

**THE RELATIONSHIP BETWEEN MATERNAL SERUM AND BREAST MILK
VITAMIN B12 CONCENTRATIONS IN A PRIMARILY SUPPLEMENTED VERSUS AN
UNSUPPLEMENTED GROUP: ASSESSED AT EIGHT WEEKS POSTPARTUM**

by

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B.Sc., The University of British Columbia, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

March 2015

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Abstract

BACKGROUND: Vitamin B12 is an essential nutrient for adequate infant growth and development and breast milk is the only dietary source of vitamin B12 to the exclusively breast-fed infant. Little is known on the relationship between maternal serum vitamin B12 and breast milk vitamin B12. A better understanding of this relationship will provide insight into the determinants of milk vitamin B12 concentrations, including maternal vitamin B12 supplementation, for optimal infant vitamin B12 status.

OBJECTIVE: To determine the association between maternal serum and breast milk vitamin B12 concentrations at eight weeks postpartum in both a supplemented and unsupplemented population and to explore these associations with infant serum vitamin B12 concentrations in the supplemented group.

METHODS: Vitamin B12 concentrations were measured in infant serum, maternal serum and breast milk samples (n=186) collected from two previous studies; in Canada (supplemented) and New Zealand (unsupplemented). Supplemented participants were provided with a prenatal supplement containing 12µg of vitamin B12 during pregnancy to eight weeks postpartum.. Breast milk and maternal blood were available from both studies, with infant blood only available from the supplemented group. Serum samples were measured using an Elecsys immunoassay analyzer, while an IMMULITE chemiluminescence machine measured breast milk vitamin B12 concentrations.

RESULTS: The supplemented group had significantly higher serum vitamin B12 concentrations (Geometric mean: 665pmol/l; 95%CI: 601,685) compared to the unsupplemented group (403pmol/l, 365, 445). Maternal serum vitamin B12 was associated with breast milk vitamin B12 in the supplemented and unsupplemented groups ($r=0.577$, $p<0.0001$; $r=0.414$

p=0.01, respectively). Infant serum vitamin B12 concentrations were associated with both maternal serum vitamin B12 ($r=0.299$ $p=0.001$) and breast milk B12 ($r=0.337$ $p<0.0001$) concentrations.

CONCLUSION: Supplement use during pregnancy and lactation may lead to higher vitamin B12 concentrations in maternal serum and breast milk. Infant serum vitamin B12 concentrations appear to be related to maternal status and this may be mediated by enhanced breast milk vitamin B12. Further RCT are needed to investigate the effects of maternal vitamin B12 supplementation on infant vitamin B12 status.

Preface

Under the supervision of Dr. Tim Green, I Philip Chebaya, have solely written this thesis. I with Dr. Tim Green developed the research protocol, in collaboration with Dr. David Kitts and Dr. Yvonne Lamers. The study is a cross sectional secondary analysis of samples obtained from a CIHR funded study collected by Master's graduates Kaitlin March and Nancy Chen in Vancouver, Canada, and a similar study conducted in Otago, New Zealand by PhD student Rose Stamm and her supervisor Dr. Lisa Houghton. Graduate students and lab technicians were recruited to assist in the analyzing of samples and entry of data.

I was solely in charge of the organization and processing of all the samples (serum and breast milk) utilized in the study. Vitamin B12 concentrations in the serum samples were measured at the University of Otago New Zealand (Rose Stamm), and vitamin B12 concentrations in breast milk were measured at UC Davis (Setareh Shahab Ferdows). It is my responsibility to understand the procedures of these methods and justify their use within our study. Portions of this thesis will be used for future publication in scientific nutrition journals.

Ethical approval for this study was granted by the Children's and Women's Research Ethics Board (H13-01971).

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List of Abbreviations

AI	Adequate Intake
apoHc	Apo-haptocorrin
BCH	British Columbia Children's Hospital
CIHR	Canadian Institute of Health Research
CNS	Central nervous system
CS	Cobinamide sepharose
CV	Coefficient of variance
DTT	Dithiothreitol
FFQ	Food Frequency Questionnaire
Hc	Haptocorrin
HoloTC	Holotranscobalamin
IF	Intrinsic Factor
MM-CoA	Methylmalonyl - CoA
MTHF	Methyltetrahydrofolate
pmol/l	Picomoles per litre
RCT	Randomized control trial
TCII	Transcobalamin II
THF	Tetrahydrofolate
WHO	World Health Organization

Acknowledgements

First and foremost I would like to thank my supervisor Dr. Tim Green for his constant support and guidance throughout my research. His novel methods of teaching and his ability to motivate have been key factors in expanding my already present interest in nutrition research. I would also like to thank my committee members Dr. Yvonne Lamers and Dr. David Kitts for their additional support and respective expertise.

Furthermore, I would like to thank Seterah Shahab-Ferdows, Dr. Lindsey Allen, Rose Stamm, and Dr. Lisa Houghton for their help in measuring vitamin B12 in the blood and breast milk samples in my study.

I would also like to thank the Faculty of Land and Food Systems as well as the Faculty of Graduate and Postdoctoral Studies for the bursaries they have awarded me to help fund my education and research. In addition to the financial support provided by these faculties, I also wanted to thank the staff members involved who have assisted me in assuring all the paper work and logistics of my research ran smoothly.

Finally, I would like to thank my Lab mates Aviva, Abeer, Amynah, Crystal, Kyly, Kristina, Vashti and Zach for their companionship and willingness to be of help throughout my project. I would also like to thank Amy Liu for her hard work in the lab and time spent teaching me valuable laboratory techniques. Special thanks to my family especially my parents for their continued support.

Chapter 1: Introduction

Vitamin B12 is a water-soluble vitamin found predominantly in animal source foods. It functions as a co-factor in two essential biochemical pathways in the body ¹. Consequences of vitamin B12 deficiency can include diarrhea, gastric discomfort and lethargy. In cases of severe vitamin B12 deficiency, megaloblastic anemia and demyelination of nerves in the central nervous system (CNS) occur, which can potentially lead to death if not treated ². Exclusively breastfed infants are at heightened risk of vitamin B12 deficiency as rapid growth and development occur during this period of the life span, specifically the myelination of the nervous system in which vitamin B12 plays an important role. The risk of vitamin B12 deficiency is particularly high in the infants of vitamin B12 deficient mothers and/or when appropriate complementary foods (containing vitamin B12) are not introduced in a timely or adequate manner ³. Infants in developing countries where maternal diets often lack animal source foods are at even higher risk of deficiency than those in developed countries ⁴, however instances of vitamin B12 deficient infants in developed countries have also been documented ⁵. In recent years, there has been a focus on quantifying vitamin B12 concentrations in breast milk, to better understand the association between vitamin B12 concentrations in the milk and maternal and infant vitamin B12 status ⁶. The extent to which either maternal serum status during pregnancy or breast milk B12 concentrations determine infant's vitamin B12 status during the first six month of life is not completely understood ³. Breast milk vitamin B12 concentrations are known to vary among women with similar maternal serum vitamin B12 status ⁷. While Emmett et al. (1997) attributed these variations to dietary vitamin B12 intake, as the main determinant of vitamin B12 concentration in breast milk; however, there is little evidence to support this and research findings to date have been contradictory ⁷. More recently, no significant relation ($r=0.2$,

$p > 0.05$) was found between maternal serum vitamin B12 and breast milk vitamin B12 concentrations at four months postpartum among Danish women ($n=25$)⁸. In contrast, a study among Guatemalan women ($n=183$) showed a strong correlation ($r=0.9$, $p < 0.05$) between maternal vitamin B12 status and breast milk vitamin B12 at two months postpartum³. The difference in the times of evaluation and sample size of these studies may explain the observed discrepancies in findings.

The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life to ensure that infants receive all essential vitamins and minerals, in addition to providing protective effects to the infant and mother which are associated with breastfeeding⁴. Therefore, in cases of inadequate vitamin B12 concentrations in breast milk due to poor maternal nutritional status, alternate strategies are needed to ensure that an infant will receive adequate vitamin B12 to meet their needs for proper growth and development. Oral infant supplementation using liquid drops has been shown to be effective in trials of vitamin D supplementation and it is therefore possible that drops could be a potential vehicle for vitamin B12 supplementation⁹; however, distribution and administration associated with this form of supplementation can be costly, and it may not be a practical intervention tool for many of the developing countries where it is needed most¹⁰. A different preventative approach is to provide adequate vitamin B12 supplements to the mother ensuring an adequate maternal vitamin B12 status. This approach may be more feasible as vitamin B12 could be added to the iron/folic acid supplements that are already distributed in many countries. Fortified complementary foods and infant formulas are another option as they commonly contain higher concentrations of vitamin B12, however, introduction of these may prevent the child from obtaining the protective benefits gained by exclusive breastfeeding for the first six months of life⁴.

Randomized controlled trials (RCT) are warranted to determine whether supplementation of breastfeeding mothers will increase vitamin B12 concentrations in breast milk, and whether maternal supplementation translates into improved infant B12 status. In order to justify an RCT, further examination of the relationship between breast milk and maternal/infant serum is required. The aim of this thesis was to determine the associations between vitamin B12 concentrations in breast milk, infant serum and maternal serum. The analyses were performed on two populations of lactating women and their infants, one in Canada (supplemented mothers) and the other in New Zealand (unsupplemented mothers). The two groups differed in vitamin B12 supplementation use, due in part to different dietary recommendations for each country. This allowed for an exploration of the potential effects of vitamin B12 supplementation during pregnancy and lactation on maternal serum and breast milk vitamin B12 concentrations.

1.1 Digestion and Absorption of Vitamin B12

Human absorption of vitamin B12 is a complex multistep process which is primarily dependent on three specialized scaffold proteins: haptocorrin (Hc), transcobalamin II (TCII) and intrinsic factor (IF) ¹¹. Upon entry into the digestive tract, vitamin B12 is bound to Hc, which is secreted in the oral cavity prior to entering the esophagus ¹¹. The Hc-B12 complex travels through the digestive tract until it enters the distal stomach, where vitamin B12 is cleaved from Hc and binds to IF secreted from parietal cells in the stomach ¹¹. When vitamin B12 is bound to IF, it is absorbed into the body at the terminal ileum. Absorption at this location occurs through specialized cubam-mediated endocytosis ¹¹. Prior to entering the circulatory system, vitamin B12 molecules are cleaved from IF through specialized lysosome degradation and bound to TCII to form holotranscobalamin (HoloTC). It is at this point while bound to TCII, that the vitamin

B12 will be available to the cells in the body. HoloTC travels through the portal circulation, transporting vitamin B12 into the cytoplasm of liver cells by receptor mediated endocytosis ¹¹. Vitamin B12 required in other tissues of the body is released from liver cells through ATP dependent transporters and then binds to TCII, which transfers it to its designated site in the body¹¹. Free vitamin B12 in the cell is then transformed into the biologically active forms necessary to perform each of its physiological functions in the body, methylcobalamin and adenosylcobalamin.

