 individuals from North India and the lowest SB12 levels were observed in individuals with the GG genotype (p<0.001).

Overall, little is known about the potential impact of these genetic variants on the metabolism of B vitamins and health consequences related to B vitamin inadequacy. Allele frequencies for genetic variants associated with altered B vitamin metabolism and the association with NTD risk appears to be different in SA and European populations. Therefore, it is possible that the association of genetic factors with impaired B vitamin status may differ by ethnicity.

2.9.6   Oral Contraceptives

There have been numerous reports of low B12 concentrations in oral contraceptive (OC) users (213–220). However, many of the older studies examined the effect of first generation OCs, which had much higher hormone concentrations than the newer formulations and may not be representative of what is currently prescribed.

Many researchers have noted that the drop in SB12 measurement associated with OC use is not reflected by any metabolic or clinical symptoms, or changes in B12 absorption (221). Riedel *et al* reported 25% lower SB12 concentrations and an increased risk for OC users to fall

in the highest quartile of MMA concentrations but there were no differences in MMA or tHcy concentrations between OC users and non-OC users (194).

Some researchers have indicated that either changes in HC concentrations, or redistribution of B12 may be responsible for the observed B12 depletion (215,217). It has been hypothesized that the hormones in OCs may have an effect on reducing granulocyte secretion of HC, akin to what is observed in pregnancy. The reduction in HC concentrations in pregnancy leads to a significant drop in circulating SB12 without adverse effects on B12 dependent metabolism (176). Hormonal effects on HC concentrations outside the context of pregnancy were not confirmed when investigated in elderly females on hormone replacement therapy (222). Given that many of the analytical methods utilized in the early studies on this topic have been replaced by more sensitive assays, it would be of interest to explore the relationship between OC use and B12 status using modern methodology and multiple biomarkers. The biochemical mechanism behind the low SB12 concentrations observed in OC users is not fully understood.

2.10 Summary and Rationale

Population projections suggest that the number of SAs in Canada will rise to 1.8 million by 2017 (223), giving justification for interventions addressing the unique health risks this population faces. It will be necessary to assess rates of chronic disease and identify preventable risk factors in an effort to improve the health of SAs and reduce healthcare costs.

It is now understood that B12 adequacy is important for healthy pregnancies and the long-term health of the child. B12 deficiency may not be uncommon in Canadian women of childbearing age, and it is prevalent in SA populations worldwide. Given the significant SA immigrant population in Metro Vancouver, it is an ideal geographic area to assess the rate of B12 deficiency in this ethnic group.

Optimal B12 assessment includes multiple biomarkers as the use of a single biomarker has significant limitations for accurately assessing B12 status. Few Canadian studies have used multiple biomarkers to assess B12 status. In addition, determinants of B12 status have not yet been assessed in Canadian women. There is evidence that factors beyond diet such as genetic variation and lifestyle may be associated with impaired B12 status and that these factors may vary by ethnic group.

This study aims to assess rates and determinants of B12 deficiency in SA and European women aged 19-35 using both functional and direct biomarkers of B12 status.

Chapter 3: Study Design and Methodology

The overall aim of the present study is to assess the rate and determinants of B12 status[14] in women aged 19-35 of SA and European ethnicity in Metro Vancouver using multiple biomarker analysis. The study is organized into primary and exploratory objectives, which are subdivided into specific objectives where applicable, and is powered based on the primary objective.

3.1   Primary Objective and Hypothesis

The primary objective is to assess whether women of SA descent have lower B12 status than women of European descent. We hypothesize that SA subjects will have lower SB12 concentrations and higher rates of chronic and marginal deficiency, as defined by SB12 cut-offs, than European subjects.

3.2   Exploratory Objectives

The exploratory objectives are **[1]** to assess the association of established B12 deficiency risk factors with B12 status, and **[2]** to assess the influence of using multiple biomarker analysis on the outcome of B12 status assessment.

Exploratory Objective 1: Determinants of Vitamin B12 Deficiency

To assess the association of established B12 deficiency risk factors with B12 status.

Rationale: Many factors have the potential to explain risk for B12 deficiency and the goal was to determine which of these established factors are associated with B12 status in this specific study population. This information could provide insight into potential routes for intervention and may indicate whether specific subgroups require enhanced monitoring.

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[14] defined by SB12 concentration and rate below the SB12 concentration cut-offs for chronic (SB12 <148 pmol/L) and marginal deficiency (SB12 148-220 pmol/L)
This definition for '**B12 Status**' will apply throughout the thesis

Exploratory Objective 2: Multiple Biomarker Assessment

To assess the influence of using multiple biomarker analysis on the outcome of B12 status assessment.

Specific objective 2-1:

To examine the correlations among SB12, MMA and holoTC concentrations, and determine whether dividing subjects by SB12 cutoffs results in differences in holoTC and MMA concentrations between adequate, marginal deficiency, and chronic deficiency groups.

Rationale:

Holotranscobalamin and MMA concentrations are proposed to strongly correlate with SB12 concentrations. This objective will allow us to determine the strength of the linear relationship among B12 biomarkers in this study sample. For instance, whether low SB12 concentrations consistently correspond to low holoTC concentrations. It will also address whether the proposed cut-offs for SB12 divide subjects into distinct categories in terms of their MMA and holoTC concentrations. For example, whether the marginally deficient group has higher MMA concentrations than the adequate group.

Specific objective 2-2:

To determine the rate of combined metabolic deficiency[15] and disagreement[16], and assess whether individuals with disagreement between biomarkers better fit the profile of adequacy or deficiency based on their holoTC concentrations, hematological status, and established risk factors for B12 deficiency.

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[15] Defined as simultaneously: **[1]** low serum B12 (based on 'conventional' or 'Bailey' cut-offs) or holoTC concentrations and **[2]** high MMA concentrations
[16] Defined as disagreement between biomarker classification for placing an individual in a category of deficiency (e.g. low SB12 and low MMA)

Rationale: The use of multiple biomarkers is suggested to improve diagnostic accuracy in B12 status assessment. Assessing rates of concurrent deficiency will enable us to observe whether the choice of biomarker combination greatly impacts the overall rate of deficiency that we observe. Further, by nature of applying multiple biomarkers, certain individuals may not satisfy the expanded criteria for combined metabolic deficiency and may fall in categories of disagreement. It is important to determine whether the use of conventional cut-offs for SB12 in combination with MMA result in the exclusion of individuals who may be at risk of developing B12 deficiency.

3.3 Design Overview

The study was a descriptive cross-sectional survey assessing the B12 status of a target sample size of n=300 non-pregnant women aged 19-35 y of SA and European descent. The age group of 19-35 y was chosen because 35 and older is considered 'advanced maternal age' and is associated with age related pregnancy risks (224). Although the present study does not assess pregnant women or pregnancy outcomes it aimed to assess women in the age range considered low risk for adverse pregnancy outcomes. Enrolment occurred between July 2012 and July 2013. Participants attended one clinic visit in Vancouver, BC or Surrey, BC at **[1]** the University of British Columbia Food Nutrition and Health building, **[2]** the Clinical Research and Evaluation Unit, Child and Family Research Institute, **[3]** the South Community Health Centre, Vancouver Coastal Health, or **[4]** the Newton Public Health Unit, Fraser Health. Fasting (~10 hours) venous and finger prick blood samples were obtained from participants. Participants completed **[1]** a demographic questionnaire (DQ) (**Appendix A**), **[2]** the "International Physical Activity Questionnaire – Short Last 7 Day Self-Administered Format" (IPAQ) **(Appendix B)**, and **[3]** a semi-quantitative Food Frequency Questionnaire (FFQ) "*Your dietary intake*" (Shatenstein *et al*, Université de Montréal) **(**guide included in **Appendix C,** full questionnaire unavailable due to

copyright). Research staff took anthropometric measurements, including height, weight, and waist circumference. A summary of study day collections appears in **Table 3–1**. The Clinical Research Ethics Board at the University of British Columbia (#H11-01216), the Vancouver Coastal Health Ethics Board (#V11-01216) and the Fraser Health (#FHREB 2013-016) Ethics Board gave ethical approval for conduct of this study.

Table 3-1- Biological and Questionnaire Data Collected During Clinic Session

Item	Description of variables and analytes
Anthropometric	• Weight • Height • Waist Circumference
Questionnaires Demographic Questionnaire International Physical Activity Questionnaire Food Frequency Questionnaire	 • Age • Ethnicity • Immigration and location • Income and education • Lifestyle • Pregnancy history • Oral contraceptive and menstrual cycle history • Lactose intolerance • Supplement use • Level of physical activity • Frequency and portion of 78 food items, food practices, lifestyle and activity questions

Fasting Blood Sample	• Complete blood count
	• Serum vitamin B12
	• Serum holotranscobalamin
	• Plasma methylmalonic acid
	• Red blood cell folate
	• Plasma folate
	• Genotype
	   o *MTHFR* C677T
	   o *TCN2* C776G
	   o *TCN2* A67G
	   o *FUT2* G893A

3.4   Study Participants

Sample size was calculated (**Equation 1**) based on a sufficient group size to detect a clinically relevant difference in SB12 concentrations between SA and European groups.

Equation 1 Sample Size Calculation

$$n = \frac{2(\sigma)^2 (Z_\beta + Z_{\alpha/2})^2}{(\mu_{1-}\mu_2)^2}$$

Differences in SB12 concentration observed when comparing SA populations to other ethnic groups range from 87 pmol/L to 124 pmol/L (**Table 2-9** for references). Based on these differences a group sample size ranging from n=16 to 33 would be appropriate. To account for the possibility of greater variation and smaller differences in SB12 concentration between ethnic groups in the study population we set a sample size of n=150 for both ethnic groups (n=300 total) that would allow for the detection of a 40 pmol/L decrease in SB12 assuming β=0.8, α=0.05, a SD of 126.9 pmol/L, and choosing Europeans as the reference group (2).

Eligibility of research participants was determined using a screening questionnaire **(Appendix D)**, administered over the phone. This questionnaire assessed multiple factors[17] to identify individuals that fit the target demographic. If conflicts were identified, YL and I further examined these exclusion factors during a second assessment to come to consensus on the participant's eligibility.

Participants were recruited by passive and active methods in Metro Vancouver. Paper-based recruitment notices in English, Punjabi and Hindi (8.5" x 11") **(Appendix E-H)**, brochures (8.5" x 11") **(Appendix I)** and advertisement cards (8.5" x 5.5") **(Appendix J)** were produced and placed in areas of high public traffic (e.g. coffee shops, libraries, community centers) and distributed by research assistants and community contacts. Online advertisements were placed through craigslist, kijiji, SA websites (e.g. Schema Magazine Online), Facebook and Twitter pages (e.g. Be Your Own Best Friend Network for SA Women), academic and professional email listservs (e.g. The University of British Columbia Graduate Student Society list serve), SA club listservs (e.g. Tamil Society of Vancouver), and a study website and social media.

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[17] In brief: **Participants must** 1) be aged 19-35, 2) have at least 3 grandparents from the same ethnic background (either European or South Asian), and 3) currently reside in Metro Vancouver. **Participants must not** 1) suffer from a chronic condition for which they are receiving treatment or medication, 2) currently take certain prescription medications performance enhancing or recreational drugs, or over the counter medications, 3) be pregnant, breastfeeding or lactating, or 4) suffer from an active gastrointestinal condition or have undergone gastric bypass surgery

Newspaper advertisements were placed in The Vancouver Sun and The Province. In addition, articles were published about the study in selected newspapers including VancouverDesi Newspaper, The Province, and Langley Today. Recruitment booths were set up at community events (e.g. Vaisakhi Parade 2013) and community centers (e.g. Roundhouse Community Center). I gave verbal announcements at group fitness classes at Vancouver area community centers and other fitness facilities (e.g. Gold's Gym), and in undergraduate and graduate classes at the University of British Columbia (Vancouver campus) and Simon Fraser (Surrey campus) and volunteered at a private food bank (CityReach Vancouver) in exchange for weekly recruitment opportunities. Community outreach was conducted in SA temples, at SA events (e.g. Punjab Mela), and through conference and community presentations (e.g. Destination South Asia Conference). South Asian public health nurses and key figures within the SA community distributed brochures to their community groups (e.g. South Vancouver Neighborhood House) and clients. Several pharmacies with SA client bases (e.g. Wescana Pharmacy, Naz's Pharmacy) distributed pamphlets to potentially eligible individuals.

All participants provided written informed consent to participate, and most provided written informed consent **[1]** to be contacted for follow up studies, **[2]** to receive results, and **[3]** participate in optional tissue banking for future research **(Appendixes K and L)**.

3.5 Methods for Enrolment and Anthropometric, Questionnaire and Biological Sample Collection

All appointments followed a standard protocol **(**blood collection summarized in **Appendix M)** on which each research participant was briefed at the research site of their choosing. Research staff ensured that participants read and signed all informed consent documents, which were provided at least 24 hours ahead of the briefing, and that there were no outstanding questions about subject responsibilities. Each participant was assigned an

identification number (i.e. 001 to 300) that was recorded on all questionnaires and biological

samples.

Anthropometric measurements were completed with subjects wearing light clothing and

no shoes for height (to nearest 0.1 cm), weight (to nearest 0.1 kg), and waist circumference

(midpoint between the lowest rib and iliac crest, to nearest 0.1 cm) using a stadiometer,

calibrated digital scale, and flexible tape measure, respectively. Duplicate measurements were

performed and recorded. If the values varied by greater than 0.1 units an additional measurement

was taken followed by an averaging of the two closest values. Body mass index was calculated

using a standard equation (**Equation 2**).

Equation 2 Body Mass Index Calculation

$$BMI = \ Weight\ (kg)\ /\ Height\ (m)^2$$

BMI classification is presented in **Table 3-2**, and waist circumference in **Table 3-3**.

Table 3-2 Body Mass Index Classification by Ethnicity

	European Cut-offs (kg/m^2)	South Asian Cut-offs (kg/m^2)
Underweight	<18.5	<18.5
Normal	18.5-24.9	18.5-23.9
Overweight	25.0-29.9	24-26.9
Obese	>30.0	>27
(225,226)		

Table 3-3 Waist Circumference Categorization by Ethnicity

	European Cut-offs (cm)	South Asian Cut-offs (cm)
Normal	0-88 cm	0-80 cm
At Risk (elevated)	>88 cm	>80 cm
(227)		

Research participants completed the DQ (**Appendix A**), physical activity (IPAQ)

(**Appendix B)**, and semi-quantitative FFQ (**Appendix C**) under the supervision of research

personnel.

Participants were asked to bring in nutritional supplement bottles. I collected information

on supplement use from all participants. Each participant was classified as a user of nutritional

supplements (Y/N), B12 supplements (Y/N), and folate supplements (Y/N). Descriptive data was

taken on all supplements used (i.e. brand name, product name, dose, frequency of dose) and

quantitative values were recorded for B12 and folate supplement intake (µg /day). From this

information and the estimated dietary B12 intake determined from the FFQ, a new variable: B12

intake from food and supplements (µg/day), was created.

Research staff doubly entered data from the demographic, food frequency, and physical

activity questionnaires. The DQ was pre-coded with discrete (0 (yes), 1 (no)), nominal (e.g.

Vancouver), continuous (e.g. 29 days), or ordinal (1 to 5 for education level) inputs

corresponding to each variable. Some exceptions that required further calculation are explained

in **Table 3-4.**

Table 3-4 Coding Criteria for Select Variables

Variable	Criteria
Total household income	Lowest (1), Lower-middle (2), Upper-middle (3), Highest (4) (detail in **Appendix O)**
Total vitamin B12 and folate intake supplements (µg/day)	Dose of supplement (µg) x Number of doses per day x daily frequency of supplement use (i.e. 2 x week)
Days since last period	Number of days counted forward from the last day of menstruation
Level of physical activity	Low (1), Moderate (2), High (3) **(detail in Appendix P)**

A semi-quantitative FFQ developed by Shatenstein *et al* **(**guide, **Appendix C)** was

administered using a standard protocol by personnel who had undergone training provided by

research staff from Dr. Bryna Shatenstein's lab, Université de Montréal, via phone conference.

The questionnaire has been validated for use in adult pregnant and non-pregnant Canadian

women against 4-day non-consecutive food records mean (r_s =0.41, range 0.32 to 0.58, p<0.001)

(228,229).

A line item representing 'Simulated meat products (meatless cold cuts, hotdogs, burgers,

chicken, beef, fish…)' was generated to ensure that B12 intake from fortified vegetarian sources

was represented. The final modified questionnaire included 78 food items and was intended to

represent intakes over a 12-month reference period. Results were entered into Microsoft Access™ software using systematic double entry. Six frequency categories of consumption, and three portion categories (smaller, similar, larger) were possible for each food item. Lifestyle question responses were entered based on selected options from nominal lists. Height and weight were manually entered according to the values obtained during anthropometric measurements. The data and physical questionnaires were checked for disagreement between entries. The final Access™ file was exported to Microsoft Excel™ and sent to the Université de Montréal. Data was then transferred to PASW (PASW Statistics 18, Chicago, IL) for analysis and plausibility assessment by Dr. Shatenstein's team. Nutrient intakes were computed by multiplying the frequency response by the nutrient content of specified portion sizes using a program developed at the Université de Montréal. The database for calculating nutrient intakes used information from the Canadian Nutrient File (2007b, Health Canada, 1982).

The FFQ produces results on 50 nutrients and number of servings of the four Canada's Food Guide (Health Canada, 2007) food groups. Dietary assessment data derived from the FFQ was extracted to establish values for select variables **(Table 3-5)**

Table 3-5 Dietary Intake Variables Extracted from Food Frequency Questionnaire

Item	Measure
Total caloric intake	kcal
Animal protein intake	grams
Servings of meat	# of servings
Servings of dairy and alternatives	# of servings
B12 intake	μg
Servings of red meat	# of servings
Total protein intake	grams

I collected the majority of blood samples following a standard venipuncture and finger prick protocol **(Appendix M)**. A research nurse was present to assist with blood draws for the one-day pilot run of the protocol. Following a 10-hour fast, 24 mL of blood was collected from

each participant in three tubes: **[1]** serum (10 mL), **[2]** ethylenediaminetetraacetic acid (4 mL), and **[3]** aluminum foil wrapped lithium heparin (10 mL) prepared glass vacutainers (BD Vacutainer ®) using butterfly needles (25, 23, or 21 G x 0.75 inch needle x 12 in tubing BD Safety-Lok™ blood collection set). The serum tube was left to sit upright for 30 minutes at room temperature. The ethylenediaminetetraacetic acid tube was placed in a biohazard bag with a requisition for CBC analysis and delivered within four hours to the BC Children's Hospital Department of Pathology and Laboratory Medicine for processing. The lithium heparin tube was immediately placed onto ice and covered to prevent light exposure. Sample processing was completed according to a standard protocol **(Appendix M)** and is explained in brief below.

For each participant, two 100 µL aliquots of whole blood were pipetted from the lithium heparin tube (BD Vacutainer ® LH 158 USP Units REF 367880, Sterile) into one mL of freshly prepared 10% ascorbic acid solution (L-Ascorbic acid, Sigma Life Science ®) in amber cryotubes (Fisher Brand Cat. No. 05-407-15). This solution was vortexed for ~five seconds (VWR Mini Vortexer ®) and placed in a water bath (Fisher Scientific Isotemp ®) at 37°C for three hours. The tube was then centrifuged at 3000 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5702 R ®) for 15 minutes at four ^{o}C. Eight 500 µL plasma samples, and one buffy coat sample were aliquoted into cryotubes (SARSTEDT ® REF 72.692) labeled and stored at –80°C. After removal of the buffy coat, the tube was filled with room temperature phosphate buffered saline and the tube was centrifuged for ten minutes at 3000 rpm at four ^{o}C. Phosphate buffered saline waste was discarded and the tube was refilled with PBS and spun again for ten minutes. This process was repeated and after the final spin, PBS waste was discarded and three 100 µL aliquots of packed red cells were pipetted into one mL 10% ascorbic acid solution in amber cryotubes. These samples were vortexed for ~five seconds and placed in a 37°C water

bath for three hours. Post-incubation, blood samples were placed in 96 sample cryoboxes in a -80°C freezer in a locked room.

After 30 minutes, the serum tube was spun for 15 minutes at 3000 rpm in a refrigerated centrifuge. Eight 500 μL aliquots of serum were placed in 1.5 mL cryotubes (SARSTEDT ®) and stored in 96 sample cryoboxes in the -80°C freezer.

I obtained all dried blood spot samples. The digitus medius of the non-dominant hand was wiped with alcohol and let dry for 60 seconds. A SARSTEDT® Super Safety-Lancet was used to puncture the fingertip and five x 50 μL drops were collected onto Whatman® 903 filter paper. To avoid contamination with tissue cells, the first drop of blood was discarded using a cotton swab. Collection cards were labeled with subject ID and date, and let dry for 24 hours at room temperature, then placed in sealable biohazard bags with desiccant and stored at -80°C.

We provided research participants with a five dollar gift card to a coffee shop (i.e. Starbucks® or Bean Around the World®) to defray the costs of transportation. After all analyses had been completed, results of SB12 measurements, and B12 and folate intake were released to the subject in the form of an ethics approved report, presenting the individual's results in reference to the Dietary Reference Intakes (35) and vitamin status based on conventional cut-off values for SB12 (**Appendix N**).

3.6   Summary of Laboratory Analysis

Samples underwent no freeze-thaw cycles and were stored for up to six months before primary analysis **(Table 3-6)**. Additional aliquots will be stored for up to ten years for future research purposes. Research assistants completed genotype analysis of the first 170 participants, all SB12 and holoTC assays, and operation of the LC-MS/MS for the MMA assay. I completed all other laboratory analyses.

Table 3-6 Summary of Methods Used for Laboratory Analysis

Analyte/Method	Assay
Serum vitamin B12	Microparticle enzyme immunoassay[a]
Serum holotranscobalamin	Microparticle enzyme intrinsic factor assay[a]
Plasma methylmalonic acid	Liquid chromatography tandem mass spectrometry[b]
Genotyping	TaqMan® real time polymerase chain reaction[c]
Complete blood count Analysis	Fluorescent flow cytometry[d]

Location: [a]Lab of Dr. Sheila Innis, [b]Lab of Dr. Yvonne Lamers, [c]Lab of Dr. Angela Devlin, [d]BC Children's and Women's Hospital Department of Pathology and Laboratory Medicine

3.6.1 Vitamin B12 Biomarker Assessment

We chose to measure SB12, holoTC, and MMA in our sample. We excluded Hcy because of its low specificity as a functional biomarker. SB12 and holoTC were quantified using microparticle-enzyme immunoassays designed for the AxSYM analyzer[18]. The AxSYM is an automated random-access immunoassay analyzer. The approach we used to classify B12 deficiency is outlined in **Table 3-7**.

Table 3-7 Biomarker Cut-off Values used to Classify Vitamin B12 Deficiency

Analyte	Deficient	Marginally Deficient	Adequate
Serum vitamin B12[a]	<148 pmol/L	148-220 pmol/L	>220 pmol/L
Serum vitamin B12[b]	<126 pmol/L	126-287 pmol/L	>287 pmol/L
Holotranscobalamin	<35 pmol/L	Insufficient evidence to set cut-off	≥35 pmol/L
Methylmalonic acid[c]	>260 nmol/L	210-260 nmol/L	<210 nmol/L

[a] 'Conventional' cut-offs [b] 'Bailey' cut-offs (80)

To determine the rate of deficiency, SB12 was measured in duplicate by microparticle enzyme immunoassay using the **AxSYM ® B-12 Assay** (Abbott Laboratories, Abbott Park, Illinois) **(230).** This assay operates on a traditional microparticle enzyme immunoassay principle. Vitamin B12 is bound to an IF coated microparticle that subsequently binds to a matrix cell. The concentration of B12 bound to the microparticles is quantified by adding 4-methylumbelliferyl phosphate to the matrix and measuring the fluorescent product methylumbelliferone.

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[18] Protocols available from: http://www.ilexmedical.com/files/PDF/B12_AXS.pdf, http://www.ilexmedical.com/files/PDF/AXS_ActiveB12Holot.pdf

The holoTC assay (**AxSYM Active B-12 ®**, Abbott Laboratories, Abbott Park, Illinois) is a traditional MEIA utilizing a monoclonal antibody against holoTC (231). This assay operates by combining dilute serum with microparticles containing anti-holoTC monoclonal antibody. Human holoTC antigen binds these microparticles forming a larger particle that then binds to a matrix. 4-methylumbelliferyl phosphate is added to the matrix and quantification is completed as the fluorescence of 4-methylumbellifuerone is measured following the removal of a phosphate group from the substrate. Samples were analyzed in duplicate.

Analysis of MMA was carried out in duplicate using LC-MS/MS and following the protocol of Blom *et al* (232) with enhanced sample cleanup**.** Briefly, 100 µL of plasma was mixed with 100 µL of d_3-MMA (0.4 µM) and deproteinized by ultracentrifugation (mass cut-off 3 kDa). The filtrate underwent liquid-liquid-extraction with 600 µL of hexane followed by acidification with formic acid. One hundred µL of the cleaned extract was plated and 10 µL was injected. Chromatography was carried out using an Agilent® 1260 LC with a reversed phase C-18 column (2.1 x 50 mm, 3.5 µm, 120 Å; InertSustain®) and C-18 guard column (2.1 x 10 mm; Fortis®). Methylmalonic acid and its isomer succinic acid were separated at 40°C and elution was achieved with an isocratic flow of 0.4% formic acid in 5% methanol at a flow rate of 200 µL /min followed by a wash step. Run time was 12 minutes/sample. Analytes in the effluent were quantified using an ABSciex® API4000 triple-quadrupole MS. The electrospray ionization probe was operated in negative mode. The selected reaction monitoring of the carbonyl loss of MMA and d_3-MMA recorded the transitions m/z 117$\rightarrow$73 for unlabeled and m/z 120$\rightarrow$76 for labeled MMA, respectively. MMA concentrations were quantified following the isotope dilution principle using the isotope ratio (analyte peak area / internal standard peak area). **Analyst®**

Software (Version 1.5.2; ABSciex®) was used for LC-MS/MS control, data acquisition, and data processing.

3.6.2 Hematological Assessment

Hematological parameters were assessed on a SYSMEX® XE 2100 instrument using fluorescent flow cytometry by the Pathology and Laboratory Services Department at BC Children's Hospital (233,234). This analysis included measurements for CBC and differential count and we pulled Hb, Hematocrit, and MCV values for each subject. Suspected anemia was defined as Hb <12.0 g/dL as per the World Health Organization guidelines (235), macrocytosis as MCV >99 fL (236), and macrocytic anemia if both criteria were met.

3.6.3 Genotyping

Genomic DNA was extracted from frozen buffy coat using a QIAGEN® DNA Blood Mini Kit (QIAGEN®, Hilden, Germany). Four µL of RNase was applied during extraction to remove contaminating RNA for future DNA methylation analysis. Concentration was determined on a NanoDrop (NanoVue^{TM} Plus Spectrophotometer 2100 (GE, Montreal)) based on the assumption that an optical density of 1.0 @ 260 nm is equivalent to 50 µg/µL of genomic DNA. Genomic DNA purity was determined by a A_{260}/A_{280} ratio of >1.8.

Samples were prepared on 96 well plates (MicroAMP® Optical 96 Well Reaction Plate) using Taqman Master Mix (Taqman ® Genotyping Master Mix – Mini Pack 1 mL) and allele-specific primers **(Table 3-8)** (Taqman ® Single Nucleotide Polymorphism Genotyping Assay, Human SM) **(Appendix Q)**.

Table 3-8 Genetic Variants and Corresponding rs numbers

Variant	rs number
MTHFR C677T	1801133
TCN2 C776G	1801198
TCN2 A67G	9606756
FUT2 A893G	602662

Plates were sealed using MicroAmp ® Optical Adhesive Film. Using extracted DNA samples genotyping was conducted following a Taqman® Polymerase Chain Reaction (PCR) protocol utilizing the Applied Biosystems® 7500 Real Time PCR System.

3.7   Final Variables Included in Analysis

Final variables from all questionnaires and biological assessment included in analysis are summarized in **Table 3-9.**

Table 3-9 Final Outcome Variables and Coding Method

Variable	Type	Unit or coding	Variable	Type	Unit or coding
Height	Continuous	cm	**Holotrascobalamin**	Continuous	pmol/L
Weight	Continuous	kg		Categorical	<35=Chronic deficiency, ≥35=Adequate
Waist circumference	Continuous	cm	***MTHFR* C677T**	Categorical	0=CC, 1=CT, 2=TT
	Categorical	0=< risk cut-off, 1=≥ risk cut-off	***TCN2* C776G**	Categorical	0=CC, 1=CG, 2=GG
Body mass index	Continuous	Kg/m^2	***TCN2* A67G**	Categorical	0=AA, 1=AG, 2=GG
Obesity	Categorical	0=Normal, 1=Obese	***FUT2* G893A**	Categorical	0=AA, 1=GA, 2=GG
Physical activity level	Categorical	1=Low, 2=Moderate, 3=High	**Hemoglobin**	Continuous	g/dL
Age	Continuous	Years		Categorical	<120=Anemic, ≥120=Not Anemic
Ethnicity	Categorical	0=European, 1=South Asian	**Hematocrit**	Continuous	%
Immigration	Categorical	0=>1st gen, 1=1st gen	**Mean corpuscular volume**	Continuous	fL
Income	Categorical	1=Low, 2=Low-middle 3=Middle-high, 4=High		Categorical	<99=Normal, ≥99=Macrocytosis
Smoker	Categorical	0=No, 1=Yes	**Macrocytic Anemia**	Categorical	0=No, 1= Yes (Hb<120, MCV≥99)
Caffeinated beverages	Categorical	0=No, 1=Yes	**Total caloric intake**	Continuous	Kilocalories
Oral contraceptive use	Categorical	0=No, 1=Yes	**Total protein intake**	Continuous	Grams
Supplement Use	Continuous	μg/day	**Animal protein intake**	Continuous	Grams
	Categorical	0=No, 1=Yes	**Intake food alone**[c]	Continuous	μg
Lactose intolerance	Categorical	0=No, 1=Yes	**Intake food and supplements**[c]	Continuous	μg
Methylmalonic acid	Continuous	μmol/L	**Food Group Servings**	Continuous	Servings
	Categorical	<210 = Adequate 210-260=Marginal deficiency >260=Chronic deficiency	**Vegetarian**	Categorical	0=No, 1=Yes
Serum vitamin B12	Continuous	pmol/L	**Vegetarian+pescatarian**	Categorical	0=No, 1=Yes
	Categorical (1)[a]	>220=Adequate 148-220=Marginal deficiency <148=Chronic deficiency	**Non-red meat eater**	Categorical	0=No, 1=Yes
	Categorical (2)[b]	>287=Adequate 128-287=Marginal deficiency <128=Chronic deficiency			

[a]Conventional cut-offs, [b]Cut-offs proposed by Bailey et al [c]Dietary vitamin B12 intake
Abbreviations: gen=generation

The majority of the outcome variables were not normally distributed except for height and Hb concentrations. Transformation of non-normal continuous variables was largely successful (**Table 3-10**). Variables that were non-transformable included holoTC and age, B12 intake from food and supplements, B12 supplement intake and MCV.

Table 3-10 Data Transformation Applied to Normalize Skewed Variables

Transformation	Variables
Log	Serum vitamin B12, vitamin B12 intake from food alone, fat intake, carbohydrate intake, vegetable protein intake, total protein intake, total energy intake
1/square	Body mass index, waist circumference
1/√	Plasma methylmalonic acid, weight
Inverse	Hematocrit
N/A[a]	Holotranscobalamin, age, mean corpuscular volume, vitamin B12 intake from food and supplements

[a]These variables were not successfully transformed. All analysis was completed using non-parametric tests.

3.8   Statistical Analysis

3.8.1   Subject Characteristics including Dietary and Genotype Summaries

Statistical analyses were performed using STATA® 12 software (StataCorp LP, TX, USA). Normality was assessed visually (i.e. histogram) and using Shapiro Wilks, Kolmogorow Smirnov, and D'Agostino Pearson tests. Normally distributed variables were reported as mean ± SD. Non-normally distributed variables were transformed (according to **Table 3-10**) and reported as means ±95% CI. If not normal after transformation they were reported as medians (IQR). Ethnic group differences were assessed using a chi-square (χ^2) test for categorical variables, and a Students t-test for continuous variables, or the non-parametric equivalent when necessary. Two-tailed significance was set at p<0.05.

The protocol recommended questionnaire exclusion from analysis if there was more than one full blank page, ten percent or more food items had missing answers for frequency or portion, or if energy intakes were less than 800 kcal/d or greater than 4000 kcal/d. None of the questionnaires we collected had enough missing data to warrant exclusion. Further,

questionnaires were highlighted for review if they had intake values >3SD from the mean intake of nutrients or food groups of interest. If the questionnaire had been flagged for potential plausibility issues it was excluded. Means ± SD or medians (25/75%ile) for dietary variables were presented stratified by ethnicity and by supplement use to assess whether these factors affected dietary intakes.

Spearman's correlation coefficients were calculated for the relationship between dietary intake variables to assess whether dietary factors associated with B12 intake had a linear relationship with absolute B12 intake. The prevalence of dietary B12 inadequacy (% below the EAR) was presented in the following categories **[1]** diet only in non-supplement users, **[2]** food only in supplement users, **[3]** diet and supplements in supplement users. Unadjusted linear models were assessed to determine whether dietary intake variables were associated with total energy intake. As B12 intake and total energy intake were correlated, energy adjusted nutrient intakes were computed using the residual method **(Equation 3).**

Equation 3 Dietary Residual Method

$$\textbf{Dietary B12 intake } = \ \beta o + \beta(\textbf{Total Caloric Intake})$$

$$\textbf{Dietary B12 intake} = \ -\textbf{0.22} + \textbf{0.002} \ (\textbf{Total Caloric Intake})$$

The residual method is a procedure used to adjust nutrient intake for energy intake by computing a regression model with total caloric intake as the independent variable and absolute nutrient intake as the dependent variable (237). The residuals provide a measure of nutrient intake that is not correlated with total energy intake. The resulting residuals are then multiplied by a constant (the predicted B12 intake corresponding to the median energy intake of the study population) for interpretability. Based on an average energy intake of 1608 Kcal/day the predicted B12 constant was 2.89 µg/day. This new variable (energy adjusted dietary B12 intake) was applied in all further dietary analyses.

Statistical analyses pertaining to genotype and allele frequencies for genetic variants were stratified by ethnic group. Genotype and allelic frequency were calculated for each gene variant and presented as proportions (%). Compliance with Hardy-Weinberg equilibrium was tested using a χ^2 test. Genotypes with a p value <0.001 were further assessed for minor allele frequency and removed if <2%. To determine if the frequency of the minor homozygous genotype for all variants differed for SA and European women contingency tables were created for ethnicity by genotype and χ^2 tests were performed to test the null hypothesis that there are no differences in genotype frequency depending on ethnicity.

3.8.2 Primary Objective: Rate of Deficiency

Objectives are restated in the shaded areas followed by the approach used for hypothesis testing.

Primary Objective: The primary objective is to assess whether young adult women of SA ethnicity have lower B12 status than European women aged 19-35.

Differences in SB12 concentration between ethnic groups were assessed using a Mann Whitney U test. Overall rate of deficiency was quantified and differences in rates of deficiency were tested using a χ^2 test or Fisher's exact test between ethnic groups and supplement and non-supplement users. Differences in holoTC and MMA status were assessed following the same method.

3.8.3 Exploratory Objective 1: Determinants of Vitamin B12 Deficiency

Exploratory Objective 1: To assess the association of established B12 deficiency risk factors with B12 status.

A) PRELIMINARY UNIVARIATE ANALYSIS

Simple linear and logistic regression was used to identify variables with a potential influence on B12 biomarker concentrations and deficiency classification. Variables were carried forward to multivariate analyses if $p \leq 0.2$.

Example:

Linear model:

e.g. SB12 concentration $= \beta o + \beta 1(\text{ethnicity})$

Logistic model:

e.g. Chronic or marginal deficiency $= \beta o + \beta 1(\text{ethnicity})$

Dietary univariate analysis

i. Correlations among dietary B12 intakes and B12 biomarker concentrations were assessed by calculating Spearman's correlation coefficients.

ii. Median dietary B12 intakes for deficiency categories defined by SB12, holoTC and MMA concentration cut-offs were calculated and compared using Kruskal Wallis tests with Bonferroni adjustments, or in the case of holoTC with only two deficiency levels, a Wilcoxon rank sum test.

iii. The influence of dietary B12 intakes on SB12 concentration was assessed using simple linear regression models adjusted for energy and supplement intake.

Genotype univariate analysis

Differences in B12 biomarker concentration and rate of deficiency defined by SB12, holoTC, and MMA concentrations cut-offs between variant genotypes were compared using Kruskal Wallis tests with Bonferroni adjustments, or logistic regression with the dominant genotype as the reference group. For the *TCN2* A67G variant, which was collapsed into two groups, Wilcoxon rank sum tests or χ^2 tests were used.

B) MULTIVARIATE MODEL CONSTRUCTION

Multiple linear and logistic models were created with SB12, holoTC, and MMA concentration
and rate of deficiency by each biomarker as the dependent variables, respectively.

   i.   As stated above, univariate analysis was used to assess which variables were significantly
associated with concentration and rate of deficiency. If the p-value was <0.2 the variable
was carried forward to the step-wise regression models.

  ii.   Variables were assessed for collinearity by the variance inflation factor and tolerance,
and identified for review if variance inflation factor was >10 and tolerance was <0.1.

 iii.   Forward stepwise linear regression was used to establish the best-fit models with SB12
concentration as an outcome variable.

C) MULTIVARIATE MODEL SELECTION CRITERIA

   i. R^2 and adjusted R^2 were compared between models

  ii.   F tests were conducted to identify the smallest subset of terms where F_m was not
significant

 iii.   Mallows C_m was assessed with a criteria of $C_m<$# of variables in model+1

D) REGRESSION ANALYSIS DIAGNOSTIC PROTOCOL

Influential data points were identified by assessing leverage and studentized residuals by
calculating Cook's distance and DFITS (a measure of how unusual an outcome is). Data points
were considered for removal if Cook's distance was >4/207 and if the absolute value of DFITS
was > 2 $\sqrt{(n)}$.

To assess influence of individual data points on regression coefficients DFBETA (a measure
which identifies specific data points (or subjects) whose values affect the regression coefficient
inordinately) was calculated and data points were removed if DFBETA was >2/$\sqrt{(207)}$.
Normality of the models was assessed with kernel density plots, P-P plots, and Q-Q plots and
the Shapiro Wilks test. **Homoscedasticity** was tested using the Breusch-Pagan test and White's
test. **Linearity** was assessed using an augmented partial residual plot by visual assessment.
Model specification errors were assessed using a Ramsey Reset test and link test. Logistic
regression models were assessed for goodness of fit using the Hosmer Lemeshow goodness of fit
test.

3.8.4   Exploratory Objective 2: Multiple Biomarker Assessment

To assess the influence of using multiple biomarker analysis on the outcome of B12 status assessment.

> **Specific objective 2-1**:
> To examine the correlations among SB12, MMA and holoTC concentrations, and determine whether dividing subjects by SB12 cutoffs results in differences in holoTC and MMA concentrations between adequate, marginal deficiency, and chronic deficiency groups.

The correlation among biomarkers was tested by calculating the Spearman's correlation coefficient. Median concentrations of holoTC and MMA were compared between SB12 deficiency categories using a Kruskal Wallis test and Bonferroni adjustment for multiple comparisons.

> **Specific objective 2-2:**
> To determine the rate of combined metabolic deficiency[19] and disagreement[20], and assess whether misclassified individuals better fit the profile of adequacy or deficiency based on their holoTC concentrations, hematological status, and established risk factors for B12 deficiency.

Subjects were categorized as having combined metabolic chronic or marginal deficiency **(Figure 3-1)**. Rates were observed and compared to the overall rate of deficiency determined using SB12 alone. Rates of disagreement were also assessed.

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[19] Defined as simultaneously: **[1]** low serum B12 (based on 'conventional' or 'Bailey' cut-offs) or holoTC concentrations and **[2]** high MMA concentrations
[20] Defined as disagreement between biomarker classification for placing an individual in a category of deficiency (e.g. low SB12 and low MMA)

Figure 3-1 Approach for Classifying Moderate and Gross Disagreement between Serum Vitamin B12 and Methylmalonic Acid Deficiency Categories

Combined metabolic deficiency (chronic)	Marginal MMA, chronic SB12	Adequate MMA, chronic SB12
Marginal SB12, chronic MMA	Combined metabolic deficiency (marginal)	Adequate MMA, marginal SB12
Adequate SB12, chronic MMA	Marginal MMA, adequate SB12	Concurrent adequacy

LEGEND		
	Moderate disagreement		Gross disagreement

To visualize the incidence of combined metabolic deficiency and disagreement, locally weighted scatterplot smoothing (LOWESS) curve plots with reference lines placed at both chronic and marginal deficiency cutoffs were created.