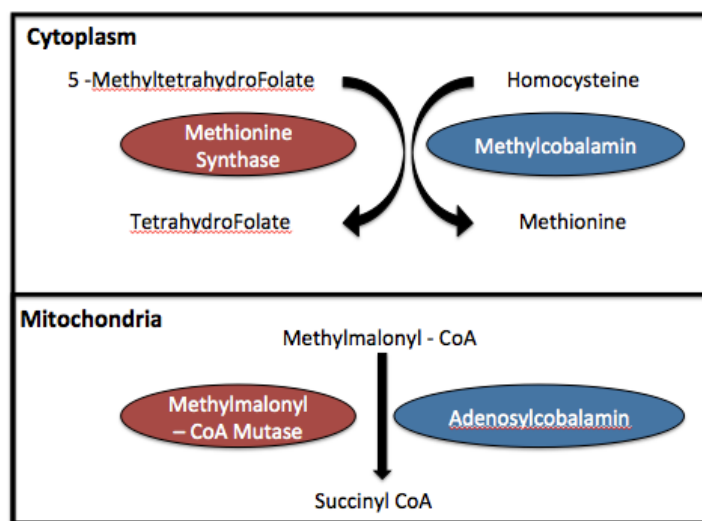
1.2 Physiological Roles of Vitamin B12

It is upon entry into the cytoplasm of the cell that vitamin B12 participates in the two reactions in which it is required as a co-factor ¹. The first reaction is catalyzed by the enzyme methionine synthase EC:2.1.1.13. In this reaction, vitamin B12 cleaves the methyl group from 5-methyltetrahydrofolate (5-MTHF), converting 5-MTHF into tetrahydrofolate (THF) - the active form of folate ¹¹. After this, the newly formed methylcobalamin transfers its adopted methyl group to homocysteine forming methionine an essential amino acid (**Figure 1**) ¹¹. Methionine is further converted into S-adenosylmethionine, the key methylation agent in the body. The other reaction vitamin B12 participates in occurs in the mitochondria of the cell..Vitamin B12 as adenosylcobalamin participates in the conversion of methylmalonyl CoA (MM-CoA) to succinyl CoA; a crucial intermediate in the Krebs cycle (**Figure 1**) ¹¹. This is an isomerase reaction catalyzed by methylmalonyl-CoA mutase ¹¹. This reaction is required for the body to completely metabolize odd chain fatty acids and certain amino acids ¹¹.

A lack of vitamin B12 inhibits these reactions leading to elevated circulating concentrations of methylmalonic acid (MMA) and homocysteine ¹⁴. Therefore, high plasma concentrations of

these metabolites can be indicators of vitamin B12 deficiency. For example, an evaluation of the reliability of vitamin B12 indicators in 259 pregnant women, found a significant ($p < 0.0001$) inverse relationship ($r = -0.4658$) between serum vitamin B12 and MMA (Section 1.5.2) ¹². Homocysteine was also assessed; however, a significant relationship between it and serum vitamin B2 was not observed. Explanation of this result may lie in methionine synthase's requirement of both vitamin B12 and folate to convert homocysteine to methionine decreasing its specificity ¹². Observations of these metabolite concentrations can act as early indicators to detect vitamin B12 when there is a risk of deficiency, preventing further harm.

Figure 1.1: Simplified schematic of reactions vitamin B12 dependent pathways



1.3 Vitamin B12 Deficiency

Vitamin B12 deficiency can lead to a number of adverse health outcomes throughout the lifespan¹³. Critically, symptoms associated with vitamin B12 deficiency occur as a continuum as severity of deficiency increases¹⁴. Initially, subclinical vitamin B12 deficiency is characterized by a decrease in serum vitamin B12 and elevated concentrations of the functional biomarkers

homocysteine and MMA, which may not be accompanied by symptoms¹⁵. The severity of deficiency increases when liver and body stores of vitamin B12 are depleted. Symptoms such as lethargy, poor cognitive performance and megaloblastic anemia may then occur¹⁶. Pernicious anemia is a particular form of vitamin B12 deficiency worth noting. It is an autoimmune disorder that leads to a loss of parietal cells, which are responsible for the release of IF. Oral consumption of vitamin B12 in the case of pernicious anemia will not always reverse the consequences as absorption is impaired, instead intramuscular injection are suggested. Those consuming vegan diets (no animal source foods) are also at higher risk of vitamin B12 deficiency, as animal source foods are the key dietary source of vitamin B12¹⁷. Megaloblastic anemia caused by vitamin B12 deficiency is characterized by high mean corpuscular volume, low hemoglobin and low vitamin B12 concentrations in the blood. Due to the fact that adults can store up to 4mg of vitamin B12 at a time while only excreting <0.25µg/day (due to the enterohepatic circulation)¹¹, it can take over five years for vitamin B12 deficiency to occur¹⁸. Unlike adults, infants can only store approximately 25µg of vitamin B12 during pregnancy, which lasts for only the first month of life if not replenished, putting them at increased risk of deficiency¹⁹. In this literature review the focus will be on infant deficiency, and its consequences.

1.3.1 Consequences of Vitamin B12 Deficiency in Infants

Vitamin B12 deficiency leads to two well-defined consequences, megaloblastic anemia and neuropathy. Prior to the onset of these symptoms, less severe symptoms may arise which can act as early indicators of vitamin B12 deficiency²⁰. In infants, early symptoms include apathy, diarrhea, paleness of skin and loss of appetite and gastric discomfort. These symptoms do not

usually develop until four months of age ⁵, with the delay in the onset of symptoms attributed to the availability of small amounts of vitamin B12 from stores obtained during gestation⁴.

Fortunately, most symptoms that occur in this period can be reversed with supplementation and/or direct intramuscular injection of vitamin B12 ⁸. For example, in a recent case study of a 10-month old infant with serum vitamin B12 concentrations of 145pmol/l, symptoms of lethargy and apathy were reversed after administration of a one milligram intramuscular injection of vitamin B12 ²¹. The timing of the injection may be important in determining the effectiveness of supplementation and reversal of symptoms²¹. As such, studies are needed to identify the optimal timing and method of supplementation for the reversal of symptoms associated with vitamin B12 deficiency in both infants and adults ⁴. Megaloblastic anemia in infants is rare, since proper folate concentrations can prevent its occurrence⁴. Instead, infants tend to experience neurological damage, characterized by decreased motor control and minor delays in recognition. These symptoms tend to be more subtle until severity increases ²², though if untreated can produce long term negative effects ². The behavior of infants deficient in vitamin B12 needs to be monitored continually to observe changes in cognitive queues such as lack of motor skills and coordination ²³.

As mentioned above, untreated vitamin B12 deficiency can lead to irreversible neurological damage ^{3,4}. While the precise causal mechanisms of this are not well defined; researchers argue that the neurological damage is due to the integration of improper fatty acids during the crucial myelination period that occurs during gestation and the first year of life. In brief, deficiency of vitamin B12 inhibits optimal methylmalonyl-CoA mutase activity, this in turn increases the production of the precursor propionyl CoA ²⁴. The elevated concentration of propionyl CoA may contribute to odd chain fatty acids ¹¹ becoming inappropriately incorporated into the

myelin, which decreases the efficiency of signal propagation and leads to poorer neurological function²⁵. Observation of the irreversible neurological consequences of vitamin B12 deficiency was first seen in case studies where full cognitive ability was not regained after administration of vitamin B12 to the infant^{26,27}. In addition, an experimental study performed in Eastern Europe examined symptoms of vitamin B12 deficiency such as anorexia, apathy, vomiting and cognitive delay in 40 infants born to vitamin B12 deficient mothers²⁸. After these infants were administered an injection of vitamin B12, a significant ($p < 0.05$) improvement was observed in all categories except cognitive function²⁸. In order to determine if the cognitive damage due to infant vitamin B12 deficiency was in fact irreversible, a long term follow up study in rural India was performed by Bhate et al². Infants who suffered from vitamin B12 deficiency at birth were followed until the age of nine; cognitive ability was tested using a validated color trial test. At nine years of age a significant ($p < 0.05$) difference was observed in the time required to identify specific colors between the infants born to vitamin B12-deficient mothers and those who were not, 182 and 153 seconds respectively². According to this WHO, children who are deficient in vitamin B12 ($< 150 \text{ pmol/l}$) are delayed in reaching developmental milestones such as reading and writing compared to those children who were vitamin B12 replete ($> 220 \text{ pmol/l}$)⁴.

1.4 Vitamin B12 in Breast milk

1.4.1 Composition in Breast Milk

Vitamin B12 is found in breast milk primarily bound to haptocorrin, and its concentration is variable during the different stage of lactation. Assessment of breast milk vitamin B12 concentrations have shown that concentrations can significantly vary within individual feedings as well at different times after birth^{7,8}. Significant differences in milk vitamin B12

concentrations have been found at 2 weeks (720pmol/l), 4 weeks (290pmol/l) and 9 months (440pmol/l) postpartum in the breast milk of 25 lactating Danish women⁸. The relatively higher concentrations of vitamin B12 observed at two weeks postpartum were attributed to the higher concentration of protein found in colostrum (milk <14 weeks postpartum) in comparison to mature milk⁸. Within individual feedings milk is also released in two stages, the first milk to leave the breast is known as foremilk, and is more watery due to the absence of fat cells which stick to mammary glands of the breast prior to excretion⁷. By the end of the feeding the milk is known as hindmilk; it contains most of the fat in which was originally attached to the mammary gland⁷. Of note, the Danish study described above also examined the differences in vitamin B12 concentrations between hindmilk and foremilk at different points of time postpartum.

Significantly higher concentrations of vitamin B12 in hindmilk ($p<0.05$) were observed at all three time periods (2 weeks, 4 months, and 9 months) compared to foremilk⁸. Therefore, when evaluating vitamin B12 concentration in breast milk, it is essential to attempt to receive a full expression consisting of both hind and foremilk.