Individuals were placed in one of the four profiles of metabolic deficiency defined by Bailey *et al* based on their MMA and SB12 concentrations **(Figure 3-2)** and holoTC, hematological and established risk factors were assessed for differences by profile using one-way ANOVA for continuous variables with a Bonferroni adjustment, and logistic regression for categorical variables. Assessing these differences will assist in determining whether individuals in categories of disagreement are more characteristically similar to deficient or adequate groups.

Figure 3-2 Approach for Classifying Profiles of Combined Metabolic Deficiency

A) Profile 1 SB12 (<148 pmol) and MMA (>260 pmol/L) deficient, represents true deficiency	**B) Profile 3** SB12 ($\geq$148 pmol/L) adequate, MMA (>260 pmol/L) deficient
C) Profile 2 SB12 (<148 pmol/L) deficient, MMA ($\leq$260 pmol/L) adequate	**D) Profile 4** SB12 ($\geq$148 pmol/L) and MMA($\leq$260 pmol/L) adequate, represents true adequacy

Chapter 4: Results

The results are presented in four main subsections: **[1]** Subject Characteristics, **[2]** Rates of Deficiency, **[3]** Determinants of Deficiency and **[4]** Multiple Biomarker Assessment. **Subsection 1** begins with a summary of the sample characteristics and an assessment of group differences based on ethnic group and supplement use. It also includes an in-depth summary of subject dietary intakes and genetic variants to provide context for the multivariate analysis that follows. **Subsection 2** is comprised of the main results of the primary objective of the present study and presents the rates of deficiency for each measured biomarker along with hematological outcomes.

The final sections cover the exploratory objectives of the present study. **Subsection 3** begins with the results of univariate analysis to assess the association of known risk factors with B12 status, with an extra focus on the relationship between dietary and genetic variables and B12 status. This is followed by outcomes of multiple linear and logistic regression models used to assess the association between lifestyle and genetic variables on B12 biomarker concentration and B12 deficiency status in the study population. **Subsection 4** shows the results of using different combinations of biomarkers and cut-offs to determine deficiency classification and ultimately define B12 status. This includes a comparison of biomarker concentrations at different cut-off levels (e.g. MMA concentration of individuals grouped by SB12 cut-off values), assessment of the rates of combined metabolic deficiency and disagreement, and assessment of differences in subject characteristics when grouped by combined metabolic profiles of functional deficiency.

4.1   Subject Characteristics: Demographics and Lifestyle Variables

The final study population (n=207) included 56 SA and 151 European women. There was one completely unsuccessful venipuncture in the European group. Due to insufficient blood volume collection, failed analysis, or unavailable questionnaire data the final sample size for biomarker, genotype, and dietary analyses varied and is indicated throughout the results as well as in **Figure 4-1.**

Figure 4-1 Final Sample Size Flowchart for Survey and Biological Data
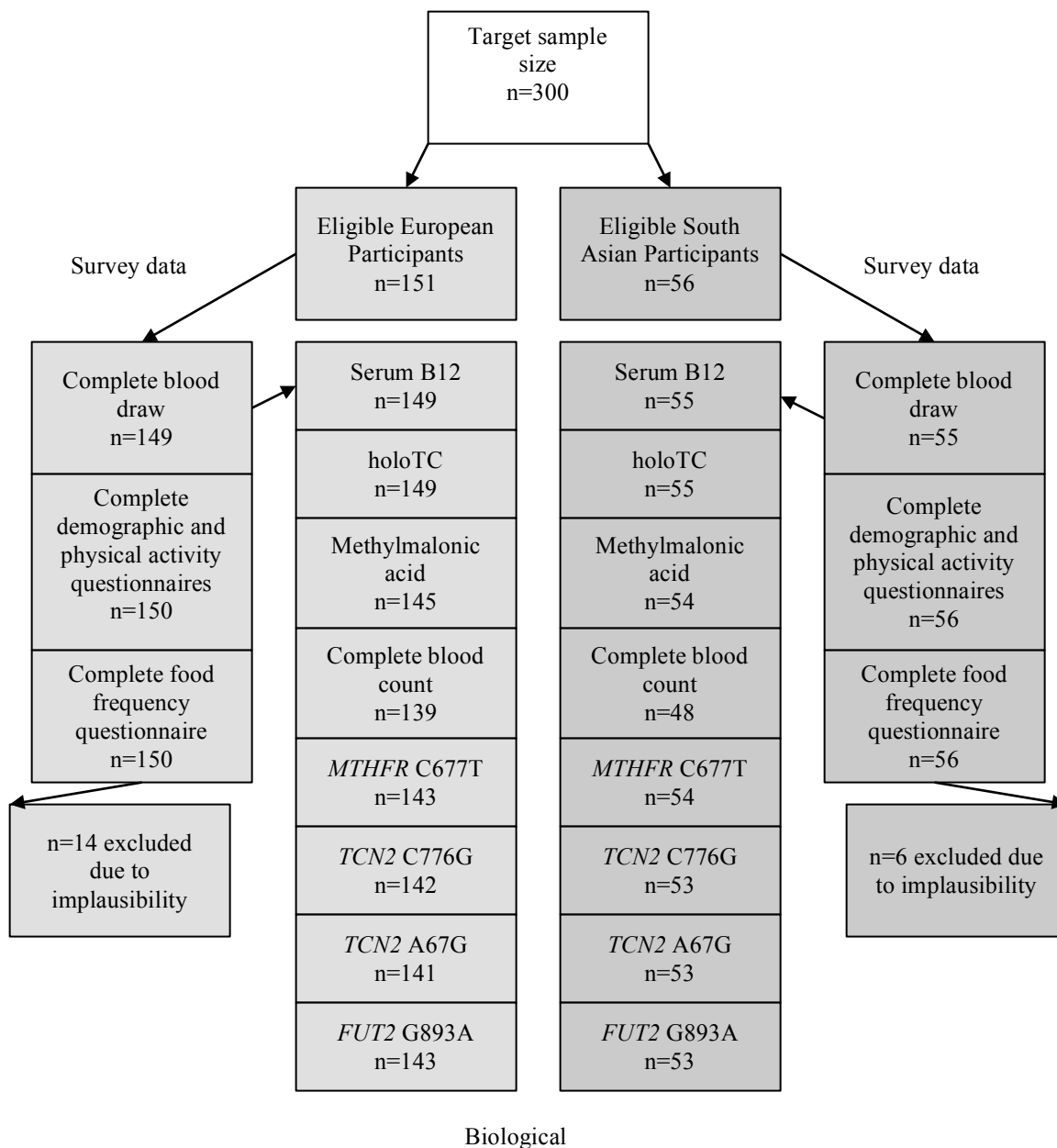
This figure depicts the final sample size obtained for survey and biological data divided by ethnic group
Abbreviations: *MTHFR*= Methylenetetrahydrofolate reductase, *TCN2*=Transcobalamin 2, *FUT2*= fucosyltransferase 2

Subject characteristics are summarized in **Table 4-1.** Most individuals in the sample were of European descent (73%). The median age of the sample was 27 y (IQR: 23, 30). Women of SA descent were significantly younger than European women (p=0.03). Women of SA descent had a higher median BMI but this difference was not statistically significant (p=0.07). There were more obese subjects in the SA group (20%) compared to the European group (3%) (p<0.0001), based on ethnic specific cut-offs. The median waist circumference was higher in SA individuals but this difference was not statistically significant. The proportion of SA individuals (31%) with waist circumference above the cut-off for central adiposity was greater than that of Europeans (8%) (p<0.0001).

Table 4-1 Subject Age and Physical Characteristics

Variable	Total	SA (n=55)	Euro (n=151)	p-value
Age (years)	**27(23,30)**	25(22,29)	27(24,30)	0.03
Height* (m)	**1.66(0.1)**	1.62(0.1)	1.68(0.1)	**<0.0001**
Weight (kg)	**62(56, 69)**	62(53, 70)	62(57, 69)	0.65
BMI (kg/m^2)	**22(21, 24)**	23(21, 27)	22(20, 24)	0.07
Risk cut-offs[a] (n, (%))				
Underweight	11(5)	3(6)	8(5)	**<0.0001**
Normal	140(68)	24(44)	116(77)	
Overweight	39(19)	17(30)	22(15)	
Obese	16(8)	11(20)	5(3)	
Waist circumference (cm)	74(70, 79)	73(69, 84)	71(70, 78)	0.58
Risk cut-off[b] (n, (%))				
Below	177(85)	38(69)	139(92)	**<0.0001**
Above	29(14)	17(31)	12(8)	

Results presented as median (inter-quartile range) or frequency (%), *except for height which is presented as mean (standard deviation)

p-values for ethnic group differences were derived for normal variables from students t-tests for continuous variables and χ^2 square tests for categorical variables. Non-normal variables were assessed for differences by ethnic group by Wilcoxon rank sum for continuous variables, and Fishers exact for categorical variables

Abbreviations: BMI= Body Mass Index, SA= South Asian

[a] South Asian cut-offs <18.5 (underweight), 18.6-23.9 (normal), 24-26.9 (overweight), >27 (obese)
European cut-offs <18.5 (underweight), 18.6-24.9 (normal), 25-29.9 (overweight), >30 (obese)
[b] Europeans >88cm, SA >80cm

Data on immigrant status, income, and education is presented in **Table 4-2.** There were a greater number of first generation immigrants in the SA group (69%) than the European group (25%) (p<0.0001). There were no differences in total annual household income or education level between ethnic groups. Over 95% of participants reported some post-secondary education and over 50% were in the upper-middle or highest bracket for total household income. Overall the sample was of higher SES based on income and education indicators.

Table 4-2 Subject Immigration Status, Income and Education

	All (n=206)	SA (n=55)	European (n=151)	p value[a]
		Data presented as frequency (%)		
Generation of immigration[b]				
First	76(36.9)	38(69)	38(25.2)	**<0.0001**
Second	26(12.6)	16(29.1)	10(6.6)	
Mixed	32(15.5)	0(0)	32(21.2)	
Third	56(27.2)	0(0)	56(37.1)	
Fourth or higher	16(7.8)	1(2)	15(9.9)	
Total annual household income[c]				
Lowest	37(19.9)	11(26.8)	26(17.9)	0.6
Lower-middle	44(23.7)	10(24.4)	34(23.5)	
Upper-middle	52(28.0)	11(26.8)	41(28.3)	
Highest	53(28.5)	9(22.0)	44(40.3)	
Education				
< Secondary (high school) education	2(1)	1(1.8)	1(0.7)	0.4
Secondary school (high school) diploma	5(2.4)	1(1.8)	4(2.6)	
Some post-secondary education[d]	53(25.7)	16(29.1)	37(24.5)	
Bachelor's degree (post-secondary education)	88(42.7)	27(49.1)	61(40.4)	
University degree or certificate > than bachelor's degree[e]	58(28.2)	10(18.2)	48(31.8)	

[a]p-value for comparisons between ethnic groups
SA= South Asian
[b]First: subject immigrated, Second: subject's parents immigrated, Mixed: one of subjects parent's immigrated, Third: subject's grandparents immigrated, Fourth or higher: subject's great grandparents or earlier generations of ancestors immigrated.
[c]Lowest: <$15000 if 1-2 people, <$20000 if 3-4 people, <$30000 if ≥5 people, Lower-middle: $15000-29999 if 1-2 people, $20000-39999 if 3 or 4 people, $30000 to 50000 if ≥5 people, Upper-middle: $30000-59999 if 1-2 people, $40000-79999 if 3-4 people, $60000-79999 if ≥5 people, Highest: >$60000 if 1-2 people, >$80000 if ≥3 people
[d]Trade certificate or diploma, apprenticeship training, or non-university education below bachelor's level (2/3 year diploma or partially completed bachelor's degree)
[e]Masters, doctorate, professional degree requiring prior post-secondary degree (e.g. Physiotherapy)

Most participants (86%) were non-smokers, consumed caffeinated beverages (86%) and 29% of participants reported OC use **(Table 4-3)**. There was no difference in the proportion of smokers or consumers of caffeinated beverages between ethnic groups. There were significantly fewer OC users in the SA group (p=0.002).

Table 4-3 Lifestyle Characteristics

	Total n=207	South Asian n=56	European n=151	p-value[a]
		Data presented as frequency (%)		
Smoker[b]				
No	177(86)	50(91)	127(84)	0.2
Yes	29(14)	5(9)	24(16)	
Caffeinated Beverages[c]				
No	28(14)	9(16)	19(13)	0.5
Yes	178(86)	46(84)	132(87)	
Oral Contraceptives				
No	146(71)	48(87)	98(65)	**0.002**
Yes	60(29)	7(13)	53(35)	

[a]p-value for comparisons between ethnic groups
[b]Answered yes to occasional, regular, or frequent smoker [c]>1 cup/day

4.1.1 Summary of Dietary Vitamin B12 Intakes and Supplement Use

Nutritional supplement use was reported by 48% of the participants **(Table 4-4)**. Folic acid supplement use was reported by 28% and B12 supplement use was reported by 30%. There was no difference between ethnic groups in the rate of reported supplement use.

Table 4-4 Nutritional Supplement Use

	Total n=206	SA n=56	Euro n=150	p-value[a]
Nutritional supplement use				
No	106(52)	30(55)	76(50)	
Yes	100(48)	25(45)	75(50)	0.6
Folic acid containing supplement use				
No	148(72)	43(78)	105(70)	0.2
Yes	58(28)	12(22)	46(30)	
B12 containing supplement use				
No	144(70)	41(75)	103(68)	0.5
Yes	62(30)	14(15)	48(32)	

[a]p-value for comparisons between ethnic groups
Abbreviations: SA=South Asian, Euro=European

Of the available dietary assessment data, four questionnaires were excluded due to implausible energy intakes. Due to implausible intakes of dietary categories of interest (B12 intake, total protein intake, animal protein intake, energy intake, servings of the four Canada's Food Guide food groups) 16 questionnaires were excluded for a final dietary data sample size of n=186.

Variables relevant for assessing B12 intakes were obtained from the questionnaire and are listed in **Table 4-5**.

Table 4-5 Final Dietary Variables from Food Frequency Questionnaire

Variable	Coding	Explanation
Vegetarian	0=No 1=Yes	Individuals who did not indicate consumption of any meat or seafood
Vegetarian + Pescatarian	0=No 1=Yes	Individuals who did not indicate consumption of any meat, but did indicate consumption of seafood
Non red meat eater	0=No 1=Yes	Individuals who did not indicate consumption of any red meat
Animal protein intake	Grams	Total protein intake from foods of animal origin
Total protein intake	Grams	Total protein intake from all food sources
Total energy intake	Kilocalories	Total energy intake from all food sources
Vitamin B12 intake	µg/day	Total vitamin B12 intake from all food sources
Vegetables and Fruit	Servings	Number of servings as defined in Canada's Food Guide
Grains	Servings	Number of servings from Canada's Food Guide
Milk and Alternatives	Servings	Number of servings from Canada's Food Guide
Meat and Alternatives	Servings	Number of servings from Canada's Food Guide

Correlations among dietary intake variables are reported in **Table 4-6.** Vitamin B12 intake from food was positively correlated with all dietary variables, which were in turn positively correlated with total caloric intake. Subsequent adjustment for energy intake was completed using the residual method **(Equation 3)** prior to inclusion of variables in multivariate analysis. Due to the strong correlation between intake variables we chose to use dietary vitamin B12 intake to represent dietary contribution rather than include additional intake variables into multivariate analysis.

Table 4-6 Correlation between Dietary Intake Variables Associated with Vitamin B12 Intake

	1	2	3	4	5	6	7
1. Dietary vitamin B12 intake food alone							
2. Dietary vitamin B12 intake food +supplements	**0.50**						
3. Total protein intake	**0.50**	**0.82**					
4. Animal protein intake	**0.46**	**0.80**	**0.84**				
5. Servings of meat and alternatives	**0.42**	**0.51**	**0.77**	**0.46**			
6. Servings of dairy and alternatives	**0.27**	**0.59**	**0.46**	**0.48**	0.09		
7. Total caloric intake (kcal)	**0.33**	**0.58**	**0.78**	**0.49**	**0.61**	**0.42**	

Values reported are Spearman's rho correlation coefficients
All **bolded** Spearmans rho values were significant at the p<0.0001 level

Total energy, protein, and animal protein intakes were not different between supplement and non-supplement users or ethnic groups **(Table 4-7)**. SA individuals consumed significantly more grain product servings, and significantly less vegetable and fruit servings than European individuals. Intake of Canada's Food Guide servings from the four food groups was not significantly different between supplement users and non-supplement users. In the overall sample there was under consumption of grains, vegetables and fruit, and milk and alternatives, and marginal over-consumption of meat based on comparing median servings to recommendations by Canada's Food Guide for Healthy Eating. Prevalence of ASF restricted dietary practices ranged from 15-33% depending on the level of restriction. A significantly higher proportion of SA participants (24%) reported vegetarian diets compared to European participants (11%), but there was no difference in the proportion reporting other ASF restricted diets. Supplement users were not more likely to report practicing ASF restricted diets.

Table 4-7 Dietary Intake and Preferences by Ethnicity and Supplement Use

	Total (n=186)	**SA** (n=50)	**European** (n=136)	**p-value**[a]	**S** (n=56)	**NS** (n=130)	**p-value**[b]
Intake category Mean (SD)							
Total energy (kcal)	1625(464)	1567(463)	1647(465)	0.30	1625(502)	1626(449)	0.98
Total protein (g)	63(21)	62(22)	63(21)	0.74	65(23)	62(20)	0.44
AP (g)	37(20)	37(18)	37(20)	0.85	38(22)	37(19)	0.78
Canada's Food Guide Servings Mean (SD)							
Grains	5.0(2.8)	5.5(2.3)	4.5(2.2)	**0.007**	4.6(2.3)	4.8(2.2)	0.55
Vegetables and fruit	5.4(2.8)	4.2(1.7)	5.4(2.2)	**0.0004**	5.0(2.2)	5.1(2.1)	0.70
Dairy	1.6(1.2)	1.4(0.9)	1.6(1.0)	0.17	1.4(0.8)	1.5(1.0)	0.21
Meat and alternatives	3.1(1.6)	2.8(1.2)	3.0(124)	0.21	3.2(1.2)	2.9(1.2)	0.10
Dietary Practices F (%)							
Lactose Intolerance	41(22)	7(14)	34(25)	0.11	17(30)	24(19)	0.07
Vegetarian	28(15)	12(24)	16(12)	**0.04**	12(21)	16(12)	0.11
Pescatarian[c]	47(25)	14(28)	33(24)	0.60	15(27)	32(25)	0.76
Non red meat eater	63(34)	22(44)	41(30)	0.08	18(32)	45(35)	0.74

Abbreviations: F (%) Frequency/Percentage, S, supplement user, NS, non supplement user, SA South Asian, V&F Vegetables and Fruit, M&A, Meats and Alternatives, AP, Animal Protein
[a]p-value for comparison of intakes and practices between ethnic groups [b]p-value for comparison of intakes and practices between supplement and non-supplement users, p values with statistical significance are in **bold** [c]Pescatarian includes all vegetarians and vegetarians who include seafood in their diet

There was a trend towards lower dietary B12 intake from food alone and from food and supplements with increasing degree of meat restriction (**Figure 4-2).**

Figure 4-2 Vitamin B12 Intakes by Dietary Pattern

ASF Restriction	Vitamin B12 Intake from Food (μg/day)	Vitamin B12 Intake from Food +Supplements (μg/day)
■ Vegetarians (n=30)	1.5	2.2
■ Vegetarian+Pescatarian (n=51)	1.8	2.4
■ Non Red Meat Eater (n=69)	2	2.9
■ Non-Vegetarians (n=137)	2.9	3.7
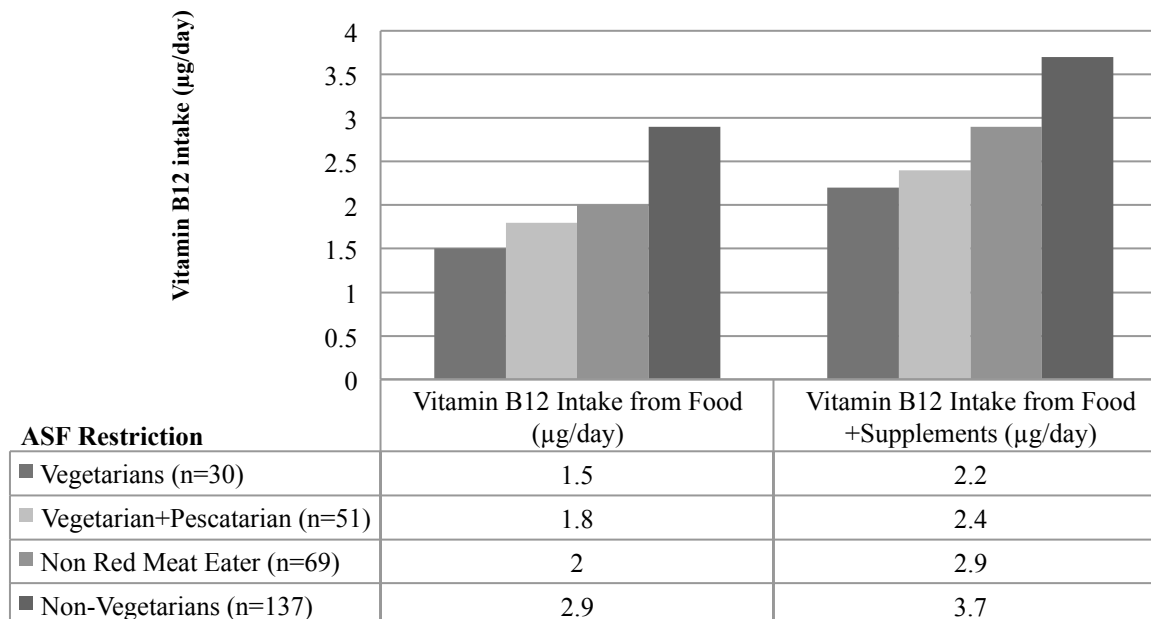
Total median vitamin B12 intakes from food alone, and from food and supplements is shown for all categories of dietary ASF restriction in μg/day.

Median dietary B12 intakes from food alone did not differ based on supplement use or ethnicity **(Table 4-8)**. Median B12 intake from food and supplements was significantly higher in supplement users (p<0.0001), as expected, and did not differ by ethnicity.

Table 4-8 Vitamin B12 Intakes from Food Alone, Food and Supplements and Supplements Alone

	Total vitamin B12 intake (μg/d) Presented as Median, IQR						
	Total	**SA**	**European**	**p-value**[a]	**S**	**NS**	**p-value**[b]
Intake category							
B12 intake from food alone	2.7 (1.9, 4.0)	2.8 (2.0,3.9)	2.6 (1.8,3.7)	0.54	2.4 (1.7,3.4)	2.8 (2.0,3.9)	0.09
B12 intake from food and supplements	3.5 (2.2,6.3)	3.7 (2.3,5.6)	3.4 (92.2,7.1)	0.69	20.5 (5.3,70.2)	2.8 (2.0,3.9)	**<0.0001**
B12 intake from supplements alone	0 (0.2)	0 (0,0.2)	0 (0,2.3)	0.55	17.4 (3.2,70.9)	0(0,0)	**<0.00001**

Abbreviations: B12=vitamin B12, S=supplement user, NS=non supplement user, SA=South Asian
[a]p-value for comparison of intakes and practices between ethnic groups [b]p-value for comparison of intakes and practices between supplement and non-supplement users

The proportion of dietary B12 inadequacy, defined as intakes below the EAR (2.0 μg/day), from food alone was higher in supplement users (34%) vs. non-supplement users (27%) but this difference was not statistically significant (p=0.29). The proportion of dietary B12 inadequacy in supplement users was significantly lower when considering B12 intake from supplements as part of their total dietary B12 intake (4%) compared to B12 intake from food sources alone (34%) (p=0.04) **(Table 4-9).**

Table 4-9 Rate of Dietary Vitamin B12 Inadequacy from Food Intake Alone, Food and Supplement Intake and Supplement Intake Alone

	n	% <EAR	p-value
Food alone			
Total	186	53(28%)	
Non-supplement users	130	34(26%)	0.29
Supplement users	56	19(34%)	
Food and supplements			
Total	186	36(20%)	0.04[a]
Supplement users	56	2(4%)	

EAR, estimated average requirement = 2.0μg/day
NS= non supplement user, S= supplement user
[a]p-value comparing proportion of dietary inadequacy from food alone, and food and supplements in supplement users and non-supplement users

The proportion of individuals with inadequate dietary B12 intakes from food alone was higher in the chronic deficiency group but not different between adequate and marginal deficiency groups **(Table 4-10)**. When supplement intake was considered, the proportion of inadequate dietary B12 intakes was significantly higher in the chronic deficiency group compared to the adequate and marginal deficiency groups and higher in the marginal deficiency group than the adequate group.

Table 4-10 Proportion of Individuals Below the Estimated Average Requirement for Vitamin B12 Intake (from Food Alone or Food and Supplements) by Category of Vitamin B12 Status

		Category of vitamin B12 status		
		Adequate[1]	Marginal deficiency[2]	Chronic deficiency[3]
Vitamin B12 biomarker	Dietary intake category	Proportion with dietary vitamin B12 intakes below the estimated average requirement (% below 2.0 µg/day)		
Serum Vitamin B12 (n=204)	Food alone	25[a]	26[a]	**46[b]**
	Food and supplements	15[a]	**21[b]**	**36[c]**

Statistically significant differences between groups within a row are denoted by not sharing a common superscript. Differences between groups were assessed using logistic regression (p<0.05).
[1]SB12≥220, [2]SB12 148-219, [3]SB12<148, Kruskal Wallis test and Bonferroni adjustment used to assess differences among groups

4.1.2 Genetic Variants

All genetic variants with the exception of the two *TCN2* variants were in Hardy-Weinberg equilibrium based on the criteria of rejecting variants when p <0.1 on the assumption that the allele frequencies differ significantly from what is expected if they were in true Hardy-Weinberg equilibrium, however they were included in subsequent analysis **(Table 4-11)**.

Table 4-11 Hardy Weinberg Equilibrium Tests

Variant	Pearson χ^2	p-value	Likelihood ratio χ^2	p-value	Exact significance
MTHFR C677T	0.1	0.7	0.1	0.7	0.7
TCN2 C776G	5	0.02	5	0.02	**0.03**
TCN2 A67G	4	0.05	4	0.03	**0.06**
FUT2 G893A	0.03	0.86	0.03	0.86	0.88

Abbreviations: *MTHFR*= methylenetetrahydrofolate reductase, *TCN2*= transcobalamin 2, *FUT2*= fucosyltransferase 2

Ethnic specific differences in genotype frequencies for the *MTHFR* C677T and *FUT2* G893A variants were observed **(Table 4-12)**. The frequency of the T allele for the *MTHFR* C677T variant was significantly lower for SA individuals and there were significantly more SA individuals with the A allele of the *FUT2* G893A variant.

Table 4-12 Genotype and Allele Frequency by Ethnicity

Genotype	Nucleotide	AA	Group	Genotype Frequency			p-value	Allele Frequency	
								Major AF	Minor AF
				CC	CT	TT			
MTHFR	C677T	Ala -	Total	49	41	10	- - -	70	30
n=197		Val	E	36	51	13	**<0.0001**	62	38
			SA	83	15	2		91	9
				CC	CG	GG			
TCN2	C776G	Pro-	Total	25	58	17	- - -	54	46
n=195		Arg	E	26	58	16	0.58	55	45
			SA	21	58	21		50	50
				AA	AG	GG			
TCN2	A67G	Ile -Val	Total	63	36	2	- - -	81	19
n=194			E	65	33	2	0.54	82	18
			SA	57	41	2		77	23
				GG	GA	AA			
FUT2	G893A	Gly-Ser	Total	34	48	18	- - -	45	55
n=197			E	23	48	29	**0.006**	48	52
			SA	6	48	46		36	64

Abbreviations: E= European, SA= South Asian, AF= Allele Frequency, AA=Amino Acid, *MTHFR*= Methyl-tetrahydrofolate reductase, TCN2= Transcobalamin 2, *FUT2*= Fucosyltransferase 2
p-values determined using X2 or Fishers exact tests

4.2   Primary Objective: Rate of Deficiency and Vitamin B12 Biomarker Concentrations

Serum B12 concentration and rates of deficiency did not differ by ethnicity. Supplement users had a higher median SB12 concentration but rates of deficiency did not differ between users and non-users. The rate of deficiency based on SB12 concentrations was 14% for chronic deficiency and 20% for marginal deficiency, for an overall rate of 34% inadequacy in the study population **(Table 4-13)**.

The rate of elevated MMA concentrations >260 nmol/L was 9%, and of MMA concentrations 210-260 nmol/L was 11%. Most (80%) of the sample had MMA concentrations <210 nmol/L indicating adequate B12 status. There was no difference in MMA concentration or proportion of individuals below the cut-offs between ethnicity and supplement use groups. The rate of low holoTC concentrations <35 pmol/L was 8%. Holotranscobalamin concentration and the proportion of individuals below the cut-off did not differ by ethnicity but holoTC concentrations were significantly lower in non-supplement users (p=0.0003).

There was no difference in the rate of marginal or chronic deficiency defined by concentration cut-offs of any of the B12 biomarkers between ethnic groups or supplement and non-supplement users.

Table 4-13 Biomarker Concentrations and Vitamin B12 Deficiency Rates

Biomarker	Category	Total	South Asian	European	p	Supplement user	Non supplement user	p
Serum B12 (pmol/L)	Conc	269 (189, 369)	243 (174, 372)	282 (197, 369)	0.27[a]	322 (219, 446)	249 (174, 337)	**0.0011[a]**
(n=204)	<148	29(14)	12(22)	17(11)	0.17	5(8)	24(17)	0.20
	148-220	41(20)	10(18)	31(21)		11(18)	30(21)	
	>220	134(66)	33(60)	101(67)		45(74)	89(62)	
MMA (nmol/L)	Conc	150 (110, 190)	140 (110, 220)	150 (120, 180)	0.40[a]	140 (110, 180)	160 (120, 190)	0.55[a]
(n=199)	>260	18(9)	7(13)	11(8)	0.09	5(8)	13(9)	0.25
	210-260	21(11)	9(17)	12(8)		4(7)	17(12)	
	<210	160(80)	38(70)	122(84)		52(85)	108(78)	
HoloTC (pmol/L)	Conc	66 (49, 86)	60(45, 77)	67(49, 89)	0.09[a]	75(57,103)	63(45,75)	**0.0003[a]**
(n=204)	<35	17(8)	7(13)	10(7)	0.17	3(5)	14(10)	0.44
	≥35	187(92)	48(87)	139(93)		58(95)	129(90)	

[a]**Univariate analysis performed using transformed variables**
Values displayed as Median (IQR) or Frequency(%) unless otherwise specified
Abbreviations: Conc=Concentration, holoTC= holotranscobalamin, MMA=methylmalonic acid

4.2.1 Hematological Outcomes

Hemoglobin concentrations were significantly lower in SA individuals and they had a higher rate of anemia than European individuals **(Table 4-14)**. SA individuals also had lower hematocrit, and a higher incidence of low hematocrit concentrations. SA individuals had lower MCV but there was no ethnic difference in the incidence of macrocytosis. No difference was observed in hematological parameters between supplement users and non-supplement users.

Deficient individuals had significantly lower Hb, and significantly higher MCV than adequate individuals **(Table 4-15)**. However, these differences were not clinically significant. There were no differences in the rates of anemia, low hematocrit or macrocytosis across categories of deficiency

Table 4-14 Hematological Measurements Divided by Ethnicity and Supplement Use

Parameter	Total	South Asian	European	p	Supplement user	Non-supplement user	p
Hemoglobin (g/dL) (n=187)	12.7 (12.1,13.2)	12.3 (11.7,12.7)	12.9 (12.3,13.3)	**<0.0001**	12.7 (12.1,13.2)	12.7 (12.1,13.2)	0.85
Anemia (<12 g/dL)	37(18)	18(33)	19(13)	**0.001**	11(17)	26(18)	1.000
Hematocrit (%) (n=187)	38 (37, 40)	37 (35,39)	39 (37,40)	**0.0001**	38 (37,40)	38 (37,40)	0.65
Low Hematocrit <36%	28(14)	15(27)	13(9)	**0.0001**	7(11)	21(15)	0.66
MCV (fL) (n=187)	90 (87,93)	85 (82,89)	91 (88,93)	**<0.0001**	88 (87,92)	90 (86,93)	0.83
Macrocytosis (MCV ≥99 fL)	36(17)	7(13)	29(19)	0.28	12(19)	24(17)	0.69
Macrocytic Anemia[a]	0(0)	---	---	---	---	---	---

Presented as median (IQR), or frequency (%) unless otherwise specified. [a]Coincident anemia (Hb<120 g/L) and macrocytosis (MCV ≥99)
Continuous measurements compared by Wilcoxon rank sum. **Abbreviation:** MCV=Mean corpuscular volume

Table 4-15 Hematological Marker Concentrations and Incidence of Hematological Outcomes by Category of Deficiency

Parameter	Chronic deficiency	Marginal deficiency	Adequate	p
Hemoglobin (g/dL) (n=187)	12.2 (11.8,12.5)[a]	12.9 (12.0,13.3)[a,b]	12.7 (12.2,13.2)[c]	**0.03**
Anemia (<12 g/dL)	7(24)[a]	8(20)[a]	22(16)[a]	0.61
Hematocrit (%)	38 (36,39)[a]	39 (37,40)[a]	38 (37,40)[a]	0.21
Low <0.36%	5(17)[a]	8(20)[a]	15(11)[a]	0.35
MCV (fL)	89 (85,91)[a]	90 (88,92)[a,b]	88 (87,93)[b]	**0.02**
MCV >99 fL	8(28)[a]	4(9)[a]	22(16)[a]	0.15

Presented as median (IQR), or frequency (%) unless otherwise specified. Continuous measurements compared by one-way ANOVA with a Bonferroni post-hoc adjustment. Categorical variables compared by logistic regression. Statistically significant differences (p<0.05) between groups within a row are denoted by not sharing a common superscript.
Abbreviations: Hb=Hemoglobin, MCV=Mean corpuscular volume, SA=South Asian, E=European, S=Supplement user, NS=Non supplement user

4.3 Exploratory Objective 1: Determinants of Vitamin B12 Deficiency

4.3.1 Univariate Analysis

4.3.1.1 Association of Lifestyle and Social Variables with Rates of Vitamin B12 Deficiency

The purpose of univariate analysis was to identify variables that were associated with B12 biomarkers and deficiency classification in the study population. Variables were carried forward to multivariate analysis if they had borderline significance ($p \leq 0.2$) for an influence on B12 status[21]. Variables that were associated with an increased odds for SB12 concentrations <220 pmol/L included obesity and OC use, and for SB12 concentrations <148 pmol/L: obesity, waist circumference above risk cut-offs, and OCs. Variables with borderline significance ($p \leq 0.20$) included consumption of caffeinated beverages, and first generation immigrant status, both associated with an increased risk for deficiency, and high total annual household income, which was associated with a decreased risk for deficiency **(Table 4-16)**.

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[21] Smoking status was excluded from all further analysis due to the low rate of tobacco use in the study population.

Table 4-16 Association of Lifestyle and Social Variables with Serum B12 Categories

Variable (n=186)	OR	95% CI	p-value
South Asian ethnicity (yes=1, no=0)			
SB12[a] <220	1.4	0.7, 2.7	0.30
SB12 <148	2.2	1.0, 4.9	**0.06†**
Obesity (yes=1, no=0)			
SB12 <220	3.6	1.2, 10.2	**0.02***
SB12 <148	4.3	1.4, 12.6	**0.009***
Elevated waist circumference (yes=1, no=0)			
SB12 <220	1.4	0.6, 3.2	0.34
SB12 <148	2.8	1.1, 7.1	**0.03***
Total annual household income[a] (low=1, high=0)			
SB12 <220	0.9	0.5, 1.7	0.80
SB12 <148	0.5	0.2, 1.1	0.08
Oral contraceptives (yes=1, no=0)			
SB12 <220	2.2	1.2, 4.1	**0.01***
SB12 <148	2.3	1.0, 5.1	**0.05***
Physical activity level (low=2, moderate=1, high=0)			
SB12 <220	0.9	0.6, 1.6	0.99
SB12 <148	0.7	0.4, 1.5	0.36
Caffeinated beverages (yes=1, no=0)			
SB12 <220	2.1	0.8, 5.4	**0.13†**
SB12 <148	2.4	0.5, 10.5	0.26
Lactose intolerance (yes=1, no=0)			
SB12 <220	1.4	0.7, 2.7	0.36
SB12 <148	1.14	0.5, 2.9	0.77
First generation immigrant status (yes=1, no=0)			
SB12 <220	1.7	0.95, 3.1	**0.07†**
SB12 <148	1.7	0.91, 4.4	**0.09†**

[a]Serum B12 categories are in pmol/L

SB12= Serum B12, OR=Odds ratio, determined by simple logistic regression

[a]Total household income collapsed into low income=low and low-mid and high income=mid-high, high **(Table 3-4)**

* p-values were significant at the p<0.05 level

† indicates a borderline significance sufficient for inclusion in multivariate regression

In contrast to SB12, variables associated with increased odds for MMA>260 pmol/L included only included first generation immigrant status. No variables were associated with an increased odds for MMA>210 pmol/L. Reduced odds for MMA >260 pmol/L was associated with high total annual household income. Variables that were borderline significant for an increased odds of deficiency included SA ethnicity, and obesity. Physical activity level was borderline significant for a reduced odds of elevated MMA **(Table 4-17)**.

Table 4-17 Association of Lifestyle and Social Variables with Methylmalonic Acid Categories of Deficiency

Variable (n=186)	OR	95% CI	p-value
South Asian ethnicity (yes=1, no=0)			
MMA>210	1.7	0.6,5.0	0.97
MMA>260	1.9	0.9,4.0	**0.09†**
Obesity (yes=1, no=0)			
MMA>210	2.7	0.7,10.7	**0.16†**
MMA>260	0.8	0.2,3.1	0.78
Elevated waist circumference (yes=1, no=0)			
MMA>210	1.4	0.4,3.5	0.60
MMA>260	1.3	0.5,3.4	0.55
Total annual household income[a] (low=1, high=0)			
MMA>210	0.4	0.1,1.1	**0.07†**
MMA>260	0.5	0.2,1.0	**0.04***
Oral contraceptives (yes=1, no=0)			
MMA>210	1.1	0.4,3.3	0.88
MMA>260	0.6	0.3,1.4	0.27
Physical activity level (low=2, moderate=1, high=0)			
MMA>210	0.6	0.2,1.4	**0.20†**
MMA>260	0.8	0.4,1.6	0.55
Caffeinated beverages (yes=1, no=0)			
MMA>210	2.3	0.3,18.4	0.43
MMA>260	0.7	0.3,1.7	0.40
Lactose intolerance (yes=1, no=0)			
MMA>210	1.2	0.4,3.9	0.77
MMA>260	1.2	0.6,2.8	0.61
First generation immigrant status (yes=1, no=0)			
MMA>210	2.2	0.8,6.2	**0.14†**
MMA>260	2.1	1.0,4.3	**0.04***

OR=Odds Ratio, determined by simple logistic regression
[a]Total household income collapsed into low income=low and low-mid and high income=mid-high, high **(Table 3-4)**
* p-values were significant at the p<0.05 level
† indicates a borderline significance sufficient for inclusion in multivariate regression

Variables associated with an increased odds of holoTC <35 pmol/L included obesity, waist circumference above the risk cut-offs, and consumption of caffeinated beverages. Variables that were borderline significant for an association with increased odds of holoTC <35 pmol/L included OC use and lactose intolerance. No variables were associated with reduced odds of holoTC <35 pmol/L **(Table 4-18)**.

Table 4-18 Association of Lifestyle and Social Variables with Holotranscobalamin Category of Deficiency

Variable (n=186)	**OR**	**95% CI**	**p-value**
South Asian ethnicity (yes=1, no=0)			
holoTC<35	1.7	0.6,4.9	0.34
Obesity (yes=1, no=0)			
holoTC<35	6.5	1.9,22.0	**0.003***
Elevated waist circumference (yes=1, no=0)			
holoTC<35	4.2	1.4,12.8	**0.01***
Total annual household income[a] (low=1, high=0)			
holoTC<35	0.6	0.2,1.6	0.3
Oral contraceptives (yes=1, no=0)			
holoTC<35	2.7	0.9,7.5	**0.07†**
Physical activity level (low=2, medium=1, high=0)			
holoTC<35	0.7	0.2,1.7	0.4
Caffeinated beverages (yes=1, no=0)			
holoTC<35	0.04	0.00,0.3	**0.002***
Lactose intolerance (yes=1, no=0)			
holoTC<35	2.4	0.80,7.0	**0.1†**
First generation immigrant status (yes=1, no=0)			
holoTC<35	1.3	0.5,3.6	0.4

OR=Odds ratio, determined by simple logistic regression
[a]Total household income collapsed into low income=low and low-mid and high income=mid-high, high **(Table 3-4)**
*p-values were significant at the p<0.05 level
† indicates a borderline significance sufficient for inclusion in multivariate regression

4.3.1.2   Association of Lifestyle and Social Variables with Vitamin B12 Biomarker Concentration

To address the arbitrary nature of biomarker cut-offs and the limitations they impose in regards to properly assessing individuals that fall close to the division point, simple linear regression was used to assess the association of selected variables on biomarker concentration.