Breast milk and serum are relatively similar in composition, yet, they are very different in regards to the concentration of certain key components⁷. Breast milk contains approximately 100 times more protein than serum, specifically Hc which is the protein that the majority of vitamin B12 present in breast milk is bound to²⁹. While the reason for this is not fully understood, some have argued its presence is due to its ability to act as antimicrobial agent³⁰, though the bulk of recent research has refuted this once accepted hypothesis³¹. TCII's role as the active protein carrier of vitamin B12 has led to the belief that it would carry vitamin B12 to the mammary glands prior to excretion. Yet, this theory has been questioned, as TCII is found in low concentrations in breast milk³². As such the mechanisms of TCII in vitamin B12's transfer

to breast milk are not understood, as not enough research has been performed to determine if any factors may affect its concentration. These unique characteristics of breast milk, make it challenging to determine the most accurate and reproducible laboratory method for measuring vitamin B12, as discussed below²⁹

1.4.2 Methods for Measuring Vitamin B12 in Breast Milk

The similar composition of breast milk to serum prompted the use of automatic serum analyzer and/or immunoassay kits to measure breast milk vitamin B12 concentrations in early research^{3,29}. As the amount of research evaluating the concentrations of vitamin B12 in breast milk increased, poor reproducibility and high variations in vitamin B12 concentrations were observed between similar studies using these methods³³. As research progressed, the large variation observed in breast milk vitamin B12 concentrations, was attributed to interference from the extremely high concentrations of unbound Hc (apoHc) found in breast milk²⁹. After these findings, new methods for measuring vitamin B12 in breast milk, focused on the utilization of a pre-treatment to remove the of excess apoHc prior to directly measuring vitamin B12 concentrations^{8,34}. Lildballe et al.²⁹ evaluated the interfering effects of apoHc by measuring vitamin B12 concentrations in the breast milk of 25 healthy women. Breast milk vitamin B12 concentrations were measured using a COBRA serum analyzing system; each sample was run with and without cobinamide sepharose pretreatment, which acts to removes the excess apoHc. Results showed that samples not exposed to the cobinamide sepharose pre-treatment produced inconsistent results and had large inter-variability (CV=28-200%) compared to samples which received cobinamide sepharose pretreatment (CV=3.9-6.7%)²⁹. Furthermore, the bulk of the research assessing vitamin B12 concentrations in breast milk is performed on vitamin B12 deplete

populations where concentrations of vitamin B12 in breast milk are regularly below the accurate detectable range of the serum analyzer used ($<50\text{pmol/l}$)³. This limitation led to the exploration of methods that were capable of making accurate measurements at low concentrations.

One such method is the use of microbial assays. Microbiological assays have been recognized for their ability to enable very low concentrations of vitamin B12 to be detected, due to the bacteria's requirement for this vitamin to grow³⁵. The strain of bacteria used for microbiological assessment of vitamin B12 is *Lactobacillus leichmannii*. Because this strain is dependent on vitamin B12 to grow and thrive, the microbial assay is less affected by the elevated apoHc concentrations found in the breast milk³⁵. Another potential method, attempted by our lab at University of British Columbia is a Japanese microbiological assay published by Sakauri et al³⁵. After producing promising results in early trials (not published) we ran into continued contamination. Further investigation attributed antibiotic interference as the reason for contamination, however determining the proper enzyme to remove these antibiotics only brought about more challenges. It is the tremendous precision required for microbiological methods that have led to the evaluation of alternative methods, which are less prone to human error.

In 2013, a group from UC Davis measured vitamin B12 concentrations in both Bangladeshi and Californian women using a tabletop chemiluminescence machine known as IMMULITE³³.

They argued that using their method, the laborious process of removing excess apoHc was not needed to obtain reliable results. Identical breast milk samples were run through both the IMMULITE machine and a competitive protein-binding assay. Prior to measurement of vitamin B12 using the competitive protein binding assay the samples were run through a sepharose pretreatment process. Vitamin B12 concentration results between the two methods showed no significant difference³³. The IMMULITE machine demonstrated an average recovery rate of

83%, after running 450 samples over a period of three months. Haptocorr was said not interfere with their machine as they obtained similar vitamin B12 concentrations using a pretreated (with cobinamide sepharose) and untreated version of the same sample³³. It has recently been utilized in an RCT published in the American Journal of Nutrition, evaluating vitamin B12 concentrations in an Indian population of pregnant and lactating women and their infants³⁶. Further studies using this method, such as my own, will allow researchers to expand on limited understanding of vitamin B12 concentrations in breast milk.

1.4.3 Transfer of Vitamin B12 from the Mother to the Infant

During gestation, changes occur in the maternal metabolism to accommodate the developing fetus. Many changes in nutrient absorption that occur during pregnancy are not understood, one of those being vitamin B12³⁷. Early animal studies assessing vitamin B12 concentrations during pregnancy were the first to observe increased maternal vitamin B12 absorption during pregnancy³⁸. However, even with increased absorption, serum vitamin B12 concentrations over the course of pregnancy have shown to decrease from the first to the third trimester. The main argument to explain this is that the fetus has an increased affinity for obtaining stores of the vitamin, and so the growing fetus seems to be receive much of the maternal serum vitamin B12³⁸. Conversely, a current RCT assessing the behavior of vitamin B12 indicators throughout pregnancy in 39 healthy women concluded that the poor status observed in the mother may be due to the use of improper indicators to account for vitamin B12 status during gestation³⁷. The inappropriate vitamin B12 indicators for infant vitamin B12 concentration has also occurred. In a cross sectional evaluation, 40 exclusively breast fed infants of vitamin B12 supplemented women assessed at six month after, were categorized as deficient in vitamin B12 (serum vitamin B12

<150pmol/l)²⁶. Some argue the infants' poor status is due to a lack of appropriate proteins to absorb vitamin B12, as the infant is still adapting to oral feeding^{25,39}. The lack of confidence in currently used indicator cut offs of vitamin B12 indicators during gestation and lactation has posed challenges in determining the appropriate vitamin B12 concentration required in the mother to assure the infant may obtain adequate stores.

1.5 Assessment of Vitamin B12 Concentrations in the Serum of Pregnant/Lactating Women and their Infants

A range of indicators are used for evaluating vitamin B12 status however, there, is still debate as to which is the most appropriate⁴⁰. Two categories of indicators will be discussed in this section: direct indicators and functional indicators. Direct indicators contain a form of vitamin B12, such as holoTC and serum vitamin B12. Functional indicators such as MMA and homocysteine do not contain vitamin B12, but instead are reflection of intercellular vitamin B12 deficiency, as they are substrates that depend on vitamin B12. Thus, elevations in their concentration are correlated with a decreases in vitamin B12 concentrations⁴¹.

1.5.1 Direct Indicators of Vitamin B12 Status

Serum vitamin B12 - the concentration of total vitamin B12 found in the blood – is a direct indicator commonly used for the measurement of vitamin B12 status in the entire population⁶. Its consistency and reproducibility in the literature have led to its use as the international standard for determining vitamin B12 status^{4,42}. However, certain characteristics of serum vitamin B12 lead to difficulty in its interpretation⁴³. Firstly, the majority of vitamin B12 in the body is stored in the liver and muscle tissues, so depletion in these store sites may go unnoticed

if serum vitamin B12 concentrations remain normal ¹¹. Secondly, not all vitamin B12 found in serum will be utilized by the cells of the body; only vitamin B12 bound to TCII is considered bioavailable ⁴⁴. Serum vitamin B12's efficacy as an indicator was tested in an experimental study evaluating serum vitamin B12 concentrations in 39 healthy pregnant women throughout pregnancy. A significant ($p < 0.0001$) decrease in serum vitamin B12 concentrations from the first to the third trimester was observed, but homocysteine concentrations were not elevated as normally observed during deficiency, instead they remained constant ³⁷. The author concluded that these discrepancies may have been due to the improper use of reference values in research on pregnant women ³⁷. In a separate longitudinal follow up study, the decreased serum vitamin B12 values returned back to normal ranges at three weeks postpartum and remained consistent throughout lactation ⁴⁵.

In this same study, the infant's serum vitamin B12 concentrations values did not change when environmental and dietary conditions remained the same. This finding are consistent with the fact that fewer discrepancies in serum vitamin B12 in infants as an indicator of deficiency are observed ¹³. Despite its limitations many researchers still argue that serum vitamin B12 is the preferred direct biomarker at this time ^{5,19}.

Vitamin B12 bound to TCII is known as holoTC. HoloTC is the active form of vitamin B12 that is most available to tissue cells in the body ²⁵ and is a more sensitive biomarker for acute vitamin B12 deficiency than serum vitamin B12 during pregnancy. One reason for this is that unlike serum vitamin B12, holoTC concentrations remain constant during the hormonal and homeostatic fluctuations that occur throughout pregnancy and lactation ⁴⁶. Secondly, TCII has less affinity than other vitamin B12 binding proteins to vitamin B12 analogues, preventing any over or underestimation of vitamin B12 concentrations ⁴⁷. In a laboratory assessment of serum

samples (n = 49) from vitamin B12 deficient participants, holoTC concentrations were found to be below the established cut off, while serum vitamin B12 concentrations were in the range of adequate vitamin B12 status (i.e., >220pmol/L)⁴⁴. However, this improved sensitivity was only seen in less than 5% of the samples within the study. HoloTC's short half life may cause it to reflect daily changes in vitamin B12 which would not necessarily be relevant for B12 deficiency⁴⁴. Regardless of some of the possible benefits of utilization of holoTC, research examining the accuracy of holoTC as an indicator of vitamin B12 status in pregnant/lactating woman and their infants is still limited in comparison to experimental support provided for serum vitamin B12⁴⁴. In addition, the relatively higher cost required to measure holoTC may be unachievable for many laboratories in a clinical setting.

1.5.2 Functional Indicators of Vitamin B12 Status

MMA and homocysteine are the functional indicators of vitamin B12 deficiency. They can assist in the early detection of vitamin B12 deficiency as elevated concentrations of these indicators can be present prior to serum vitamin B12 concentration falling below 150pmol/L⁴⁸. In the absence of vitamin B12, methylation of homocysteine is interrupted preventing its conversion to methionine by the vitamin B12 dependent enzyme methionine synthase¹². An experimental study in rural India among pregnant women with poor vitamin B12 status (n=139), observed an inverse relationship between homocysteine and vitamin B12 concentrations when vitamin B12 supplementation was provided¹⁰. In contrast, a study evaluating the efficacy of homocysteine as a functional indicator for vitamin B12 deficiency in pregnant women found no significant inverse correlation between homocysteine and vitamin B12, however, a significant ($p>0.0007$, $r=0.39$) correlation between homocysteine and red cell folate was seen¹².

Homocysteine's requirement of both folate and vitamin B12 for its conversion to methionine may explain this result and is why it can be rendered an ambiguous indicator⁴. Unlike the many precursors in the one carbon metabolism cycle which may elevate homocysteine, MMA is primarily dependent on vitamin B12, and therefore MMA is thought to be a more reliable indicator of vitamin B12 deficiency^{12 42}. In an experimental study of infants consuming a macrobiotic diet, the ability of homocysteine and MMA to act as functional indicators of vitamin B12 deficiency were compared; MMA showed a stronger inverse correlation ($r = -0.765$, $p < 0.001$) with serum vitamin B12 than did Homocysteine ($r = -0.741$, $p < 0.001$)⁴⁹. Functional indicators play an important role in evaluating vitamin B12 status, due to the “grey area” created by non-definitive cut offs currently used for pregnant/lactating women and infants⁴. When values are within the “grey area” functional indicators such as MMA can be used as an additional tool to help determine and whether interventions are warranted to prevent the negative consequences of deficiency⁴².

1.6 Recommendations and Guidelines

1.6.1 Current Serum vitamin B12 Cutoffs

The WHO has categorized serum vitamin B12 concentrations for the healthy average individual into three categories: deficient status ($<150\text{pmol/l}$), marginal status ($150\text{-}225\text{pmol/l}$) and adequate status ($>225\text{pmol/l}$). Research is still limited on the appropriate vitamin B12 cut offs for a lactating or pregnant woman and therefore the use of guidelines designated for normal healthy individuals in this context may be inappropriately utilized⁴. Homeostatic changes that occur during pregnancy such as hemodilution may take part in the large variation observed in vitamin B12 concentrations between the first and third trimester. In a cross sectional evaluation

of 25 women performed by the Women and their Children association (WATCH), a 16% decrease in the plasma vitamin B12 of pregnant women from 20 to 36 weeks gestation was observed⁵⁰. All participants in the third trimester fell within the category of marginal vitamin B12 status (150-225pmol/L) established by the WHO, even though they had been regularly consuming adequate amounts of vitamin B12³⁷. Similar confusion is present in the current cut offs used for assessing infant vitamin B12 status.

Prior to the recent reevaluation of guidelines, vitamin B12 deficiency in infants was based on the appearance of observed symptoms; however, at this time deficiency may have been already present for months in the infant¹⁷. Based on new research examining infant vitamin B12 status, the WHO released technical briefs proposing an increase in the 150pmol/L cut off currently used for infants, on the basis that there is a possibility that neurological damage could occur prior to serum B12 falling below this concentration. This calls for the revaluation of current recommendations and guidelines currently stated¹⁷.

1.6.2 Current Recommendations for Vitamin B12 During Lactation

In order to compensate for the increased requirement of vitamin B12 during lactation and pregnancy, increases in the Recommended Dietary Allowance (RDA) for vitamin B12 have been set forth. RDAs are defined as recommendations that are sufficient to meet the nutrient requirements of approximately 98% of the total population in a particular life stage and gender group. Determination of an RDA for a specific vitamin requires an ample amount of scientific research assessing the vitamin's requirement during that specific life period⁵¹. Health Canada sets the RDA for vitamin B12 at 2.6mcg/day for woman in gestation and 2.8mcg/day for woman in lactation (**Table 1.1**)⁵¹. As mentioned above, the RDA for vitamin B12 during pregnancy is

increased from 2.4mcg/day to accommodate for the increased affinity for vitamin B12 the infant displays through placental transfer in gestation and breast milk during lactation ⁴. Vitamin B12 concentrations of the mother are transferred down a concentration gradient towards the fetus, reinforcing the importance of adequate maternal vitamin B12 status ⁵².

By contrast, research present on evaluating infant status in the first six month of life, is too premature to establish a RDA for vitamin B12. In its place, an Adequate Intake (AI) is utilized, which is defined as an experimentally observed average of appropriate intakes of a group (or groups) of apparently healthy people ¹⁸. The AI for this life stage was determined by measuring the average concentration of vitamin B12 in the breast milk of three groups of women, vitamin B12 supplemented mothers, vegan and vegetarian mothers, and non-supplemented mothers in good vitamin B12 status. A value of 0.43mcg/L was found to be the best representative of the average population. This average value was then multiplied by 0.78L/day, which is considered the average amount of milk consumed per day by an exclusively breast fed infant during the first six month of life. This led to a value of 0.33mcg/day which was then rounded to 0.4mcg/day⁵¹. An increase in AI is observed with age: at 0-6 month the AI is 0.4mcg/day and at 6-12 month it is 0.5mcg/day, as the toddler begins to build their bodily stores ⁵¹. As exclusive breastfeeding is recommended until six month of age, an improved understanding on the impact of maternal vitamin B12 status on vitamin B12 in breast milk to infants is crucial to ensure the infant receives adequate stores ⁶.

Table 1.1⁶⁰: Recommended Dietary Allowances for Vitamin B12 (* Signifies Adequate Intake)

Age	Male	Female	Pregnancy	Lactation
0-6 months	0.4 mcg*	0.4 mcg*		
7-12 months	0.5 mcg*	0.5 mcg*		
1-3 years	0.9 mcg	0.9 mcg		
4-8 years	1.2 mcg	1.2 mcg		
9-13 years	1.8 mcg	1.8 mcg		
14 + years	2.4 mcg	2.4 mcg	2.6 mcg	2.8mcg

1.6.3 Assessment of Possible Vitamin B12 Interventions and their Implications

As mentioned above, there has been an increasing appreciation of the importance of proper maternal vitamin B12 status during pregnancy and lactation to prevent the incidence of vitamin B12 deficiency⁴. In developed countries, vitamin B12 rich foods are readily available, however religious beliefs and vegetarian diets may inhibit the consumption of animal source food which pose a challenge. In addition, abiding by these recommendations in developing countries has shown to be particularly challenging especially since vitamin B12 food sources may not be available. When vitamin B12 rich foods are not always readily available, or culturally acceptable, obtaining proper vitamin B12 status may be unattainable.

Effective interventions are therefore needed and must take in account the differences in both a developing and developed country context as well as the effect eating preferences and malabsorption conditions which pose a further challenge⁵³. In 2003, the WHO released a projected budget for the provision of fortified complementary foods to six month old infants in developing countries⁵³. Cost ranged from seven cents to twenty-one cents per 100g of these

foods, depending on the country. The cost of these foods depended on product availability and production within the specific region and could change over time^{54 55}. Lack of established RDA's and limited research, created a struggle in obtaining proper funding so that the intervention may be implemented.

In comparison, no supplementation or fortification system has been proposed for children ages 0-6 month, as the WHO discourages complementary feeding during this time period²⁰. As maternal serum vitamin B12 is thought to be a primary determinant of infant and breast milk vitamin B12 concentrations, regions where maternal deficiency is common need to be monitored closely⁵⁶. In a population of Guatemalan women, Deegan et al observed a significant difference ($p > 0.05$) between the serum vitamin B12 concentrations of infants born to deficient mothers ($< 150 \text{ pmol/l}$) and those who were not (mother B12 $> 220 \text{ pmol/l}$)³. In addition, in an experimental analysis of vitamin B12 deficient infants, when vitamin B12 supplementation was provided, a significant ($p < 0.05$) score increase on the score of the Alberta Infants Motor Scale (AIMS) was observed²². Closely monitoring maternal and infant vitamin B12 status and vitamin B12 consumption is important, otherwise symptoms of deficiency in the infants may occur during lactation prior to consumption of complementary or fortified foods after 6 months.

If proper dietary sources are not available, supplementation needs to be provided to the mother prior and during gestation so that infant will have accumulated appropriate stores before birth⁶.

In support of this notion, a RCT was published evaluating the efficacy of vitamin B12 supplementation in rural India among pregnant and lactating women and their infants. Women were randomly assigned to be given either a placebo or a supplement containing 50mcg of vitamin B12 (randomization was blind)³⁶. A significant ($p < 0.05$) increase was observed between vitamin B12 concentrations in the breast milk of supplemented mothers compared to

those in the placebo group at 6 weeks (136pmol vs. 87pmol/l), 3 month (97pmol/l vs. 68pmol/l) and 6 month (106pmol/l vs. 80pmol/l) postpartum³⁶. However, a cross sectional evaluation of 25 healthy Danish woman observed contradictory results, documenting no association between concentrations of vitamin B12 in maternal serum and in their breast milk⁴⁵. This result may be due to Danish woman within this study having already containing adequate stores of vitamin B12, and therefore, are losing the excess vitamin B12 supplementation through urinary excretion¹⁹.

As infant storage capacity for vitamin B12 is much less than in adults, without proper replenishment from the breast milk in the first six month of life infants may not obtain adequate vitamin B12 status and thus are at risk of deficiency²⁵. Understanding the transfer and concentrations of vitamin B12 in breast milk is crucial for knowing the concentration of vitamin B12 supplementation the mother would require. It is important that the mothers obtain the appropriate amount of vitamin B12 so that her breast milk may provide her infant with adequate amounts of vitamin B12.

1.7 Prevalence of Deficiency In Mothers and their Infants

1.7.1 Demographics at Risk

The WHO considers pregnant woman and their infants (0-6 month old) part of the populations who are at highest risk of B12 deficiency, especially if their diets do not contain vitamin B12 rich foods⁴. In the following section, I will focus on prevalence of deficiency in pregnant and lactating women and their infants⁵.

1.7.2 Developing Countries

Developing countries are regions where living standards are considered low. An important factor regularly missing in these areas is food security; the populations access to food and food products in which allow them to sustain life ⁵⁴. Vitamin B12's near exclusivity in animal source foods makes it particularly challenging to obtain the required amounts to prevent deficiency. The consequences of this are reflected in the prevalence of vitamin B12 deficiency in infants and their mothers in these regions (**Table 1.2**). For example, the Mexico Collaborative Research Program documented that up to 41% of pregnant woman in rural Mexico were considered deficient by which they defined by a serum vitamin B12 concentrations below 147pmol/l ⁵⁷. In regions such as Guatemala an increase in vitamin B12 deficiency has been seen in study participants from 1997 to 2012 (**Table 2**). The lack of proper recommendations poses challenge to lowering deficiency rates in developing countries ⁴.