Positive associations with SB12 concentrations were not observed for any variables. Variables that showed a borderline positive association (p<0.2) on SB12 included total household income. SB12 concentration was negatively associated with first generation immigrant status (p=0.013). Variables that showed a borderline negative association (p<0.2) on SB12 concentrations included obesity and OC use **(Table 4-19)**.

Table 4-19 Associations of Lifestyle and Social Variables with Serum B12 Concentration

Variable (n=186)	**Sub-category**	**Median Serum B12 (pmol/L)**	**p-value**
Ethnicity	European	296.9	0.61
	South Asian	285.2	
Obese	No	298.9	**0.07†**
	Yes	232.8	
Waist circumference	Below	298.6	0.23
	Above	264.2	
Total annual household income	Low and low middle	259.4	**0.17†**
	Middle-upper and high	267.2	
Oral contraceptives	No	304.1	**0.10†**
	Yes	268.3	
Physical activity level	Low	309.3	0.69
	Moderate	301.3	
	High	284.5	
Caffeinated beverages	No	305	0.64
	Yes	291	
Lactose intolerance	No	293.1	0.90
	Yes	296.1	
First generation immigrant status	No	312.7	**0.013***
	Yes	261.8	

Significance determined by simple linear regression
* p-values were significant at the p<0.05 level
† indicates a borderline significance (p≤0.2) sufficient for inclusion in multivariate regression

No variables were negatively associated with MMA concentrations. First generation immigrant status was associated with increased MMA concentrations. Borderline significant variables included obesity and SA ethnicity for a negative association, and high total household income for a positive association on MMA concentrations (**Table 4-20**).

Table 4-20 Association of Lifestyle and Social Variables with Methylmalonic Acid Concentration

Variable (n=186)	**Sub-category**	**Median MMA (nmol/L)**	**p-value**
Ethnicity	European	154	**0.13†**
	South Asian	140	
Obese	No	154	**0.19†**
	Yes	137	
Waist circumference	Below	152	0.28
	Above	143	
Total annual household income	Low and low middle	162	**0.09†**
	Middle-upper and high	145	
Oral contraceptives	No	149	0.69
	Yes	158	
Physical activity level	Low	143	0.97
	Moderate	138	
	High	164	
Caffeinated beverages	No	164	0.70
	Yes	150	
Lactose intolerance	No	151	0.21
	Yes	164	
First generation immigrant status	No	148	**0.01***
	Yes	166	

Significance determined by simple linear regression
* p-values were significant at the $p<0.05$ level
† indicates a borderline significance ($p≤0.2$) sufficient for inclusion in multivariate regression

Negative influence over holoTC concentrations was observed for waist circumference measurements above the risk cut-off, and first generation immigrant status. Variables with a borderline negative influence included SA ethnicity and obesity **(Table 4-21)**.

Table 4-21 Association of Lifestyle and Social Variables with Holotranscobalamin Concentrations

Variable (n=186)	Sub-category	Median holotranscobalamin (pmol/L)	p-value
Ethnicity	European	66.5	**0.13†**
	South Asian	58.3	
Obese	No	65.9	**0.11†**
	Yes	54.7	
Waist circumference	Below	66.2	**0.03***
	Above	55.1	
Total annual household income	Low and low middle	66.2	0.46
	Middle-upper and high	65.9	
Oral contraceptives	No	65.6	0.56
	Yes	63.1	
Physical activity level	Low	82.7	0.54
	Medium	63.5	
	High	65.6	
Caffeinated beverages	No	70.0	0.59
	Yes	64.4	
Lactose intolerance	No	65.3	0.57
	Yes	63.8	
First generation immigrant status	No	68.1	**0.03***
	Yes	58.9	

Significance determined by simple linear regression
Total household income collapsed into low income=low and low-mid and high income=mid-high, high **(Table 3-4)**
* p-values were significant at the p<0.05 level
† indicates a borderline significance (p≤0.2) sufficient for inclusion in multivariate regression

4.3.1.3 Association of Dietary Intakes with Vitamin B12 Status

Dietary intake from food alone was positively correlated with SB12 (p=0.02) **(Figure 4-3)** and holoTC (p=0.003) concentrations and negatively correlated with MMA concentrations (p=0.01) [22]. Dietary intake from food and supplements was positively correlated with SB12 concentrations (p<0.0001) and holoTC (p<0.0001) concentrations and negatively correlated with MMA (p=0.05) concentrations **(Figure 4-4)**.

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[22] Plots only shown for serum vitamin B12

Figure 4-3 Correlation between Dietary Vitamin B12 Intakes from Food Alone and Serum Vitamin B12 Concentration
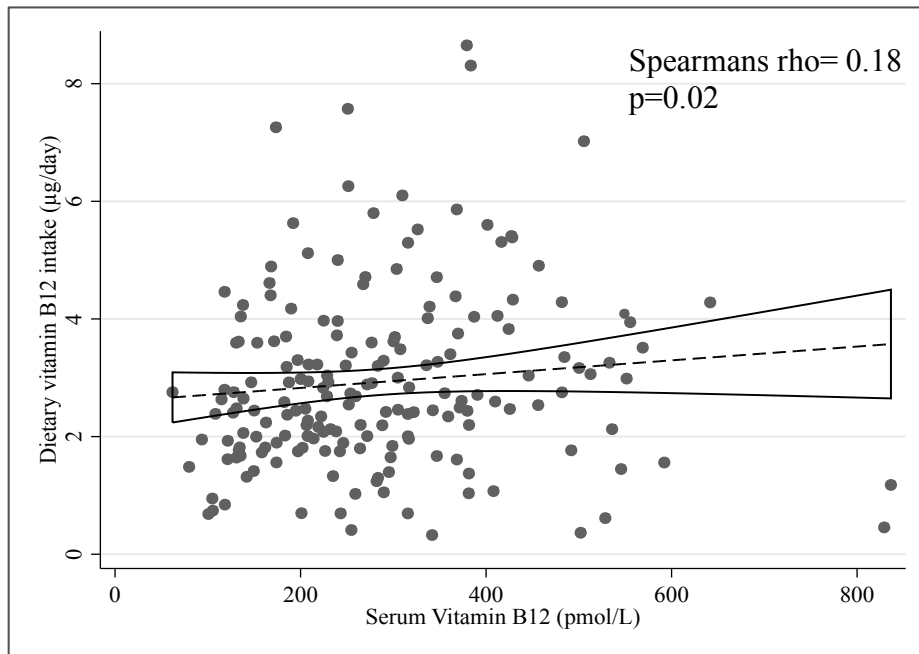
This figure depicts the relationship between dietary vitamin B12 intake from food alone and serum vitamin B12 concentration

Figure 4-4 Correlation between Dietary Vitamin B12 Intakes from Food and Supplements and Serum Vitamin B12 Concentration
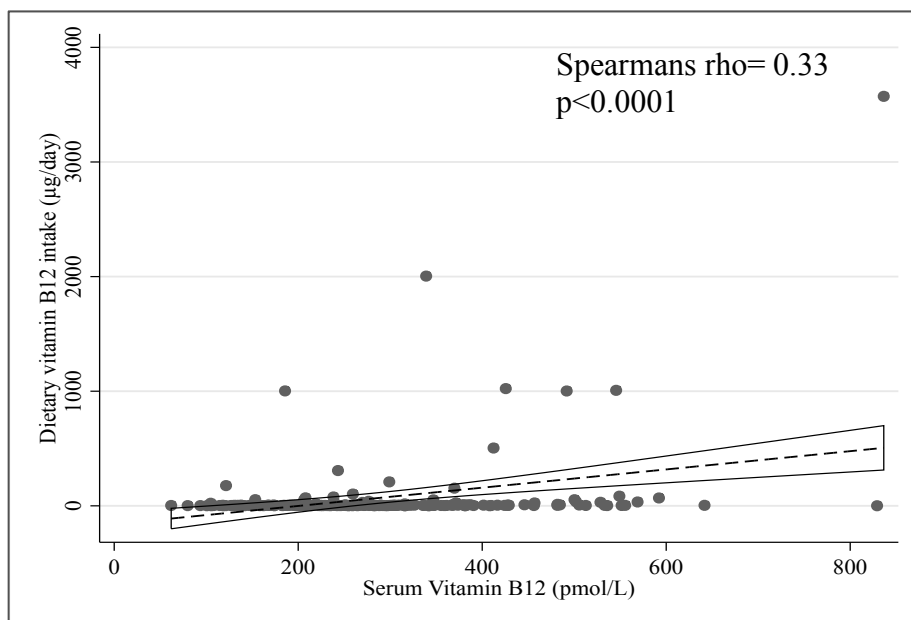
This figure depicts the relationship between dietary vitamin B12 intake from food and supplements and serum vitamin B12 concentration

The group with chronic deficiency based on SB12 concentration cut-offs had

significantly lower B12 intakes from food alone (p=0.02) than the other groups, but the marginal

deficiency group did not have different B12 intakes from the adequate group **(Table 4-22)**.

Based on SB12 concentration cut-offs the chronic deficiency group had the lowest B12

intakes from food and supplements (p=0.002) followed by the marginally deficient group

(p=0.01), and differences between groups were all statistically significant.

Table 4-22 Difference in Dietary Vitamin B12 Intakes from Food Alone and from Food and Supplements by Category of Deficiency

		Category of Deficiency		
		Adequate (n=118)	Marginal Deficiency (n=38)	Chronic Deficiency (n=28)
Biomarker	**Intake category**	**Vitamin B12 intake (µg/day) Median(IQR)**		
Serum B12	Food	2.8(2.0,4.0)[a]	2.5(2.0,3.6)[a,b]	**2.2(1.6,2.8)[b]**
	Food+Supplements	3.9(2.5,8.4)[a]	**3.3(2.0,5.6)[b]**	2.5(1.7,3.6)[c]

This table displays the vitamin B12 intakes from food alone, and from food and supplements for groups divided by vitamin B12 status as determined by SB12 concentration category
Difference between groups tested by Kruskal Wallis test and Bonferroni post-hoc test, or by Wilcoxon rank sum test
Statistically significant differences (p<0.05) between groups within a row are denoted by not sharing a common superscript.

After adjustment for supplement use energy adjusted dietary B12 intake was associated

with higher B12 concentrations **(Table 4-23)**. Unadjusted B12 intake was not associated with

SB12 concentration before or after correction for supplement use.

Table 4-23 Influence of Dietary B12 Intake on Serum B12 Concentration

Intake Variable	Unadjusted models β_{intake} (95% CI)	t	p-value	R^2 (AR^2)	Supplement adjusted models β_{intake} (95% CI)	t	p-value	R^2 (AR^2)
B12 intake (EA)	5.6 (-0.06 to 11.4)	1.95	0.05	0.02 (0.01)	7.53 (1.95 to 13.1)	2.66	**0.008**	0.09 (0.08)
B12 intake	8.0 (-5.4 to 21.3)	1.19	0.24	0.007 (0.002)	11.2 (-1.9 to 24.1)	1.7	0.09	0.07 (0.06)

Abbreviations: B12=vitamin B12, EA= Energy adjusted, AR^2=Adjusted R^2
n=186

4.3.1.4 Association of Genetic Variants with Vitamin B12 Status

Subject characteristics for individuals grouped by *MTHFR* C677T genotype are presented in **Table 4-24**. The MMA concentration of individuals with the CT genotype was significantly lower than those with the CC and TT genotype. The proportion of individuals with MMA concentrations >210 nmol/L, and >260 nmol/L was significantly lower in the CT group. No other differences in biomarker concentrations, rates of deficiency, or hematological outcomes were observed between genotype groups.

Table 4-24 Subject Characteristics Grouped by *MTHFR* C677T Genotype

	Genotype		
	CC (n=97)	CT (n=81)	TT (n=19)
SB12 (pmol/L)	283.3[a]	305.4[a]	294.5[a]
SB12 <148 pmol/L (%)	19[a]	11[a]	5[a]
SB12 <220 pmol/L (%)	36[a]	36[a]	21[a]
holoTC (pmol/L)	66.5[a]	70.2[a]	68.5[a]
holoTC <35 pmol/L (%)	11[a]	7[a]	0[a]
holoTC: SB12 Ratio (%)	26.2[a]	26.1[a]	23.3[a]
MMA (nmol/L)	191**[a]**	**146[b]**	192[a]
MMA >210 (%)	27[a]	**9[b]**	37[a]
MMA >260 (%)	13[a]	**3[b]**	17[a]
Anemia (%)	23[a]	14[a]	16[a]
Macrocytosis (%)	18[a]	15[a]	5[a]

Abbreviations: SB12=Serum vitamin B12, HoloTC=holotranscobalamin, MMA=methylmalonic acid
Difference between groups assessed for continuous variables using a Kruskal Wallis test followed by a Bonferroni post-hoc assessment ($p<0.05$), and for categorical variables using logistic regression with the CC genotype as the reference group
Statistically significant differences between groups within a row are denoted by not sharing a common superscript.
Continuous variables presented as medians, categorical as percentage/genotype

There were no differences in B12 biomarker concentrations, deficiency classification, or hematological status between genotypes for the *TCN2* C776G variant **(Table 4-25)**.

Table 4-25 Subject Characteristics Grouped by *TCN2* C776G Genotype

	Genotype		
	CC (n=48)	CG (n=113)	GG (n=34)
SB12 (pmol/L)	287.9[a]	297.9[a]	276.4[a]
SB12 <148 pmol/L (%)	17[a]	14[a]	12[a]
SB12 <220 pmol/L (%)	40[a]	33[a]	32[a]
HoloTC (pmol/L)	69.3[a]	68.6[a]	64.0[a]
holoTC <35 pmol/L (%)	8[a]	8[a]	12[a]
HoloTC: SB12 Ratio (%)	26.9[a]	25.5[a]	25.9[a]
MMA (nmol/L)	164[a]	171[a]	197[a]
MMA >210 (%)	15[a]	21[a]	26[a]
MMA >260 (%)	8[a]	8[a]	15[a]
Anemia (%)	19[a]	20[a]	12[a]
Macrocytosis (%)	13[a]	17[a]	12[a]

Abbreviations: SB12=Serum vitamin B12, HoloTC=holotranscobalamin, MMA=methylmalonic acid
Difference between groups assessed for continuous variables using a Kruskal Wallis test (p<0.05), and for categorical variables using logistic regression with the CC genotype as the reference group.
Statistically significant differences between groups within a row are denoted by not sharing a common superscript.
Continuous variables presented as medians, categorical as percentage/genotype

Due to the low cell count of individuals with the GG genotype for the *TCN2* A67G variant, GG and AG individuals were combined into one group and compared with the AA genotype reference group.

HoloTC levels and holoTC to SB12 ratio was significantly lower in the combined AG+GG genotype group. There was a higher rate of macrocytosis in individuals with the G allele **(Table 4-26).**

Table 4-26 Subject Characteristics Grouped by *TCN2* A67G Genotype

	Genotype	
	AA (n=122)	**AG+GG** (n=72)
SB12 (pmol/L)	279.8	311.6
SB12 <148 pmol/L (%)	12	18
SB12 <220 pmol/L (%)	35	33
HoloTC (pmol/L)	70	64
holoTC <35 pmol/L (%)	6	**14***
HoloTC: SB12 Ratio (%)	27.4	**23.4***
MMA (nmol/L)	176	171
MMA >210 (%)	8	11
MMA >260 (%)	21	19
Anemia (%)	18	19
Macrocytosis (%)	11	**22***

Abbreviations: SB12=Serum vitamin B12, HoloTC=holotranscobalamin, MMA=methylmalonic acid
*Difference between groups assessed for continuous variables using students t-test or Mann Whitney U test **(p<0.05)**, and for categorical variables using a χ^2 test
Continuous variables presented as medians, categorical as percentage/genotype

There was a higher rate of chronic deficiency based on SB12 for the AA and AG

genotype groups compared to the GG genotype group for the *FUT2* G893A variant but these

differences were not significant (p=0.08) **(Table 4-27)**. There was a significantly lower rate of

marginal deficiency in the GG genotype group compared to the AA and AG genotype groups

(p=0.03).

Table 4-27 Subject Characteristics Grouped by *FUT2* G893A Genotype

	Genotype		
	GG	**AG**	**AA**
	(n=36)	(n=95)	(n=66)
SB12 (pmol/L)	318.3[a]	292.1[a]	281.8[a]
SB12 <148 pmol/L (%)	3[a]	17[a]	17[a]
SB12 <220 pmol/L (%)	**17[a]**	37[b]	41[b]
HoloTC (pmol/L)	67.4[a]	70[a]	66[a]
holoTC <35 pmol/L (%)	3[a]	8[a]	12[a]
HoloTC: SB12 Ratio (%)	22.5[a]	22.6[a]	26.7[a]
MMA (nmol/L)	148[a]	178[a]	181[a]
MMA >210 (%)	3[a]	11[a]	11[a]
MMA >260 (%)	8[a]	22[a]	24[a]
Anemia (%)	19[a]	19[a]	17[a]
Macrocytosis (%)	19[a]	15[a]	14[a]

Abbreviations: SB12=Serum vitamin B12, HoloTC=holotranscobalamin, MMA=methylmalonic acid
Difference between groups assessed for continuous variables using One-way ANOVA (p<0.05), and for categorical variables using logistic regression with the AA genotype as the reference group.
Statistically significant differences between groups within a row are denoted by not sharing a common superscript.
Continuous variables presented as medians, categorical as percentage/genotype
Note that the homozygous dominant genotype is GG.

4.3.2   Multivariate Analysis

4.3.2.1   Determinants of B12 Deficiency Status using Multiple Logistic Regression

To construct multiple logistic regression models a standard set of procedures was followed and is summarized in **Table 4-28**.

Table 4-28 Procedures for Constructing Multivariate Logistic Regression Models

Step 1 – Full Model

Vitamin B12 Deficiency[a] = $\beta_o + \beta_1$(dietary intake)$+\beta_2$(B12 supplement use$+\beta$(consumption of caffeine)$+\beta$(lactose intolerance$+\beta$(first generation immigrant status)$+\beta$(income status)$+\beta$(OC use)$+\beta$(obesity)$+\beta$(central obesity)$+\beta$(physical activity level)$+\beta$(*MTHFR* C677T)$+\beta$(*TCN2* C776G)$+\beta$(*TCN2* A67G)$+\beta$(*FUT2* G893A)

Step 2 – Non significant variables removed

Step 3 Regression Diagnostics

Pre-model fitting: **Influential data points** were identified by assessing leverage and studentized residuals by calculating Cook's distance and DFITS (a measure of how unusual an outcome is taking into account leverage and studentized residuals). Data points were considered for removal if Cook's distance was >4/207 and if the absolute value of DFITS was > 2 $\sqrt{(n)}$.

To assess influence of individual data points on regression coefficients cox regression algorithms (DFBETA) was calculated and data points were removed if DFBETA was >2/$\sqrt{}$ (207). **Normality** of the models was assessed with kernel density plots, P-P plots, and Q-Q plots and the Shapiro Wilks test. **Homoscedasticity** was tested using White's test. **Model specification errors** were assessed using a Ramsey Reset test and link test. Logistic regression models were assessed for goodness of fit using the Hosmer Lemeshow goodness of fit test.

Final Model Fit (Example shown for SB12)

Chronic deficiency (Y/N) = $\beta_o + \beta_1$ (dietary B12 intake) $+\beta_2$ (obese) $+\beta_3$ (OC use) $+\beta_4$ (B12 supplement use) $+\beta_5$ (ethnicity)

[a]= Based on Serum vitamin B12, Methylmalonic acid or holotranscobalamin cut-offs

Five separate models were constructed to assess the determinants of marginal and chronic deficiency **(Table 4-29)**. Dietary intake and B12 supplement use were associated with reduced odds of marginal deficiency based on SB12 levels, whereas OC use, and first generation immigrant status were associated with increased odds. For chronic deficiency based on SB12 levels, dietary B12 intake and B12 supplement use were associated with reduced odds. Oral contraceptive use, and SA ethnicity were associated with increased odds of chronic deficiency.

For marginal deficiency defined by MMA levels, first generation immigrant status was associated with increased odds of elevated MMA. No variables of interest were significant determinants of MMA levels indicative of chronic deficiency. For chronic deficiency defined by holoTC levels, obesity and OC use were both associated with a increased odds for deficiency. Due to low cell counts, logistic regression models were not stratified by ethnicity or supplement use.

Table 4-29 Multiple Logistic Regression Models Assessing the Influence of Lifestyle and Genetic Factors on the Odds for Deficiency Classification by Vitamin B12 Biomarkers

Independent Variable	Dependent Variable				
	SB12<220 pmol/L	SB12<148 pmol/L	MMA>210 nmol/L[a]	MMA>260 nmol/L[b]	holoTC <35 nmol/L
	OR (95% CI)				
Dietary intake (energy adjusted) (µg/day)	0.8(0.7, 0.9)**	0.8(0.7, 0.9)**	0.9(0.8, 1.0)		
First generation	2.0(1.0, 4.1)*		2.6(1.2, 5.5)*		
FUT2 G893A			0.6(0.3, 1.0)		
Oral contraceptive use	4.1(1.9, 8.9)****	4.9(1.7, 13.6)**			3.3(1.1, 9.6)*
Obesity	2.7(0.9, 8.8)	2.8(0.8, 9.7)			7.1(2.0, 25.5)**
B12 supplement use	0.4(0.2, 0.9)*	0.3(0.1, 0.9)*			
Ethnicity		3.9(1.3, 11.0)*			
Constant	0.07(0.03, 0.2)	0.5(0.2, 1.0)	0.3(0.1, 0.5)		0.05(0.02, 0.1)
n	177	177	177		177
LR χ^2	25.6	30.9	13.1		11.7
Prob>χ^2	0.0001	<0.0001	0.004		0.003
Pseudo R^2	0.17	0.13			0.11

Abbreviations: SB12= Serum vitamin B12, MMA=Methylmalonic acid, holoTC=holotranscobalamin, LR= likelihood ratio test

Significance: $p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$**** [a]Marginal vitamin B12 deficiency [b]Chronic vitamin B12 deficiency

Shading indicates variables that were not included in the final model for specific dependent variables.

Number of observations: n=184

4.3.2.2   Determinants of B12 Biomarker Concentrations Using Multiple Linear Regression

To assess the contribution of different influential variables to biomarker concentration stepwise multiple linear regression analysis was used. The final variable pool established from preliminary univariate analysis to compose the full model included dietary B12 intake, B12 supplement use, the 4 genotypes (*MTHFR* C677T, *TCN2* C776G, *TCN2* A67G, and *FUT2* G893A), ethnicity, obesity, waist circumference above risk cut-offs, OC use, consumption of caffeine, first generation immigrant status, income level, and physical activity level. Before entry into the models all variables included in the full model were tested for collinearity, which was not detected. A set of standard procedures was followed for fitting all multivariate models **(Table 4-30)**.

Table 4-30 Example of Multivariate Linear Regression Model Fit Procedure

Step 1 – Full Model

Biomarker[a]= β_o+β_1(dietary intake)+β_2(B12 supplement use+β(consumption of caffeine)+β(lactose intolerance+β(first generation immigrant status)+β(income status)+β(OC use)+β(obesity)+β(central obesity)+β(physical activity level)+β(*MTHFR* C677T)+β(*TCN2* C776G)+β(*TCN2* A67G)+β(*FUT2* G893A)

Step 2 – Non significant variables removed

Step 3 Regression Diagnostics

Pre-model fitting: **Influential data points** were identified by assessing leverage and studentized residuals by calculating Cook's distance and DFITS (a measure of how unusual an outcome is taking into account leverage and studentized residuals). Data points were considered for removal if Cook's distance was >4/207 and if the absolute value of DFITS was > 2 $\sqrt{(n)}$.

To assess influence of individual data points on regression coefficients cox regression algorithms (DFBETA) was calculated and data points were removed if DFBETA was >2/$\sqrt{(207)}$. **Normality** of the models was assessed with kernel density plots, P-P plots, and Q-Q plots and the Shapiro Wilks test. **Homoscedasticity** was tested using a Breusch-Pagan test, and White's test. Linearity was assessed using an augmented partial residual plot by visual assessment. **Model specification errors** were assessed using a Ramsey Reset test and link test.

Final Model Fit

(Example shown for SB12)

SB12 = β_o+β_1 (non red meat eater) +β_2 (obese) +β_3 (OC use) +β_4 (first generation) +β_5 (total protein intake) +β_6 (dietary and supplemental B12 intake) +β_7 (*FUT2* G893A genotype)

[a]= Serum vitamin B12, Methylmalonic acid or holotranscobalamin

The final model suggests that SB12 concentration is positively influenced by supplement use and dietary intake of B12 and negatively influenced by OC use and first generation immigrant status **(Table 4-31)**.

Table 4-31 Determinants of Serum B12 Concentration

Variable	β Coefficient (95%CI)	SE	t	p-value
Vitamin B12 supplement use	80.4 (38.8, 122.2)	21.1	3.8	**<0.0001**
Oral contraceptive use	-45.3 (-87.5, -3.0)	21.4	-2.1	**0.04**
Dietary intake (energy adjusted) (µg/day)	7.4 (2.0, 12.7)	2.7	2.7	**0.007**
First generation immigrant	-43.5 (-82.7, -4.2)	19.9	-2.2	**0.03**
Constant	291.9 (260.8, 322.9)	15.7	18.6	<0.0001
Number of observations=184, **Model p-value** = <0.0001 R^2=0.13, **Adj** R^2=0.11, **Root MSE**=128.25				

This model explains 13 percent of the variation in SB12 concentrations suggesting that other factors contribute significantly to determining SB12 concentration in this study population.

MMA was not influenced by any variables with the exception of first generation immigrant status, which was associated with increased MMA concentrations. This model did not meet diagnostic specifications so it was concluded that MMA concentrations were not influenced by any of the variables we investigated. Using log transformed MMA as the dependent variable did not improve model fit or change the outcome.

HoloTC concentration was influenced positively by dietary B12 intake, and B12 supplement use (**Table 4-32**). First generation immigrant status and OC use were both associated with lower holoTC concentrations though the association with OC use was not statistically significant.

Table 4-32 Determinants of Holotranscobalamin Concentration

Variable	β Coefficient (95%CI)	SE	t	p-value
Dietary intake (energy adjusted) (μg/day)	1.6(0.5, 2.4)	0.5	3.0	**0.003**
First generation immigrant status	-8.3(-15.3, -1.3)	3.6	-2.3	**0.02**
B12 supplement use	14.1(6.6, 21.5)	3.8	3.7	**<0.0001**
Oral contraceptive use	-6.5(-14.1, 1.0)	3.8	-1.7	0.09
Constant	68.2(62.6, 73.7)	2.8	24.2	<0.0001
Number of observations=184, **Model p-value** = <0.0001 **R^2**=0.13, **Adj R^2**=0.11, **Root MSE**=23.0				

4.4   Exploratory Objective 2: Multiple Biomarker Assessment

4.4.1   Specific Aim 2-1

Correlation coefficients were calculated to test for linear relationships between biomarkers. Serum vitamin B12 (**Figure 4-5,** next page**)** and holoTC **(Figure 4-6,** next page**)** concentrations showed a weak inverse correlation with MMA concentrations. Serum B12 and holotranscobalamin concentrations showed a moderate positive correlation **(Figure 4-7,** page 107**).**

Figure 4-5 Correlation between Serum Vitamin B12 and Methylmalonic Acid Concentrations

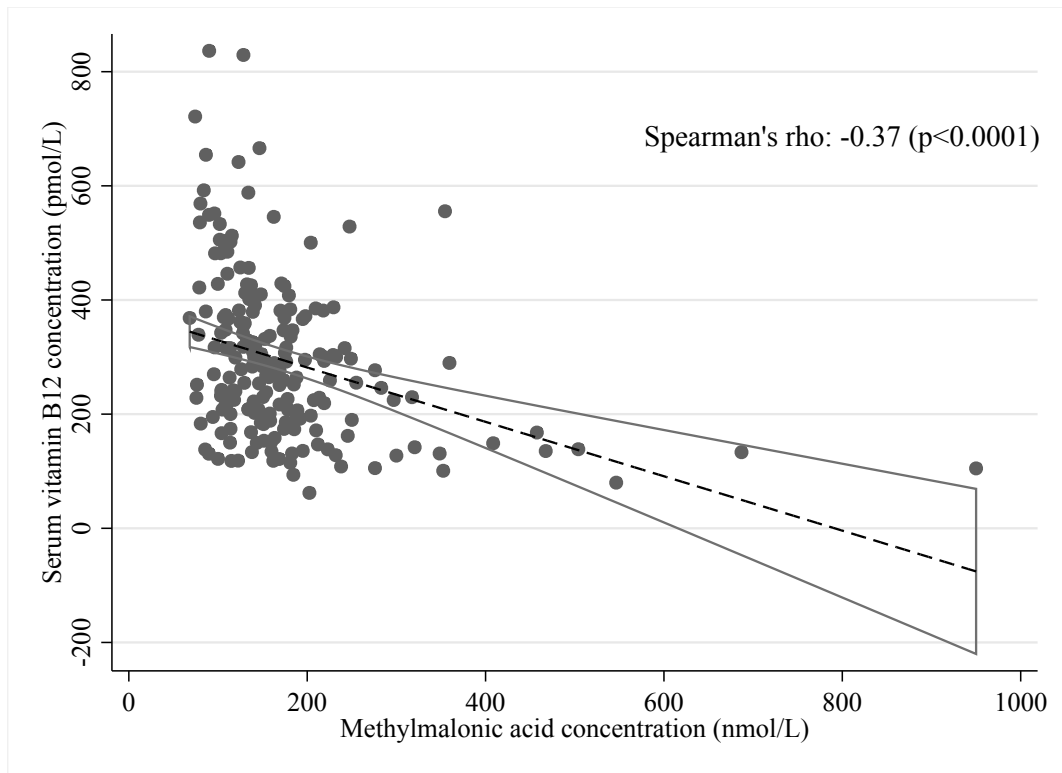Spearman's rho: -0.37 (p<0.0001)

Figure 4-6 Correlation between Holotranscobalamin and Methylmalonic Acid Concentrations

Spearman's rho: -0.35 (p<0.0001)
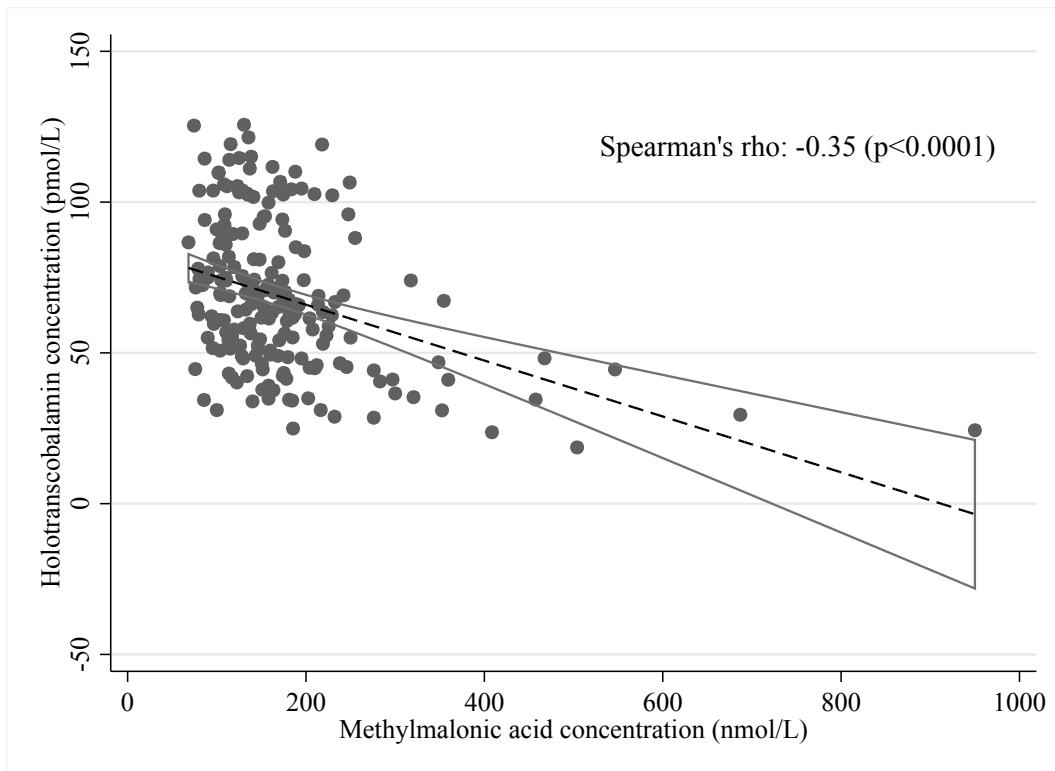
Figure 4-7 Correlation between Serum Vitamin B12 and Holotranscobalamin Concentrations
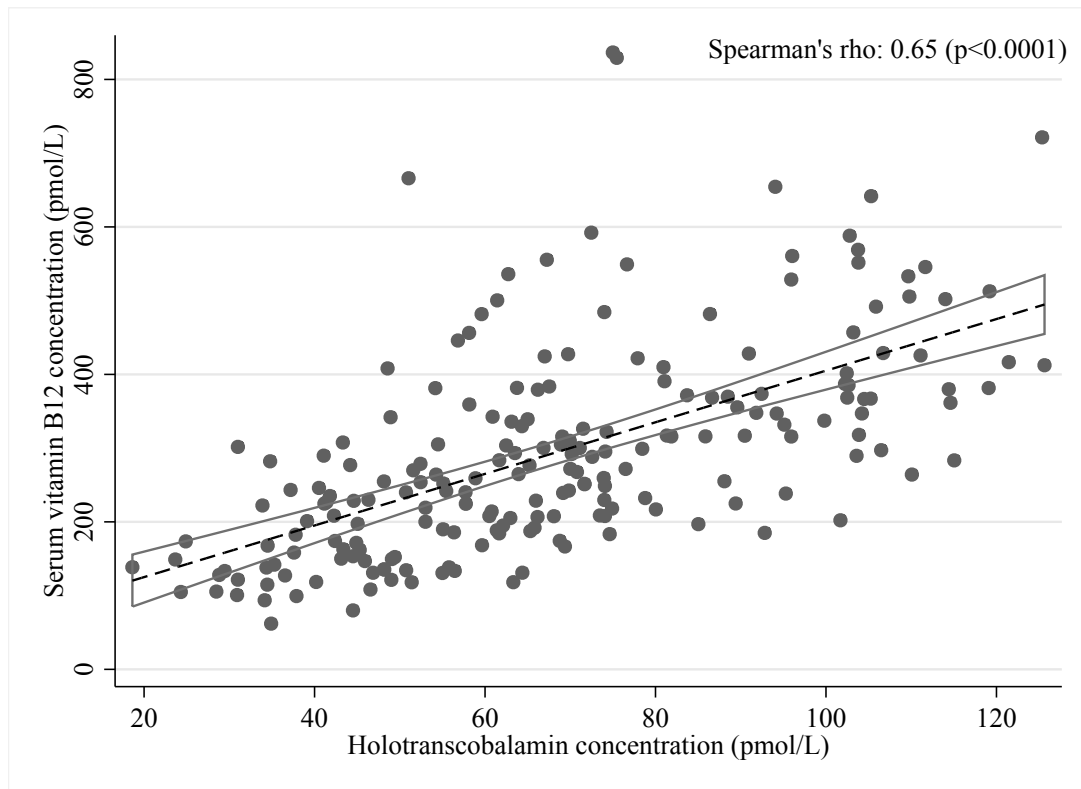
Using the 'conventional' SB12 cut-offs to define deficiency groups, the chronic deficiency group had significantly lower holoTC concentrations than the marginal deficiency group who in turn had significantly lower holoTC concentrations than adequate group **(Table 4-33)**. Concentrations of MMA did not differ between the adequate and marginal deficiency groups but were significantly higher in the chronic deficiency group in comparison to both adequate and marginal deficiency groups.

When the 'Bailey' cut-offs were applied a similar trend was observed for differences in holoTC concentrations. Concentrations of MMA were only significantly different in the chronic deficiency group compared to the marginal deficiency and adequate group.

Using the Bailey cut-offs did result in slightly larger differences in average holoTC concentration between deficiency groups and resulted in greater differences between MMA concentration of the marginal deficiency and adequate groups.

Table 4-33 Median Biomarker Concentration Divided by "Conventional" and "Bailey" Serum Vitamin B12 Cut-offs

		Holotranscobalamin (pmol/L)	p-value	Methylmalonic acid (nmol/L)	p-value
Serum Vitamin B12 cut-off (pmol/L)		Results displayed as median (interquartile range)			
	Conventional				
n=134	SB12 <148	**42.3 (32.6,49.9)**[a]	<0.0001	**207.3(161.0,334.5)**[a]	<0.0001
n=41	SB12 148-220	**58.0 (44.5,68.1)**[b]		152.5(134.0,188.0)[b]	
n=29	SB12 >220	**72.5 (59.6,95.3)**[c]		139.0(110.3,176.5)[b]	
	Bailey				
n=95	SB12 <126	**34.9(31.0,46.6)**[a]	<0.0001	**184.5(162.0,276.0)**[a]	0.0001
n=97	SB12 126-287	**55.1(44.2,69.2)**[b]		159.0(127.8,199.5)[b]	
n=14	SB12 >287	**81.2(66.9,103.4)**[c]		134.8(108.8,175.8)[b]	

Abbreviations: SB12=Serum vitamin b12
Difference between groups tested using *Kruskal Wallis* test and Bonferroni post-hoc test. Statistically significant differences between groups (p<0.05) within a row are denoted by not sharing a common superscript.

4.4.2 Specific Aim 2-2

Overall, the rates of concurrent marginal and chronic deficiency as assessed using two biomarkers were lower than the rates of deficiency determined by single biomarker analysis **(Chapter 4.2)**.

The rates of concurrent marginal deficiency were similar using the 'conventional' cut-offs and the 'Bailey' cut-offs **(Table 4-34).**

Table 4-34 Rates of Concurrent Marginal Vitamin B12 Deficiency

	Secondary Biomarker
	Methylmalonic acid >210 nmol/L Results presented as frequency (%)
Biomarker	**n=199**
Serum Vitamin B12 148-220 pmol/L[a]	21(11)
Serum Vitamin B12 126-287 pmol/L[b]	30(15)

Results are presented as frequency(%) for the proportion of individuals with concurrent marginal deficiency
[a]conventional cut-off [b] 'Bailey' cut-off

The rates of concurrent chronic deficiency were similar (3-5%) when comparing different combinations of biomarkers and different cut-offs for SB12 **(Table 4-35)**.

Table 4-35 Rates of Concurrent Chronic Deficiency

	Secondary Biomarker	
	Methylmalonic acid >260 nmol/L	Holotranscobalamin <35 pmol/L
	Results presented as frequency (%)	
Biomarker	**n=199**	**n=204**
Serum Vitamin B12 <148 pmol/L[a]	10(5%)	
Serum Vitamin B12<126 pmol/L[b]	5(3%)	
Methylmalonic acid >260 nmol/L		7(4%)

Results are presented as frequency(%) for the proportion of individuals with concurrent chronic deficiency
[a]conventional cut-off [b] 'Bailey' cut-off

Using 'conventional' SB12 cut-offs and MMA cut-offs, gross disagreement[23] occurred in 10% of individuals, and moderate disagreement[24] occurred in 37% of individuals **(Figure 4-8, page 111).** This is illustrated graphically in **Figures 4-9 and 4-10** (page 112-113). Using the 'Bailey' SB12 cut-offs and MMA cut-offs, gross disagreement occurred in 5% of individuals and moderate disagreement occurred in 48% of individuals **(Figures 4-11 and 4-12,** page 114-115). This suggests that the distinction between marginal and adequate individuals is difficult to discern, even when using multiple biomarkers. Overall, there was less gross disagreement based on use of the 'Bailey' cut-offs in combination with the MMA cut-offs, compared to the 'conventional' cut-offs. However, there was more moderate disagreement using the 'Bailey' cut-offs in combination with MMA cut-offs, compared to the 'conventional' cut-offs.

---

[23] Defined as having chronic deficiency based on one biomarker and adequate status based on another

[24] Defined as either **[1]** chronic deficiency based on one biomarker and marginally deficient based on another, or **[2]** marginally deficient based on one biomarker and adequate based on another

Figure 4-8 Incidence of Disagreement Resulting from Multiple Biomarker Determination of Marginal and Chronic Deficiency Rates

	Conventional				Bailey		
	MMA Adequate	**MMA Marginal**	**MMA Deficient**		**MMA Adequate**	**MMA Marginal**	**MMA Deficient**
SB12 Adequate	112(56)	13(7)	6(3)		80(40)	72(36)	8(4)
SB12 Marginal	34(17)	4(2)	2(1)		10(5)	10(5)	1(1)
SB12 Deficient	14(7)	4(2)	10(5)		2(1)	12(6)	4(2)

This figure depicts the frequency and percentage of:

🟩	Concurrent adequacy	🟥	Concurrent chronic deficiency	🟨	Concurrent marginal deficiency
⬛	Gross disagreement	⬜	Moderate disagreement		

Definitions:
Conventional: SB12 adequate = >220 pmol/L, SB12 marginal = 148-220 pmol/L, SB12 chronic = <148 pmol/L
Bailey: SB12 adequate = >287 pmol/L, SB12 marginal= 126-287 pmol/L, SB12 chronic= <126 pmol/L
MMA adequate: = <210 pmol/L, MMA marginal = 210-260 pmol/L, MMA chronic = >260 pmol/L
Concurrent adequacy = adequate SB12 and MMA concentrations
Concurrent chronic deficiency= chronic SB12 and MMA concentrations
Concurrent moderate deficiency= marginal SB12 and MMA concentrations
Gross disagreement: either **[1]** chronic SB12 concentration and adequate MMA concentration, or **[2]** adequate SB12 concentration and chronic MMA concentration
Moderate disagreement: either **[1]** moderate MMA and adequate SB12 concentrations, **[2]** moderate MMA and chronic SB12 concentrations, **[3]** moderate SB12 and adequate MMA concentrations, **[4]** moderate SB12 and chronic MMA concentrations

Figure 4-9 Rates of Disagreement and Concurrent Marginal Vitamin B12 Deficiency (Conventional Serum Vitamin B12 Cut-offs)
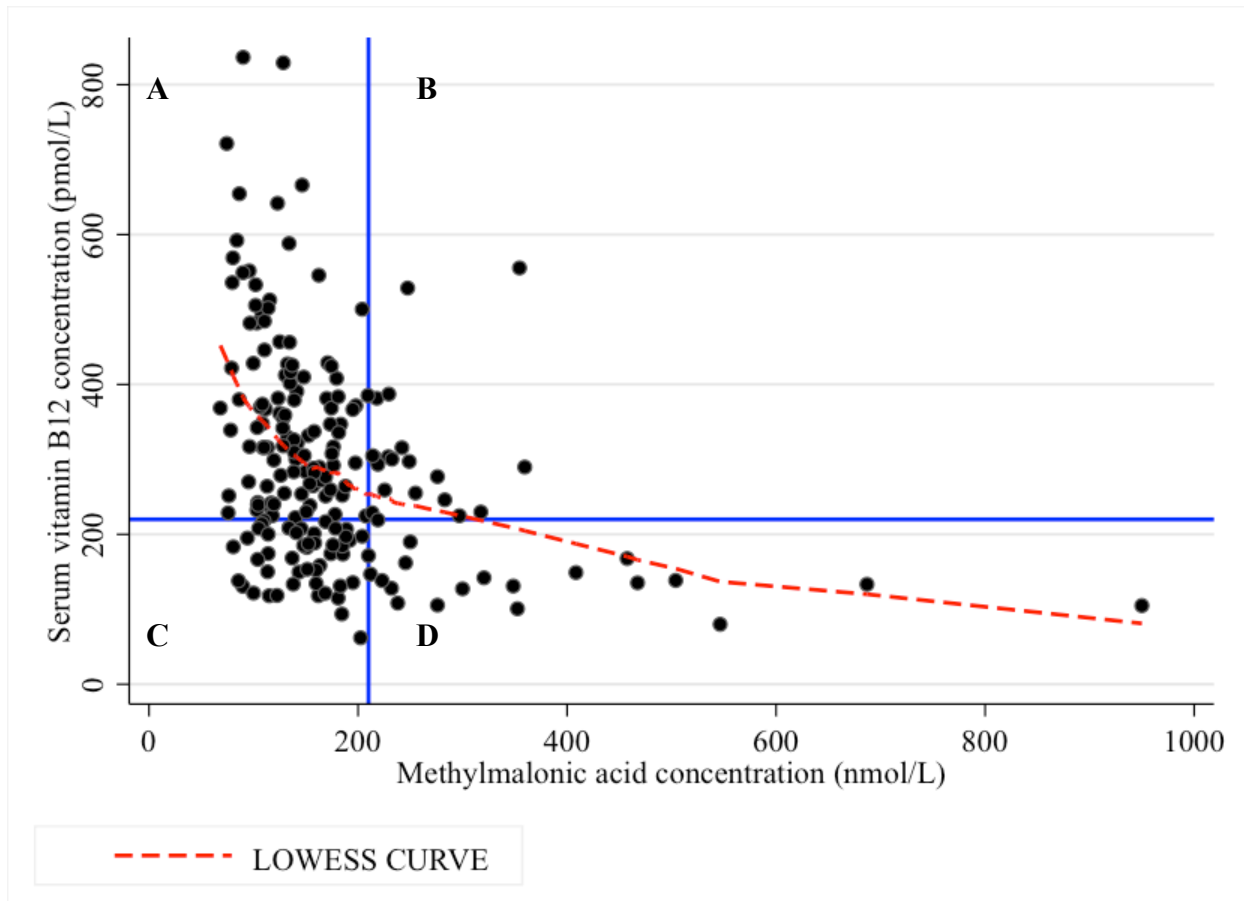
Serum vitamin B12 and Methylmalonic acid cut-offs for marginal vitamin B12 deficiency depicted in BLUE

A Concurrent adequacy

B Disagreement

C Disagreement

D Concurrent deficiency

LOWESS CURVE= locally weighted scatterplot smoothing curve (depicts locally weighted regression of the y variable over the x variable)

Figure 4-10 Rates of Disagreement and Concurrent Marginal Vitamin B12 Deficiency (Bailey Serum Vitamin B12 Cut-offs)

Serum vitamin B12 and Methylmalonic acid cut-offs for marginal vitamin B12 deficiency depicted in BLUE
A Concurrent adequacy
B Disagreement
C Disagreement
D Concurrent deficiency
LOWESS CURVE= locally weighted scatterplot smoothing curve (depicts locally weighted regression of the y variable over the x variable)

Figure 4-11 Rates of Disagreement and Concurrent Chronic Vitamin B12 Deficiency (Conventional Serum Vitamin B12 Cut-offs)

Serum vitamin B12 and Methylmalonic acid cut-offs for marginal vitamin B12 deficiency depicted in BLUE
A Concurrent adequacy
B Disagreement
C Disagreement
D Concurrent deficiency
LOWESS CURVE= locally weighted scatterplot smoothing curve (depicts locally weighted regression of the y variable over the x variable)

Figure 4-12 Rates of Disagreement and Concurrent Chronic Vitamin B12 Deficiency (Bailey Serum Vitamin B12 Cut-offs)

Serum vitamin B12 and Methylmalonic acid cut-offs for marginal vitamin B12 deficiency depicted in BLUE
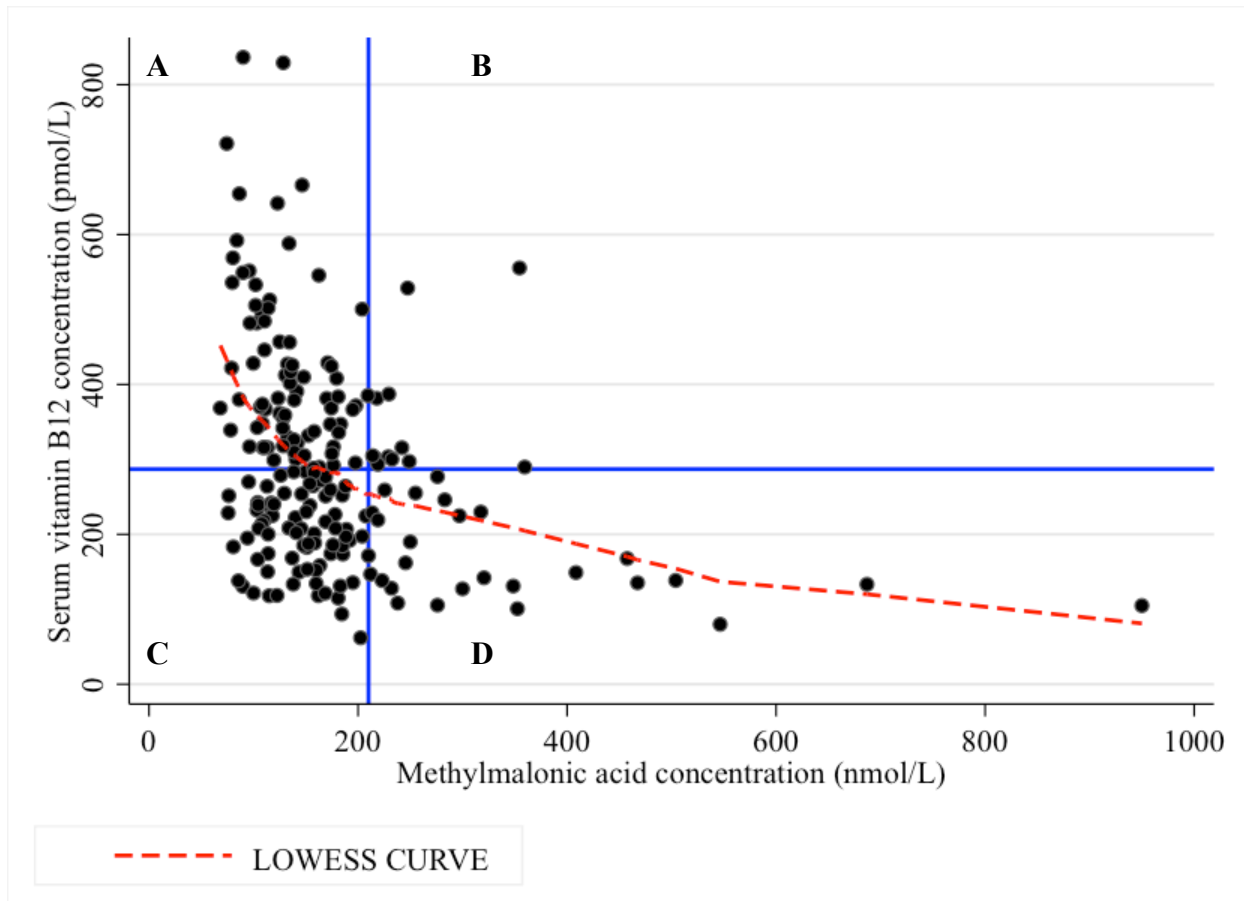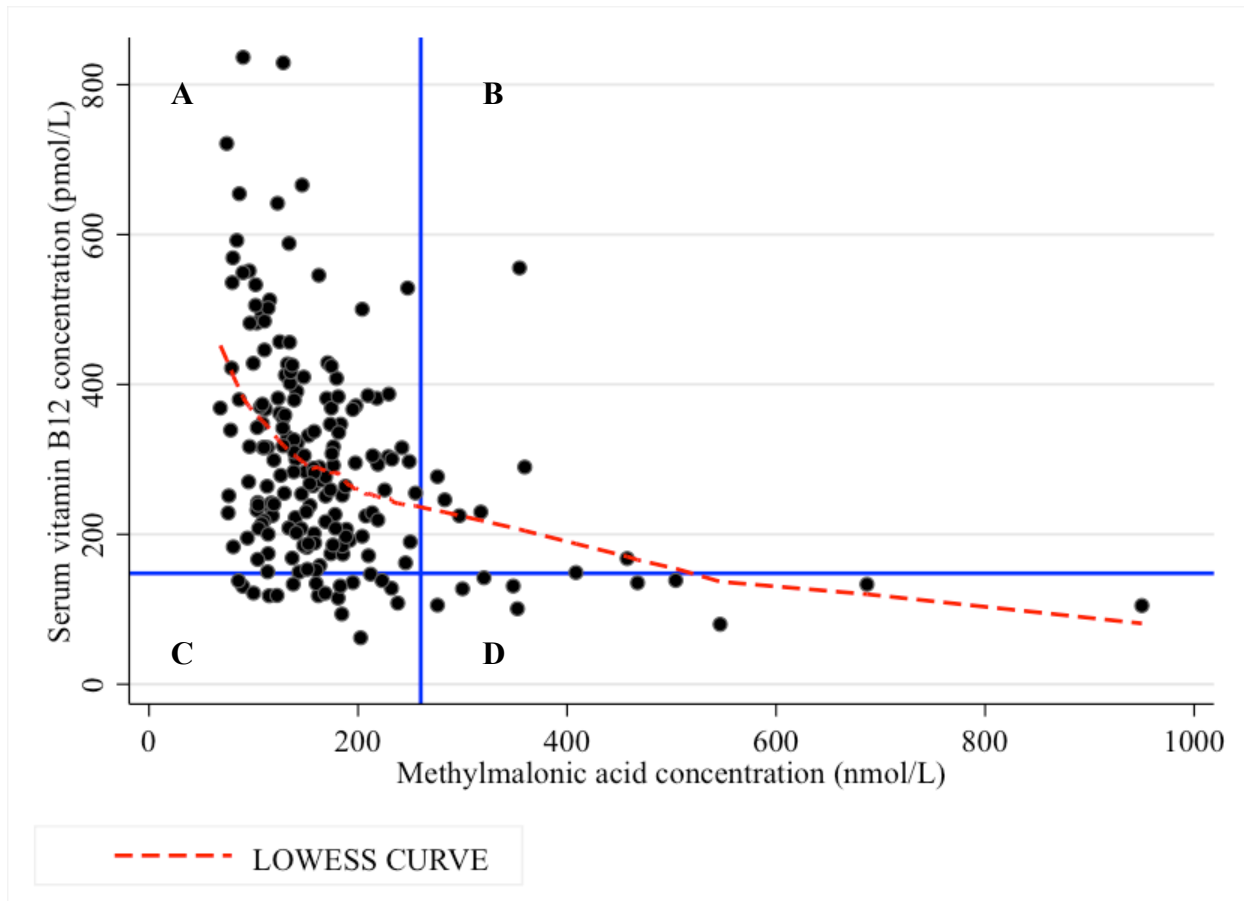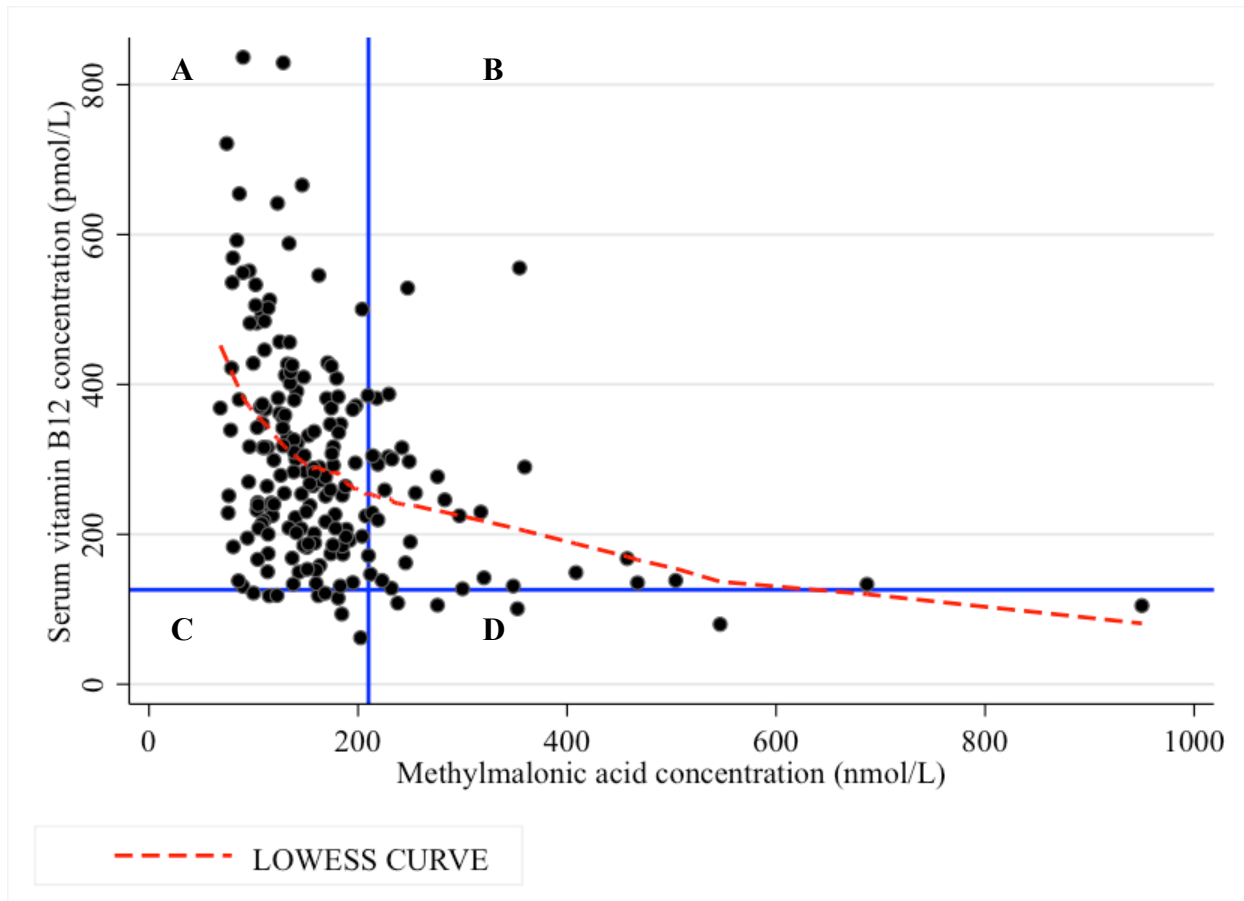**A** Concurrent adequacy
B Disagreement
C Disagreement
D Concurrent deficiency
LOWESS CURVE= locally weighted scatterplot smoothing curve (depicts locally weighted regression of the y variable over the x variable)

To assess the characteristics of the misclassified groups, subjects were grouped by the four metabolic profiles established by Bailey *et* al (106) **(Table 4-36)**. These profiles use a combined measure of normal or abnormal SB12 and MMA concentrations (106).

Low SB12 and elevated MMA (profile 1) occurred in 5% of individuals. This profile had very low SB12, very high MMA, the lowest holoTC concentrations, the highest (50%) proportion of individuals below the holoTC cut-off of 35 pmol/L and is the group most likely to reflect true B12 deficiency.

Low SB12 and normal MMA (profile 2) occurred in 9% of individuals. These individuals had the lowest SB12 concentrations, normal MMA concentrations, moderate holoTC concentrations, and a moderate proportion of individuals (32%) below the holoTC cut-off of 35 pmol/L.

Normal SB12 and elevated MMA (profile 3) occurred in 4% of individuals. These individuals had moderate SB12 concentrations, very high MMA concentrations, moderate holoTC concentrations, and a moderate proportion of individuals (25%) below the holoTC cut-off of 35 pmol/L.

Normal SB12 and normal MMA (profile 4) occurred in 82% of individuals. These individuals had the highest SB12 concentrations, the lowest MMA concentrations, significantly higher holoTC concentrations than the other profiles, and a significantly lower proportion (2%) of individuals below the holoTC cut-off of 35 pmol/L than the other profiles. Profile 4 was the group most likely to represent true B12 adequacy.

In terms of non-biochemical characteristics: supplement use was significantly lower in profile 2 than profiles 1 and 4 but did not differ among the other groups; dietary B12 intake from food and supplements was significantly lower in profile 2 than profile 4 but did not differ among

the other groups, and B12 intake from food alone was significantly lower in profile 1 than profile 4 but did not differ among the other groups. There was no difference in the proportion of OC users among profiles, despite a trend towards the highest proportion of use in profile 1 individuals. There was no difference in the rate of anemia or macrocytosis among profiles but there was a trend towards a greater proportion of individuals having evidence of macrocytosis in the profile 1 group. There were no differences in the proportion of first generation immigrants among profiles but a trend towards less first generation immigrants in the profile 4 group. The proportion of obese individuals was significantly higher in profile 2 than profile 4, and there were no other differences among groups despite a trend towards a higher proportion of obesity in profile 1.

Overall, there was evidence to suggest that profile 1 represents true deficiency, profile 4 represents true adequacy, and that profile 2 and 3 individuals may still be at risk for B12 deficiency. Profile 2 individuals, despite having normal MMA concentrations, appeared to have similar characteristics indicative of deficiency risk to profile 1 individuals. Profile 3, despite having normal SB12 concentrations, appeared to have certain characteristics indicative of deficiency risk although these observations were not as profound as for profile 2.

Table 4-36 Summary of Subject Characteristics for Profiles of Functional Vitamin B12 Deficiency

	Profile 1[a]	Profile 2[b]	Profile 3[c]	Profile 4[d]	p-value
No. of subjects	10	19	8	167	
Subjects per group (%)	5	9	4	82	
Demographic characteristics					
Age	26.5 (24.0,33.0)[a]	26.0(23.0,31.0)[a]	28.5(25.0,33.0)[a]	27.0(23.0,29.0)[a]	0.28
South Asian (frequency,%)	3(30)	9(19)	4(50)	39(23.4)	
Methylmalonic Acid (nmol/L)	410.0(320.5,546.5)[a]	174.5(122.3,202.5)[b]	336.0(290.0,384.0)[a]	141.0(111.2,176.0)[b]	**0.0001**
Serum B12 (pmol/L)	129.1(104.9,135.3)[a]	121.5(114.8,134.3)[a]	237.8(196.2,283.3)[b]	300.2(224.9,383.5)[b]	**0.0001**
Holotranscobalamin (pmol/L)	33.1(28.5,44.5)[a]	44.6(34.5,55.0)[a]	41.1(37.5,55.7)[a]	70.0(57.7,92.5)[b]	**0.0001**
Low <35 pmol/L(%)	50[a]	32[a]	25[a]	2[b]	
Diet					
B12 supplement user (%)	40[a]	5[b]	13[a,b]	33[a]	**0.02**
B12 intake (μg/d) food/supplements	2.0(1.3,3.6)[a,b]	2.8(2.0,3.6)[b]	3.4(2.0,4.6)[a,b]	4.3(2.5,9.0)[a]	**0.04**
B12 intake (μg/d) food alone	1.6(0.9,2.4)[a]	2.6(1.9,3.1)[a,b]	2.5(1.5,4.2)[a,b]	2.8(2.0,4.2)[b]	**0.02**
Medications					
Oral Contraceptives (%)	40[a]	26[a]	13[a]	29[a]	0.6
Hematological outcomes					
Anemia (%)	10[a]	32[a]	13[a]	17[a]	0.4
Macrocytosis (%)	30[a]	26[a]	13[a]	15[a]	0.4
Social and lifestyle characteristics					
First generation immigrant status (%)	50[a]	53[a]	63[a]	34[a]	0.1
Obesity (%)	20[a,b]	21[b]	13[a,b]	5[a]	**0.02**

[a] Low serum B12 (SB12) high plasma methylmalonic acid (MMA)[b] Low SB12 normal MMA[c] normal SB12 and high MMA[d] normal SB12 and normal MMA
Continuous variables compared using one-way ANOVA or Kruskal Wallis followed by Bonferroni post-hoc test. Categorical variables compared logistic regression.
p-values represent overall significance test for all associations. Statistically significant differences (p<0.05) between groups within a row are denoted by not sharing a common superscript. **Abbreviations:** B12 = Vitamin B12

Chapter 5: Discussion

The discussion will be presented in nine subjections: **[1]** characteristics of the study population, **[2]** comment on recruitment, **[3]** rates of deficiency, **[4]** clinical outcomes, **[5]** determinants of B12 status, **[6]** multiple biomarker analysis, **[7]** study limitations, **[8]** suggestions for future research, and **[9]** final conclusions.

Sections 1 and 2 provide a commentary on the study population and our recruitment process to provide context to the reader necessary to follow sections 3 through 6, which present my interpretation of the study results in the context of previous research findings and hypotheses. Section 7 addresses the relevant limitations of the study design necessary for the reader to clarify the main findings. Section 8 lists my suggestions for future research to address knowledge gaps in this research area, and to build upon the work of this thesis. Section 9 provides a summary of this discussion chapter and closing statements.

In summary, the major results of this thesis support that in a highly educated and health conscious convenience sample of women aged 19-35, with a high rate of supplement use and ASF restricted diets:

[1] There is a substantial rate of biochemical B12 deficiency based on SB12 concentrations and women of SA and European ethnicity may be vulnerable to marginal and chronic B12 deficiency. However, these rates are reduced substantially when multiple biomarkers are considered and there was no incidence of macrocytic anemia and confounding hematological parameters. Neurological symptoms were not assessed making it impossible to comment on the rate of true clinical B12 deficiency.

[2] There is a substantial rate of dietary B12 inadequacy (intakes below the EAR) that may be attributable to prevalent meat restricted diets and is reduced by B12 supplement intake.

[3] Common genetic variants in B12 related genes are not associated with B12 status in the present study sample as evidenced by the lack of association in multivariate models. However, given the observations of other researchers and the isolated univariate associations we observed in this present study, the association of genetic variants with impaired B12 status warrants further investigation.

[4] Vitamin B12 biomarker concentrations are associated with obesity, ethnicity, OC use, B12 supplement use, first generation immigrant status, and dietary intake of B12. These variables are potential candidates to explain susceptibility to changes in B12 biomarker concentrations suggestive of B12 deficiency in women of childbearing age and are of interest to further investigate as modifiable risk factors in the prevention of B12 deficiency

[5] The choice of biomarker cut-off and combination of biomarkers in multiple biomarker assessment has a significant impact on the rate of combined metabolic deficiency that is quantified and the rate of disagreement. Newly proposed cut-offs by Bailey may reduce the rate of gross disagreement.

[6] The use of biochemical profiles that consider multiple biomarkers may refine assessment of B12 status by reducing the rate of false positives. However, evidence from the present study suggests that when using conventional cut-offs to define these profiles, individuals classified as deficient by only one biomarker, and therefore classified as adequate, may still be at risk of deficiency.

5.1 Characteristics of Study Population

The study population was a group of healthy women aged 19-35 with no self-reported existing health conditions and/or medication use known to affect B12 status, other than OCs. There was a lower proportion of obesity in our sample compared to national (18%) and British Columbia (14%) averages but we did observe significantly higher rates in the SA group

compared to the European group. In the SA group 50% had BMI values above the cut-off for overweight or obesity, when ethnic specific cut-offs were applied. In addition to reports of higher rates of obesity and central adiposity, SAs have a lower proportion of lean body mass (238,239). This translates to increased adiposity at lower BMI and is what has prompted researchers to propose ethnic specific cut-off values to properly assess risk (225,226).

Over one third of the study sample reported first generation immigrant status, with a significantly higher proportion of first generation immigrants within the SA group (69%). Although seemingly high, this proportion among the South Asian group is consistent with national estimates, which are four times higher than the proportion of immigrants in the general Canadian population (19). Over 95% of subjects in the present study reported greater than high school education and although education levels in Metro Vancouver are slightly higher (56% > high school education) than national averages (51% > high school education), the present study population was relatively highly educated, which could reflect a proportion of recruitment occurring in academic and healthcare settings (240). Reported income was fairly consistent across groups with over 50% reporting middle-high to high income. The large percentage of students who participated in the study may have contributed to an underestimation of the rate of highly affluent participants, as sources of income such as scholarships, student loans, and parental support were not considered in the income calculation. Impaired vitamin B12 status has been associated with low SES in Colombia and India, and use of nutritional supplements is reduced in Canadians with lower income and education (241). If the association between impaired B12 status and low SES is present in the Metro Vancouver population then the present study may have underestimated the true rate of deficiency, given the higher SES of the sample.

However, we cannot make this conclusion, as there is no data on the influence of SES on B12 status in the Canadian population.

In contrast, a high proportion of meat-restricted diet practices were reported by the study participants, which may have led to an overestimation of deficiency rates compared to populations with lower rates of meat-restriction. However, 29-43% of these individuals reported B12 supplement use compared to 28%-31% of individuals not practicing ASF restricted diets. Supplement use may have attenuated the risk for impaired B12 status associated with vegetarian practices to some extent.

Overall, given the sampling methods and demographic characteristics we observed we cannot conclude that this sample is representative of the general female population in Metro Vancouver but rather that it is a good representation of women aged 19-35 of SA and European descent who are nutritionally conscious and highly educated.

5.2 Comment on Recruitment

There were substantially more European women successfully recruited to participate than SA women despite a concentrated recruitment strategy. The lack of success in obtaining the target sample size is not necessarily a reflection on the recruitment process, which used a comprehensive and evidence-based approach to targeting the SA population (**Chapter 3.4.3**). Difficulty recruiting SA women has been previously reported by other researchers conducting clinical research on SA populations (242–245). Further, SA women are widely reported to experience hesitance when engaging with western healthcare systems. Many of the SAs residing in Vancouver are first or second generation immigrants and may not have had sufficient time to acclimatize to an appropriate level of familiarity with health research, and the Canadian healthcare system. The most widely reported strategies to combat these difficulties include involving physicians and SA health care workers in the recruitment process, and using

community recruitment based approaches (246). We chose not to pursue physician-assisted recruitment as we were targeting a healthy population. One reason for the low enrolment numbers may be the lack of face-to-face counseling sessions (e.g. free dietetic counseling) provided—such sessions have been shown to increase enrolment of SA women (242). The low monetary incentive may also have contributed to low SA enrolment. For example, many prospective participants declined to participate once the monetary value of the gift-card provided as compensation was disclosed during the informed consent process. Unfortunately we did not collect data on subject retention so the percentage of dropouts pre-assessment is unknown.

5.3   Rates of Deficiency

There was a substantial rate of SB12 concentrations indicative of risk for both marginal and chronic deficiency in the study population. The overall 34% inadequacy rates appear marginally higher than what has been observed by other Canadian studies investigating B12 deficiency in women of childbearing age, such as the study by Ray *et al* which observed an overall 26% inadequacy rate, and the general Canadian population which had a ~23.5% inadequacy rate based on CHMS data. In particular the rate of chronic deficiency (14%) was somewhat higher than the general Canadian population (~5%) and Canadian women of childbearing age (~7%).

We did not observe differences in the concentration of SB12 between ethnic groups contrary to the findings of other studies comparing B12 status of SA groups to other ethnic groups. Median SB12 concentrations were 39 pmol/L lower in SA participants in the present study compared to European participants, compared to differences of 87-124 pmol/L observed by others (**see Table 2-9).** Due to the low numbers of SA participants we did not have statistical power to detect this clinically insignificant difference in SB12 concentration. Despite the lack of difference in concentration there was a greater proportion of the SA group classified as having

chronic deficiency (SB12 <148 pmol/L) (p=0.06) suggesting that SA women of childbearing age may be more susceptible to chronic deficiency than their European counterparts.

5.4   Clinical Outcomes

To assess clinical consequences of low B12 status we looked at hematological parameters. There was no incidence of concurrent macrocytosis and anemia ("macrocytic anemia") and no significant differences in the concentration of Hb or MCV between chronically deficient, marginally deficient and adequate groups (based on their SB12 concentrations). In addition there was no difference in rates of anemia or macrocytosis between groups, despite both being slightly higher in the chronically deficient group. This is similar to the findings of Bailey *et al* who found no differences in the prevalence of macrocytosis across B12 status groupings in the NHANES (1999-2004) population (106). These observations suggest that biochemical evidence of deficiency was not translated into increased risk for hematological clinical outcomes in the study population.

We did observe a higher rate of anemia in the SA group. Rates of anemia in SA may be attributed to other nutritional anemias. Iron deficiency anemia (microcytic anemia) for instance is common in SA women of childbearing age but were unlikely to act as confounders in our study population (247). Carmel suggests that MCV is a better indicator of B12 deficiency as it precedes the development of anemia by months and is not confounded by co-existing non-macrocytic anemias. Mean corpuscular volume, however, is affected by alcoholism, liver disease, many pharmaceutical treatments, and pregnancy; factors that must be considered in assessment (248). South Asian participants had significantly lower MCV than Europeans (p<0.0001), which was contrary to our hypothesis of greater impairment of B12 status in SA participants. It has been observed that MCV does not correlate well with deficiency as defined by SB12 values and that other indices should be combined in assessment, which could suggest that

without a comprehensive clinical assessment, accurate identification of deficiency may be difficult (249). A comprehensive clinical assessment might potentially include measurement of neutrophil segmentation (proposed as the most sensitive peripheral blood cell marker of B12 deficiency (250)) and measures of neurological impairment (e.g. spinal magnetic resonance imaging, nerve conduction studies, motor evoked potential, visual evoked potential, tibial somatosensory evoked potential (251)) though cost and availability may limit the applicability of these assessment tools. In B12 status assessment it may also be prudent to include measures of iron deficiency to enhance hematological observation clarity (81). In summary, we cannot make any conclusions about the ability of the biochemical measurements we made to predict true clinical deficiency, due to the absence of clinical assessment tools from the present study. The absence of macrocytic anemia would suggest that biochemical measurements may overestimate rates of deficiency, or at least rates of hematological impairment due to B12 deficiency, but without full hematological parameters and neurological assessment this could not be determined.

5.5   Determinants of Vitamin B12 Status

To address the limitations imposed by the use of finite cut-off values to define deficiency, determinants of B12 status were assessed using both multiple logistic regression models to investigate which variables influence categorization as deficient, and multiple linear regression models to investigate which variables influence biomarker concentration. The variables that influenced B12 status and concentration are summarized below.

5.5.1   Vitamin B12 Intake from Food and Supplements

Overall, greater dietary B12 intakes appeared to support optimal B12 status. Dietary B12 intake was positively correlated with select dietary intake variables including total protein intake, animal protein intake, servings of meat and alternatives, and servings of dairy and alternatives in the present study. These observations were similar to a study on preadolescent Indian girls,

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which reported higher B12 intakes in meat eaters attributed to consumption of fish, beef, chicken, milk and dairy, and for Indian pregnant women in whom fish and yoghurt consumption was associated with better B12 status (140,252). In the present study, consumption of meat and milk products as well as total and animal protein was associated with reduced odds of dietary inadequacy.

Median dietary B12 intakes from food alone (2.7 µg/day) in our study sample were similar to that of females aged 19-30 (n=176) measured in the British Columbia Nutrition Survey (BCNS) (1999) (2.3 µg/day) (253), and somewhat lower than the median intakes of females aged 19-30 in the British Columbia population (n=208) (3.7 µg/day), and the general Canadian population (n=1854) (3.1 µg/day) as measured in the CCHS. Median dietary intake from food and supplements was 3.7 µg/day in females aged 19-30 in the BCNS, similar to the intake of 3.5 µg/day measured in the present study.

The observation that dietary intake and supplement use is associated with higher B12 biomarker concentrations is supported by other evidence that providing sufficient intakes contributes to adequacy in healthy populations. In populations without malabsorption or other potential risk factors for B12 deficiency, consuming adequate B12 from the diet or other sources should be an effective means of maintaining adequate status. After adjustment for supplement use, B12 intake, total protein intake, and servings of meat were associated with higher SB12 concentration. It has been observed that plasma B12 is positively associated with dietary patterns that include frequent intake of beef, chicken, and dairy products in a dose response manner (p=0.008) (241). It has also been reported that diets low in meat, such as lacto-vegetarian diets are negatively associated with SB12 concentration (254). Daily intake of B12 from food and supplements analyzed as a continuous variable was not associated with SB12 concentrations in

multivariate models. It is possible that several subjects with very high intakes of B12 supplements influenced this outcome. SB12 concentration does not increase in a dose-dependent manner with chronically high intakes due to its saturable absorption and transport mechanisms.

The rate of inadequate dietary intake from food alone (26-34% depending on supplement use) was substantially higher than the national estimates of 12% for non-supplement users, and 8% for supplement users reported from the CCHS (Cycle 2.2) suggesting that the study population would be at relatively higher risk for deficiency than the general Canadian population based on dietary B12 intakes alone (185). However, the rate of inadequacy (from food alone) that we measured was similar to that measured (38%) for females 19-30 in the BCNS(1999) so it is plausible that the intakes we measured are not low but rather typical of a British Columbia population.

The high rate of dietary inadequacy from food alone may be attributable in part to the rate of meat restricted dietary patterns, as they have been associated with lower B12 intakes. The rate of ASF restriction ranged from 15% with full meat restriction, to 34% with restriction of only red meat in the present study. The rate of meat-restricted diets in the present study was higher than the rate of 6% observed in the general British Columbia population (182). In a convenience sample of vegetarian and non-vegetarian women aged 18-50 in Vancouver, vegetarians had significantly lower mean B12 intakes (1.68 ± 1.6 µg/day) compared to non-vegetarians (3.51 ± 3.29 µg/day) (p<0.0001) (255). Similarly, in another Vancouver convenience sample of women aged 20-40 recruited for a study on the frequency of ovulatory disturbances, vegetarians had a mean dietary B12 intake of 1.56 ± 0.53 µg/day compared to non-vegetarians who had a mean intake of 3.79 ± 1.60 µg/day (256). This trend is similar to what we observed although the magnitude of difference was smaller in our study.

In the present study, SA participants reported higher intakes of grain products (5.9 vs. 4.7 in European group p=0.004) and lower intakes of vegetables and fruit (4.7 vs. 5.6 in European p=0.03). In adults in Beijing, China, consumption of refined cereal (associated with high carbohydrate content) was associated with a 5.2 x greater risk of low SB12 concentrations (<221 pmol/L), compared to a fruit and milk dietary pattern (257). It is possible that the displacement of animal protein sources by increased refined carbohydrate intake could contribute to reduced B12 intake. It would be of interest to investigate whether certain dietary patterns are associated with poor micronutrient status in the Metro Vancouver population, especially given that the expected associations of vegetarian status with poor B12 status were not observed in the present study.

5.5.2   Supplement Use

The rate of low SB12 concentrations we observed is especially concerning given the rate of B12 containing supplement use (30%) in the study population. We assessed rates of deficiency in supplement users and non-supplement users given that supplement intake is likely to result in improvements in B12 biomarkers in healthy populations. Supplement use was associated with reduced odds for deficiency based on SB12 concentration cut-offs and higher SB12 and holoTC concentrations, although it was not associated with the functional biomarker MMA. This observation is consistent with NHANES 2003-2006 results, which showed that B12 supplement use was associated with a 21% increase in SB12 concentrations over non-supplement use (258). B12 inadequacy was slightly less prevalent in supplement users (<5%) compared to non-supplement users (8%) in the Canadian population. Prevalence of nutritional supplement use in the study population was similar to national rates. Use was reported by 48% of the study population (vs. 42% Canada-wide in women 19-50), and the use of nutritional supplements containing B12 was reported by 30% of the subjects (vs. 27% Canada-wide, CCHS 2.2 data)

(185). Dietary inadequacy from diet alone was similar in supplement users (34%) and non supplement users (26%) in the present study (p=0.29) and when comparing supplement users (12%) and non supplement users (8%) Canada wide. The rate of inadequacy from food and supplements was only 4% in supplement users, similar to the <5% rate of inadequacy observed in the CCHS, and substantially lower than the 21% inadequacy rate measured in the BCNS (1999) for females aged 19-30 (253). Supplemental B12 from nutritional supplements or food sources may be an effective means of improving or maintaining B12 status in individuals with traditional risk factors for deficiency, such as ASF restricted diets.

Within the supplement use group, five individuals had SB12 <148 pmol/L suggesting that reported supplement use is not a foolproof determinant of B12 adequacy. We did not assess supplement compliance or duration of supplement use but rather asked about the dose and frequency of consumption. If there was non-compliance or if supplementation had been recently initiated it may partially explain the presence of deficiency in some supplement users. On the other hand these individuals could potentially have underlying malabsorption or other factors counteracting the benefit of supplement intake.

5.5.3   Genetic Variants

In general, the collective outcomes of studies investigating the association of the four variants we assessed with impaired B12 status have been inconsistent. In the present study, these genetic variants did not show an association with B12 status. This result may suggest a negligible effect of these variants on B12 biomarker concentrations in healthy women aged 19-35, or stem from an insufficient sample size to detect differences and potential confounding by supplement use.

MTHFR C677T Variant

The frequency of the *MTHFR* C677T T allele was significantly lower in the SA group (9% vs 34% (European group)). This is consistent with the allele frequencies observed in other SA populations compared to European populations (259,260). The TT genotype was not associated with lower B12 biomarker concentrations. In univariate analysis it was observed that the *MTHFR* C677T CT group but not TT group had lower MMA concentrations, and a tendency towards higher SB12 concentrations. This has not been previously observed and may be partially attributable to both the small sample size in the present study and the cut-off value implemented to assess B12 status. It is unlikely that this variant would have a profound effect on MMA concentrations, as it would only have an indirect impact through reductions in B12 availability. Overall, this variant did not significantly impact B12 status of the present study population based on multivariate analysis.

TCN2 Variants

Transcobalamin 2 genetic variants are associated with lower holoTC concentrations rather than an effect on SB12 concentrations (209). In univariate analysis, there were no differences in the subject or biomarker characteristics between genotypes for the *TCN2* C776G variant. Although holoTC levels were lowest and MMA levels were highest in the GG group these differences were not significantly different from the other genotypes. Similarly, Riedel *et al* did not observe any differences in holoTC concentrations between CC (54 ± 0.67 pmol/L), CG (54 ± 1.2 pmol/L), and GG (56 ± 1.8 pmol/L) genotypes.