Table 1.2: Summary table identifying baseline characteristics of participants in vitamin B12 study in developing regions

Author /Year	(n)	<150pmol/l	<221pmol/l	Country
Duggan et al. 2014	366	51% of women	-	India
Deegan et al. 2012	188	40% of women 50 % of infants	70% woman	Guatemala
Stewart et al. 2011	544	27 % of women	-	Nepal
Taneja et al. 2007	2252	17% of infants	34% of infants	India
Garcia et al. 2005	5658	21% of infants 61% of women	-	Venezuela
Shimohara et al. 2002	69	-	60 % of infants 75% of infants	Brazil
Casterline et al. 1997	113	13% of women	33% of women	Guatemala

1.7.3 Developed Countries

Most of the populations living in developed or industrialized countries have access to more than their recommended requirement of vitamin B12. Yet in developed countries such as Canada and the USA, five percent and two percent respectively are still considered biochemically deficient (<150pmol/l) as indicated by the Center of Disease Control ¹⁶. Increase in the popularity of vegetarians diets, are thought to contribute to the slight increase in vitamin B12 deficiency seen in the past few years ³. However, overall vitamin B12 deficiency rates in developed countries are quite low, and the resources present in these countries that play an important role in determining guidelines and solutions to evaluate deficiency in developing countries ¹⁷. The wealth and food security of these nations allow experimental research to be performed to examine vitamin B12 concentrations throughout pregnancy and lactation. Therefore, it is important to assure that deficiency is not unnecessarily increasing in these developed countries.

1.7.4 Efficacy of B12 Supplementation During Pregnancy and Lactation

As mentioned above, the bulk of the research has been based in developing countries on where vitamin B12 deficiency is most common. Participants within this population are normally separated by vitamin B12 status determined by current WHO cut offs ³. Studying the efficacy of supplementation during pregnancy and lactation would be a positive first step in addressing this issue. Recently research in this area has taken another step forward with the release of a vitamin B12 supplementation RCT consisting of 200 pregnant and lactating woman and their infants in rural India. During pregnancy woman were randomly assigned to either receive a placebo or a vitamin B12 supplement (50mcg) in which they would consume daily throughout pregnancy and lactation. The infants of the women who received the supplement containing vitamin B12

(199pmol/l) had significantly higher ($p<0.01$) serum vitamin B12 concentrations at six weeks postpartum than those who were given a placebo (139pmol/l)³⁶. Concentrations of the functional biomarkers homocysteine and MMA were also lower in the supplemented group compared to placebo group³⁶. The promising results of this study further motivate the importance of proper vitamin B12 status in the mother.

1.7.5 Rationale

My cross sectional study will assess the differences in maternal serum and breast milk vitamin B12 concentration between a supplemented and an. unsupplemented population from two developed nations. It will also explore the associations present between the mother's serum, infant serum (Canada only) and breast milk concentration of vitamin B12. This will aid in identifying the primary predictor of infant B12 status during the early stages of lactation.

Associations between breast milk and maternal serum vitamin B12 concentrations may provide evidence that breast milk vitamin B12 concentrations can be manipulated by changing concentrations of vitamin B12 in maternal serum.

Chapter 2: Objectives and Methods

2.1 Purpose

The purpose of this study was to determine the association between maternal serum vitamin B12 and breast milk vitamin B12 in both a supplemented and unsupplemented cohort and explore their association with infant serum vitamin B12 (supplemented group only) at eight weeks postpartum. These findings will indirectly build on the current lack of research data assessing maternal breast milk vitamin B12 concentrations. These findings will aid in determining the importance of vitamin B12 supplementation during pregnancy and lactation for an infant to maintain adequate vitamin B12 status during the lactation period..

2.2 Research Hypothesis and Objectives

H_0 : Vitamin B12 status in breast milk of an exclusively breastfed infant, as assessed by circulating B12 at eight weeks postpartum, are not associated with maternal serum vitamin B12 concentration of the mother's serum.

H_a : Vitamin B12 status in breast milk of an exclusively breastfed infant, as assessed by circulating B12 at eight weeks postpartum, is associated with vitamin B12 concentration of the mother's serum

Primary Objective

1. To determine the association between vitamin B12 concentrations in maternal serum and breast milk at eight weeks post-partum in a primarily vitamin B12 supplemented population (Canada) and non-supplemented population (New Zealand)
2. To determine if a statistically significant difference is present between the mean (95%CI) serum vitamin B12 concentrations of mothers from the New Zealand (non supplemented) and Vancouver (supplemented) group at eight weeks postpartum.

Secondary Objectives

3. To examine the association between vitamin B12 concentrations in maternal and infant serum in a supplemented Canadian population.
4. To determine if vitamin B12 supplementation can be associated with a higher concentration of vitamin B12 in breast milk.

2.3 Methods

2.3.1 Project Overview

This cross sectional study measured vitamin B12 concentrations in blood and breast milk samples collected at eight weeks postpartum from two previous studies. The first of the two studies was a randomized control trial (RCT) carried out in Vancouver, Canada between 2010 and 2012. The RCT examined mothers from 13-22 weeks of gestation to eight weeks postpartum. All mothers were provided and advised to consume a prenatal multivitamin and mineral supplement that contained 12µg of vitamin B12 and randomly assigned to one of three

vitamin D supplement groups (400IU, 2000IU, or 4000IU). The second study was conducted at the University of Otago and included women recruited from Dunedin New Zealand. This was a cross sectional study designed “to assess one carbon metabolism and nutrition status in healthy term infants in relation to breast milk intake and maternal status at eight weeks postpartum”; no prenatal supplement was provided or recommended. Procedures for sample collection and storage were similar in both studies (**Table 2.1**).

Maternal and infant serum vitamin B12 concentrations were measured at eight weeks postpartum in the laboratory of Dr. Lisa Houghton, University of Otago, New Zealand, Breast milk vitamin B12 concentrations were measured in the Western Human Nutrition Research Center (USDA lab) at the University of California, Davis.

2.4 Primary Studies

2.4.1 Overview

The Canadian study examined the effects of varying doses of vitamin D supplements on serum 25-hydroxyvitamin D concentrations in mothers from pregnancy to eight weeks postpartum and their infants. As prenatal supplementation is recommended for all pregnant woman by Health Canada, all participants were provided with a prenatal multivitamin to consume throughout the study⁵⁸. For the purpose of my study the group of Canadian women were considered the vitamin B12 supplemented group, as the prenatal multivitamin contained 12µg of B12 (Natural Factors Ltd.), which is well above the RDA for vitamin B12 (**Table 1.1**).

In comparison, the New Zealand observational study focused on one-carbon metabolite concentrations and their transfer from the breast milk to the infant. No supplementation was

provided or recommended to the study participants. This group is considered here as the vitamin B12-unsupplemented group.

The maternal serum, infant serum and breast milk samples used in our study were collected at eight weeks postpartum in both studies. Due to limited sample availability, there was no blood available for infants from the New Zealand unsupplemented group.

2.4.2 Participant Recruitment

Participants in the Canadian study consisted of a convenient sample recruited from Greater Vancouver, British Columbia. Participant recruitment occurred between June 2010 and August 2011. Researchers recruited participants directly from the ultrasound clinic at BC Women's Hospital and indirectly by brochures, newspaper advertisements and word of mouth. The inclusion criteria included women aged 18 to 42 years who were between 13 to 24 weeks pregnant (based on last missed menstrual period) expecting a singleton low risk birth. Women were not eligible to participate in the study if they had one/or more of the following conditions: gestational diabetes, preeclampsia, tuberculosis, cardiac and/or renal disease, hypertension, autoimmune disease, or any other disease that would categorize the pregnancy as high risk. In addition, participants were excluded if they had been taking vitamin D supplements ($>15\mu\text{g/day}$) and/or had any conditions that may be associated with vitamin D malabsorption. Upon entry into the study, written consent was obtained from all participants.

Participants in New Zealand study were recruited between May 2012 and December 2013 using local advertising. If interest was shown, women were provided information sheets and/or contacted by phone to determine if they would be eligible for participate in the study. The inclusion criteria was similar to that of Canadian study, except that women in the New Zealand

study were only assessed at eight weeks postpartum. Also, instead of excluding participants who had conditions effecting vitamin D absorption, the New Zealand study excluded women who had folate malabsorption.

2.4.3 Supplementation

All participants in the Canadian study regardless of vitamin D supplementation dose were given a prenatal supplement containing 12µg of vitamin B12 (cyanocobalamin). No supplementation was assigned to the New Zealand participants. A vitamin and mineral questionnaire form was provided upon entry into each primary study and this captured those taking vitamin and mineral supplements outside of those provided in the studies. If a New Zealand women consumed vitamin B12 supplements in excess of six micrograms a day throughout pregnancy and lactation, they were reassigned to the supplemented group for analysis.

2.4.4 Serum Sample Collection

Blood collection procedures were similar in the Canadian and New Zealand studies. Fasting (12 hour) blood samples were collected in the New Zealand study, whereas non-fasting blood samples were collected in the Canadian study. In the Canadian study, blood samples were collected at BC Children's Hospital (outpatient lab). In the New Zealand study blood collection was performed either at participant homes or at the Nutritional Department Clinic, University of Otago. Venipuncture was used to obtain the blood from mothers and infants in the Canadian study and mothers in New Zealand. At both sites blood was collected into 10ml vacutainers containing Increased Silica Act Clot Activator which were allowed to stand for 15-20 minutes,

and centrifuged for 10 minutes (3000rpm). Serum was then transferred into 1.5ml tubes and stored at -80°C until analysis.

2.4.5 Breast Milk Sample Collection

In both studies, breast milk samples were expressed from one breast, two or more hours after the previous feed. Women were requested to provide a full expression of breast milk (in an attempt to obtain both foremilk and hindmilk) whenever possible. An electric breast pump (Medela Swing™ Breast pump) was used during clinic visits to aid in milk expression. In the Canadian study, participants were allowed to provide a breast milk sample collected the morning prior to the clinic. Participants were advised to store milk sample in the refrigerator prior to the study clinic visit. The New Zealand protocol allowed milk samples that were collected and refrigerated within three days of clinic visit to be used, if stored properly.

In both studies, if participants had produced more than one tube of breast milk, the samples were gently mixed before being stored at -80°C for subsequent analysis.

Table 2.1: Procedure comparisons between primary studies.

Variable	Folate Study (New Zealand)	Vitamin D Study (Canada)
Blood Collection	At 8 week postpartum: infant venipuncture or heel prick; mother venipuncture	At 8 weeks postpartum: mother and infant venipuncture
Blood Sample	Serum	Serum
Breast Milk Collection	1) By electric pump at visit 2) Within three days of clinic visit if sample is stored in patient fridge prior.	1) By electric pump at visit 2) Same day as clinic visit if refrigerated prior.
Breast Milk Sample	Complete expression of one breast (>2 hours after previous feed)	Complete expression of one breast (>2 hours after previous feed)

2.5 Thesis Project

2.5.1 Serum Preparation and Measurement

Serum samples from the Canadian study were removed from storage in the -80°C freezer at BC Children's Hospital, packaged in dry ice and sent to Otago University in New Zealand to measure vitamin B12 concentrations together with the New Zealand study serum samples ⁸. The serum samples were run through on an Elecsys® 2010 (Roche Diagnostics, Switzerland) automated electrochemiluminescence immunoassay to measure serum B12 content (detection range 22 -1495pmol/l) ²⁹. Validation control samples were in the recommended range provided by manufacturer and the inter-assay CV based on a pooled serum was 8.3% (n=13).

2.5.2 Breast Milk Sample Measurement

Breast milk samples from the New Zealand study were sent to Canada on dry ice. Prior to analysis, individual milk samples were thawed and shaken to assure a homogenous mixture (including fat). Thawed samples were divided into 4ml aliquots and placed back in the -80°C freezer at BC Children's Hospital. The method used for measure breast milk vitamin B12 content was an amplified chemiluminescence assay validated by Hampel et al ³³. Milk samples were analyzed at the Western Human Nutrition Research Centre in the lab of Dr. Lindsay Allen at the University of California, Davis. Validation data for this method exhibited an inter-assay variation and intra assay precision of 5.6%-7.6% and 4.6%-7.4% respectively, using 400 samples run over a period of three months. Overall recovery rate was 88.4%_with a CV of 3.14%. The IMMULITE machine used for this method is a tabletop amplified chemiluminescence machine; its intensive washing process and quick boiling time have shown to be effective in accurately measuring vitamin B12 concentrations in breast milk. Prior to being placed in the IMMULITE

machine milk samples were boiled in dithiothreitol (DTT) and potassium cyanide to release the vitamin B12 from its binding protein and inactivate antibodies of IF that may be present. After the samples were cooled in an ice bath, they were inserted into the IMMULITE 1000 Immunoassay machine for analysis. The IMMULITE machine uses a polystyrene bead that contains phosphatase linked IF binding sites. Only labeled vitamin B12 analogs bound to the bead will produce a reaction to allow a luminescent reading to occur. Therefore, the concentration in the sample is inversely related to the value measured by the machine. The raw data produced by the machine is in counts, which were then converted to pmol/l.

2.5.3 Sample Size

A total of 186 women were recruited in this study: 50 from the New Zealand study consisting of a maternal serum and breast milk; 136 from the Canadian study consisting of maternal serum, infant serum and breast milk. The 136 samples chosen from the Canadian study were selected because they contained all serum and breast milk samples we required. No calculation was performed to determine appropriate sample size due to the exploratory nature of the study.

2.5.4 Study Ethics and Re-consent

The initial vitamin D study was approved by the Children's and Women's Research Ethics Board. The ethics board also approved the secondary analysis of vitamin B12 in the breast milk and blood. Participants had to re-consent to be included in the study.

Participants were sent letters containing information on the study and a new consent form (**Appendix 1**). If participants agreed to participate, they were required to sign the consent form and return it by mail. If participant preferred they could also provide consent by email to Dr. Tim

Green (**Appendix A.1**). The University of Otago Ethic's Board approved the New Zealand study.

2.5.5 Data Analysis

Statistical analyses were performed using SPSS Statistics 20.00 for Macintosh (SPSS Inc., Chicago, IL 2012). Frequencies and/or means \pm SD were determined for baseline demographics such as age, ethnicity, income and education in both populations. The normality of the data was tested visually using a histogram/QQ plots and statistically using a Shapiro -Wilks test. A $p < 0.05$ on the Shapiro-Wilks test provided evidence that the serum and milk samples of our participants were not normally distributed. Following this observation data was assessed using the natural log of the continuous variables. All statistical tests were performed using the natural log transformed data. Differences between these characteristics were determined using an independent t test. Entry characteristic were placed in a linear regression model to determine if they had any significant effect when infant serum vitamin B12 or breast milk vitamin B12 was the dependent variable. Entry characteristics were only included in the final analysis if they were considered statistically significant, defined by a $p < 0.05$. The natural log transformed mean B12 concentrations in maternal serum and breast milk were compared between the unsupplemented and supplemented population using an independent t test. Results were considered significantly different if $p < 0.05$.

Maternal serum vitamin B12 was separated into three even quartiles based on the sample group maternal serum vitamin B12 concentrations. The concentration ranges ($< 310 \text{ pmol/l}$, $310\text{--}624 \text{ pmol/l}$ and $> 624 \text{ pmol/l}$) were generated to observe if any difference was present between the strength of associations of maternal serum, infant serum and breast milk at these different

concentrations. A Pearson's correlation was used to calculate the association between maternal and infant serum in the Canadian samples.

Chapter 3: Results

3.1 Sample Collection and Utilization

From the 227 participants recruited in the Canadian study, 172 women attended the third clinic visit, which was scheduled at eight weeks postpartum. From those 172 participants, we were able to obtain re-consent from 136 women. From the New Zealand study, we received approval to use all 50 participants samples collected. **Figure 3.1** shows the samples utilization of the 186 collected from the Canadian and New Zealand studies. In brief, all 186 maternal serum samples from both the Canadian (n=136) and New Zealand (n=50) study were used in our analysis. Infant serum samples were not available from the New Zealand infants and from only 112 of 136 Canadian infants. Of the 136 women who participated in the Canadian study, ten breast milk samples were not collected and six were lost to method validation, leaving 120 breast milk samples for our analysis (**Figure 3.1**). Breast milk samples were obtained from all 50 New Zealand women.

3.2 Comparison of Baseline Demographic Characteristics Between Study Participant Groups

No significant differences ($p > 0.05$) were found in the participant characteristics between the New Zealand and Canadian women, other than average household income being significantly ($p = 0.04$) higher in the Canadian population (**Table 3.1**). Canadian women were on average 5.2 years older than the New Zealand women, but this did not reach significance ($p = 0.08$) (**Table 3.1**). Participants were well educated with 79.7% and 88.6% of the Canadian and New Zealand women respectively, reported having completed secondary education ($p = 0.96$) (**Table 3.1**).

The majority of women in both studies were European (78%) followed by Asian, Indian, Aboriginal or other ethnic backgrounds (**Table 3.2**). As there were few non-Europeans, ethnicities we separated only into two categories: European and non-European in the remaining analyses.

Figure 3.1: Flow diagram of study sample use (n=186).

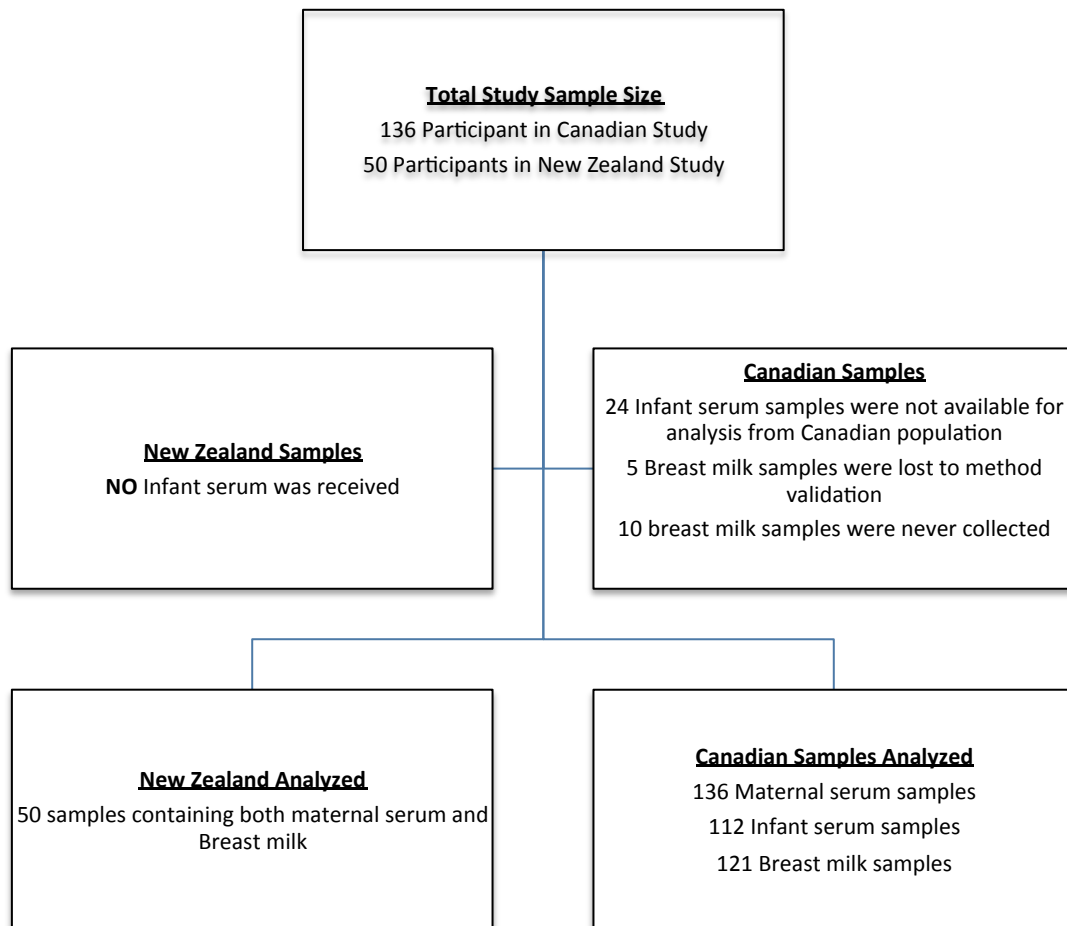


Table 3.1: Characteristics of participants in the Canadian and New Zealand studies.
All characteristics are shown as n (%) unless otherwise stated.