There was a very low occurrence of the GG genotype for the *TCN2* A67G variant. Due to this low cell count, the *TCN2* A67G genotype was analyzed by grouping the GG and AG genotypes together and comparing against the AA genotype. The rate of low holoTC

concentrations (<35 pmol/L) was higher in the AG+GG group, the holoTC to B12 ratio was lower, and there was more a higher rate of macrocytosis. This genotype has been previously observed to influence susceptibility to low holoTC concentrations as well as reduced holoTC:B12 ratio (212). Riedel *et al* observed a significantly lower holoTC concentration in 67AG (55 ± 0.75 pmol/L) and 67GG genotypes (48 ± 2.1 pmol/L) compared to 67AA (62 ± 0.67 pmol/L). These results suggest that the G allele of the *TCN2* A67G genotype may have a moderately negative influence on holoTC concentrations. The *TCN2* variants did not show an association with B12 status when assessed in multivariate models.

FUT2 G893A Variant

The variant we investigated (*FUT2* G893A) has previously shown the strongest association with SB12 levels in genome wide association studies and was associated with reduced SB12 concentrations in SA individuals (212,261). In the study population, there was a lower proportion of individuals homozygous for the G allele for the *FUT2* G893A variant classified as deficient (p=0.02). These individuals also had the highest SB12 concentrations compared to AG and AA individuals thought this difference was not significant (p=0.2). This is contrary to the more common observation that the GG genotype is associated with impaired B12 status and that the AA genotype is associated with higher plasma B12 concentration. Our observation does not fit with the mechanistic principle behind the influence of this variant on B12 status. H-antigen synthesis by FUT2, as well as *FUT2* variants, are reported to mediate *H.pylori* infection (262). *H.pylori* infection can result in atrophic gastritis and reduced B12 status and is more prevalent in individuals with low SB12. We did not assess *H.pylori* infection rates in the present study and cannot make conclusions about the association of the *FUT2* variant with infection rates or the influence of *H.pylori* infection rates on deficiency in the study population.

The rate of *H.pylori* has been observed to be as high as 80% in SA countries (263). It would be of interest to determine the prevalence of *H.pylori* in Canadian SAs. Investigating the association of the *FUT2* G893A variant with impaired B12 status in a population with a larger sample size than the present study may provide clarification its role in determining risk of B12 deficiency.

5.5.4   South Asian Ethnicity

South Asian ethnicity was not associated with an increased odds for marginal deficiency, high MMA, or low holoTC but the increased odds for chronic deficiency in SA individuals is in line with several studies showing highly prevalent deficiency in SA populations (**See Chapter 2.3, Table 2-7, Table 2-8**). SA ethnicity was a risk factor for having SB12 concentrations <148 pmol/L independent of dietary intakes and supplement intake suggesting that other factors may be at play in this subgroup, such as *H.pylori* infections, underlying GI conditions, and genetic variants. The lack of association of SA deficiency rates with diet may be attributable to inadequacies in the dietary assessment tool, which will be discussed at length within the study limitations section (**Chapter 5.7**). No provisions were made to accommodate the heterogeneous nature of religious and regional variation in the SA diet. These accommodations may be important for successful application of FFQs in this ethnic group.

5.5.5   Obesity

Elevated BMI has been previously associated with low B12 biomarker concentrations (1,191,192,258,264). This association was not strong in the present study as obesity was not associated with SB12 or MMA concentration or status in multivariate models, despite significantly higher odds of low holoTC concentrations <35 pmol/L in the obese group. Many studies focus on the risk for deficiency in patients that have undergone bariatric surgery. In these individuals the increased risk for B12 deficiency can be assigned to malabsorption imposed by physical alterations of the GI tract. For obese patients who have not undergone surgery, lower

B12 concentrations have been observed compared to normal weight subjects. Children, adolescents and adult obese participants in the CHMS were more likely to be B12 deficient than non-obese individuals. Data from this survey showed that significantly fewer obese adults (20-79 y) (66.7%) had adequate SB12 concentrations (>220 pmol/L) than individuals in the overweight (79.2%) or normal (76.9%) categories (1). In addition in the NHANES 2003-2006 population, increased BMI was associated with reduced SB12 concentrations and higher SB12 concentrations were observed in individuals who were in the highest category of physical activity (>1000 metabolic equivalence of task minutes/week) (258). Macfarlane *et al* suggested that the association of increasing BMI with lower SB12 concentrations could be attributed to poor diet quality and lower rate of supplement use in obese individuals (1). The rate of B12 supplement use in obese individuals in the present study was 25% compared to 31% in the non-obese group but this difference was not statistically significant (p=0.78). Macfarlane *et al* also suggested that there may be underlying physiological changes that could contribute to poorer B12 status in obese individuals. Due to the correlations that others have observed between BMI and B12 status, it would be pertinent to assess the absorption of B12 in a case-control study comparing obese and non-obese women of childbearing age. Further, diet quality and dietary intake of B12 in obese individuals should be assessed in the Canadian population.

Obesity and B12 deficiency are both significant risk factors for poor pregnancy outcomes such as NTDs. It is not yet understood whether the presence of either factor predisposes to the alternate outcome (i.e. does B12 deficiency contribute to obesity risk and vice versa). This is important to investigate to determine whether obese women have higher B12 requirements prior to and during pregnancy. The mechanism behind the relationship between obesity and B12 deficiency should be assessed using multiple indices in addition to BMI to define obesity. As

BMI is only a surrogate measure methods such as dual energy x-ray absorptiometry or bioelectric impedance analysis would ensure a more accurate representation of obesity rates and allow for the assessment of the association of additional factors such as total lean body mass, total fat mass, and specific fat depots with B12 status.

5.5.6   Oral Contraceptives

Prevalence of OC use in this population was close to the national estimates for OC use in sexually active females (265) but it was lower in SA compared to European subjects. This reflects culturally motivated aversions to OC use, which have been documented in SA women in Metro Vancouver (266).

Historical accounts of lower B vitamin status, including low SB12, TCN1 and SB12 binding capacity have been reported in OC users (214–216,221). However, it has been observed that low SB12 concentrations do not translate into increased urinary MMA excretion suggesting that the decrease is related to the proportion of SB12 binders and does not represent true deficiency (217). Fluctuations in tHcy concentrations have been observed between the low and high hormonal phase of the menstrual cycle and represent cyclically recurrent periods of elevated tHcy in OC users (267). Hjelt *et al* conducted a case control study of women using combination OCs vs controls in which they observed that mean SB12 and B12 binding capacity was reduced in OC users (221). In this study, administration of high dose estrogen resulted in further reduced B12 binding capacity, SB12, and Hb in OC users. The applicability of these earlier studies today is limited due to the significant changes in hormonal formulations in OCs over time. The impact of first generation OCs is not representative of the third generation OCs currently prescribed. In addition, many of these studies were cross sectional in nature making it difficult to examine a cause and effect relationship.

One study on the current formulations of OCs has shown that SB12 concentrations are significantly lower in OC users compared to non-OC users (172 pmol/L vs 318 pmol/L in controls) (268). This difference persisted over 4 time points during a 12-week period. Berenson *et al* conducted a randomized controlled trial to assess the impact of progesterone (Depo-Provera) and OCs (20 µg ethniyl estradiol, and 0.15 mg desogestrel) on SB12 (269). The subjects were monitored for 3 years and the SB12 concentration in OC users was shown to sharply drop (-72 pmol/L) over the initial 6 months and then plateau (269). In progesterone users, the initial 6-month drop in SB12 was not as large (-47 pmol/L) but concentrations continued to decrease past 6 months. Overall, there was no evidence to suggest a clinically significant influence of these particular formulations of OCs on SB12 despite the drop in concentrations, as they remained above the cut-off for impaired B12 status. It has been proposed that the decrease in SB12 could be attributed to changes in the concentration of TCN1, which is associated with a falsely low B12 level in OC users and redistribution of B12 rather than actual cellular depletion (214,215,217). To address this theory, changes in all B12 biomarkers, including holoTC should be investigated in OC users.

In the present study median holoTC was lower in OC users, and median MMA was higher but we did not observe significant differences between groups. However, OC use was associated with an increased odds (OR: 3.3; 95% CI: 1.1, 9.6) of having holoTC concentrations below 35 pmol/L. Riedel *et al* observed that OC users had 25% lower holoTC concentrations than non-OC users ($p<0.01$) (220).

If OC use causes a reduction in B12 at the cellular level individuals with long-term use may be at disadvantaged B12 status. This is especially relevant for women of childbearing age who may be discontinuing OC use soon before becoming pregnant, those who become pregnant

while on OCs due to poor compliance and for individuals with long term ASF restricted diets or malabsorption and concurrent OC use. These populations may require B12 intakes above that of the general population to maintain adequate status. The effect of OCs on B12 deficiency risk should be further assessed in a range of available formulations given the vast differences in hormone content, controlling for high and low hormone periods of the menstrual cycle and assessing the impact of hormone levels on multiple biomarkers and clinical outcomes to determine whether re-distribution, or real B12 depletion is the result of the effect of OCs on B12 biomarkers.

5.5.7   First Generation Immigrant Status

In the present study, first generation immigrant status was associated with an increased risk for lower SB12 and holoTC concentrations, and increased odds for being categorized as deficient based on SB12 levels. In a study on pregnant immigrants in Britain, SA patients had significantly lower mean SB12 (175-202 pmol/L) than the European reference group (286 pmol/L) (270). In Canada, mean SB12 concentrations of East Indian immigrants were measured to be 254-264 pmol/L and the rate of SB12 concentrations <118 pmol/L was 6.9 to 7.8% which is high considering the low cut-off value used to define deficiency (152).

In focus group interviews of SA immigrant women in Canada, it was reported that maintaining traditional or cultural dietary practices, which may involve exclusion of ASFs, was preferred over adopting a western diet (271). While, it has been suggested that dietary acculturation of immigrants actually decreases the healthfulness of the diet (272,273), if a traditional diet is lacking in a specific nutrient, as vegetarian diets are lacking in B12, maintenance of traditional practices may promote select nutrient inadequacies. Accordingly, lack of dietary acculturation may contribute to the persistent risk for B12 deficiency observed in immigrant SA populations (274). New immigrants may also face complex issues such as food

insecurity, language barriers, and nutrition transition, which could further increase the risk of inadequate nutrition (272). Counseling on the nutritional benefits and risks of vegetarian spectrum diets and on interpretation of Canada's Food Guide in the context of ethnic specific foods may be a positive component in the care of SA patients and new immigrants.

5.5.8   Determinants of MMA

The predictors that were investigated in the present study did not appear to influence risk for deficiency defined by MMA concentration cut-offs or MMA concentration. As a functional indicator, MMA does not decrease with dietary intake, unless in response to repletion treatment. In an assessment of elderly patients in the British National Diet and Nutrition Survey, MMA was not significantly correlated with B12 intake in elderly individuals (275). Correlates of elevated MMA are commonly advanced age, poor renal function, and low SB12 levels (276). The age of the present study's population did not extend to the range where age related declines in GI function might negatively influence B12 status. Renal function would presumably be normal in a study population of healthy young adult women; however, absence of health conditions was self-reported during the subject screening process so it would not be possible to rule out a potential influence of impaired renal function on MMA concentration, especially early benign kidney disease. The prevalence of typically asymptomatic stage 1 and 2 chronic kidney disease in Canadian women (18-79 y) is estimated to be 9.3% based on CHMS data (277). Other factors with the potential to influence MMA concentrations include intravascular volume depletion, inherited methylmalonic aciduria, increased production of propionic acid from bacterial overgrowth, and increased catabolism of cholesterol, BCAA, and odd-chain fatty acids (276). These factors were not assessed in our population but may nonetheless have the potential to explain several observed anomalies (i.e. individuals with normal SB12 or holoTC concentrations and very high MMA levels (>400 nmol/L)). MMA concentration has thus far not been reported

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to be influenced by any common variants independent of those that affect serum SB12 concentrations (276).

5.5.9   Other Determinants of B12 Status

In the NHANES (2003-2006) population B12 status was influenced by smoking status, alcohol intake, and physical activity (258). The rate of tobacco use was too low to assess differences in the present study population. Physical activity level was not associated with B12 status in the present study, however, less than 4% of the population reported low physical activity levels. This factor might be better explored in a more sedentary population or by using more accurate tools to assess physical activity such as pedometers, accelerometers, and heart rate monitors (278). Alcohol intake was not explored as a determinant of B12 status in the present study due to infrequent regular intake amongst subjects.

5.6 Multiple Biomarker Analysis

The cut-offs that we selected for B12 biomarker status assessment, which were used throughout this thesis, were the most commonly encountered cut-off values in the literature but it should be acknowledged that different researchers have proposed higher and lower cut-off values.

The rates of combined metabolic deficiency were substantially lower than the rates determined by single biomarker analysis. Overall, the rate of marginal deficiency was lowest using holoTC and MMA in combination, which would indicate that the use of SB12 likely overestimates the rate of deficiency. The rate of chronic deficiency was similar when using MMA in combination with either SB12 or holoTC cut-offs. If these combined metabolic deficiency rates are more representative of true deficiency, then any study that reports deficiency rates based on SB12 as the sole biomarker could be over reporting on the prevalence of deficiency. This applies to current data on the general Canadian population from the CHMS.

5.6.1 Rates of Disagreement

Gross disagreement, defined as having chronic deficiency levels in one biomarker and adequate levels in another, was less frequent with the use of Bailey cut-offs compared to the conventional cut-offs. However, the rate of moderate disagreement (defined as either **[1]** chronic deficiency based on one biomarker and marginally deficient based on another, or **[2]** marginally deficient based on one biomarker and adequate based on another) was higher with the Bailey cut-offs. These results show that widening the range of marginal deficiency as suggested by Bailey *et al* will result in less gross disagreement and greater moderate disagreement than the conventional cut-off values when used in combination with MMA. The new chronic deficiency cut-off (<126 pmol/L) that they set may be more accurate than the conventional (<148 pmol/L) cut-off.

The rates of disagreement we observed illustrate the importance of using multiple biomarkers to assess B12 status and also the paramount need for research on the appropriate cut-off values for all biomarkers. This is a common suggestion communicated by many researchers (65,70). Without critically evaluating deficiency with the use of more than one index (either biomarker or clinical outcome) there is a significant chance of false positives and negatives. Unfortunately, this option may not always feasible in clinical settings due to lacking infrastructure and/or lack of awareness about the necessity of multiple measurements. Often, comprehensive assessment is foregone and replaced with prophylactic B12 treatment regardless of the etiology of disease when B12 deficiency is suspected.

The ideal approach to defining appropriate cutoffs would be to conduct a clinical or repletion trial in which a quantitative relationship between biomarker concentrations and the appearance of clinical outcomes of presumed B12 inadequacy is established and in which the measurement of biomarkers is traceable to a higher order reference method (106). It would be difficult to conduct these types of studies due to ethical conflicts involved with restricting

essential nutrients or providing different levels of treatment to groups with active disease. This type of information cannot be derived from a cross sectional survey such as the study we conducted. Bailey *et al* used statistical models based on a linear splines framework representing the relationship between SB12 and MMA to estimate cut-points for deficiency (80). This method is vulnerable to choice of model, and the population from which data is derived. Selhub *et al* determined a different cut-off using a linear-linear model (75). The advantage of the Bailey model is that it estimates two knots (cut-offs), which acknowledges that the marginal deficiency state is distinct from chronic deficiency and adequate groups and offers increased biological plausibility. Generation of a model, which explores the association of changes in concentrations of B12 biomarkers with clinical outcomes may further improve on these approaches but requires data that would be difficult and expensive to obtain.

The state of B12 assessment in both clinical and research domains will remain in a precarious state until an evidence based decision can be made in regards to reference methods, cut-off values, and the handling of misclassified individuals in multiple biomarker assessment. Furthermore, until more is known about the clinical implications of marginal B12 deficiency and the significance of changes in biomarker concentrations in relation to this distinct deficiency state it will not be possible to accurately identify individuals who are truly at risk of the unique health consequences that have been proposed to accompany this condition.

5.6.2 Metabolic Profiles of Deficiency

Bailey *et al* proposed 4 metabolic profiles of combined SB12 and MMA status for the purpose of critically evaluating rates of deficiency. These profiles are re-stated in **Table 5-1**.

Table 5-1 Metabolic Profiles of Functional Deficiency

Profile	Reference cut-off [a]	Suggested status
1	SB12<148 MMA >260	True B12 deficiency
2	SB12<148 MMA≤260	Falsely low SB12
3	SB12 ≥148 MMA >260	Marginal deficiency or independently elevated MMA
4	SB12 ≥148 MMA ≤260	B12 adequacy

[a]Bailey *et al (80)*

In the present study, profile 1 was characterized as representative of true functional B12 deficiency and based on the group characteristics we can confidently conclude that this group is the most representative of true deficiency out of the four profiles.

Profile 2 individuals had low SB12 and low MMA concentrations, as well as the lowest holoTC levels of all profiles. Bailey *et al* suggested that this profile was suggestive of falsely low SB12 levels because MMA and tHcy levels were within the normal range. The authors suggested that this group might include individuals with TCN1 deficiency. TCN1 deficiency is thought to be a common contributor to SB12 depletion without the cellular consequences (64,83). Concurrently low holoTC levels in the profile 2 group in the present study provides evidence against the conclusion that deficiency in this group is only attributable to falsely low concentrations and suggests they might be at risk of true depletion.

Profile 3 in the Bailey study was indicative of potential early metabolic changes related to marginal deficiency or elevated MMA unrelated to B12 deficiency. In the present study profile 3 individuals fit that profile with normal but not high SB12, and a lower incidence of elevated MMA concentrations than profile 1. As discussed previously a range of factors may contribute to independently elevated MMA, including most prominently, renal dysfunction, which was not assessed in the present study. This group had a lower rate of risk factors previously determined

to influence B12 status in the study population including OC use and obesity, and dietary intakes which were more similar to that of Profile 4 individuals.

Profile 4 fit the profile for B12 adequacy, all biomarkers falling within normal ranges. It should be noted that there was 15% macrocytosis and 2% low holoTC concentrations in this group, which suggests that a very small proportion of individuals with adequate SB12 and MMA concentrations may still be at risk for deficiency and that with the use of biomarkers alone it may not be possible to absolutely rule out false negatives.

The most significant divergence between our observations and those of Bailey *et al* was that Profile 2 did not suggest falsely low SB12 due to the simultaneously low holoTC levels. HoloTC levels were not measured in the Bailey study so it cannot be determined whether the low SB12 concentrations would have predicted low holoTC concentrations. In our study population supplement use was lower in this group than all other profiles, which may have contributed to the higher rate of low holoTC.

In summary, profiles 1, 2, and 3 all have characteristics suggestive of potentially impaired B12 status. Classifying individuals as deficient (profile 1) or marginally deficient (profile 2 or 3) based on the combined biomarker profiles may reduce the rate of false negatives that results from using a single biomarker. This approach may however, increase the rate of false positives, and could be addressed by the assessment of concentrations of a third biomarker.

5.7   Study Limitations

The cross sectional design of the present study limits our ability to make causal statements about any of the outcomes we observed. In addition, as this was a convenience sample, the study has very limited generalizability to the Metro Vancouver and Canadian populations of women aged 19-35. The final sample size was significantly lower than our initial goal for the SA group. This limits our ability to make statistical comparisons between the ethnic

groups with sufficient power and as such all conclusions regarding ethnicity should be interpreted with caution. This study did not critically assess clinical outcomes. While we did include hematological profile as an outcome variable, no neurological or cognitive measurements were made. Biomarkers can be used to objectively assess the risk of deficiency, and to indicate the depletion of stores or appearance of functional metabolites; however, they cannot definitively predict clinical outcomes. Therefore, we are able to make conclusions about risk for low B12 status but not about confirmed deficiency.

5.7.1 Limitations of Dietary Assessment Tool

One major limitation of our dietary tool was the lack of ethnic specific foods and common food items potentially consumed by our study population. Also, the FFQ was designed to look at the past year's intake. This timeframe may pose a risk for increased recall error, especially for season specific intake, and may inaccurately reflect drastic dietary changes (e.g. change to vegetarian diet during timeframe of questionnaire). Although FFQs appropriate for Canadian SA populations exist (279) we decided on the Shatenstein questionnaire, which has been validated for micronutrient intake in multiple Canadian populations including women of childbearing age. The Shatenstein *et al* tool included a comprehensive list of B12 food sources and we updated the questionnaire to include meatless meat products (e.g. soy based hot dogs) to reflect the important potential contribution of these foods to B12 intake in ASF restricted diets and to attempt to capture intake of B12 from all food sources. However, if an individual consumed B12 containing foods as mixed meals and did not properly consider mixed meals in their calculation of specific line items then consumption of B12 containing foods may have been missed. Conversely, if they incorrectly estimated serving sizes for B12 containing foods they might have wrongly estimated B12 intake. Similarly, the combination of similar foods into single line items may have introduced 'cognitively complex' challenges to the responder as frequency

and portion sizes of all items are not necessarily consistent. The Keleman *et al* survey mentioned above differs from the one we used in that: it includes specific versions of the FFQ for individuals of SA, Asian, and European descent, incorporates open ended response options for consumption frequencies, asks about type of fat used in mixed meals (e.g. curries) and frequency of consumption of four types of multivitamins, and is available in native languages. The SA version includes 167 food items of which 37% are unique. The unique food items were determined by: assessing commonly consumed foods reported by SA participants in diet recalls during a pilot study, consulting SA community members, and assessing common SA restaurant items. In retrospect this tool may have been a more appropriate choice, however logistical challenges prevented its use.

South Asian individuals reported similar intakes of B12 from food as Europeans despite a higher rate of ASF restricted diets than the European group. This may suggest that the dietary tool was not successful in capturing B12 intake accurately, and that over-estimation may have occurred due to improper selection of portion sizes applied to mixed meals. SA diets are notoriously heterogeneous and vary largely based on the regional origin and religion of the individual (e.g. Hindus typically do not eat beef, and Muslims typically do not eat pork) (280). An article on the development of a SA specific FFQ for use in the UK reported the incorporation of extensive composite ethnic specific dishes into the questionnaire (280). To account for variation in recipes due to ethnic origin they modified the calculated recipe for each line item on an individual basis based on the respondent's regional and religious background. It is apparent that significant accommodations may help to accurately assess the diet of SA individuals (281). In general, due to the multiple opportunities for internal and external errors associated with FFQ analysis, the results from the FFQ can only be regarded as an 'estimate' of dietary intake and

may inaccurately represent quantitative indicators of dietary intake, such as median B12 intake for both the SA and European population. Due to the cross-sectional nature of the present study multiple dietary assessments were not possible and the FFQ was the most appropriate tool to estimate dietary intake. In future research, to get a more accurate estimate of B12 intake in multiethnic women, a more specific dietary assessment approach involving multiple pass measurements, such as repeated 24-hour recalls or weighed food records could be used. For example, the United States national data on folate intake was obtained using two-time 24-hour recalls with the United States Department of Agriculture 'Automated Multiple Pass Method' (282).

5.8   Suggestions for Future Research

Several suggestions for investigations to build upon this research and to provide further understanding about B12 deficiency in women of childbearing age include:

[1] Establishment of holoTC cut-off values, using the statistical modeling approach of Bailey *et al* against MMA inflection points, that are appropriate for defining marginal B12 deficiency to improve the ability to evaluate the clinical implications of marginal B12 deficiency and adverse pregnancy outcomes in women of childbearing age.

[2] Investigation of the rate of B12 deficiency in a broader sample of women of childbearing age in British Columbia, with adequate representation from all socioeconomic and demographic groups and including adequate clinical outcomes, such as neutrophil segmentation and nerve conduction studies in addition to multiple biomarkers to determine true deficiency rates, risk factors, and to assess the relationship between biomarker concentrations and clinical outcomes.

[3] Assessment of dietary contributions to B12 status and the risk attributed to varying degrees of ASF restriction using multiple ethnic specific dietary assessment tools.

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[4] Assessment of the contributions of other potential risk factors observed to affect B12 status, such as *Helicobacter pylori* infection, benign GI disease, smoking, alcohol intake, and physical activity using appropriately sensitive tools given that these associations have been made in the general United States population.

[5] Investigation of strategies to encourage successful recruitment of SA women to health research studies and encourage retention.

5.9 Conclusions

Through the work conducted for this thesis we have observed that based on using SB12, which persists as the reference biomarker in clinical settings, B12 deficiency is common in highly educated and health conscious women aged 19-35 of SA and European descent in Metro Vancouver. Variation in B12 status and concentration defined by 3 different biomarkers separately or in tandem can be partially explained by dietary B12 intake, supplement use, OC use, obesity, first generation immigrant status, and ethnicity. The rates of deficiency and combined metabolic decreased moderately to substantially when different combinations of biomarkers and cut-off values were applied. When we applied the recent cut-off values proposed by Bailey *et al* they allowed for slightly improved distinction between marginal deficiency and chronic deficiency, and resulted in a lower rate of gross disagreement between biomarkers compared to the conventional cut-offs, in our study population. Furthermore, by observing profiles of metabolic deficiency we illustrated that misclassified individuals may still be at risk for deficiency.

In conclusion, the outcomes of this cross sectional study, while not generalizable to all women aged 19-35 in Metro Vancouver, show cause for concern regarding the B12 status of women in this region and potentially elsewhere in Canada. Given the numerous potential impacts of B12 deficiency during pregnancy, it is important that women are B12 adequate prior to

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conception. Ideally, potential at risk populations such as pregnant women following extreme ASF-restricted diets, using OCs, and individuals of SA descent or first generation immigrant status, should be adequately screened for vulnerability to B12 deficiency using multiple biomarkers. It is also necessary that the methods used to assess B12 status are refined and deficiency classification approaches consolidated so that accuracy and precision are optimized, consistency is maintained, and misclassification is minimized. Ultimately the aim is for researchers to produce data on B12 status that is truly representative of prevalence and risk for B12 deficiency. This will hopefully enable the development of interventions such as targeted supplementation or enhanced pre-natal counseling to support the nutritional status of women of childbearing age and the health of their offspring.

References

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1. MacFarlane AJ, Greene-Finestone LS, Shi Y. Vitamin B-12 and homocysteine status in a folate-replete population: results from the Canadian Health Measures Survey. Am J Clin Nutr. 2011 Oct;94(4):1079–87.

2. Ray JG, Goodman J, O'Mahoney PRA, Mamdani MM, Jiang D. High rate of maternal vitamin B12 deficiency nearly a decade after Canadian folic acid flour fortification. QJM Mon J Assoc Physicians. 2008 Jun;101(6):475–7.

3. De Wals P, Tairou F, Van Allen MI, Uh S-H, Lowry RB, Sibbald B, et al. Reduction in neural-tube defects after folic acid fortification in Canada. N Engl J Med. 2007 Jul 12;357(2):135–42.

4. Ray JG, Meier C, Vermeulen MJ, Boss S, Wyatt PR, Cole DEC. Association of neural tube defects and folic acid food fortification in Canada. Lancet. 2002 Dec 21;360(9350):2047–8.

5. Ray JG, Wyatt PR, Thompson MD, Vermeulen MJ, Meier C, Wong P-Y, et al. Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. Epidemiol Camb Mass. 2007 May;18(3):362–6.

6. Molloy AM, Kirke PN, Troendle JF, Burke H, Sutton M, Brody LC, et al. Maternal vitamin B12 status and risk of neural tube defects in a population with high neural tube defect prevalence and no folic Acid fortification. Pediatrics. 2009 Mar;123(3):917–23.

7. Black MM. Effects of vitamin B12 and folate deficiency on brain development in children. Food Nutr Bull. 2008 Jun;29(2 Suppl):S126–131.

8. Refsum H. Folate, vitamin B12 and homocysteine in relation to birth defects and pregnancy outcome. Br J Nutr. 2001 May;85 Suppl 2:S109–113.

9. Furness D, Fenech M, Dekker G, Khong TY, Roberts C, Hague W. Folate, vitamin B12, vitamin B6 and homocysteine: impact on pregnancy outcome. Matern Child Nutr. 2013 Apr;9(2):155–66.

10. Krishnaveni GV, Hill JC, Veena SR, Bhat DS, Wills AK, Karat CLS, et al. Low plasma vitamin B12 in pregnancy is associated with gestational "diabesity" and later diabetes. Diabetologia. 2009 Nov;52(11):2350–8.

11. Yajnik CS, Deshpande SS, Jackson AA, Refsum H, Rao S, Fisher DJ, et al. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia. 2008 Jan;51(1):29–38.

12. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, et al. Biomarkers of vitamin B-12 status in NHANES: a roundtable summary. Am J Clin Nutr. 2011 Jul;94(1):313S–321S.

13. McLean E, de Benoist B, Allen LH. Review of the magnitude of folate and vitamin B12 deficiencies worldwide. Food Nutr Bull. 2008 Jun;29(2 Suppl):S38–51.

14. Gupta AK, Damji A, Uppaluri A. Vitamin B12 deficiency. Prevalence among South Asians at a Toronto clinic. Can Fam Physician Médecin Fam Can. 2004 May;50:743–7.

15. Refsum H, Yajnik CS, Gadkari M, Schneede J, Vollset SE, Orning L, et al. Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. Am J Clin Nutr. 2001 Aug;74(2):233–41.

16. Yajnik CS, Deshpande SS, Lubree HG, Naik SS, Bhat DS, Uradey BS, et al. Vitamin B12 deficiency and hyperhomocysteinemia in rural and urban Indians. J Assoc Physicians India. 2006 Oct;54:775–82.

17. Antony AC. Vegetarianism and vitamin B-12 (cobalamin) deficiency. Am J Clin Nutr. 2003 Jul;78(1):3–6.

18. Antony AC. Prevalence of cobalamin (vitamin B-12) and folate deficiency in India--audi alteram partem. Am J Clin Nutr. 2001 Aug;74(2):157–9.

19. Lindsay C. The South Asian Community in Canada [Internet]. Ottawa, Ontario: Social and Aboriginal Statistics Division, Statistics Canada; 2001. Available from: http://www.statcan.gc.ca/pub/89-621-x/89-621-x2007006-eng.htm#1

20. Tran K, Kaddatz J, Allard P. South Asians in Canada: Unity through diversity [Internet]. Statistics Canada; 2005 Sep. Available from: http://www5.statcan.gc.ca/bsolc/olc-cel/olc-cel?catno=11-008-X20050028455&lang=eng

21. Hilton BA, Grewal S, Popatia N, Bottorff JL, Johnson JL, Clarke H, et al. The desi ways: traditional health practices of South Asian women in Canada. Health Care Women Int. 2001 Sep;22(6):553–67.

22. Smith E. The discovery and identification of vitamin B12. Br J Nutr. 1952;6(3):295–9.

23. Gräsbeck R. Hooked to vitamin B12 since 1955: a historical perspective. Biochimie. 2013 May;95(5):970–5.

24. Martens JH, Barg H, Warren MJ, Jahn D. Microbial production of vitamin B12. Appl Microbiol Biotechnol. 2002 Mar;58(3):275–85.

25. Watanabe F. Vitamin B12 Sources and Bioavailability. Exp Biol Med. 2007 Nov 1;232(10):1266–74.

26. Mozafar A. Enrichment of some B-vitamins in plants with application of organic fertilizers. Plant Soil. 1994 Dec 1;167(2):305–11.

27. Hill MJ. Intestinal flora and endogenous vitamin synthesis. Eur J Cancer Prev. 1997 Mar;6 Suppl 1:S43–45.

28. Armstrong BK. Absorption of vitamin B12 from the human colon. Am J Clin Nutr. 1968 Apr;21(4):298–9.

29. Nielsen MJ, Rasmussen MR, Andersen CBF, Nexø E, Moestrup SK. Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. Nat Rev Gastroenterol Hepatol. 2012 Jun;9(6):345–54.

30. Ueta K, Takenaka S, Yabuta Y, Watanabe F. Broth from canned clams is suitable for use as an excellent source of free vitamin B12. J Agric Food Chem. 2011 Nov 23;59(22):12054–8.

31. Bennink MR, Ono K. Vitamin B12, E and D Content of Raw and Cooked Beef. J Food Sci. 1982;47(6):1786–92.

32. Matte JJ, Guay F, Girard CL. Bioavailability of vitamin B12 in cows' milk. Br J Nutr. 2012 Jan;107(1):61–6.

33. Government of Canada, Health Canada. Canadian Nutrient File, version 2010 [Internet]. 2010 [cited 2013 Aug 19]. Available from: http://www.hc-sc.gc.ca/fn-an/nutrition/fiche-nutri-data/index-eng.php

34. Watanabe, Abe, Fujita, Goto, Hiemori, Nakano. Effects of Microwave Heating on the Loss of Vitamin B(12) in Foods. J Agric Food Chem. 1998 Jan 19;46(1):206–10.

35. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline [Internet]. Washington (DC): National Academies

Press (US); 1998 [cited 2014 Jan 13]. Available from:
http://www.ncbi.nlm.nih.gov/books/NBK114310/

36. Doscherholmen A, McMahon J, Ripley D. Inhibitory effect of eggs on vitamin B12 absorption: description of a simple ovalbumin 57Co-vitamin B12 absorption test. Br J Haematol. 1976 Jun;33(2):261–72.

37. Levine AS, Doscherholmen A. Vitamin B12 bioavailability from egg yolk and egg white: relationship to binding proteins. Am J Clin Nutr. 1983 Sep;38(3):436–9.

38. Doets EL, In 't Veld PH, Szczecińska A, Dhonukshe-Rutten RAM, Cavelaars AEJM, van 't Veer P, et al. Systematic review on daily vitamin B12 losses and bioavailability for deriving recommendations on vitamin B12 intake with the factorial approach. Ann Nutr Metab. 2013;62(4):311–22.

39. Agriculture and Agri-Food Canada. Vitamin and Mineral Addition [Internet]. 2009 [cited 2013 Aug 19]. Available from: http://www.agr.gc.ca/eng/industry-markets-and-trade/food-regulations/food-policy-and-regulatory-issues/current-food-policy-and-regulatory-issues/vitamin-and-mineral-addition/?id=1172254614356

40. Government of Canada PHA of C. Folic Acid and Prevention of Neural Tube Defects - Public Health Agency of Canada [Internet]. 2008 [cited 2014 Mar 4]. Available from: http://www.phac-aspc.gc.ca/fa-af/fa-af08-eng.php

41. Allen LH, Rosenberg IH, Oakley GP, Omenn GS. Considering the case for vitamin B12 fortification of flour. Food Nutr Bull. 2010 Mar;31(1 Suppl):S36–46.

42. Carmel R. Efficacy and safety of fortification and supplementation with vitamin B12: biochemical and physiological effects. Food Nutr Bull. 2008 Jun;29(2 Suppl):S177–187.

43. Green R. Is it time for vitamin B-12 fortification? What are the questions? Am J Clin Nutr. 2009 Feb;89(2):712S–6S.

44. Herrmann W, Obeid R. Causes and early diagnosis of vitamin B12 deficiency. Dtsch Ärztebl Int. 2008 Oct;105(40):680–5.

45. Seetharam B, Alpers DH. Absorption and transport of cobalamin (vitamin B12). Annu Rev Nutr. 1982;2:343–69.

46. Stabler SP. Vitamin B12 Deficiency. N Engl J Med. 2013;368(2):149–60.

47. Hughes CF, Ward M, Hoey L, McNulty H. Vitamin B12 and ageing: current issues and interaction with folate. Ann Clin Biochem. 2013 Jun 18;50(4):315–29.

48. Abels J, Vegter J, Woldring M, Jans J, Nieweg H. The physiologic mechanism of vitamin B12 absorption. Acta Med Scand. 1959 Oct 16;165:105–13.

49. Shils ME, Shike M. Modern Nutrition in Health and Disease. Lippincott Williams & Wilkins; 2006. 2342 p.

50. Beedholm-Ebsen R, van de Wetering K, Hardlei T, Nexø E, Borst P, Moestrup SK. Identification of multidrug resistance protein 1 (MRP1/ABCC1) as a molecular gate for cellular export of cobalamin. Blood. 2010 Feb 25;115(8):1632–9.

51. Quadros EV, Regec AL, Khan KM, Quadros E, Rothenberg SP. Transcobalamin II synthesized in the intestinal villi facilitates transfer of cobalamin to the portal blood. Am J Physiol. 1999 Jul;277(1 Pt 1):G161–166.

52. Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, et al. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A. 1998 Mar 17;95(6):3059–64.

53. Takahashi-Iñiguez T, García-Hernandez E, Arreguín-Espinosa R, Flores ME. Role of vitamin B12 on methylmalonyl-CoA mutase activity. J Zhejiang Univ Sci B. 2012 Jun;13(6):423–37.

54. Gruber K, Kratky C. Methylmalonyl CoA Mutase. Handbook of Metalloproteins [Internet]. Online: John Wiley & Sons, Ltd; 2006 [cited 2013 Aug 19]. Available from: http://onlinelibrary.wiley.com/doi/10.1002/0470028637.met172/abstract

55. Gropper SS, Smith JL. Advanced Nutrition and Human Metabolism, 6th ed. CA, USA: Cengage Learning; 2012. 606 p.

56. Herrmann W, Obeid R. Vitamins in the Prevention of Human Diseases. Walter de Gruyter; 2011. 745 p.

57. Allen LH. Bioavailability of vitamin B12. Int J Vitam Nutr Res. 2010 Oct;80(4-5):330–5.

58. Adams JF, Tankel HI, MacEwan F. Estimation of the total body vitamin B12 in the live subject. Clin Sci. 1970 Jul;39(1):107–13.

59. Grasbeck R. Physiology and pathology of vitamin B12 absorption, distribution, and excretion. Adv Clin Chem. 1960;3:299–366.

60. Darby WJ, Bridgforth EB, le Brocquy J, Clark SL, Oliveira D, Dutra J, et al. Vitamin B12 requirement of adult man. Am J Med. 1958 Nov;25(5):726–32.

61. Quay T, Lamers Y. Food Fortification with Vitamin B12: Potential Benefits and Open Questions. Sight Life. 2012;26(2).

62. Loikas S, Koskinen P, Irjala K, Löppönen M, Isoaho R, Kivelä S-L, et al. Renal impairment compromises the use of total homocysteine and methylmalonic acid but not total vitamin B12 and holotranscobalamin in screening for vitamin B12 deficiency in the aged. Clin Chem Lab Med. 2007;45(2):197–201.

63. Quadros EV, Sequeira JM. Cellular uptake of cobalamin: transcobalamin and the TCblR/CD320 receptor. Biochimie. 2013 May;95(5):1008–18.

64. Carmel R. Mild transcobalamin I (haptocorrin) deficiency and low serum cobalamin concentrations. Clin Chem. 2003 Aug;49(8):1367–74.

65. Carmel R. Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. Am J Clin Nutr. 2011 Jul;94(1):348S–358S.

66. Nexo E, Hoffmann-Lücke E. Holotranscobalamin, a marker of vitamin B-12 status: analytical aspects and clinical utility. Am J Clin Nutr. 2011 Jul;94(1):359S–365S.

67. Zironi E, Gazzotti T, Barbarossa A, Devicienti C, Scardilli M, Pagliuca G. Technical note: development and validation of a method using ultra performance liquid chromatography coupled with tandem mass spectrometry for determination of vitamin B12 concentrations in milk and dairy products. J Dairy Sci. 2013 May;96(5):2832–6.

68. Morkbak AL, Hvas A-M, Milman N, Nexo E. Holotranscobalamin remains unchanged during pregnancy. Longitudinal changes of cobalamins and their binding proteins during pregnancy and postpartum. Haematologica. 2007 Dec;92(12):1711–2.

69. Murphy MM, Molloy AM, Ueland PM, Fernandez-Ballart JD, Schneede J, Arija V, et al. Longitudinal study of the effect of pregnancy on maternal and fetal cobalamin status in healthy women and their offspring. J Nutr. 2007 Aug;137(8):1863–7.

70. Miller JW, Garrod MG, Rockwood AL, Kushnir MM, Allen LH, Haan MN, et al. Measurement of total vitamin B12 and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. Clin Chem. 2006 Feb;52(2):278–85.

71. Lloyd-Wright Z, Hvas A-M, Møller J, Sanders TAB, Nexø E. Holotranscobalamin as an indicator of dietary vitamin B12 deficiency. Clin Chem. 2003 Dec;49(12):2076–8.

72. Bor MV, von Castel-Roberts KM, Kauwell GP, Stabler SP, Allen RH, Maneval DR, et al. Daily intake of 4 to 7 microg dietary vitamin B-12 is associated with steady concentrations of vitamin B-12-related biomarkers in a healthy young population. Am J Clin Nutr. 2010 Mar;91(3):571–7.

73. Lindgren A, Kilander A, Bagge E, Nexø E. Holotranscobalamin - a sensitive marker of cobalamin malabsorption. Eur J Clin Invest. 1999 Apr;29(4):321–9.

74. Refsum H, Nurk E, Smith AD, Ueland PM, Gjesdal CG, Bjelland I, et al. The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr. 2006 Jun;136(6 Suppl):1731S–1740S.

75. Selhub J, Jacques PF, Dallal G, Choumenkovitch S, Rogers G. The use of blood concentrations of vitamins and their respective functional indicators to define folate and vitamin B12 status. Food Nutr Bull. 2008 Jun;29(2 Suppl):S67–73.

76. Lamers Y. Indicators and methods for folate, vitamin B-12, and vitamin B-6 status assessment in humans: Curr Opin Clin Nutr Metab Care. 2011 Sep;14(5):445–54.

77. Stabler SP, Marcell PD, Podell ER, Allen RH, Lindenbaum J. Assay of methylmalonic acid in the serum of patients with cobalamin deficiency using capillary gas chromatography-mass spectrometry. J Clin Invest. 1986 May;77(5):1606–12.

78. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Diagnosis of cobalamin deficiency I: usefulness of serum methylmalonic acid and total homocysteine concentrations. Am J Hematol. 1990 Jun;34(2):90–8.

79. Lindenbaum J, Savage DG, Stabler SP, Allen RH. Diagnosis of cobalamin deficiency: II. Relative sensitivities of serum cobalamin, methylmalonic acid, and total homocysteine concentrations. Am J Hematol. 1990 Jun;34(2):99–107.

80. Bailey RL, Durazo-Arvizu RA, Carmel R, Green R, Pfeiffer CM, Sempos CT, et al. Modeling a methylmalonic acid–derived change point for serum vitamin B-12 for adults in NHANES. Am J Clin Nutr. 2013 Aug 1;

81. Carmel R. How I treat cobalamin (vitamin B12) deficiency. Blood. 2008 Sep 15;112(6):2214–21.

82. Allen LH. How common is vitamin B-12 deficiency? Am J Clin Nutr. 2009 Feb;89(2):693S–6S.

83. Pfeiffer CM, Johnson CL, Jain RB, Yetley EA, Picciano MF, Rader JI, et al. Trends in blood folate and vitamin B-12 concentrations in the United States, 1988 2004. Am J Clin Nutr. 2007 Sep;86(3):718–27.

84. Bottiglieri T. Folate, vitamin B12, and neuropsychiatric disorders. Nutr Rev. 1996 Dec;54(12):382–90.

85. Koury MJ, Ponka P. New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annu Rev Nutr. 2004;24:105–31.

86. Weir DG, Scott JM. Brain function in the elderly: role of vitamin B12 and folate. Br Med Bull. 1999;55(3):669–82.

87. Chanarin I. Megaloblastic anaemia, cobalamin, and folate. J Clin Pathol. 1987 Sep;40(9):978–84.

88. Shorvon SD, Carney MW, Chanarin I, Reynolds EH. The neuropsychiatry of megaloblastic anaemia. Br Med J. 1980 Oct 18;281(6247):1036–8.

89. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Metabolic abnormalities in cobalamin (vitamin B12) and folate deficiency. FASEB J Off Publ Fed Am Soc Exp Biol. 1993 Nov;7(14):1344–53.

90. Schatz RA, Stramentinoli G, Sellinger OZ. Decreased cerebral catabolism of [3H]histamine in vivo after S-adenosylmethionine administration. J Pharmacol Exp Ther. 1981 Jan;216(1):118–24.

91. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr. 2007 Jan;85(1):193–200.

92. Carmel R. Does high folic acid intake affect unrecognized cobalamin deficiency, and how will we know it if we see it? Am J Clin Nutr. 2009 Dec 1;90(6):1449–50.

93. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr. 2007 Jan;85(1):193–200.

94. Colapinto CK, O'Connor DL, Tremblay MS. Folate status of the population in the Canadian Health Measures Survey. Can Med Assoc J. 2011 Feb 8;183(2):E100–106.

95. Carmel R. Cobalamin, the stomach, and aging. Am J Clin Nutr. 1997 Oct;66(4):750–9.

96. Rochtchina E, Wang JJ, Flood VM, Mitchell P. Elevated serum homocysteine, low serum vitamin B12, folate, and age-related macular degeneration: the Blue Mountains Eye Study. Am J Ophthalmol. 2007 Feb;143(2):344–6.

97. Herrmann M, Umanskaya N, Traber L, Schmidt-Gayk H, Menke W, Lanzer G, et al. The effect of B-vitamins on biochemical bone turnover markers and bone mineral density in osteoporotic patients: a 1-year double blind placebo controlled trial. Clin Chem Lab Med. 2007;45(12):1785–92.

98. Swart KMA, van Schoor NM, Lips P. Vitamin B12, Folic Acid, and Bone. Curr Osteoporos Rep. 2013 Jul 20;

99. Karnaze DS, Carmel R. Neurologic and evoked potential abnormalities in subtle cobalamin deficiency states, including deficiency without anemia and with normal absorption of free cobalamin. Arch Neurol. 1990 Sep;47(9):1008–12.

100. Spence JD. Homocysteine-lowering therapy: a role in stroke prevention? Lancet Neurol. 2007 Sep;6(9):830–8.

101. Carmel R, Sarrai M. Diagnosis and management of clinical and subclinical cobalamin deficiency: advances and controversies. Curr Hematol Rep. 2006 Mar;5(1):23–33.

102. Carmel R. Subclinical cobalamin deficiency. Curr Opin Gastroenterol. 2012 Mar;28(2):151–8.

103. Stabler SP. Screening the older population for cobalamin (vitamin B12) deficiency. J Am Geriatr Soc. 1995 Nov;43(11):1290–7.

104. Schroder T, Quay T, Lamers Y. Methylmalonic acid concentration in dried blood spots - validation of a novel method for vitamin B12 status assessment. FASEB J. 2013 Apr;Meeting Abstract Supplement:1077.

105. Wagner DA, Schatz R, Coston R, Curington C, Bolt D, Toskes PP. A new 13C breath test to detect vitamin B12 deficiency: a prevalent and poorly diagnosed health problem. J Breath Res. 2011 Dec;5(4):046001.

106. Bailey RL, Carmel R, Green R, Pfeiffer CM, Cogswell ME, Osterloh JD, et al. Monitoring of vitamin B-12 nutritional status in the United States by using plasma methylmalonic acid and serum vitamin B-12. Am J Clin Nutr. 2011 Aug;94(2):552–61.

107. Pérez-Sepúlveda A, España-Perrot PP, Fernández B X, Ahumada V, Bustos V, Arraztoa JA, et al. Levels of key enzymes of methionine-homocysteine metabolism in preeclampsia. BioMed Res Int. 2013;2013:731962.

108. Yajnik CS, Deshpande SS, Panchanadikar AV, Naik SS, Deshpande JA, Coyaji KJ, et al. Maternal total homocysteine concentration and neonatal size in India. Asia Pac J Clin Nutr. 2005;14(2):179–81.

109. Muthayya S, Kurpad AV, Duggan CP, Bosch RJ, Dwarkanath P, Mhaskar A, et al. Low maternal vitamin B12 status is associated with intrauterine growth retardation in urban South Indians. Eur J Clin Nutr. 2006 Jun;60(6):791–801.

110. Monagle PT, Tauro GP. Infantile megaloblastosis secondary to maternal vitamin B12 deficiency. Clin Lab Haematol. 1997 Mar;19(1):23–5.

111. Louwman MW, van Dusseldorp M, van de Vijver FJ, Thomas CM, Schneede J, Ueland PM, et al. Signs of impaired cognitive function in adolescents with marginal cobalamin status. Am J Clin Nutr. 2000 Sep;72(3):762–9.

112. Graham SM, Arvela OM, Wise GA. Long-term neurologic consequences of nutritional vitamin B12 deficiency in infants. J Pediatr. 1992 Nov;121(5 Pt 1):710–4.

113. Dror DK, Allen LH. Effect of vitamin B12 deficiency on neurodevelopment in infants: current knowledge and possible mechanisms. Nutr Rev. 2008 May;66(5):250–5.

114. Bhate V, Deshpande S, Bhat D, Joshi N, Ladkat R, Watve S, et al. Vitamin B12 status of pregnant Indian women and cognitive function in their 9-year-old children. Food Nutr Bull. 2008 Dec;29(4):249–54.

115. Thompson MD, Cole DEC, Ray JG. Vitamin B-12 and neural tube defects: the Canadian experience. Am J Clin Nutr. 2009 Feb;89(2):697S–701S.

116. Wallingford JB, Niswander LA, Shaw GM, Finnell RH. The Continuing Challenge of Understanding, Preventing, and Treating Neural Tube Defects. Science. 2013 Mar 1;339(6123):1222002.

117. Heseker HB, Mason JB, Selhub J, Rosenberg IH, Jacques PF. Not all cases of neural-tube defect can be prevented by increasing the intake of folic acid. Br J Nutr. 2009 Jul;102(2):173–80.

118. Czeizel AE, Dudás I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med. 1992 Dec 24;327(26):1832–5.

119. Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. Folate levels and neural tube defects. Implications for prevention. JAMA J Am Med Assoc. 1995 Dec 6;274(21):1698–702.

120. Wilson RD, Johnson J-A, Wyatt P, Allen V, Gagnon A, Langlois S, et al. Pre-conceptional vitamin/folic acid supplementation 2007: the use of folic acid in combination with a multivitamin supplement for the prevention of neural tube defects and other congenital anomalies. J Obstet Gynaecol Can. 2007 Dec;29(12):1003–26.

121. Blom HJ, Smulders Y. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis. 2011 Feb;34(1):75–81.

122. Afman LA, Van Der Put NMJ, Thomas CMG, Trijbels JMF, Blom HJ. Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects. QJM. 2001 Mar 1;94(3):159–66.

123. Zhao W, Mosley BS, Cleves MA, Melnyk S, James SJ, Hobbs CA. Neural tube defects and maternal biomarkers of folate, homocysteine, and glutathione metabolism. Birt Defects Res A Clin Mol Teratol. 2006 Apr;76(4):230–6.

124. Gardiki-Kouidou P, Seller MJ. Amniotic fluid folate, vitamin B12 and transcobalamins in neural tube defects. Clin Genet. 1988 Jun;33(6):441–8.

125. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DC, Scott JM. Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. QJM. 1993 Nov 1;86(11):703–8.

126. Suarez L, Hendricks K, Felkner M, Gunter E. Maternal Serum B12 Levels and Risk for Neural Tube Defects in a Texas-Mexico Border Population. Ann Epidemiol. 2003 Feb;13(2):81–8.

127. Wang Z-P, Shang X-X, Zhao Z-T. Low maternal vitamin B(12) is a risk factor for neural tube defects: a meta-analysis. J Matern-Fetal Neonatal Med Off J Eur Assoc Perinat Med Fed Asia Ocean Perinat Soc Int Soc Perinat Obstet. 2012 Apr;25(4):389–94.

128. Bavdekar A, Yajnik CS, Fall CH, Bapat S, Pandit AN, Deshpande V, et al. Insulin resistance syndrome in 8-year-old Indian children: small at birth, big at 8 years, or both? Diabetes. 1999 Dec;48(12):2422–9.

129. Bond TA, Joglekar CV, Marley-Zagar E, Lubree HG, Kumaran K, Yajnik CS, et al. Maternal vitamin B12 and folate status during pregnancy and insulin resistance and body composition in the offspring at 12 years in a rural Indian birth cohort: data from the Pune Maternal Nutrition Study. Proc Nutr Soc. 2013;72((72) OCE4):E306.

130. Pilia S, Casini MR, Foschini ML, Minerba L, Musiu MC, Marras V, et al. The effect of puberty on insulin resistance in obese children. J Endocrinol Invest. 2009 May;32(5):401–5.

131. Stewart CP, Christian P, Schulze KJ, Arguello M, LeClerq SC, Khatry SK, et al. Low Maternal Vitamin B-12 Status Is Associated with Offspring Insulin Resistance Regardless of Antenatal Micronutrient Supplementation in Rural Nepal. J Nutr. 2011 Oct 1;141(10):1912–7.

132. Van Sande H, Jacquemyn Y, Karepouan N, Ajaji M. Vitamin B12 in pregnancy: Maternal and fetal/neonatal effects—A review. Open J Obstet Gynecol. 2013;3:599–602.

133. Thamm M, Mensink GB, Thierfelder W. [Folic acid intake of women in childbearing age]. Gesundheitswesen Bundesverb Ärzte Öffentl Gesundheitsdienstes Ger. 1999 Dec;61 Spec No:S207–212.

134. Laillou A, Pham TV, Tran NT, Le HT, Wieringa F, Rohner F, et al. Micronutrient deficits are still public health issues among women and young children in Vietnam. PloS One. 2012;7(4):e34906.

135. Thuesen BH, Husemoen LLN, Ovesen L, Jørgensen T, Fenger M, Linneberg A. Lifestyle and genetic determinants of folate and vitamin B12 levels in a general adult population. Br J Nutr. 2010 Apr;103(8):1195–204.

136. Gammon CS, von Hurst PR, Coad J, Kruger R, Stonehouse W. Vegetarianism, vitamin B12 status, and insulin resistance in a group of predominantly overweight/obese South Asian women. Nutrition. 2012 Jan;28(1):20–4.

137. House JD, March SB, Ratnam S, Ives E, Brosnan JT, Friel JK. Folate and vitamin B12 status of women in Newfoundland at their first prenatal visit. Can Med Assoc J. 2000 May 30;162(11):1557–9.

138. Jiang T, Christian P, Khatry SK, Wu L, West KP Jr. Micronutrient deficiencies in early pregnancy are common, concurrent, and vary by season among rural Nepali pregnant women. J Nutr. 2005 May;135(5):1106–12.

139. García-Casal MN, Osorio C, Landaeta M, Leets I, Matus P, Fazzino F, et al. High prevalence of folic acid and vitamin B12 deficiencies in infants, children, adolescents and pregnant women in Venezuela. Eur J Clin Nutr. 2005 Sep;59(9):1064–70.

140. Samuel TM, Duggan C, Thomas T, Bosch R, Rajendran R, Virtanen SM, et al. Vitamin B(12) intake and status in early pregnancy among urban South Indian women. Ann Nutr Metab. 2013;62(2):113–22.

141. Abraham A, Mathews JE, Sebastian A, Chacko KP, Sam D. A nested case-control study to evaluate the association between fetal growth restriction and vitamin B12 deficiency. Aust N Z J Obstet Gynaecol. 2013 Aug;53(4):399–402.

142. Statistics Canada. Immigration and Ethnocultural Diversity in Canada, National Household Survey, 2011 [Internet]. [cited 2013 Dec 4]. Available from: http://www12.statcan.gc.ca/nhs-enm/2011/as-sa/99-010-x/99-010-x2011001-eng.pdf

143. Government of Canada SC. Statistics Canada: Ethnocultural Portrait of Canada Highlight Tables, 2006 Census [Internet]. 2008 [cited 2013 Aug 20]. Available from: http://www12.statcan.gc.ca/census-recensement/2006/dp-pd/hlt/97-562/index.cfm?Lang=E

144. Jiwani Y, (Center) F, Kelly D. Intersecting Inequalities: Immigrant Women of Colour, Violence and Health Care. Vancouver, Canada: Feminist Research Education Development & Action Centre; 2001. 88 p.

145. Auluck R, Iles P. The Referral Process: A Study of Working Relationships between Antenatal Clinic Nursing Staff and Hospital Social Workers and their impact on Asian Women. Br J Soc Work. 1991 Feb 1;21(1):41–61.

146. Bowes A m., Domokos TM. Pakistani women and maternity care: raising muted voices. Sociol Health Illn. 1996;18(1):45–65.

147. Anand SS, Yusuf S, Vuksan V, Devanesen S, Teo KK, Montague PA, et al. Differences in risk factors, atherosclerosis, and cardiovascular disease between ethnic groups in Canada: the Study of Health Assessment and Risk in Ethnic groups (SHARE). Lancet. 2000 Jul 22;356(9226):279–84.

148. Chiu M, Austin PC, Manuel DG, Tu JV. Comparison of cardiovascular risk profiles among ethnic groups using population health surveys between 1996 and 2007. Can Med Assoc J. 2010 May 18;182(8):E301–310.

149. Allen LH. Folate and vitamin B12 status in the Americas. Nutr Rev. 2004 Jun;62(6):S29–S34.

150. McLean ED, Allen LH, Neumann CG, Peerson JM, Siekmann JH, Murphy SP, et al. Low plasma vitamin B-12 in Kenyan school children is highly prevalent and improved by supplemental animal source foods. J Nutr. 2007 Mar;137(3):676–82.

151. Siekmann JH, Allen LH, Bwibo NO, Demment MW, Murphy SP, Neumann CG. Kenyan school children have multiple micronutrient deficiencies, but increased plasma vitamin B-

12 is the only detectable micronutrient response to meat or milk supplementation. J Nutr. 2003 Nov;133(11 Suppl 2):3972S–3980S.

152. Bindra GS, Gibson RS, Berry M. Vitamin B12 and folate status of East Indian immigrants living in Canada. Nutr Res. 1987 Apr;7(4):365–74.

153. Statistics Canada. 2011 National Household Survey Profile - Census subdivision [Internet]. 2013 [cited 2013 Nov 18]. Available from: http://www12.statcan.gc.ca/nhs-enm/2011/dp-pd/prof/details/page.cfm?Lang=E&Geo1=CSD&Code1=5915022&Data=Count&SearchText=Vancouver&SearchType=Begins&SearchPR=01&A1=All&B1=All&Custom=&TABID=1

154. Carmel R, Green R, Jacobsen DW, Rasmussen K, Florea M, Azen C. Serum cobalamin, homocysteine, and methylmalonic acid concentrations in a multiethnic elderly population: ethnic and sex differences in cobalamin and metabolite abnormalities. Am J Clin Nutr. 1999 Nov;70(5):904–10.

155. Stabler SP, Allen RH, Fried LP, Pahor M, Kittner SJ, Penninx BW, et al. Racial differences in prevalence of cobalamin and folate deficiencies in disabled elderly women. Am J Clin Nutr. 1999 Nov 1;70(5):911–9.

156. Chanarin I, Malkowska V, O'Hea AM, Rinsler MG, Price AB. Megaloblastic anaemia in a vegetarian Hindu community. Lancet. 1985 Nov 23;2(8465):1168–72.

157. Carmel R. Ethnic and racial factors in cobalamin metabolism and its disorders. Semin Hematol. 1999 Jan;36(1):88–100.

158. Chandalia M, Abate N, Cabo-Chan AV Jr, Devaraj S, Jialal I, Grundy SM. Hyperhomocysteinemia in Asian Indians living in the United States. J Clin Endocrinol Metab. 2003 Mar;88(3):1089–95.

159. Stabler SP, Allen RH. Vitamin B12 deficiency as a worldwide problem. Annu Rev Nutr. 2004;24:299–326.

160. Wun Chan JC, Yu Liu HS, Sang Kho BC, Yin Sim JP, Hang Lau TK, Luk YW, et al. Pernicious anemia in Chinese: a study of 181 patients in a Hong Kong hospital. Medicine (Baltimore). 2006 May;85(3):129–38.

161. Misra A, Vikram NK, Pandey RM, Dwivedi M, Ahmad FU, Luthra K, et al. Hyperhomocysteinemia, and low intakes of folic acid and vitamin B12 in urban North India. Eur J Nutr. 2002 Apr;41(2):68–77.

162. Gamble MV, Ahsan H, Liu X, Factor-Litvak P, Ilievski V, Slavkovich V, et al. Folate and cobalamin deficiencies and hyperhomocysteinemia in Bangladesh. Am J Clin Nutr. 2005 Jun;81(6):1372–7.

163. Saritaben Mangukiya, Bhautik Modi, Pallavi Chaurasia, Krushna Modi, Rita M Shah. Distribution of vitamin B12 level according to socio-demographic characteristics in patients of Dhiraj General Hospital, Pipariya, Gujarat. Natl J Med Res. 2011;1(2):54–6.

164. Shobha V, Tarey SD, Singh RG, Shetty P, Unni US, Srinivasan K, et al. Vitamin B12 deficiency & levels of metabolites in an apparently normal urban south Indian elderly population. Indian J Med Res. 2011 Oct;134(4):432–9.

165. Mahalle N, Kulkarni MV, Garg MK, Naik SS. Vitamin B12 deficiency and hyperhomocysteinemia as correlates of cardiovascular risk factors in Indian subjects with coronary artery disease. J Cardiol. 2013 Apr;61(4):289–94.

166. Taneja S, Bhandari N, Strand TA, Sommerfelt H, Refsum H, Ueland PM, et al. Cobalamin and folate status in infants and young children in a low-to-middle income community in India. Am J Clin Nutr. 2007 Nov;86(5):1302–9.

167. Cherian A, Seena S, Bullock RK, Antony AC. Incidence of neural tube defects in the least-developed area of India: a population-based study. Lancet. 2005 Sep 10;366(9489):930–1.

168. Allen LH. Causes of vitamin B12 and folate deficiency. Food Nutr Bull. 2008 Jun;29(2 Suppl):S20–34; discussion S35–37.

169. Elmadfa I, Singer I. Vitamin B-12 and homocysteine status among vegetarians: a global perspective. Am J Clin Nutr. 2009 May;89(5):1693S–1698S.

170. Messina V, Melina V, Mangels AR. A new food guide for North American vegetarians. Can J Diet Pract Res. 2003;64(2):82–6.

171. Position of the American Dietetic Association: Vegetarian Diets. J Am Diet Assoc. 2009 Jul;109(7):1266–82.

172. American Dietetic Association, Dietitians of Canada. Position of the American Dietetic Association and Dietitians of Canada: vegetarian diets. Can J Diet Pract Res. 2003;64(2):62–81.

173. Pawlak R, Parrott SJ, Raj S, Cullum-Dugan D, Lucus D. How prevalent is vitamin B(12) deficiency among vegetarians? Nutr Rev. 2013 Feb;71(2):110–7.

174. Herrmann W, Schorr H, Purschwitz K, Rassoul F, Richter V. Total homocysteine, vitamin B(12), and total antioxidant status in vegetarians. Clin Chem. 2001 Jun;47(6):1094–101.

175. Gilsing AMJ, Crowe FL, Lloyd-Wright Z, Sanders TAB, Appleby PN, Allen NE, et al. Serum concentrations of vitamin B12 and folate in British male omnivores, vegetarians and vegans: results from a cross-sectional analysis of the EPIC-Oxford cohort study. Eur J Clin Nutr. 2010 Sep;64(9):933–9.

176. Koebnick C, Heins UA, Dagnelie PC, Wickramasinghe SN, Ratnayaka ID, Hothorn T, et al. Longitudinal concentrations of vitamin B(12) and vitamin B(12)-binding proteins during uncomplicated pregnancy. Clin Chem. 2002 Jun;48(6 Pt 1):928–33.

177. Löwik MR, Schrijver J, Odink J, van den Berg H, Wedel M. Long-term effects of a vegetarian diet on the nutritional status of elderly people (Dutch Nutrition Surveillance System). J Am Coll Nutr. 1990 Dec;9(6):600–9.

178. Premkumar M, Gupta N, Singh T, Velpandian T. Vitamin B12-homocysteine interaction and the efficacy of B12 therapy in relation to anemia and neurological disease in a North Indian population. Int J Nutr Pharmacol Neurol Dis. 2012;2(1):61.

179. Prakash R, Kumar A. Urban poverty and utilization of maternal and child health care services in India. J Biosoc Sci. 2013 Jul;45(4):433–49.

180. Donaldson MS. Metabolic vitamin B12 status on a mostly raw vegan diet with follow-up using tablets, nutritional yeast, or probiotic supplements. Ann Nutr Metab. 2000;44(5-6):229–34.

181. Herrmann W, Obeid R, Schorr H, Hübner U, Geisel J, Sand-Hill M, et al. Enhanced bone metabolism in vegetarians--the role of vitamin B12 deficiency. Clin Chem Lab Med. 2009;47(11):1381–7.

182. Bedford JL, Barr SI. Diets and selected lifestyle practices of self-defined adult vegetarians from a population-based sample suggest they are more "health conscious."Int J Behav Nutr Phys Act. 2005 Apr 13;2(1):4.

183. Sharma S, Sheehy T, Kolonel LN. Contribution of meat to vitamin B(12), iron and zinc intakes in five ethnic groups in the USA: implications for developing food-based dietary guidelines. J Hum Nutr Diet Off J Br Diet Assoc. 2013 Apr;26(2):156–68.

184. Government of Canada HC. Do Canadian Adults Meet their Nutrient Requirements through Food Intake Alone? [Health Canada, 2012] [Internet]. 2010 [cited 2013 Aug 19]. Available from: http://www.hc-sc.gc.ca/fn-an/surveill/nutrition/commun/art-nutr-adult-eng.php

185. Shakur YA, Tarasuk V, Corey P, O'Connor DL. A Comparison of Micronutrient Inadequacy and Risk of High Micronutrient Intakes among Vitamin and Mineral Supplement Users and Nonusers in Canada. J Nutr. 2012 Mar 1;142(3):534–40.

186. Bianchetti A, Rozzini R, Carabellese C, Zanetti O, Trabucchi M. Nutritional intake, socioeconomic conditions, and health status in a large elderly population. J Am Geriatr Soc. 1990 May;38(5):521–6.

187. Torheim LE, Ferguson EL, Penrose K, Arimond M. Women in resource-poor settings are at risk of inadequate intakes of multiple micronutrients. J Nutr. 2010 Nov;140(11):2051S–8S.

188. Brusque A., Borba Rosa R, Schuck P., Dalcin K., Ribeiro CA., Silva C., et al. Inhibition of the mitochondrial respiratory chain complex activities in rat cerebral cortex by methylmalonic acid. Neurochem Int. 2002 Jun;40(7):593–601.

189. Melo DR, Kowaltowski AJ, Wajner M, Castilho RF. Mitochondrial energy metabolism in neurodegeneration associated with methylmalonic acidemia. J Bioenerg Biomembr. 2011 Feb 1;43(1):39–46.

190. Elshorbagy AK, Nurk E, Gjesdal CG, Tell GS, Ueland PM, Nygård O, et al. Homocysteine, cysteine, and body composition in the Hordaland Homocysteine Study: does cysteine link amino acid and lipid metabolism? Am J Clin Nutr. 2008 Sep 1;88(3):738–46.

191. Pinhas-Hamiel O, Doron-Panush N, Reichman B, Nitzan-Kaluski D, Shalitin S, Geva-Lerner L. Obese children and adolescents: A risk group for low vitamin b12 concentration. Arch Pediatr Adolesc Med. 2006 Sep 1;160(9):933–6.

192. Baltaci D, Kutlucan A, Turker Y, Yilmaz A, Karacam S, Deler H, et al. Association of vitamin B12 with obesity, overweight, insulin resistance and metabolic syndrome, and body fat composition; primary care-based study. Med Glas Zenica. 2013 Aug;10(2):203–10.

193. Wang X, Wu H, Qiu X. Methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism and risk of preeclampsia: an updated meta-analysis based on 51 studies. Arch Med Res. 2013 Apr;44(3):159–68.

194. Yang P, Li H-L, Wang C-Y. FUT2 Nonfunctional Variant: A "Missing Link" Between Genes and Environment in Type 1 Diabetes? Diabetes. 2011 Nov 1;60(11):2685–7.

195. Banerjee R, Rosenblatt D. Chemistry and Biochemistry of B12 - Inborn errors of cobalamin metabolism. New York, NY: Wiley; 1999. 958 p.

196. Finnell RH, Shaw GM, Lammer EJ, Rosenquist TH. Gene-nutrient interactions: importance of folic acid and vitamin B12 during early embryogenesis. Food Nutr Bull. 2008 Jun;29(2 Suppl):S86–98; discussion S99–100.

197. Speer MC, Nye J, McLone D, Worley G, Melvin EC, Viles KD, et al. Possible interaction of genotypes at cystathionine beta-synthase and methylenetetrahydrofolate reductase (MTHFR) in neural tube defects. NTD Collaborative Group. Clin Genet. 1999 Aug;56(2):142–4.

198. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci. 2001 Apr;22(4):195–201.

199. Brattström L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. Circulation. 1998 Dec 8;98(23):2520–6.

200. Lamers Y, Prinz-Langenohl R, Moser R, Pietrzik K. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am J Clin Nutr. 2004 Mar;79(3):473–8.

201. Guttormsen AB, Ueland PM, Nesthus I, Nygård O, Schneede J, Vollset SE, et al. Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (> or = 40 micromol/liter). The Hordaland Homocysteine Study. J Clin Invest. 1996 Nov 1;98(9):2174–83.

202. D'Angelo A, Coppola A, Madonna P, Fermo I, Pagano A, Mazzola G, et al. The role of vitamin B12 in fasting hyperhomocysteinemia and its interaction with the homozygous C677T mutation of the methylenetetrahydrofolate reductase (MTHFR) gene. A case-control study of patients with early-onset thrombotic events. Thromb Haemost. 2000 Apr;83(4):563–70.

203. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995 May;10(1):111–3.

204. Godbole K, Deshmukh U, Yajnik C. Nutrigenetic determinants of neural tube defects in India. Indian Pediatr. 2009 Jun;46(6):467–75.

205. Namour F, Olivier J, Abdelmouttaleb I, Adjalla C, Debard R, Salvat C, et al. Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood. Blood. 2001 Feb 15;97(4):1092–8.

206. Hazra A, Kraft P, Lazarus R, Chen C, Chanock SJ, Jacques P, et al. Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. Hum Mol Genet. 2009 Dec 1;18(23):4677–87.

207. Castro R, Barroso M, Rocha M, Esse R, Ramos R, Ravasco P, et al. The TCN2 776CNG polymorphism correlates with vitamin B(12) cellular delivery in healthy adult populations. Clin Biochem. 2010 May;43(7-8):645–9.

208. Swanson DA, Pangilinan F, Mills JL, Kirke PN, Conley M, Weiler A, et al. Evaluation of transcobalamin II polymorphisms as neural tube defect risk factors in an Irish population. Birt Defects Res A Clin Mol Teratol. 2005;73(4):239–44.

209. Riedel BM, Molloy AM, Meyer K, Fredriksen A, Ulvik A, Schneede J, et al. Transcobalamin polymorphism 67A->G, but not 776C->G, affects serum holotranscobalamin in a cohort of healthy middle-aged men and women. J Nutr. 2011 Oct;141(10):1784–90.

210. Borén T, Falk P, Roth KA, Larson G, Normark S. Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens. Science. 1993 Dec 17;262(5141):1892–5.

211. Dickey W, Collins JS, Watson RG, Sloan JM, Porter KG. Secretor status and Helicobacter pylori infection are independent risk factors for gastroduodenal disease. Gut. 1993 Mar;34(3):351–3.

212. Hazra A, Kraft P, Selhub J, Giovannucci EL, Thomas G, Hoover RN, et al. Common variants of FUT2 are associated with plasma vitamin B12 levels. Nat Genet. 2008 Oct;40(10):1160–2.

213. Sütterlin MW, Bussen SS, Rieger L, Dietl J, Steck T. Serum folate and Vitamin B12 levels in women using modern oral contraceptives (OC) containing 20 microg ethinyl estradiol. Eur J Obstet Gynecol Reprod Biol. 2003 Mar 26;107(1):57–61.

214. Costanzi JJ, Young BK, Carmel R. Serum vitamin B12 and B12-binding protein levels associated with oral contraceptives. Tex Rep Biol Med. 1978;36:69–77.

215. Shojania AM. Oral contraceptives: effect of folate and vitamin B12 metabolism. Can Med Assoc J. 1982 Feb 1;126(3):244–7.

216. Wertalik LF, Metz EN, LoBuglio AF, Balcerzak SP. Decreased serum B 12 levels with oral contraceptive use. J Am Med Assoc. 1972 Sep 18;221(12):1371–4.

217. Gardyn J, Mittelman M, Zlotnik J, Sela BA, Cohen AM. Oral contraceptives can cause falsely low vitamin B(12) levels. Acta Haematol. 2000;104(1):22–4.

218. Lussana F, Zighetti ML, Bucciarelli P, Cugno M, Cattaneo M. Blood levels of homocysteine, folate, vitamin B6 and B12 in women using oral contraceptives compared to non-users. Thromb Res. 2003;112(1-2):37–41.

219. Green TJ, Houghton LA, Donovan U, Gibson RS, O'Connor DL. Oral contraceptives did not affect biochemical folate indexes and homocysteine concentrations in adolescent females. J Am Diet Assoc. 1998 Jan;98(1):49–55.

220. Riedel B, Bjørke Monsen A-L, Ueland PM, Schneede J. Effects of oral contraceptives and hormone replacement therapy on markers of cobalamin status. Clin Chem. 2005 Apr;51(4):778–81.

221. Hjelt K, Brynskov J, Hippe E, Lundström P, Munck O. Oral contraceptives and the cobalamin (vitamin B12) metabolism. Acta Obstet Gynecol Scand. 1985;64(1):59–63.

222. Carmel R, Howard JM, Green R, Jacobsen DW, Azen C. Hormone replacement therapy and cobalamin status in elderly women. Am J Clin Nutr. 1996 Dec;64(6):856–9.

223. The Daily, Tuesday, March 22, 2005. Study: Canada's visible minority population in 2017 [Internet]. [cited 2013 Dec 15]. Available from: http://www.statcan.gc.ca/daily-quotidien/050322/dq050322b-eng.htm

224. Heffner LJ. Advanced maternal age--how old is too old? N Engl J Med. 2004 Nov 4;351(19):1927–9.

225. Lear SA, Humphries KH, Kohli S, Birmingham CL. The use of BMI and waist circumference as surrogates of body fat differs by ethnicity. Obes Silver Spring Md. 2007 Nov;15(11):2817–24.

226. WHO expert consultation. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. The Lancet. 2004 Oct 1;363:157–73.

227. World Health Organization. Waist Circumference and Waist-hip Ratio: Report of a WHO Expert Consultation, Geneva, 8-11 December 2008. World Health Organization; 2011. 39 p.

228. Shatenstein B, Xu H, Luo Z-C, Fraser W. Relative validity of a food frequency questionnaire for pregnant women. Can J Diet Pract Res. 2011;72(2):60–9.

229. Shatenstein B, Nadon S, Godin C, Ferland G. Development and validation of a food frequency questionnaire. Can J Diet Pract Res. 2005;66(2):67–75.

230. Wilson DH, Yu J, Karian A, Kozlowski J, O'Reilly S. Development and multisite evaluation of an automated assay for B12 on the Abbott AxSYM analyzer. Clin Chem. 1999 Mar;45(3):428–9.

231. Brady J, Wilson L, McGregor L, Valente E, Orning L. Active B12: a rapid, automated assay for holotranscobalamin on the Abbott AxSYM analyzer. Clin Chem. 2008 Mar;54(3):567–73.

232. Blom HJ, van Rooij A, Hogeveen M. A simple high-throughput method for the determination of plasma methylmalonic acid by liquid chromatography-tandem mass spectrometry. Clin Chem Lab Med. 2007;45(5):645–50.

233. Briggs C, Harrison P, Grant D, Staves J, Chavada N, Machin SJ. Performance Evaluation of the Sysmex XE-2100TM Automated Haematology Analyser. Sysmex J Int. 1999;9(2).

234. Briggs C, Longair I, Kumar P, Singh D, Machin SJ. Performance evaluation of the Sysmex haematology XN modular system. J Clin Pathol. 2012 Nov;65(11):1024–30.

235. United Nations Children's Fund, United Nations University, World Health Organization. Iron Deficiency Anaemia: Assessment, Prevention, and control, A guide for programme managers [Internet]. Geneva, Switzerland: World Health Organization; 2001 [cited 2013 Dec 31]. Available from: http://www.who.int/nutrition/publications/micronutrients/anaemia_iron_deficiency/WHO_NHD_01.3/en/

236. Carmel R, Karnaze DS. Physician response to low serum cobalamin levels. Arch Intern Med. 1986 Jun;146(6):1161–5.

237. Willett W. Nutritional Epidemiology. New York, NY: Oxford University Press; 1998. 538 p.

238. Lear SA, Humphries KH, Kohli S, Chockalingam A, Frohlich JJ, Birmingham CL. Visceral adipose tissue accumulation differs according to ethnic background: results of the Multicultural Community Health Assessment Trial (M-CHAT). Am J Clin Nutr. 2007 Aug 1;86(2):353–9.

239. Sulistyoningrum DC, Green TJ, Lear SA, Devlin AM. Ethnic-Specific Differences in Vitamin D Status Is Associated with Adiposity. PLoS ONE. 2012 Aug 29;7(8):e43159.

240. Government of Canada SC. Statistics by subject: Education, training and learning - Educational attainment - Census tables [Internet]. 2003 [cited 2013 Oct 9]. Available from: http://www5.statcan.gc.ca/subject-sujet/result-resultat.action?pid=1821&id=1825&lang=eng&type=CENSUSTBL&pageNum=1&more=0

241. Villamor E, Mora-Plazas M, Forero Y, Lopez-Arana S, Baylin A. Vitamin B-12 status is associated with socioeconomic level and adherence to an animal food dietary pattern in Colombian school children. J Nutr. 2008 Jul;138(7):1391–8.

242. MacNeill V, Nwokoro C, Griffiths C, Grigg J, Seale C. Recruiting ethnic minority participants to a clinical trial: a qualitative study. BMJ Open. 2013 Jan 1;3(4):e002750.

243. Sheikh A, Halani L, Bhopal R, Netuveli G, Partridge MR, Car J, et al. Facilitating the Recruitment of Minority Ethnic People into Research: Qualitative Case Study of South Asians and Asthma. PLoS Med. 2009 Oct 13;6(10):e1000148.

244. Douglas A, Bhopal RS, Bhopal R, Forbes JF, Gill JMR, Lawton J, et al. Recruiting South Asians to a lifestyle intervention trial: experiences and lessons from PODOSA (Prevention of Diabetes & Obesity in South Asians). Trials. 2011;12:220.

245. Samsudeen BS, Douglas A, Bhopal RS. Challenges in recruiting South Asians into prevention trials: health professional and community recruiters' perceptions on the PODOSA trial. Public Health. 2011 Apr;125(4):201–9.

246. Krupp K, Madhivanan P, Karat C, Chandrasekaran V, Sarvode M, Klausner J, et al. Novel recruitment strategies to increase participation of women in reproductive health research in India. Glob Public Health. 2007;2(4):395–403.

247. Samuel TM, Thomas T, Finkelstein J, Bosch R, Rajendran R, Virtanen SM, et al. Correlates of anaemia in pregnant urban South Indian women: a possible role of dietary intake of nutrients that inhibit iron absorption. Public Health Nutr. 2013;16(02):316–24.

248. Oosterhuis WP, Niessen RW, Bossuyt PM, Sanders GT, Sturk A. Diagnostic value of the mean corpuscular volume in the detection of vitamin B12 deficiency. Scand J Clin Lab Invest. 2000 Feb;60(1):9–18.

249. Jain R. M.C.V. should not be the only criteria to order vitamin B12 for anemia under evaluation. Open J Gastroenterol. 2012;02(04):187–90.

250. Carmel R, Green R, Jacobsen DW, Qian GD. Neutrophil nuclear segmentation in mild cobalamin deficiency: relation to metabolic tests of cobalamin status and observations on ethnic differences in neutrophil segmentation. Am J Clin Pathol. 1996 Jul;106(1):57–63.

251. Misra U, Kalita J, Das A. Vitamin B12 deficiency neurological syndromes: a clinical, MRI and electrodiagnostic study. Electromyogr Clin Neurophysiol. 2002 Dec;43(1):57–64.

252. Rush EC, Chhichhia P, Hinckson E, Nabiryo C. Dietary patterns and vitamin B(12) status of migrant Indian preadolescent girls. Eur J Clin Nutr. 2009 Apr;63(4):585–7.

253. Forster-Coull L, Levy Milne R, Barr S. British Columbia Nutrition Survey, Report on Energy and Nutrient Intakes. Health Canada, BC Ministry of Health; 2004.

254. Kehoe SH, Krishnaveni GV, Veena SR, Guntupalli AM, Margetts BM, Fall CHD, et al. Diet patterns are associated with demographic factors and nutritional status in South Indian children. Matern Child Nutr. 2013 Jul 2;10(1):145–58.

255. Barr SI, Broughton TM. Relative Weight, Weight Loss Efforts and Nutrient Intakes among Health-Conscious Vegetarian, Past Vegetarian and Nonvegetarian Women Ages 18 to 50. J Am Coll Nutr. 2000;19(6):781–8.

256. Janelle K, Barr S. Nutrient Intakes and Eating Behaviors of Vegetarian and Nonvegetarian Women. J Am Diet Assoc. 1995 Feb;95(2):180–9.

257. Gao X, Yao M, McCrory MA, Ma G, Li Y, Roberts SB, et al. Dietary pattern is associated with homocysteine and B vitamin status in an urban Chinese population. J Nutr. 2003 Nov;133(11):3636–42.

258. Pfeiffer CM, Sternberg MR, Schleicher RL, Rybak ME. Dietary supplement use and smoking are important correlates of biomarkers of water-soluble vitamin status after adjusting for sociodemographic and lifestyle variables in a representative sample of U.S. adults. J Nutr. 2013 Jun;143(6):957S–65S.