Characteristics	Canada n (%)	New Zealand n (%)
Age (mean \pm SD)	37.2 \pm 4.095	32.0 \pm 5.038
Ethnicity		
European	106 (78.5)	39 (78.5)
Aboriginal/Maori	1 (.7)	1 (2)
Chinese	11 (7.4)	0
East Indian	3 (2.2)	0
Other	15 (11.2)	10 (19.5)
Household Income (USD)		
<\$20,000	5 (3.8)	1 (2)
\$20,000-\$50,000	19 (13.7)	14 (27.5)
\$50,000-\$100,000	45 (32.8)	19 (39.2)
>\$100,000	67 (49.7)	16 (31.3)
Education		
High School	5 (3.8)	4 (7.5)
College	23 (16.5)	2 (3.8)
Undergraduate	51 (37.6)	26 (52.2)
Graduate	57 (42.1)	18 (36.5)
Alcohol during pregnancy*		
Yes	32 (23.3)	-
No	104 (76.7)	-

*Alcohol use was not recorded

3.3 Evaluation of Maternal Serum and Breast Milk B12 Concentrations

An objective of this research was to examine if there was a statistically significant difference in maternal serum vitamin B12 and breast milk vitamin B12 concentrations at eight weeks postpartum between the supplemented and unsupplemented groups. The supplemented group comprised of all participants from the Canadian study plus one participant from the New Zealand study who had taken B12 supplementation containing 6µg of B12 once a day during pregnancy and lactation. However, data from the transferred participant had no effect on the results. The overall Geometric mean (95% CI) maternal serum vitamin B12 concentration was 572 (538,602) pmol/l (**Table 3.7**). Geometric mean maternal vitamin B12 concentration was higher in the supplemented than the unsupplemented group, 665, (601,685) pmol/l, 403, (365,445) pmol/l respectively (**Table 3.3**), with a difference of 262 pmol/l between the two means. Distributions of the maternal serum vitamin B12 concentrations in each of the groups are illustrated in a side-by-side histogram (**Figure 3.3**).

Based on the WHO serum vitamin B12 cutoffs there was little evidence of vitamin B12 deficiency or insufficiency in either the supplemented and unsupplemented group. Overall, only two mothers were classified as vitamin B12 deficient (<150pmol/l) and six as marginally vitamin B12 insufficient (150pmol/L-225pmol/L)(**Table 3.2**).

The breast milk B12 mean in the supplemented group (270, 245-298 pmol/l) was significantly higher ($p<0.0001$) than that of the unsupplemented group (74, 55-99 pmol/l) (**Table 3.3**). **Figure 3.2** illustrates difference between breast milk concentrations in groups as side-by-side box plots. The Geometric mean concentration of breast milk vitamin B12 (181, 161-217 pmol/l) was significantly lower than maternal serum vitamin B12 (572, 538-602 pmol/l) (**Table 3.4**).

Table 3.2: Frequency of participants in both the Canadian and New Zealand study in each of the maternal serum vitamin B12 concentrations groups designated by WHO in both

Samples	< 150pmol/l	150- 220 pmol/l	>220pmol/l
Vancouver n (%)	1 (0.7)	3 (2)	132 (97.3)
New Zealand n (%)	1 (2)	3 (6)	46 (92)

Table 3.3: Geometric Mean (CI) maternal serum vitamin B12 and breast milk B12 concentrations of Supplemented and Unsupplemented population. * Significance displayed represents an independent two-sample T test comparing means between groups using log transformed data

Concentration of B12	Supplmented (n=136)	Unsupplemented (n= 50)	Sig*
Maternal Serum (pmol/l) Median B12 (IQ)	665 (601-685)	403 (365-445)	p<0.0001
Breast milk (pmol/l) Median B12 (IQ)	270 (245-298)	73.6 (55-99)	p<0.0001

Table 3.4: Geometric means of maternal serum vitamin B12 and breast milk B12 of study participants as a whole

	Maternal Serum vitamin B12 Mean (CI)	Breast milk B12 Mean (CI)
Total Sample Group (n=186) (Supplemented and Unsupplemented together)	572 pmol/l (538-602)	181 pmol/l (161- 227)

Figure 3.2: Side by side box plot representation of breast milk B12 concentration (X axis) between the supplemented and unsupplemented population

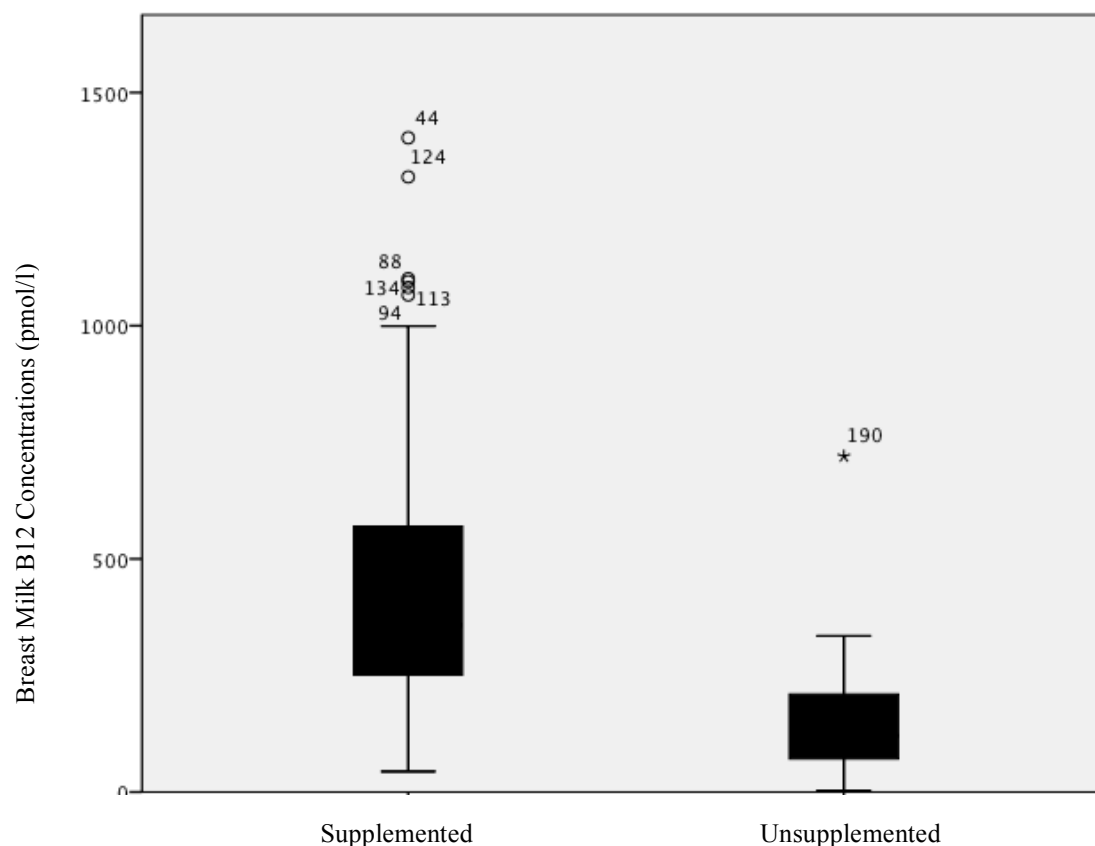
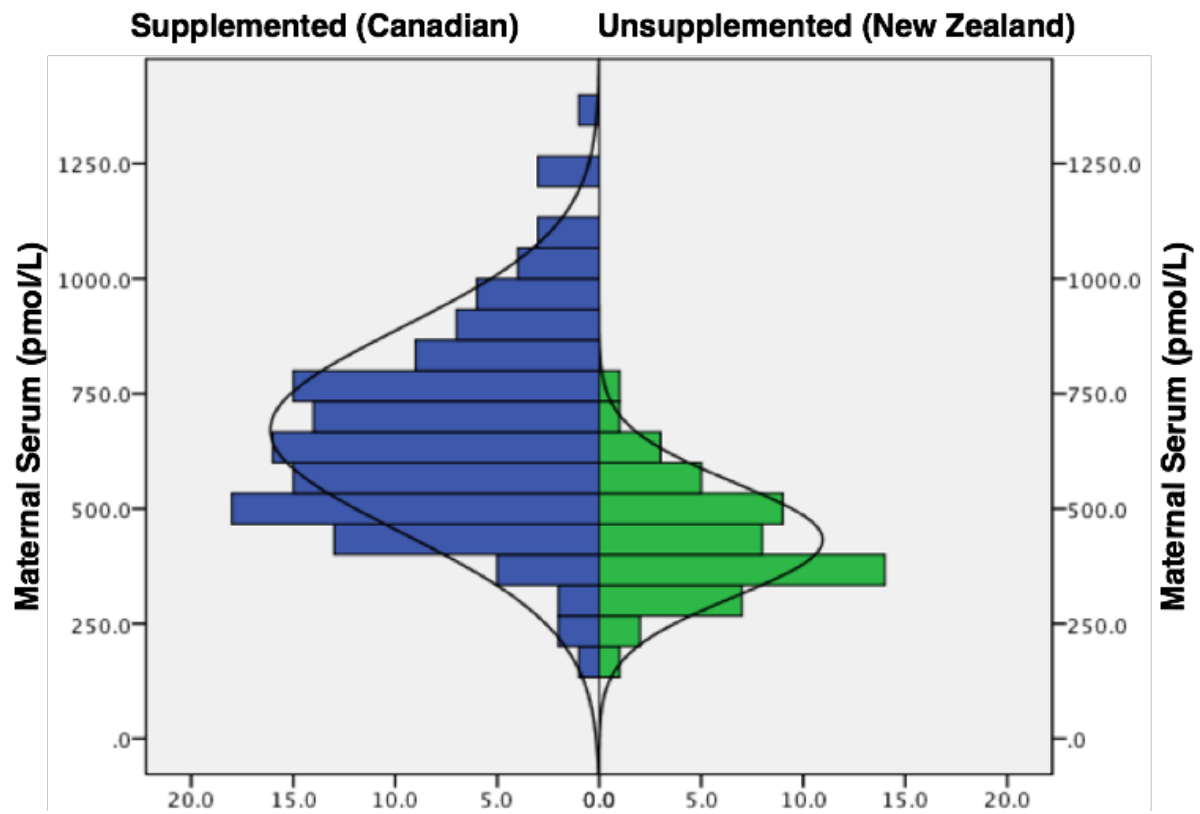


Figure 3.3: Histograms displaying the differences between maternal serum vitamin B12 concentrations in the supplemented and unsupplemented group. Maternal serum is measured in pmol/l on the y-axis.



3.4 Associations between Maternal Serum and Breast milk

The relationship between maternal serum, and breast milk was determined in both the vitamin B12 supplemented (n= 137) and unsupplemented (n= 49) groups separately and together, to explore the difference between B12 concentrations in a supplemented and unsupplemented groups.

When the supplemented and unsupplemented groups were evaluated as one group (n=186), a Pearson's correlation of $r=0.665$, $p<0.0001$ was found between the natural logarithmically transformed maternal serum and breast milk B12 concentrations.

A Pearson's correlation of $r=0.577$, $p<0.0001$ was found between maternal serum vitamin B12 and breast milk B12 concentrations in the supplemented group (n=120) compared to $r=0.414$, $p=0.01$ in the non-supplemented (n=49) group (**Table 3.5**).