259. Schneider JA, Rees DC, Liu YT, Clegg JB. Worldwide distribution of a common methylenetetrahydrofolate reductase mutation. Am J Hum Genet. 1998 May;62(5):1258–60.

260. Rai V, Yadav U, Kumar P. Prevalence of methylenetetrahydrofolate reductase C677T polymorphism in eastern Uttar Pradesh. Indian J Hum Genet. 2012;18(1):43–6.

261. Tanwar VS, Chand MP, Kumar J, Garg G, Seth S, Karthikeyan G, et al. Common variant in FUT2 gene is associated with levels of vitamin B(12) in Indian population. Gene. 2013 Feb 15;515(1):224–8.

262. Oussalah A, Besseau C, Chery C, Jeannesson E, Guéant-Rodriguez R-M, Anello G, et al. Helicobacter pylori serologic status has no influence on the association between

fucosyltransferase 2 polymorphism (FUT2 461 G->A) and vitamin B-12 in Europe and West Africa. Am J Clin Nutr. 2012 Feb;95(2):514–21.

263. Thirumurthi S, Graham DY. Helicobacter pylori infection in India from a western perspective. Indian J Med Res. 2012 Oct;136(4):549–62.

264. Kimmons JE, Blanck HM, Tohill BC, Zhang J, Khan LK. Associations between body mass index and the prevalence of low micronutrient levels among US adults. Medscape Gen Med. 2006;8(4):59.

265. Canadian Contraception Consensus (Part 1 of 3) [Internet]. [cited 2013 Oct 9]. Available from: http://sogc.org/guidelines/canadian-contraception-consensus-part-1-of-3-replaces-131aug-2003-and-49-sept-1996/

266. Shoveller J, Chabot C, Soon JA, Levine M. Identifying barriers to emergency contraception use among young women from various sociocultural groups in British Columbia, Canada. Perspect Sex Reprod Health. 2007 Mar;39(1):13–20.

267. Steegers-Theunissen RP, Boers GH, Steegers EA, Trijbels FJ, Thomas CM, Eskes TK. Effects of sub-50 oral contraceptives on homocysteine metabolism: a preliminary study. Contraception. 1992 Feb;45(2):129–39.

268. McArthur J, Tang H, Petocz P, Samman S. Biological Variability and Impact of Oral Contraceptives on Vitamins B6, B12 and Folate Status in Women of Reproductive Age. Nutrients. 2013 Sep 16;5(9):3634–45.

269. Berenson AB, Rahman M. Effect of hormonal contraceptives on vitamin B12 level and the association of the latter with bone mineral density. Contraception. 2012 Nov;86(5):481–7.

270. Roberts PD, James H, Petrie A, Morgan JO, Hoffbrand AV. Vitamin B 12 status in pregnancy among immigrants to Britain. Br Med J. 1973 Jul 14;3(5871):67–72.

271. Vallianatos H, Raine K. Consuming Food and Constructing Identities among Arabic and South Asian Immigrant Women. Food Cult Soc Int J Multidiscip Res. 2008 Sep 1;11(3):355–73.

272. Sanou D, O'Reilly E, Ngnie-Teta I, Batal M, Mondain N, Andrew C, et al. Acculturation and Nutritional Health of Immigrants in Canada: A Scoping Review. J Immigr Minor Health. 2014;16:24–34.

273. Gilbert PA, Khokhar S. Changing dietary habits of ethnic groups in Europe and implications for health. Nutr Rev. 2008 Apr;66(4):203–15.

274. Satia-Abouta J, Patterson R, Neuhouser M, Elder J. Dietary acculturation: Applications to nutrition research and dietetics. J Am Diet Assoc. 2002 Aug;102(8):1105–18.

275. Bates CJ, Schneede J, Mishra G, Prentice A, Mansoor MA. Relationship between methylmalonic acid, homocysteine, vitamin B12 intake and status and socio-economic indices, in a subset of participants in the British National Diet and Nutrition Survey of people aged 65 y and over. Eur J Clin Nutr. 2003 Feb;57(2):349–57.

276. Vogiatzoglou A, Oulhaj A, Smith AD, Nurk E, Drevon CA, Ueland PM, et al. Determinants of plasma methylmalonic acid in a large population: implications for assessment of vitamin B12 status. Clin Chem. 2009 Dec;55(12):2198–206.

277. Arora P, Vasa P, Brenner D, Iglar K, McFarlane P, Morrison H, et al. Prevalence estimates of chronic kidney disease in Canada: results of a nationally representative survey. Can Med Assoc J. 2013 Jun 11;185(9):E417–423.

278. Butte NF, Ekelund U, Westerterp KR. Assessing physical activity using wearable monitors: measures of physical activity. Med Sci Sports Exerc. 2012 Jan;44(1 Suppl 1):S5–12.

279. Kelemen LE, Anand SS, Vuksan V, Yi Q, Teo KK, Devanesen S, et al. Development and evaluation of cultural food frequency questionnaires for South Asians, Chinese, and Europeans in North America. J Am Diet Assoc. 2003 Sep;103(9):1178–84.

280. Fieldhouse P. Food & Nutrition: Customs & Culture. London, UK: Croom Helm; 1986. 262 p.

281. Sevak L, Mangtani P, McCormack V, Bhakta D, Kassam-Khamis T, Silva I dos S. Validation of a food frequency questionnaire to assess macro- and micro-nutrient intake among South Asians in the United Kingdom. Eur J Nutr. 2004 Jun 1;43(3):160–8.

282. Bailey RL, Dodd KW, Gahche JJ, Dwyer JT, McDowell MA, Yetley EA, et al. Total folate and folic acid intake from foods and dietary supplements in the United States: 2003-2006. Am J Clin Nutr. 2010 Jan;91(1):231–7.

a place of mind
THE UNIVERSITY OF BRITISH COLUMBIA

ADEQUACY OF VITAMIN B12 STATUS IN WOMEN OF CHILDBEARING AGE
DEMOGRAPHIC QUESTIONNAIRE

*Please note that all information on this questionnaire is labelled with an ID number only, and stored separately from your name and contact details. All information collected will be stored securely, and personally identifiable information will be accessible to no one except Dr Yvonne Lamers.

1| **Today's Date** __ __/ __ __ / __ __ __ __(DD/MM/YYYY)

2| **Study Personnel ID Number** __ __ (##)

3| **Location of Subject Visit:**
 ☐ Food Nutrition and Health Building, University of British Columbia
 ☐ Clinical Research and Evaluation Unit, Child and Family Research Institute
 ☐ South Community Health Centre, Vancouver Coastal Health
 ☐ Newton Public Health Unit, Fraser Health, Surrey

4| **Subject ID Number** __ __ __ (###)
*Please repeat this field at the top right corner of every separate page

BODY COMPOSITION

5| **Height** __ __ cm *(researcher to fill in)*

6| **Weight** __ __ __ kg *(researcher to fill in)*

7| **Waist Circumference** __ __ __ cm *(researcher to fill in)*

Please ensure that all 8 pages have been filled out

PERSONAL INFORMATION

8| What is your age? __ __ (Years)

ETHNIC BACKGROUND

9| What ethnic group do you identify as:

Please check the MOST appropriate box
☐ Caucasian (01)
☐ South Asian (02)

10| To what generation of immigration do you belong to?

☐ First-generation (01): *You immigrated to Canada. You were NOT born in Canada.*
☐ Second-generation (02): *Your parents immigrated to Canada. You WERE born in Canada.*
☐ Mixed-generation (05): *One of your parents immigrated to Canada, the other one was born in Canada. You WERE born in Canada.*
☐ Third-generation (03): *Your grandparents immigrated to Canada. You and your parents WERE born in Canada.*
☐ Fourth-generation or higher (04): *Your great-grandparents or more distant (generationally) relatives immigrated to Canada. You, your parents, AND your grandparents WERE born in Canada.*

11| What area of Metro Vancouver are you from?

Abbotsford, Anmore, Belcarra, Bowen Island, Burnaby, Coquitlam, Delta, Langley – City, Langley – Township, Lions Bay, Maple Ridge, Metro Vancouver, New Westminster, North Vancouver – City, North Vancouver – District, Pitt Meadows, Port Coquitlam, Port Moody, Richmond, Surrey, Vancouver, West Vancouver, White Rock, Electoral Area A

_____ (Please fill in 1 from the above list)

SOCIO-DEMOGRAPHIC

12| How many people live in your household? __ __ (##)
Please include only individuals (i.e. spouse, partner, family member) who you share income and expenses with (e.g. do not list roommates if expenses are largely unshared).

13| What is your best estimate of the total income, before taxes and deductions, of all household members from all sources in the past 12 months? *Only list combined income of individuals (i.e. spouse, partner, other family members) who contribute jointly to the household income and share expenses*

☐ Less than $15,000 (01)
☐ $15,001 to less than or equal to $20,000 (02)
☐ $20,001 to less than or equal to $30,000 (03)
☐ $30,001 to less than or equal to $40,000 (04)
☐ $40,001 to less than or equal to $50,000 (05)
☐ $50,001 to less than or equal to $60,000 (06)
☐ $60,001 to less than or equal to $70,000 (07)
☐ $70,001 to less than or equal to $80,000 (08)
☐ $80,000 or more (09)

☐ Don't know (10)

EDUCATION

14| Please list the highest level of education you have completed to date.

☐ Less than secondary (high school) education (01)
☐ Secondary school (high school) diploma (02)
☐ Some post-secondary education
Trade certificate or diploma, apprenticeship training, or non-university certificate or diploma from community college, CEGEP etc., university education below bachelor's level (2/3 year diploma or partially completed bachelor's degree)(03)
☐ Bachelor's degree (post-secondary education) (04)
☐ University degree or certificate higher than bachelor's degree
(ie Master, Doctorate, professional degree) (05)

ID [__ __ __]

☐ Don't know or decline to respond (06)

LIFESTYLE

15| Are you a smoker?

☐ Yes, Current frequent smoker (01)
   Smoke more than 20 cigarettes per day
☐ Yes, Current regular smoker (02)
   Smoke between 10 and 19 cigarettes per day
☐ Yes, Current occasional smoker (03)
   Smoke between 1 and 9 cigarettes per day
☐ Former smoker (YES) (04)
☐ No, Non smoker (NO) (05)

16| Do you drink coffee, tea, or other caffeinated beverages?

☐ Yes - Coffee (01)
   Please specify numbers of cups of coffee per day:__ __ (##)
☐ Yes - Tea (02)
   Please specify numbers of cups of tea per day: __ __ (##)
☐ Yes - other caffeinated beverages, for example energy drinks, caffeinated pop, iced coffee drinks) (03)
   Please specify numbers of other caffeinated beverages per day:
   __ __ (##)
☐ No, none of the above (04)

17| Have you donated blood in the last 6 months?
☐ Yes (01)
☐ No (02)

If yes, how many times__ __(##)

18| It is important for this study to know whether or not you are pregnant. Please check one of the following:

☐You know 100% that you ARE pregnant (01)
☐You know 100% that you are NOT pregnant (02)
☐You are unsure whether you are pregnant or not (03)

19| **Have you previously been pregnant**
☐Yes, full term (01)
☐Yes, not full term (02)
☐No (03)
☐Prefer not to answer (04)

20| **If you answered *'Yes, full term'* to the previous question, did you breastfeed?**
☐Yes (01)
☐No (02)
☐Prefer not to answer (03)

21| **If you have had children - What was the birth weight of your child(ren)?**
☐Low (1-5.5 lbs, 0.45-2.5 kg) (01)
☐Normal (5.5-9 lbs, 2.5-4 kg) (02)
☐High (greater than 9 lbs, greater than 4 kg) (03)

ORAL CONTRACEPTIVES

22| **Are you currently taking hormonal contraceptives?**
i.e. birth control pill, birth control patch, depo provera etc.?
☐Yes (01)
☐No (02)

23| **If you answered YES to the last question - Approximately how long have you been taking oral contraceptives?**

__ __(years)/__ __ (months)(##/##)

24| **If you answered YES to question 22 - Do you take hormonal contraceptives on a one month or three month cycle?**
☐ One-month cycle (01)
☐ Three months cycle (02)
☐ Other (03) Please specify number of months__ __(##)

25| What was the approximate date of your last period (the last date that you menstruated)?

Day/Month __ __/__ __(##/##)
(for example, 13/01 for the 13th of January)

26| Approximately how long is your typical menstrual cycle - in <u>days?</u>

__ __(##)
(for example: 28 days)

SUPPLEMENT USE

If you have forgotten to bring in your supplement bottles, please refer to the reference bottles available along with the assistance of study personnel to fill out this section –If you have brought your bottles in, please consult study personnel at this time to assist in filling out this aspect of the questionnaire.

27| Do you use nutritional vitamin or mineral supplements?
- ☐ Yes (01)
- ☐ No (02)

28| Do you use Folate (Folic Acid, Vitamin B9) Supplements?
- ☐ Yes (01)
- ☐ No (02)

29| Do you use Vitamin B12 (Cobalamin) Supplements?
- ☐ Yes (01)
- ☐ No (02)

Total Folate Supplement Use _____micrograms Total B12 Supplement Use _____micrograms   **To be filled out by researcher**

1. **Type of Supplement:**
☐ Multivitamin supplement (01)
☐ Vitamin supplement (02) i.e. vitamin B12 supplements
☐ Food Supplement or Meal Replacement (03)
i.e. Red Star™ nutritional yeast, meal replacement drinks (ie. Ensure™),
meal replacement bar (ie. Clifbar™), vitamin containing drinks (ie. Vitamin water™)
☐ Pre-natal vitamin (04) i.e. Materna™

☐ Folate containing (01)
☐ B12 containing (02)

Product (Brandname)

…………………………………………

How often do you consume it?

……………………………………………

Dose

………………………………Folate (in micrograms)

………………………………Vitamin B-12 (in micrograms)

Number of Doses/Day:…………………

---

2. **Type of Supplement:**
☐ Multivitamin supplement (01)
☐ Vitamin supplement (02) i.e. vitamin B12 supplements
☐ Food Supplement or Meal Replacement (03)
i.e. Red Star™ nutritional yeast, meal replacement drinks (ie. Ensure™),
meal replacement bar (ie. Clifbar™), vitamin containing drinks (ie. Vitamin water™)
☐ Pre-natal vitamin (04) i.e. Materna™

☐ Folate containing (01)
☐ B12 containing (02)

Product (Brandname)

…………………………………………

ID [__ __ __]

How often do you consume it?

……………………………………………

Dose

………………………………Folate (in micrograms)

………………………………Vitamin B-12 (in micrograms)

Number of Doses/Day:…………………

---

3. **Type of Supplement:**
   ☐ Multivitamin supplement (01)
   ☐ Vitamin supplement (02) i.e. vitamin B12 supplements
   ☐ Food Supplement or Meal Replacement (03)
   i.e. Red Star™ nutritional yeast, meal replacement drinks (ie. Ensure™), meal replacement bar (ie. Clifbar™), vitamin containing drinks (ie. Vitamin water™)
   ☐ Pre-natal vitamin (04) i.e. Materna™

   ☐ Folate containing (01)
   ☐ B12 containing (02)

Product (Brandname)

………………………………………

How often do you consume it?

……………………………………………

Dose

………………………………Folate (in micrograms)

………………………………Vitamin B-12 (in micrograms)

Number of Doses/Day:…………………

---

4. **Type of Supplement:**

☐ Multivitamin supplement (01)
☐ Vitamin supplement (02) i.e. vitamin B12 supplements
☐ Food Supplement or Meal Replacement (03)
i.e. Red Star™ nutritional yeast, meal replacement drinks (ie. Ensure™),
meal replacement bar (ie. Clifbar™), vitamin containing drinks (ie. Vitamin
water™)
☐ Pre-natal vitamin (04) i.e. Materna™

☐ Folate containing (01)
☐ B12 containing (02)

Product (Brandname)

…………………………………………

How often do you consume it?

……………………………………………

Dose

…………………………………Folate (in micrograms)

…………………………………Vitamin B-12 (in micrograms)

Number of Doses/Day:…………………

FOOD ALLERGIES/PREFERENCES

30| **Are you Lactose Intolerant?**

☐ Yes (01)
(Complete restriction of milk (dairy) products)
☐ Moderately (02)
(Incomplete restriction of milk (dairy) products)
☐ No (03)
(No (Zero) restriction of milk (dairy) products)

End of questionnaire – Thank you for your participation.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE
(August 2002)

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ
The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ
Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation
Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ
International collaboration on IPAQ is on-going and an *International Physical Activity Prevalence Study* is in progress. For further information see the IPAQ website.

More Information
More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

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INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1.   During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

     _____ **days per week**

     ☐ No vigorous physical activities   ➡ ***Skip to question 3***

2.   How much time did you usually spend doing **vigorous** physical activities on one of those days?

     _____ **hours per day**

     _____ **minutes per day**

     ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3.   During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

     _____ **days per week**

     ☐ No moderate physical activities   ➡ ***Skip to question 5***

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

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4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

[ ] Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

[ ] No walking ➞ **Skip to question 7**

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

[ ] Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

[ ] Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

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Brief Guide to the Food Frequency Questionnaire: 'Your dietary evaluation'

The food frequency questionnaire, *'Your dietary evaluation – Version 3.23A.NB (2012-09-19)'*, created by Dr. Bryna Shatenstein *et al* was used to assess dietary intake of the study population. Due to copyright restrictions the questionnaire is not included in this thesis and this guide covers the format and content.

The questionnaire was 16 pages, booklet style printed on 8.5x11 paper.
Title page Contained a blank for 'Participant ID' number, recorded by the candidate, and a brief introduction to the questionnaire, which was read aloud by the participant before completion of the questionnaire.
Copyright page This questionnaire is © Shatenstein, B. 2003. All photos © American Dietetic Association, 1997.
Instruction page Instructions were read aloud to the participant for filling out the frequency and portion columns for food items. These instructions also included a verbal explanation of how to interpret serving sizes against the images provided, and how to calculate frequency of consumption for foods eaten irregularly or seasonally. In addition the candidate also instructed the respondent on how to read the questionnaire, what order (sequential) to respond to questions in, and who to address with questions during completion.
Body The body of the questionnaire included facing pages, which included, on the left page: pictures of common food items for the subject to use as reference for portion sizes (*e.g. picture of a plate of French fries indicating a smaller portion on the left and a larger portion on the right with the guide (250 to 500 ml, 1 to 2 cups, or 50 to 100 g))*, and on the right page: a list of food items *(e.g. High fiber breakfast cereals (All Bran, 100% Bran, Bran Flakes…))* divided into broader categories *(e.g. 'Breads and cereals and related foods')*. There were spaces in each food item row to respond to two questions regarding: **[1]** frequency of consumption, and **[2]** portion size. In total there were 78 food items covered by the questionnaire. **For frequency of consumption**: The respondent was asked to choose between 6 frequency options including: **[1]** Never or rarely **[2]** 1 to 3 times per month **[3]** 1 to 2 times per week **[4]** 3 to 5 times per week **[5]** Once per day **[6]** 2 times or + per day

The above options were for all food items with the exception of the categories '*Juices and drinks*', '*Milk and similar beverages*', and '*Coffee, water, alcoholic drinks and related items*', for which the 6 options included:
[1] Never or rarely
[2] 1 to 7 times per month
[3] 2 to 6 times per week
[4] Once per day
[5] 2 to 3 times per day
[6] 4 times or + per day.

For typical portion size:
The respondent was asked to choose between 3 options including:
[1] smaller (-)
[2] similar (=)
[3] larger (+).

For each food item the respondent was directed towards a visual aid and instructed to decide whether their portion size was 'larger than the larger portion'=larger, 'equal to or in the range between the smaller and the larger portion'=equal, or 'smaller than the smaller portion'=smaller. Several food items were not linked to a reference picture. In these cases the item was easily identifiable (e.g. *1 to 2 slices of commercial sliced whole wheat breads…*) and the subject was instructed to choose larger if their typical portion size was at least 2x larger than the portion stated, smaller if their typical portion size was at least 50% smaller the portion stated, and equal if their typical portion size fell in the range between those two values. Using the example of *commercial sliced whole wheat breads* a respondent would choose larger if they consumed 4 slices or more, and smaller if they consumed half a slice or less, anything in the range of 0.5-4 slices would be considered similar.

Food habits and life style habits
A list of 18 questions pertaining to food habits (e.g. *type of fat in cooking*), physical activity, dietary practices, weight, height, lifestyle (e.g. *smoking status*), sociodemographics (e.g. *gender*, *highest level of education*) were asked. Due to overlap with our Demographic Questionnaire **(Appendix 1)** these responses were not included in our analysis but were used by Dr. Shatenstein's team during analysis of the questionnaire.

Closing statements
The subject was instructed to record the date of completion, and read through a final checklist to ensure that they followed the directions to the best of their ability.

Final check
The questionnaire, upon completion, was checked visually by the candidate to ensure that no responses were missing, that there wasn't blatant repetition (e.g. the same frequency of consumption for all food items) that the subject could not explain. Following this process the subject was asked if they had any issues completing the questionnaire and if any questions were posed (e.g. *"Does the soy milk line item include other plant based milks like almond milk?"*) they were clarified by the candidate. A space for comments was included on the last page of the questionnaire and respondents were invited to give feedback.

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Vitamin Research Fund

Food, Nutrition, and Health
Faculty of Land and Food Systems
UBC

ADEQUACY OF B12 STATUS
IN WOMEN OF CHILDBEARING AGE
– SCREENING QUESTIONNAIRE – BY TELEPHONE

Study Personnel ID # __ __ (##)

Subject ID # __ __ __ __ (####)*
Please repeat this field at the top right corner of every separate page

*Please note that all information on this questionnaire is labelled with an ID number only, and stored separately from your name and contact details. All information collected will be stored securely, and personally identifiable information will be accessible to no one except Dr Yvonne Lamers and her research associates.

Today's Date (DD/MM/YYYY) ___ ___ /___ ___ /___ ___ ___ ___

PERSONAL INFORMATION

WHAT IS YOUR AGE? ___ ___ (years) (exclude if not 19-35)

ETHNIC BACKGROUND

WHAT ETHNIC 'GROUP' DO YOU IDENTIFY AS:
Please check the MOST appropriate box
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

Subject:

FROM WHAT ETHNIC GROUP ARE YOUR ANCESTORS:
MOTHER
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

FATHER
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

Parents:

Mother

Father

MATERNAL GRANDMOTHER (your mother's mother)
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

MATERNAL GRANDFATHER (your mother's father)
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

PATERNAL GRANDMOTHER (your father's mother)
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

PATERNAL GRANDFATHER (your father's father)
☐ Caucasian (01)
☐ South Asian (02)
☐ Other (03), please specify: _____

| **Grandparents:** |
| MGM _____ |
| MGF _____ |
| PGM _____ |
| PGF _____ |

*Scoring of ethnicity: Exclude subject if she does not primarily identify as Caucasian or South Asian; i.e. exclude if less than three grandparents are from the same ethnicity (either 01 or 02). If no data on grandparents available, exclude if mother and father score 01 and 02 or 02 and 01, respectively, and exclude if parents have a total ethnicity score of >4.

HEALTH and PHARMACEUTICAL USE

DO YOU SUFFER FROM A CHRONIC CONDITION

(For Example: Diabetes , Cardiovascular Disease , Cancer , Renal Failure/Kidney Disease, Chronic Obstructive Pulmonary Disease (COPD), Emphysema, Bronchitis, High Blood Pressure , High Cholesterol, Arthritis, Fibromyalgia, Asthma, Stroke , Thyroid Condition, Major Depression, Bipolar disorder, Dysthymia (chronic mild depression), Schizophrenia, Anxiety Disorder, Learning Disability (*Attention Deficit Disorder, Attention Deficit Hyperactivity Disorder, Dyslexia, Other*), Eating Disorder (Anorexia, Bulimia etc.), Liver Disease, Gallbladder Problems, Hepatitis, HIV/AIDS**)**

Yes (01)   No (02) (Exclude if Yes)

CURRENTLY (IN THE PAST MONTH) **DID YOU TAKE ANY PRESCRIPTION MEDICATIONS?**

(In addition to other prescription medication this includes: insulin, nicotine patches)
*Do not indicate yes if you ONLY receive a prescription for oral contraceptives (birth control)

Yes (01)   No (02)

IF YOU ANSWERED YES TO THE LAST QUESTION, DO YOU TAKE ANY OF THE FOLLOWING MEDICATIONS?
Metformin, Anti-Cancer Treatment, Antibiotics, Proton Pump Inhibitors, Antacids, Chloramphenicol

Yes (01)   No (02) (Exclude if Yes)

IF THE MEDICATION YOU TAKE IS NOT ONE OF THE PREVIOUS PLEASE SPECIFY BELOW:
……………………………………………………………
(Exclude if a medication is being taken for chronic illness or if B12 interaction exists)

CURRENTLY (IN THE PAST MONTH) **DID YOU TAKE ANY PERFORMANCE ENHANCING OR RECREATIONAL DRUGS?** (such as steroids, marijuana, cocaine etc)
Yes (01)   No (02) (Exclude if yes)

ARE YOU CURRENTLY TAKING ANY OVER THE COUNTER MEDICATION SUCH AS PAIN KILLERS, ANTACIDS, ALLERGY PILLS OR HYDROCORTISONE CREAMS?
Yes (01)   No (02) (Exclude if yes)

ARE YOU CURRENTLY TAKING HORMONAL CONTRACEPTIVES (birth control pill (oral contraceptives), birth control patch, depo provera, copper intrauterine device etc.)
Yes (01)   No (02) (Exclude if anything other than oral contraceptives)
If yes please specify what type……………………………………….

IT IS IMPORTANT FOR THIS STUDY TO KNOW WHETHER OR NOT YOU ARE PREGNANT. PLEASE CHECK ONE OF THE FOLLOWING:
You Know 100% That You Are Pregnant (01)
You Know 100% that You Are NOT Pregnant (02)
You Are Unsure Whether You Are Pregnant or Not (03) (Exclude if 01 or 03)

DO YOU SUFFER FROM ANY OF THE FOLLOWING GASTROINTESTINAL CONDITIONS?
Atrophic Gastritis, Crohn's Disease, Irritable Bowel Syndrome, Colitis, Pernicious Anaemia Celiac Disease, Acid Indigestion, Constipation, Diverticulitis/Diverticulosis, Colon Polyps, Celiac Disease, Gastro-esophageal Reflux Disorder?
    Yes (01)   No (02) (Exclude if yes)

HAVE YOU UNDERGONE ANY FORM OF GASTRIC BYPASS SURGERY?
    Yes (01)   No (02) (Exclude if yes)

Thank you for your time. If you meet the criteria for subject recruitment we will be in contact within 3-6 business days. If you do not hear from us, we apologize but we cannot include you in the study.

*Coding exists so that these characteristics can be entered for all study participants

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CURIOUS ABOUT YOUR VITAMIN B12 LEVELS?

UBC

Vitamin B12 is important for your overall health as it promotes growth and development, energy levels, and a healthy brain. It is especially important for women of childbearing age. Deficiency has been associated with an increased risk of birth complications. We want to find out what factors influence vitamin B12 status of women of **SOUTH ASIAN** and **CAUCASIAN** ethnicity.

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We welcome interested healthy, non-pregnant females (19-35) to enrol in the study!
- This single session should take less than 1 hour.
- Receive a gift voucher as a thank-you for your time!

For More Information
Contact: Teo Quay
778-879-3318 - quayt@mail.ubc.ca

Appointments held on UBC campus or at the Child and Family Research Institute (28th and Oak)

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Appendix E

UBC ਵਿਟਾਮਿਨ ਬੀ 12 ਦੀ ਤੀਵੀ ਪੜ੍ਹਾਈ

UBC ਵਿਟਾਮਿਨ ਬੀ 12 ਦੀ ਅਨੁਸੰਧਾਨ ਸਵਾਗਤ ਕਰਦੀ ਹੈ (ਪੰਜਾਬੀ , ਹਿੰਦੀ , ਤਮਿਲ) ਭਾਰਤੀ ਔਰਤਾਂ ਦਾ ਜੋ ਇਹ ਮਹੱਤਵਪੂਰਣ ਪੋਸਣਾ ਅਨੁਸੰਧਾਨ ਵਿੱਚ ਭਾਗ ਲੈ ਸਕਦੀਆਂ ਹਨ

UBC ਖੋਜਕਾਰਾਂ ਦਰ ਅਤੇ ਦੱਖਣ ਏਸ਼ੀਆਈ ਔਰਤਾਂ ਵਿੱਚ ਵਿਟਾਮਿਨ ਬੀ 12 ਦੀ ਕਮੀ ਲਈ ਜੋਖਮ ਕਾਰਕਾਂ ਦਾ ਨਰਿਧਾਰਣ ਕਰਣ ਲਈ ਇੱਕ ਪੜ੍ਹਾਈ ਦਾ ਪ੍ਰਬੰਧ ਕਰ ਰਹੇ ਹਨ . ਵਿਟਾਮਿਨ ਬੀ 12 ਦੀ ਕਮੀ ਖ਼ਰਾਬ ਗਰਭਾਵਸਥਾ ਦੇ ਨਤੀਜੀਆਂ ਦੇ ਨਾਲ ਜੁੜਿਆ ਹੋਇਆ ਹੈ ਅਤੇ ਇਹ ਮਹੱਤਵਪੂਰਣ ਹੈ ਕਿ ਮਾਤਾਵਾਂ ਦੇ ਸਮਰੱਥ ਵਿਟਾਮਿਨ ਬੀ 12 ਦੀ ਹਾਲਤ ਅਤੇ ਪੱਧਰ ਦੀ ਜਾਂਚ ਕਰੀ ਜਾਵੇ

For More Information

Contact: Teo Quay

778-879-3318 - quayt@mail.ubc.ca

ਸੰਪਰਕ quayt@mail.ubc.ca ਜਾਂ 778-879-3318 ਕ੍ਰਿਪਾ ਜਿਆਦਾ ਜਾਣਕਾਰੀ ਲਈ ਅਤੇ ਨਿਯੁਕਤੀਜੋ ਦੇ ਲਈ

ਇਹ ਪੜ੍ਹਾਈ UBC , ਬਾਲ ਅਤੇ ਪਰਵਾਰ ਅਨੁਸੰਧਾਨ ਸੰਸਥਾਨ , ਵੈਂਕੂਵਰ ਅਤੇ Surrey ਦੇ ਨਿਊਟਨ ਸਾਰਵਜਨਕ ਸਹਿਤ ਯੂਨਿਟ ਵਿੱਚ ਆਜੋਜਿਤ ਕੀਤਾ ਜਾਵੇਗਾ ਤੁਹਾਡੀ ਸਹੂਲਤ ਲਈ

ਨਿਯੁਕਤੀ 1 ਘੰਟੇ ਕੀਤੀ ਹੋਵੇਗੀ . 3 ਪ੍ਰਸ਼ਨਾਵਲੀ ਅਤੇ ਇੱਕ ਛੋਟੇ ਜਿਹ ਖੂਨ ਦੇ ਨਮੂਨੇ ਦੀ ਜਰੂਰਤ ਹੇ

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UBC वटिामनि बी 12 की महलिा अध्ययन

UBC वटिामनि बी 12 की अनुसंधान स्वागत करती है (पंजाबी, हिंदी, तमलि) भारतीय महलिाओं का जो ये महत्वपूर्ण पोषण अनुसंधान में भाग ले सकती हैं

UBC शोधकर्ताओं दर और दक्षणि एश्यियाई महलिाओं में वटिामनि बी 12 की कमी के लिए जोखमि कारकों का नर्धिारण करने के लिए एक अध्ययन का आयोजन कर रहे हैं. वटिामनि बी 12 की कमी खराब गर्भावस्था के परणिामों के साथ जुडा हुआ है और यह महत्वपूर्ण है कमिाताओं के पर्याप्त वटिामनि बी 12 की स्थतिऔर स्तर की जांच करी जाए

For More Information
Contact: Teo Quay
778-879-3318 - quayt@mail.ubc.ca
संपर्क quayt@mail.ubc.ca या 778-879-3318 कृपया अधिक जानकारी के लिए और नयिुक्तियों के लिए.

ये अध्ययन UBC, बाल और परविार अनुसंधान संस्थान, वैंकूवर और Surrey के न्यूटन सार्वजनकि स्वास्थ्य यूनटि में आयोजति कयिा जाएगा आपकी सुवधिा के लिए

नयिुक्ति 1 घंटे की होगी. 3 प्रश्नावली और एक छोटे से खून के नमूने की जरूरत हे

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Are You A **South Asian** Woman Who Is -
Curious About Your
Vitamin B12 Levels?

Vitamin B12 is important for your overall health as it promotes growth and development, energy levels, and a healthy brain. It is especially important for women of childbearing age. Deficiency has been associated with an increased risk of birth complications. We want to find out what factors influence vitamin B12 status of women of **SOUTH ASIAN** ethnicity.

We welcome healthy, non-pregnant South Asian females (19-35) to enrol in the study!

This single session should take less than 1 hour.
Receive a gift voucher and your test results as a thank-you for your time!

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For More Information
Contact: Teo Quay
778-879-3318 - quayt@mail.ubc.ca

VISIT: ubcb12study.tumblr.com

Locations: UBC campus or The Child and Family Research Institute

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LOCATIONS:

Appointments are held at:

(1) The UBC **Food Nutrition and Health Building** @ 2205 East Mall

(2) The **Child and Family Research Institute** 950 – 28th Avenue West (@ Oak)

Appointments are available on weekday and weekend mornings – we are happy to accommodate your schedule

CONTACT US HERE:

Primary Contact: Teo Quay, Graduate Student

quayt@mail.ubc.ca
778-879-3318
www.ubcb12study.tumblr.com

Are you curious about your vitamin B12 levels?

Vitamin B12

Women's Study

UBC

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PRINCIPAL INVESTIGATOR:
Dr. Yvonne Lamers
Assistant Professor & Canada Research Chair in Human Nutrition and Vitamin Metabolism
UBC - Food Nutrition and Health

WHAT IS THE STUDY ABOUT?

Vitamin B12 is important for your overall health. It promotes growth and development, adequate energy, and a healthy brain. This vitamin is especially important for women of childbearing age. Deficiency has been associated with an increased risk of birth complications. **We want to find out the risk, and risk factors, for vitamin B12 deficiency for South Asian and Caucasian women.**

WHAT DOES THE STUDY INVOLVE?

We ask that you set aside 1 hour for a **one time** session.

During the appointment you will fill out **3 questionnaires** on demographics and lifestyle, physical activity, and dietary intake. We also ask for a **fasting blood sample.**
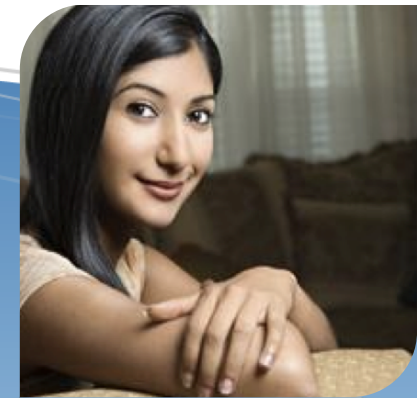
Who can participate?

Females who are:

-Non-pregnant

-Ages 19-35

-Healthy*

-South Asian or Caucasian**

No chronic or gastrointestinal disease, not currently taking prescription or over the counter medication

***At least 3 grandparents from the same ethnic background, Caucasian for the purposes of this study includes individuals from European backgrounds*

Participants will receive the **results** of their vitamin B12 and folate tests as well as information on dietary intake of both vitamins.

A **gift card** will be given as remuneration for the time commitment.

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Vitamin B12 Women's Study

Are you Curious about your vitamin levels?

Vitamin B12 is important for your overall health as it promotes growth and development, energy levels, and a healthy brain. It is especially important for women of childbearing age. Deficiency has been associated with an increased risk of birth complications. We want to determine the vitamin B12 deficiency risk factors for women of **SOUTH ASIAN** and **EUROPEAN** descent.
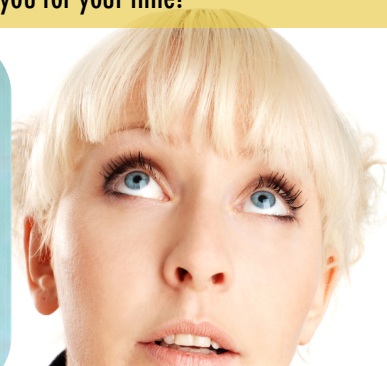
For More Information
Contact: Teo Quay
778-879-3318 - quayt@mail.ubc.ca

We welcome interested healthy, non-pregnant females (19-35) to enrol in the study!
- This single session should take less than 1 hour.
- Receive a gift voucher as a thank-you for your time!

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Grounded in Science | Global in Scope

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Subject Information and Consent Form
Adequacy of Vitamin B12 Status in Women of Childbearing Age

Principal Investigator: Yvonne Lamers, PhD
Food, Nutrition & Health
Land and Food Systems - The University of British Columbia
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- Filza Qureshi

Food, Nutrition & Health

Description: Lamers B12 Study Version 9: 2013-02-24
FHREB Approved: 2013 February 27

Page 1

1. INVITATION

You are being invited to take part in this research study because you are a healthy, non-pregnant South Asian or Caucasian woman of childbearing age (19 to 35).

2. YOUR PARTICIPATION IS VOLUNTARY

Your participation is entirely voluntary, so it is ultimately your decision whether or not to take part in this study. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study and the possible benefits, risks, and discomforts. If you wish to participate, you will be asked to sign this form. If you decide to take part in this study, you are still free to withdraw at any time. You will not be required to give a reason for your decision to withdraw. If you do not wish to participate, you do not have to provide any reason for your decision not to participate nor will you lose the benefit of any medical care to which you are entitled or are presently receiving. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide.

At the end of this consent form, there will be the option for consenting to being contacted for recruitment in follow up studies, and to receive the results of your vitamin B12 and folate tests. These options are independent of your willingness to participate in the proposed trial and it is your decision whether or not you sign the separate consent forms for these options.

In addition to this consent form there will be a separate optional form for giving consent for future use of biological materials (your blood sample). This option is independent of your willingness to participate in the proposed trial and it is your decision whether or not you sign the separate consent form for this option.

3. WHO IS CONDUCTING THE STUDY?

Researchers from the Food, Nutrition, and Health Department of the Faculty of Land and Food Systems, University of British Columbia, are conducting the study in collaboration with Vancouver Coastal Health and the Fraser Health Authority. The "University of British Columbia – Vitamin Research Fund" is funding the study. Data and biological samples collected during the duration of the study will be used as the subject of a Masters Thesis. No conflicts of interest to declare.

4. BACKGROUND

Low vitamin B-12 status in the mother was recently identified as an independent risk factor for having a neural tube defect (NTD) affected pregnancy. Neural tube defects are developmental

abnormalities that occur in the infant during pregnancy, often accompanied by physical and cognitive impairment. In addition to increased NTD risk, low but normal vitamin B-12 status, described as marginal vitamin B-12 deficiency, has been associated with an increased risk of insulin resistance, a risk factor for type 2 diabetes, in the mother and infant.

You are being invited to take part in a study that assesses the frequency, as well as potential factors contributing to vitamin B-12 deficiency, in women of childbearing age from select ethnic groups in the Greater Vancouver area.

Studies involving humans now routinely collect information on race and ethnic origin as well as other characteristics of individuals because these characteristics may influence how people respond to different diets and nutrient intakes. A person's ethnicity, for example, may impact on a range of biological factors that could potentially affect the individual's vitamin B-12 status.

We are interested in looking at only Caucasian and South Asian women because of previous research showing that individuals of these ethnicities may have lower vitamin B-12 concentrations than individuals of Asian and African descent. Providing information on your race or ethnic origin is voluntary.

5. WHAT IS THE PURPOSE OF THE STUDY?

The goal of this pilot study is to assess the difference in frequency of marginal vitamin B-12 deficiency in 300 women of childbearing age of South Asian and Caucasian ethnicity in the Greater Vancouver area and to determine potential genetic, demographic, and dietary factors influencing vitamin B-12 status in the focus population.

6. WHO CAN PARTICIPATE IN THIS STUDY?

To be eligible to participate, you must be a non-pregnant healthy woman of childbearing age (19-35 years) of South Asian or Caucasian descent.

7. WHO SHOULD NOT PARTICIPATE IN THE STUDY?

You **cannot** participate in this study if:

- **You are not aged 19 to 35 and female**
- **You have less than 3 grandparents coming from the same ethnic background (either South Asian or Caucasian).** Mixed individuals (i.e. 2 South Asian grandparents, 2
- Caucasian grandparents) are ineligible.
- **You suffer from a chronic condition that you are receiving treatment or medication for such as:** Diabetes, Cardiovascular Disease, Cancer, Renal Failure, Chronic Obstructive Pulmonary Disease, Emphysema, Bronchitis, High Blood Pressure, High Cholesterol, Arthritis, Fibromyalgia, Asthma, Stroke. Thyroid Condition, Major

- Depression, Bipolar disorder, Dysthymia (chronic mild depression), Schizophrenia, Anxiety Disorder, Learning Disability (Attention Deficit Disorder, Attention Deficit Hyperactivity Disorder, Dyslexia, Other), Eating Disorder (Anorexia, Bulimia etc.), Liver Disease, Gallbladder Problems, Hepatitis, HIV AIDS
- **You are currently (in the past month) regularly using prescription medications:** including insulin, nicotine patches, metformin, anti-cancer treatment, antibiotics, proton pump inhibitors, antacids, chloramphenicol, thyroid medication
- **You are currently (in the past month) regularly using performance enhancing or recreational drugs such as** steroids, cocaine, marijuana etc.
- **You are currently regularly over the counter medication such as** pain killers, and antacids
- **You are currently taking <u>hormonal </u>contraceptives other than oral contraceptive pills.** (For example: birth control patch, depo provera, intrauterine device)
- **You are currently pregnant or breastfeeding.**
- **You are suffering from a gastrointestinal condition such as** atrophic gastritis, Crohn's disease, irritable bowel syndrome, colitis, pernicious anemia, celiac disease, acid
- **indigestion, constipation, diverticulitis/losis, colon polyps, GERD.**
- **You have undergone gastric bypass surgery.**
- **You are unable to provide informed consent, or unable to read and write English**.

8. WHAT DOES THE STUDY INVOLVE?

The study will be conducted at (1) the Food, Nutrition, & Health Building, University of British Columbia, (2) the Clinical Research and Evaluation Unit at the Child and Family Research Institute, (3) the Courtyard Room at the South Community Health Centre (Vancouver Coastal Health), or (4) The Newton Public Health Unit (Fraser Health).

The procedures will take about 60 minutes of your time.

Before coming to the study site:

1. You will be asked to arrive at fasting state, that is, after having fasted for 10 hours. For example, if your visit is scheduled for 8 AM you should not consume anything besides water after 10 PM the night before. *You are asked to arrive fasted because eating a meal impacts some of the blood parameters.*

2. For your visit, you are asked to bring in the containers of any supplements (for example vitamin or mineral supplements, multivitamins, prenatal vitamins etc.) that you are currently taking. This includes nutritional supplements such as brewers yeast (nutritional yeast ie Red Star™), energy bars, meal replacement drinks or products etc. *We will send an email prior to the day of the study as a courtesy reminder for this request.*

At the study site:

3. You will be asked to have measured by a female study personnel your height (using a stadiometer), weight (using a scale), and waist circumference (using a tape measure). *This information will be collected in a private room and will remain confidential.*

4. You will be asked to complete a demographic questionnaire that collects information on age, ethnicity, tobacco and alcohol consumption, socioeconomic status, and intake of hormonal contraceptives. **At any time you may refuse to provide any information that you do not feel comfortable sharing.**

5. You will be asked to complete a food frequency questionnaire for assessment of total dietary intake and micronutrient intake..

6. You will be asked to complete a short questionnaire on physical activity.

7. A tube of fasting blood (less than 24 mL or 5 teaspoons) will be taken from you by a research nurse who is certified to take blood. You will also be asked for a finger prick blood sample. These blood samples will be used to measure blood levels of B-vitamins, related functional biomarkers, and presence of common genetic variants affecting vitamin B-12 status. The genetic information gathered in this study will be used for the sole purpose of investigating possible genetic influences of vitamin B-12 deficiency. No genetic information will be disclosed to participants and all information will be safeguarded and confidential.

The collection of these samples and data will allow us to investigate which factors (including age, ethnicity, or dietary habits) influence someone's vitamin B-12 status and what the prevalence of vitamin B-12 deficiency is in Metro Vancouver area. The study protocol has been reviewed and approved by the Clinical Research Ethics Board at the University of British Columbia and the Fraser Health Research Ethics Board at the Fraser Health Authority. The board aims to help protect the rights of research subjects.

9. WHAT ARE MY RESPONSIBILITIES?

We request that you review these consent forms at least 24 hours prior to your appointment. On the night before the appointment we ask that you fast for 10 hours counted backwards from your appointment time. We also ask that you bring with you the containers of any nutritional supplements or food supplements that you are currently taking on a regular basis to help us record your supplement intake. Once at the study site you will be asked to allow us to measure your height, weight, and waist circumference, provide a fasting blood sample of 24 mL, and complete 3 questionnaires.

10. WHAT ARE THE POSSIBLE HARMS AND DISCOMFORTS?

Taking a blood sample is felt to have very low risks. The needles used to take blood and blood lancet used to prick the finger might be uncomfortable and you may feel lightheadedness, and/or get some minor bruising and/or rarely an infection at the site of the blood draw or the finger-prick.

11. WHAT ARE THE POTENTIAL BENEFITS OF PARTICIPATING?

There may not be direct benefits to you from taking part in this study. We hope that the information learned from this study can be used in the future to help determine the needs for vitamin B-12 for women of childbearing age and identify influencing factors that might put individuals at risk of low vitamin B-12 status especially for the ethnicities of interest. We hope this study will provide preliminary information that can be used to facilitate further studies in the causes and consequences of low vitamin B-12 status.
Upon completion of sample analysis you will receive a detailed document describing your vitamin B-12 and folate status as well as information about your dietary vitamin B-12 and folate intake. Instructions on how to interpret the results will be included. If you have any questions about the information provided please feel free to contact us. If you have specific nutrition or health related questions upon receiving the results of the study we will recommend that you consult your family doctor and registered dietician. You will be provided with the option of receiving this information in email or paper mail format. Please indicate your preference on the corresponding signature page on Page 11.

12. WHAT ARE THE ALTERNATIVES TO THE STUDY TREATMENT?

This is an observational study and there are no treatments. As such, there is no therapy or alternative therapy for subjects. Subjects will not be asked to receive any treatment as a requirement of participation

13. WHAT IF NEW INFORMATION BECOMES AVAILABLE THAT MAY AFFECT MY DECISION TO PARTICIPATE?

You will be advised of any new information that becomes available that may affect your willingness to remain in this study.

14. WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE?

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

may decide to discontinue the study at any time, or withdraw you from the study at any time, if they feel that it is in your best interests. If you choose to enter the study and then decide to withdraw at a later time, all data and blood samples collected from you during the enrolment part of the study will be retained as unidentifiable material for analysis.

15. CAN I BE ASKED TO LEAVE THE STUDY?

If you are not able to follow the requirements of the study, meet the eligibility criteria or for any other reason, the study coordinator may withdraw you from the study. On receiving new information the study coordinator might consider it to be in your best interests to withdraw you from the study without your consent if they judge that you do not meet eligibility criteria.

16. WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

Your confidentiality will be respected. However, research records and health or other source records identifying you may be inspected in the presence of the Principal Investigator or his or her designate by the University of British Columbia and the Fraser Health Research Ethics Boards for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

You will be assigned a unique study number as a subject in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity [i.e. your name or any other information that could identify you] as a subject in this study will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor.

Questionnaires and blood samples collected at Fraser Health sites will be transported off site by the Principal Investigator, graduate student, or research assistant for secure storage at the UBC Food Nutrition & Health building under the guardianship of Dr. Yvonne Lamers. These samples will be only identifiable by assigned study number and will not be traceable back to your identity or personal information.

17. AFTER THE STUDY IS FINISHED

The collected data have to be retained for 10 years. After this period, all information including personal health information will be shredded and destroyed. If you have any questions or desire further information about the study procedures before or during participation, you may contact Dr. Yvonne Lamers, at any time, at 604-822-1490.

18. WHAT HAPPENS IF SOMETHING GOES WRONG?

Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else, and you do not release the study doctors or participating institutions from their legal and professional responsibilities.

By signing this form, you do not give up any of your legal rights and you do not release the researcher or her participating institutions from their legal and professional duties. There will be no costs to you for participation in this study. You will not be charged for any research procedures. If you become ill or physically injured as a result of participation in this study, medical treatment will be provided at no additional cost to you. The costs of your medical treatment will be paid by your provincial medical plan and/or by the study sponsor.

19. WHAT WILL THE STUDY COST ME?

There will be no costs to subjects for participation. In order to defray the costs of transport, each participant will receive a $5 gift card.

20. WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY DURING MY PARTICIPATION?

If you have any questions or desire further information about this study before or during participation, or if you experience any adverse effects, you can contact *Yvonne Lamers (Principal Investigator) at 604-822-1490.*

21. WHO DO I CONTACT IF I HAVE ANY QUESTIONS OR CONCERNS ABOUT MY RIGHTS AS A SUBJECT?

If you have any concerns or complaints about your rights as a research subject and/or your experiences while participating in this study, contact the Research Subject Information Line in the University of British Columbia Office of Research Services by e-mail at RSIL@ors.ubc.ca or by phone at 604-822-8598 (Toll Free: 1-877-822-8598).

If you have any concerns or complaints about your rights as a research subject and/or your experiences while participating in this study, contact Dr. Anton Grunfeld and/or Dr. Allan Belzberg, Research Ethics Board [REB] co-Chairs by calling 604-587-4681. You may discuss these rights with the co-chairmen of the Fraser Health REB.

22. SUBJECT CONSENT TO PARTICIPATE

Study Title:
Adequacy of Vitamin B12 Status in Women of Childbearing Age

23. SIGNATURES

My signature on this consent form means:
- I have read and understood the subject information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice if necessary.
- I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential and that the results will only be used for scientific objectives.
- I understand that my participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in
- any way the quality of care that I receive.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I understand that there is no guarantee that this study will provide any benefits to *me*

I will receive a signed copy of this consent form for my own records.

I consent to participate in this study.

_____	_____	_____
Subject's Signature	Printed name	Date

_____	_____	_____
Signature of Person Obtaining Consent	Printed name, Study Role	Date

SUBJECT CONSENT TO BE CONTACTED FOR FOLLOW UP STUDIES

My signature on this consent form means:
- I have read and understood the subject information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice if necessary.
- I have had the opportunity to ask questions and have received satisfactory responses to my questions.
- I understand that my willingness to be contacted for follow up investigation is voluntary and that I am completely free to refuse to be contacted and withdraw my consent at any time without changing in any way the quality of care that I receive.
- I am not waiving any of my legal rights as a result of singing this form, and I understand that there is no guarantee that this study will provide any benefits to me.
- I have read this form and I freely consent to be contacted for recruitment in follow up studies.
- I have received a dated and signed copy of this form for my own record.

I will receive a signed copy of this consent form for my own records.

I consent to be contacted for follow up investigations.

_____	_____	_____
Subject's Signature	Printed name	Date

_____	_____	_____
Signature of Person Obtaining Consent	Printed name, Study Role	Date

SUBJECT CONSENT TO RECEIVE RESULTS

My signature on this consent form means

- I have read and understood the subject information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice if necessary.
- I have had the opportunity to ask questions and have received satisfactory responses to my questions.
- I understand that my willingness to receive my blood vitamin and nutrient intake results is voluntary and that I am completely free to refuse to this and withdraw my consent at any time without changing in any way the quality of care that I receive.
- I am not waiving any of my legal rights as a result of singing this form, and I understand that there is no guarantee that this study will provide any benefits to me.
- I have read this form and I freely consent to be sent my blood vitamin and nutrient intake results
- I have received a dated and signed copy of this form for my own record.

I will receive a signed copy of this consent form for my own records.

I wish to receive the results by (*please check one and provide contact details*):

Email
Please print email address: _____

Postal mail
Please print mailing address: _____

I consent to receive my blood vitamin levels and dietary vitamin intake results.

| _____ | _____ | _____ |
| Subject's Signature | Printed name | Date |

| _____ | _____ | _____ |
| Signature of Person Obtaining Consent | Printed name, Study Role | Date |

THE UNIVERSITY OF BRITISH COLUMBIA

Faculty of Land and Food Systems
Grounded in Science | Global in Scope

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SUBJECT INFORMATION AND CONSENT FORM
OPTIONAL TISSUE BANKING FOR FUTURE RESEARCH
ORIGINAL STUDY TITLE:
ADEQUACY OF VITAMIN B-12 STATUS IN WOMEN OF CHILDBEARING AGE

Principal Investigator: Dr Yvonne Lamers, PhD
Food, Nutrition & Health
Land and Food Systems
The University of British Columbia
604 822 1490

Co-Investigator: Beth Snow, PhD, C.E.
Evaluation Specialist, Public Health
Fraser Health Authority
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Erin MacMillan, BSc
Filza Qureshi
Food, Nutrition & Health

1. INTRODUCTION

In addition to the main part of the research study, you are being invited to participate in optional storage (known as tissue banking) of your blood samples because you are taking part in the main research study titled "Adequacy of Vitamin B-12 Status in Women of Childbearing Age". You may take part in the main part of the study without having to agree to participate in optional tissue banking.

2. YOUR PARTICIPATION IS VOLUNTARY

Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this optional tissue banking. Before you decide, it is important for you to understand what the tissue banking involves. This consent form will tell you about the tissue banking, why it is being done, and the possible benefits and risks to you.

If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide.

If you do not wish to participate, you do not have to provide any reason for your decision not to participate nor will you lose the benefit of any medical care, education, or other services to which you are entitled or are presently receiving.

3. WHO IS CONDUCTING THIS STUDY

Researchers from the Food, Nutrition, and Health Department of the Faculty of Land and Food Systems, University of British Columbia, in collaboration with Vancouver Coastal Health and the Fraser Health Authority are conducting the study. The "University of British Columbia – Vitamin Research Fund" is funding the study. No conflicts of interest to declare.

WHO IS RESPONSIBLE FOR STORING MY BLOOD AND ANY FUTURE RESEARCH USING MY BLOOD?

Dr. Yvonne Lamers, PhD, of the Food, Nutrition, and Health Program of the Faculty of Land and Food Systems, University of British Columbia, is responsible for the storage of your blood samples and for any future research involving your samples. Any analysis conducted on these samples will not be the subject of the same Masters degree project that is associated with the primary study. However, it may be the subject of further graduate projects.

4. BACKGROUND

Left over blood samples taken from you during the primary study would normally be disposed of through the University of British Columbia (UBC) biological waste program after a required storage period of ten years. However, your remaining blood samples can be stored to be used for

future research. This is known as "tissue banking". This extra tissue, not needed for a medical diagnosis or treatment, is used by researchers to study nutritional status or disease risk factors and to find better ways to diagnose, prevent, and treat disease in the future.

Dr. Yvonne Lamers, PhD, the researcher of the primary study in which you are participating, is interested in doing research studies in the future on blood samples taken from you that may help to better understand prevention and treatment of B-vitamin related diseases or other nutritional and medical questions not related to B-vitamins. The purpose of such follow-up research would be to address similar overall goals and research areas as this study that were not feasible as part of this project.

5. WHAT IS THE PURPOSE OF THIS STUDY?

The purpose of the tissue banking is to use the blood samples in future research to learn more about the influence of different nutrients on the human body. By understanding how different nutrients levels affect our body, we hope to find new ways to reduce nutrient-related diseases.

6. WHO CAN PARTICIPATE?

Women who are enrolled in the study entitled: "Adequacy of Vitamin B-12 Status in Women of Childbearing Age" and who had blood collected during any study visit can participate in this optional tissue banking part of the primary study.

7. WHO MAY NOT PARTICIPATE IN THE STUDY?

Women who are not enrolled in the study mentioned above may not participate.

8. WHAT IS INVOLVED IN STORING MY BLOOD?

You will not be asked to specifically provide any extra blood samples apart from the 24 mL collected during the main study. Only blood left over after your blood tests in the primary study are completed will be stored.

Your blood samples will be kept under the guardianship of the primary study investigator (Yvonne Lamers), in a secure facility at the Food, Nutrition and Health Building, Room 250, at the University of British Columbia, Vancouver, British Columbia. Technical, physical and administrative safeguards are in place to prevent unauthorized access. The blood samples will be stored for 10 years, after which they will be discarded through the University of British Columbia biological waste program.

The blood samples will be used for research purposes only and will not be sold. The research done with your samples may or may not help develop commercial products. There are no plans to provide payment to you if this happens. The blood samples will only be identifiable by assigned subject number and will not be traceable to any personal information.

Any future research studies involving your blood samples will be submitted for review and approval by one of the University of British Columbia's Clinical Research Ethics Boards.

9. WHAT ARE MY RESPONSIBILITIES?

You have no responsibilities in addition to those involved with the primary study.

10. WHAT ARE THE POSSIBLE HARMS AND SIDE EFFECTS FROM STORING MY BLOOD?

There are no anticipated risks associated with storage of your blood samples.

11. WHAT ARE THE POTENTIAL BENEFITS OF STORING MY BLOOD?

No one knows whether or not you will benefit from this study. There may or may not be direct benefits to you from taking part in this study.

We hope that the information learned from this study can be used in the future to benefit other people by providing knowledge on adequate nutrient requirements and understanding potential nutrient-disease relationships.

12. WHAT ARE THE ALTERNATIVES TO CONSENTING TO STORAGE OF MY BLOOD SAMPLES?

The alternative is not to consent to the storage of your blood samples. Choosing not to consent will in no way affect the care that you receive or your participation in the primary study.

13. WHAT IF NEW INFORMATION BECOMES AVAILABLE THAT MAY AFFECT MY DECISION TO CONSENT FOR MY BLOOD TO BE STORED?

If new information were to arise that may affect your willingness to consent to storage of your blood samples, this would be made available to you.

14. WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT FOR STORAGE OF MY BLOOD SAMPLES?

Your consent for the storage of your blood samples is entirely voluntary. If you decide to consent to the blood storage and later decide to withdraw your consent, you do not have to provide any reason nor will you lose the benefit of any medical care, education, or other services

to which you are entitled or are presently receiving. Your participation in the primary study will not be affected. If you wish for your stored blood samples to be destroyed upon your withdrawal, you may contact Dr. Yvonne Lamers, PhD, by phone at 604 822 1490.

The study investigators may choose to discontinue the study at any time or withdraw you from the study at any time if they feel it is in your best interests.

If you choose to enter the study and then decide to withdraw at a later time, all data collected during your enrolment in the study and up till the time of withdrawal will be retained for analysis. By law, this data cannot be destroyed.

15. WHAT HAPPENS IF SOMETHING GOES WRONG?

Signing this consent form in no way limits your legal rights against the investigators, or anyone else, and you do not release the principal investigator or participating institutions from their legal and professional responsibilities.

16. CAN I BE ASKED TO LEAVE THE STUDY?

If you are not complying with the requirements of the study or for any other reason, the primary investigator may withdraw you from the study and will arrange for your care to continue.

17. AFTER THE STUDY IS FINISHED

After conclusion of the study results of any analysis outside of those analyses of the primary study will not be available to participants.

18. WHAT WILL THE STORING OF MY BLOOD COST ME?

You will not be paid for your blood samples and there will be no cost to you for us to store your blood.

19. WILL MY IDENTITY AND STORAGE OF MY BLOOD SAMPLES BE KEPT CONFIDENTIAL?

Your confidentiality will be respected. However, research records and health or other source records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of The University of British Columbia and the Fraser Health Research Ethics Board for the purpose of monitoring future research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent.

Your blood samples will be transported from the Fraser Health site to the UBC FNH Building by the principal investigator, graduate student, or research assistant where they will be stored

securely and will be identifiable only by a unique study code under the guardianship of Dr. Yvonne Lamers. Only this code will be used on the samples so that your identity [i.e. your name or any other information that could identify you] will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study code that is used on your blood samples will not be removed or released without your consent.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study Principal Investigator, Yvonne Lamers.

It is possible that in the future, other investigators may access your stored blood samples. To ensure your confidentiality your stored blood samples will be identified only by unique ID number, and will have no identifying information attached to it.

20. WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT STORING MY BLOOD?

If you have any questions or desire further information before or during the time your blood is being stored, you can contact Dr Yvonne Lamers, PhD, at 604-822-1490 or any other members of the study team, listed on the first page of this consent form.

21. WHO DO I CONTACT IF I HAVE ANY QUESTIONS OR CONCERNS ABOUT MY RIGHTS AS A SUBJECT?

If you have any concerns about your rights as a research subject, contact the Research Subject Information Line in the University of British Columbia Office of Research Services by e-mail at RSIL@ors.ubc.ca or by phone at 604-822-8598 (Toll Free: 1-877-822-8598).

If you have any concerns or complaints about your rights as a research subject and/or your experiences while participating in this study, contact Dr. Anton Grunfeld and/or Dr. Allan Belzberg, Research Ethics Board [REB] co-Chairs by calling 604 587 4681. You may discuss these rights with the co-chairmen of the Fraser Health REB.

22. SUBJECT CONSENT TO PARTICIPATE

OPTIONAL TISSUE BANKING FOR FUTURE RESEARCH

ORIGINAL STUDY TITLE:
ADEQUACY OF VITAMIN B-12 STATUS IN WOMEN OF CHILDBEARING AGE

23. SIGNATURE

SUBJECT CONSENT TO BLOOD STORAGE

My signature on this consent form means that:

- I have read and understood this subject information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice if necessary.
- I have had the opportunity to ask questions and have received satisfactory responses to my questions.
- I understand that willingness to allow storage and further analysis on my blood samples is voluntary and that I am completely free to refuse this and to withdraw my consent at any time without changing in any way the quality of care I receive.
- I am not waiving any of my legal rights as a result of signing this consent form,
- I understand that there is no guarantee that this study will provide any benefits to me.
- I will receive a signed copy of this consent form for my own records.
- I voluntarily give consent for my blood samples to be stored and analyzed for future research.

I will receive a signed copy of this consent form for my own records.

I consent to participate in this optional blood storage (tissue banking).

_____	_____	_____
Subject's Signature	Printed name	Date

_____	_____	_____
Signature of Person Obtaining Consent	Printed name, Study Role	Date

Protocol for Sample Collection and Initial Sample Processing

1. Night before:
   Ensure all lab materials and reagents are prepared ahead of time.
   a. Prepare 1% wt:vol aqueous ascorbic acid sol^n (1 g to 100 mL in a volumetric flask, mix well and cover with tinfoil to protect from light damage – This can be prepared the night before and put into fridge but must be left out ahead of time to bring to room temperature. Need weigh boat and spatula – millipure water for this step.
   b. Label all cryotubes for each subject label the following (name/code):
      i. 2 serum B12/holoTC (SB12HTC ###)
      ii. 2 formate (FOR ###)
      iii. 2 serum creatinine (SCR ###)
      iv. 2 plasma folate (PF ###)
      v. 2 MMA (MMA ###)
      vi. 2 RBC folate (RBCF ###)
      vii. 2 Washed red cells (WRC ###)
      viii. 2 total homocysteine (tHCY ###)
      ix. 2 buffy coat (BC ###)
      x. 2 leftover plasma (xP ###)
      xi. 2 leftover serum (xS ###)

2. Morning of:
   a. Take ascorbic acid out of fridge
   b. Turn on water bath to 37 degrees
   c. Take out falcon tube of PBS for red cell wash
   d. Set up work area:
      i. Sharps container
      ii. Biohazard
      iii. Subject packages
      iv. Subject blood collection packages
      v. Ice bucket
      vi. Tube holder

3. Greet subject and explain procedure fully – answer any outstanding questions, verify diet restrictions (fasting)

4. Properly identify subject and ensure all materials are labelled with their identification number

5. Seat patient comfortably in examination chair and properly position arm of choice

6. Examine the arm for viable vein (avoid areas with hematoma, scarring etc.) – palpate vein – and clean area thoroughly with alcohol wipe allowing area to dry before proceeding

7. Insert needle and make sample collection ensuring to use the tube with anticoagulant FIRST followed by the serum collection tube.
    a. Lithium heparin (wrapped in tinfoil jacket) *Put on ice
    b. EDTA
    c. Serum tube
    d. Record time of draw on requisition and in lab book – EDTA must be analyzed for CBC within 4 hours – put in biohazard with requisition and put in cooler for transport

8. Remove needle by pressing cotton ball and swiftly retracting it from vein – instruct the subject to press down for ~3 minutes. If any bleeding apply bandaid. Dispose of needle and any waste in appropriate biohazard/sharps container.

9. Once blood collection is completed, place all vacutainers in secondary container. Place plasma tube on immediately (only good up to 6 hours). DO NOT put EDTA on ice – insert into cooler at room temperature for transfer to CFRI

10. Dried Blood Spot Analysis
    a. Have alcohol wipe, dry wipe, paper card, dessicant, and plastic bag at hand
    b. Wipe finger tip and let dry
    c. Use lancet to pierce finger tip and wipe first blood away
    d. Massage finger tip to promote blood droplet formation and drop directly onto paper card. Do not put multiple drops on top of eachother
    e. Ensure full circle filled for 5 spots – may need to do multiple punctures
    f. Ensure blood does not soak through card
    g. Let card air dry before putting in bag and storing in fridge

11. Before centrifugation - Invert plasma tube slowly 3 times. Aliquot 900 uL of ascorbic acid solution to 100 uL of whole blood in cryotubes (pipette tip in solution but do not wash), and vortex, incubate @ 37°C for 3 hours in the dark and freeze @ -80°C until analysis.

12. Invert plasma (lithium heparin) tube 8-10 times place in refrigerated centrifuge, Don't invert serum (let clot 30 minutes @ room temp) (wait 30 minutes for clotting) *Plasma tube should not be on ice longer than 30 minutes

13. Centrifuge at 4 degrees Celsius for 15 minutes @ 3000 RPM and transfer into cryotubes using pipette, making sure to switch pipette tips and that lid is tight. Ensure the following number of cryotubes:

2 serumB12/holoTC	500 uL x 2
2 formate (for collaborator)	500 uL x 2
2 serum creatinine	500 uL x 2
2 extra serum	Whatever is left
2 plasma folate	400 uL x2
2 MMA	300 uL x2
2 tHcy	200 uL x2
2 buffy coat	All
2 RBC folate	N/A Done ahead of time
2 Washed cells	See subsequent
2 leftover plasma	Whatever is left

14. Wash erythrocytes three times with phosphate-buffered saline (PBS, can be purchased or 'home-made' See PBS Buffer Recipe):
add same volume of PBS into vacutainers as there was plasma
invert tube slowly three times
centrifuge sample at 1500 x g at 4°C for 10min
take off PBS supernatant
- *do those wash steps 2-more times* -

15. Pipette 1mL 1% ascorbic acid solution in cryotubes

16. Add 100μL washed packed erythrocytes and pipette slowly up and down to clean pipette tip

17. Close cap and vortex

18. Keep closed cryotubes at +37C in the dark for 3 hours

19. After incubation, freeze immediately at –80°C and store at –80°C until analysis

20. Place all cryotubes in holding boxes and put in -80 degree freezer

THE UNIVERSITY OF BRITISH COLUMBIA

Faculty of Land and Food Systems
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Fraser Health Research Ethics Board
Department of Evaluation and Research Services
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Dear:

Thank you for participating in the study.

1. The result from your B-vitamin screening indicated that:

Your vitamin B-12 status is adequate. Your current diet and lifestyle are supplying adequate vitamin B-12.

Your vitamin B-12 status is marginal. You may benefit from including more sources of B-12 in your diet or taking daily supplements. Please consult your family doctor or dietician.

You are deficient in vitamin B-12. You may benefit from including more sources of B-12 in your diet or taking daily supplements. Please consult your family doctor or dietician as you may be at risk for some health problems such as anemia or neurological decline.

You are critically deficient in vitamin B-12. You are at significant risk of developing anemia and neurological symptoms. Please consult your family doctor as soon as possible. You may benefit from including more sources of B-12 in your diet or taking daily supplements.

Vitamin B-12 plays a vital role in maintaining good health by contributing to a healthy brain, metabolism, and energy levels. It is protective against neurological decline, pregnancy complications, and a blood disorder called anemia. It is recommended that women of childbearing age get 2.4 µg of vitamin B-12 daily. Foods with vitamin B-12 are limited and the most potent supply is from animal sourced foods (meat, cheese, milk, eggs, seafood). If you do not consume animal foods fortified products may be a positive alternative as plants do not contain B-12.

2. The results further indicated that:

Your folate status is adequate. Your current diet and lifestyle are supplying adequate folate.

Your folate status is marginal. You may benefit from including more sources of folate in your diet or taking daily supplements. Please consult your family doctor or dietician.

You are deficient in folate. Please consult your family doctor or dietician as you may be at risk for some health problems such as anemia or pregnancy complications. You may benefit from inclding more sources of folate in your diet or taking daily supplements.

You are critically deficient in folate. You are at significant risk of developing anemia and increasing your risk for birth defects. Please consult your family doctor as soon as possible. You may benefit from including more sources of B-12 in your diet or taking daily supplements.

Folate helps produce and maintain new cells because it is needed to make DNA and RNA, the building blocks of cells. This is especially important for fast growing cells like found in blood and the skin. Adults need folate to make normal red blood cells and prevent anemia.

During pregnancy it is especially important to maintain adequate folate levels. It is involved in the healthy development of the fetus within the first three weeks of pregnancy, when most pregnancies are still not known. Folate was shown to reduce the incidence of neural tube defects (such as spina bifida), which can be developed during this time. Because 50% of the pregnancies are not planned and folate intake from food is not sufficient, it is recommended for women of childbearing age to take supplements containing 0.4 to 1.0 mg folic acid daily.

Foods high in folate are mainly mandatory fortified grain products. Leafy green vegetables (like spinach and turnip greens), fruits (like citrus fruits and juices), and dried beans and peas are all natural sources of folate.

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3. The assessment of your dietary vitamin intake showed the following:

Your average daily intake of vitamin B12 is *(in micrograms)*:

Your average daily intake of folate (vitamin B9) is (in micrograms):

The results suggest that your vitamin B12 intake is:

Below the recommended daily allowance (RDA).You are likely not getting enough B12 from your diet. Please seek out the advice of a family doctor or registered dietitian.

Meeting the RDA. Based on the output of the food frequency questionnaire, you are likely meeting the dietary requirements for vitamin B12.

The RDA for vitamin B12 for women of your age is 2.4 micrograms per day. The RDA is the level at which nearly all healthy people will meet their vitamin B12 requirement. Dietary sources of B12 include but are not limited to meat, fish, milk and other dairy products, eggs, and fortified foods.

The results further suggest that your dietary folate intake is:

Below the recommended daily allowance (RDA). You are likely not getting enough folate or folic acid from your diet. Please seek out the advice of a family doctor or registered dietitian.

Meeting the RDA. Based on the output of the food frequency questionnaire, you are likely meeting the dietary requirements for folate.

The RDA for folate for women of your age is 400 micrograms per day. If you are planning a pregnancy this increases to 600 micrograms per day. Additionally, women of childbearing age are recommended to take daily supplements containing 0.4 to 1.0 milligram folic acid to prevent adverse pregnancy outcomes.

Foods high in folate are mainly mandatory fortified grain products. Leafy green vegetables (like spinach and turnip greens), fruits (like citrus fruits and juices), and dried beans and peas are all natural sources of folate.

Please keep in mind that the quantitative food frequency questionnaire used to determine your dietary vitamin B-12 and folate intake provides a ballpark number, or estimation of your typical dietary habits. Your actual intake may vary slightly and may be different on a day-to-day basis.

Thank you again for your participation,

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Guide to total household income

Total annual household income is classified into four categories based on the number of people in the household:

Lowest (1)
less than $15,000 if 1 or 2 people
less than $20,000 if 3 or 4 people
less than $30,000 if 5 or more people
Lower-middle (2)
$15,000 to $29,999 if 1or 2 people
$20,000 to $39,999 if 3 or 4 people
$30,000 to $50,000 if 5 or more people
Upper-middle (3)
$30,000 to $59,999 if 1 or 2 people
$40,000 to $79,000 if 3 or 4 people
$60,000 to $79,999 if 5 or more people
Highest (4)
$60,000 or more if 1 or 2 people
$80,000 if 3 or more people

Categorical Variable: Rationale for Cut Point Values

There are three levels of physical activity proposed to classify populations:

1. Low
2. Moderate
3. High

Regular participation is a key concept included in current public health guidelines for physical activity.[1] Therefore, both the total volume and the number of days/sessions are included in the IPAQ analysis algorithms. The criteria for these levels have been set taking into account that IPAQ asks questions in all domains of daily life, resulting in higher median MET-minutes estimates than would have been estimated from leisure-time participation alone. The criteria for these three levels are shown below. Given that measures such as IPAQ assess total physical activity in all domains, the "leisure time physical activity" based public health recommendation of 30 minutes on most days will be achieved by most adults in a population. Although widely accepted as a goal, in absolute terms 30 minutes of moderate-intensity activity is low and broadly equivalent to the background or basal levels of activity adult individuals would accumulate in a day. Therefore a new, higher cutpoint is needed to describe the levels of physical activity associated with health benefits for measures such as IPAQ, which report on a broad range of domains of physical activity.

'High'

This category was developed to describe higher levels of participation. Although it is known that greater health benefits are associated with increased levels of activity there is no consensus on the exact amount of activity for maximal benefit. In the absence of any established criteria, the IPAQ Research Committee proposes a measure which equates to approximately at least one hour per day or more, of at least moderate-intensity activity above the basal level of physical activity Considering that basal activity may be considered to be equivalent to approximately 5000 steps per day, it is proposed that "high active" category be considered as those who move at least 12,500 steps per day, or the equivalent in moderate and vigorous activities. This represents at least an hour more moderate-intensity activity over and above the basal level of activity, or half an hour of vigorous-intensity activity over and above basal levels daily. These calculations were based on emerging results of pedometers studies.[2] This category provides a higher threshold of measures of total physical activity and is a useful mechanism to distinguish variation in population groups. Also it could be used to set population targets for health-enhancing physical activity when multidomain instruments, such as IPAQ are used.

'Moderate'

This category is defined as doing some activity, more than the low active category. It is proposed that it is a level of activity equivalent to "half an hour of at least moderate-intensity PA on most days", the former leisure time-based physical activity population health recommendation.

'Low'

This category is simply defined as not meeting any of the criteria for either of the previous categories.

5. Protocol for IPAQ Short Form

5.1 Continuous Scores

Median values and interquartile ranges can be computed for walking (W), moderate intensity activities (M), vigorous-intensity activities (V) and a combined total physical activity score. All continuous scores are expressed in MET-minutes/week as defined below.

5.2 MET Values and Formula for Computation of MET-minutes/week

The following values continue to be used
for the analysis of IPAQ data: Walking = 3.3 METs, Moderate PA = 4.0 METs and Vigorous PA = 8.0 METs. Using these values, four continuous scores are defined:
Walking MET-minutes/week = 3.3 * walking minutes * walking days
Moderate MET-minutes/week = 4.0 * moderate-intensity activity minutes * moderate days
Vigorous MET-minutes/week = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days
Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous METminutes/week scores.

5.3 Categorical Score

Category 1 Low
This is the lowest level of physical activity. Those individuals who not meet criteria for Categories 2 or 3 are considered to have a 'low' physical activity level.
Category 2 Moderate
The pattern of activity to be classified as 'moderate' is either of the following criteria:
a) 3 or more days of vigorous-intensity activity of at least 20 minutes per day
OR
b) 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day
OR
c) 5 or more days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum Total physical activity of at least 600 MET-minutes/week.
Individuals meeting at least one of the above criteria would be defined as accumulating a minimum level of activity and therefore be classified as 'moderate'. See Section 7.5 for information about combining days across categories.
Category 3 High
A separate category labelled 'high' can be computed to describe higher levels of participation.
The two criteria for classification as 'high' are:
a) vigorous-intensity activity on at least 3 days achieving a minimum Total physical activity of at least 1500 MET-minutes/week
OR
b) 7 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity of at least 3000 MET-minutes/week.

Real Time PCR Genotyping Protocol

Sample Preparation **(set aside 1 hour minimum for 1 plate)** *Following DNA extraction using Qiagen DNA and Blood Mini Kit*
Set out gloves, filter tips, primers, master mix, purified H₂O, Styrofoam ice bucket, microcentrifuge tubes
1. Put primers and samples on ice
2. Turn on PCR workstation and UV light
3. Multiply protocol by # of samples for volumes (+2 or 3 for controls) +2 for Negative Controls + 2 or 3 for Positive Controls + 2-3 as spare volume 18 µL volume/well: 9 µL Master Mix + 0.9 µL Primer + 6.1 µL H₂O + 2µL Sample
4. Put a small aliquot of purified water into smaller plastic tube *this should remain inside the PCR hood*
Note: DNA storage @ 4ᵒC, cDNA at -20ᵒC, and RNA at -80ᵒC, Taqman Genotyping Master Mix @ 4ᵒC, Primers @ -20ᵒC
5. Put the calculated volume of H₂O into a smaller plastic tube
6. Briefly vortex Master Mix and spin it down with centrifuge (small green instrument)
7. Add Master Mix into this tube, mix several times with pipette
8. Briefly vortex primer and flick it, spin down with centrifuge (small green instrument) if available and add primer to H₂O and Master Mix
9. Vortex and spin down quickly in centrifuge (small green instrument)
10. Get ABI PRISM Applied Biosystems 96 Well Plate (Optical Rxn) *code 128 part number 4306737*
11. Label plate (A1) and top left corner
12. Make paper template for 96 wells so you know what is in each well
Need 2 or 3 controls of H₂O, note if there are multiple genes being screened for you can do on the same plate but need different samples
13. Add master mix to wells (16 µL) first and sample on top
14. Add 2µL of water to the first 2 wells (Negative Controls – NC)
15. Vortex each sample before plating
16. Add 2 µL of DNA (approximately), mix with pipette, avoid bubbles
17. Use the pipette tips to map your place on the plate so that wells are not doubly filled, using the fluorescent yellow holder for stability and a plate map
18. Add 2 or 3 positive controls (samples with known genotypes) @ the end
19. Cover plate with MicroAmp Optical Adhesive Film (Applied Biosystems) press down film and use small pipette to seal rows between wells
20. Put plate in salad spinner to mix down (located in qPCR room)
21. Run PCR

7500 Real Time PCR System – Genotyping Protocol – Run PCR
7500 Software v.2.0.6
(set aside 10-20 minutes for set up)

1. Record your session in the log-book – you will need valid log-in information for the computer, *remember to log-off at the end of your session*

2. Open 7500 Real Time PCR System
3. Place your plate firmly
4. Open the program 7500 Software (on desktop) ***DO NOT USE THE INTERNET WHILE OPERATING THIS PROGRAM***
Note: A message "RNase PRun is not valid…" may appear, choose: Ignore & continue Start-up

5. Choose in Set-up: Design wizard → or for custom choose advanced set up

6. Write the experiment name, e.g. **April 04 2013 – MTHFR C677T_Name**
7. In the yellow boxes – choose:
-which instrument: **7500 (96 Wells)**
-what type of experiment: **Genotyping (second option)**
Click **NEXT**

8. Choose:
-which reagents: **Taqman Reagents**
-what type of template: **Wet DNA**
-use the standard ramp speed: **Standard**

On the bottom of the page, **tick** the following…
-**Pre-PCR Read**
-**Amplification**
Click **Next**

9. Choose:
-Which SNP Assays (*if multiple choose more than 1*)
Yes Select SNP
e.g. SNP Assay Name: e.g. **MTHFR** (gene name) Assay ID: e.g. **rs 1801133** (rs number)
Allele 1 name: **MTHFR rs 1801133C** color: green **VIC** Quencher NFQ-MGB
Allele 2 name: **MTHFR rs 1801133T** color: pink **FAM** Quencher NFQ-MGB
*Note VIC and FAM designation will be indicated for each specific primer
NOTE: check the allele's name and dye on the CD which comes with the primer
Allele 1 is *usually* VIC and Allele 2 is *usually* FAM
Click **Next**
Note: For Technical Support: 1 800 831 6844

10. Fill in:
-Number of samples: e.g. **92** [only count the number of samples excluding the NTC & PC]
-Number of replicates: e.g. 1
-Negative controls (NTC): e.g. 2
-Positive controls (optional): e.g. 3 (if possible pick 3 samples with genotypes as follows: Allele 1/Allele 1, Allele 2/Allele 1, Allele 2/Allele 2)
Set up controls:
Match your positive controls in the order they appear on the plate with their genotypes and choose the correct option from the drop down menu
Click **Next**

11. Check/Set: Temperature & Time (usually don't need to change the default unless different from below) **-60^oC; 01.00** **-95⁰C; 10.00** **-92^oC; 00.15** *Make sure that step 3 is switched from 95^oC to 92^oC!* **-60^oC; 01.00** **-60^oC; 01.00** Click **Next**
12. Fill in: Reaction volume per well: **18 µL**
13. Click on the bottom: **Finish designing the experiment**
14. Choose: **Start run for this experiment** 1st the program will ask where to save the document (e.g. My Computer_Devlin_Lamers_Genotyping) 2nd the program will start the run
15. When it is finished (approx. 2 hours) Verify the results on the graph (e.g. positive controls, negative controls, samples). Then export your data: File – Export data (exc. File) – Browse (*save in the same folder you saved the Genotyping run)* Heck in the Excel document that all results are there If not – in the program, highlight all wells in the results window and copy and waste into excel.
16. Exit the program
17. Take out plate 18. Turn off PCR Machine **(IMPORTANT – Bulb may burn out)** 19. **Send the data file (for guests); log off** 20. **Transfer file immediately to USB and send to email**