Table 3.5: Pearson's correlation between maternal serum vitamin B12, infant serum vitamin B12 and breast milk B12 in the Canadian and New Zealand populations.

	Maternal Serum vitamin B12	Infant Serum vitamin B12 (n=112)
Maternal Serum vitamin B12		
Canadian (n=136)		0.299 (p=0.001)
Breast Milk B12		
Canadian (n=121)	0.577 (p<0.0001)	0.337 (p<0.0001)
New Zealand (n=50)	0.414 (p=0.01)	
Together (n=141)	0.667 (p<0.0001)	

Table 3.6: Pearson's correlations between maternal serum vitamin B12, breast milk B12 and infant serum vitamin B12 by varying concentrations of maternal serum vitamin B12

	Breast Milk B12	Infant Serum vitamin B12
Maternal Serum vitamin B12		
<310 pmol/l (n=33)	0.370 (p=0.04)	NS (inverse)*
310-624 pmol/l (n=118)	0.363 (p<0.0001)	0.269 (p=0.021)*
>624 pg/ml (n=35)	0.379 (p=0.033)	NS (p>0.05)*

* Only evaluated in Canadian population

3.5 Association between Maternal and Infant Serum in the Canadian Study

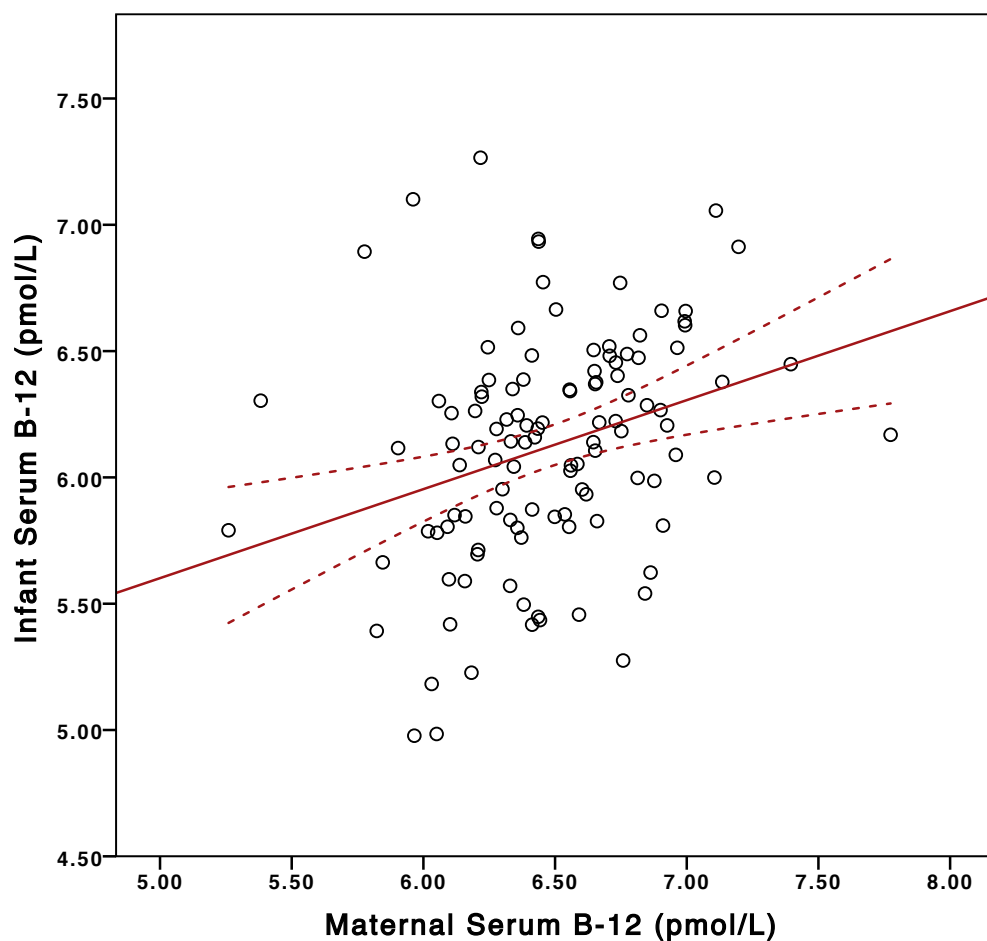
The second objective of this study was to examine the relationship between maternal and infant serum concentrations at eight weeks postpartum. A univariate general linear regression model with infant serum as the dependent variable, was adjusted for all possible explanatory variables collected at baseline; none of the characteristics showed any significance using an alpha of 0.05: age (p=0.779), ethnicity (p=0.723), income (p=0.616), education (p=0.368), smoking (p=0.483) and alcohol (p=0.062) (**Table 3.7**). All values used in these linear models were natural log transformed.

Using the natural logarithmically transformed data, the relationship between infant serum vitamin B12 and breast milk B12 (n=112) had a correlation $r=0.337$, $p<0.001$ and maternal and infant serum vitamin B12 was $r=0.299$, $p<0.001$ (**Table 3.5**).

Table 3.7: Significance values of independent variable that may affect infant serum vitamin B12

	Maternal B12	Smoke	Formula	Alcohol	Education	Income	Age	Ethnicity
Infant Serum vitamin B12 (p)	0.001	0.483	0.833	0.062	0.368	0.616	0.779	0.723

Figure 3.4: Scatter blot comparing maternal serum vitamin B12 with infant serum vitamin B12. Values displayed on x and y-axes are in pmol/l



3.6 Maternal Serum Vitamin B12 Concentration vs. Associations with Breast Milk and Infant Serum vitamin B12 (Exploratory analysis for future studies)

Following this original analysis, an exploratory analysis was performed. In this analysis the maternal serum samples from both the supplemented and unsupplemented groups were used. All samples within both groups were categorized into three even groups concentrations $<310\text{pmol/l}$ (400pg/ml), $310 - 624\text{pmol/l}$ ($400 - 800\text{pg/ml}$) and $>624\text{pmol/l}$ (800pg/ml) to determine if any difference in associations would be present at different concentrations categories. For maternal serum concentrations below 310pmol/l or $>624\text{pmol/l}$, positive correlations were found between maternal serum vitamin B12 and breast milk B12 concentrations ($r=0.370$, $p=0.04$ for B12 $<310\text{pmol/l}$ and $r=0.363$, $p<0.0001$ for $>624\text{pmol/l}$) (**Table 3.6**). For maternal serum vitamin B12 concentrations between 310 and 624pmol/l , a significant correlation was found between maternal serum vitamin B12 and breast milk B12 concentrations ($r=0.363$, $p<0.0001$) (**Table 3.6**).

Interestingly, a significant correlation between maternal and infant serum vitamin B12 was only found at maternal serum vitamin B12 concentrations between 310 and 624pmol/l ($r=0.269$, $p=0.021$) (**Table 3.6**).

Chapter 4: Discussion

An RDA for vitamin B12 for the infant during the first year of life has not been established by Health Canada, due to limited high quality research to date ⁵¹. In its place, an AI is used, which recommends consumption of 0.4mcg/day and 0.6mcg/day for 0-6 months and 6-12 months respectively; these values are based off the average concentrations of vitamin B12 found in the breast milk. Breast milk vitamin B12 is used as the reference value, as exclusively breast-feeding the infant is recommended for the first six month of life. This poses a challenge for determining whether these amounts of breast milk vitamin B12 are in fact adequate ⁴. In addition, the most significant determinant of infant vitamin B12 concentrations during this period remains unknown ⁶. Some have argued that breast milk vitamin B12 concentrations determine infant serum vitamin B12 status ⁶, while others credit the vitamin B12 stores the infant accumulates in uterine as a superior indicator of infant status ⁵. In an attempt to address these questions, an evaluation was performed among Guatemalan women and their exclusively breast fed twelve-month-old infants. Authors observed an association of $r=0.31$ ($p<0.001$) between vitamin B12 concentrations in maternal and infant serum; however no association was observed between breast milk vitamin B12 concentrations and infant serum vitamin B12 ³. These findings may be due the high prevalence of poor maternal vitamin B12 status in this region; transfer of vitamin B12 to the breast milk during lactation may be limited in women with poor status since maternal vitamin B12 stores can be depleted by the infant during pregnancy through increased placental transfer ³. This is a common observation within current research evaluating vitamin B12 concentration in mothers during pregnancy and lactation; as the bulk of research is performed among populations of vitamin B12 deplete women in developing nations. As access

to vitamin B12 rich foods in these regions is scarce, supplementation may be a practical intervention to ensure these mothers attain adequate vitamin B12 status.

At the outset of this research, no trials had attempted to evaluate the efficacy of vitamin B12 supplementation during pregnancy and lactation. In June 2014, a RCT conducted in India was published that showed effects of 50mcg supplement of vitamin B12 on mothers serum, breast milk and their infants serum throughout pregnancy and lactation. Results indicated that supplemented women and their infants displayed significantly higher concentration of vitamin B12 at six weeks postpartum³⁶. This type of study provides critical evidence on the efficacy of vitamin B12 supplementation on improving infant status through an increase in breast milk vitamin B12 concentrations. In order for interventions to be put in place, the gaps in the literature discussed above on understanding the associations between vitamin B12 concentrations in maternal serum, breast milk and infant serum need to be addressed. Performing research on this topic may help address these gaps, which may aid in the development of larger studies, which in turn will assist in the provision of funding towards vitamin B12 supplementation in regions where it is needed most.

To our knowledge, our study is the first to examine the associations between maternal serum vitamin B12, breast milk vitamin B12 and infant serum vitamin B12 concentration at eight weeks postpartum between a supplemented and unsupplemented populations residing in countries with vitamin B12 rich diets. This analysis will therefore help us to determine how vitamin B12 supplementation will affect breast milk vitamin B12 concentrations in populations in which maternal stores are sufficient or supplementation is provided.

4.1 Efficacy of B12 Supplementation During Pregnancy and Lactation

4.1.1 Maternal Serum vitamin B12 Outcomes at Eight Weeks Postpartum

Assessment of maternal serum vitamin B12 concentrations in our study found significantly higher concentrations of vitamin B12 in the supplemented (12mcg/day of B12) than the unsupplemented group. These findings are similar to those found in previous cross sectional evaluations assessing maternal vitamin B12 status during pregnancy and lactation ^{10,59}. A recently published RCT that compared maternal serum vitamin B12 concentration at six weeks postpartum between vitamin B12 supplemented (50mcg/day) women and women consuming a placebo (control) in rural India obtained similar results. The women in the vitamin B12 supplemented group displayed significantly higher ($p<0.05$) maternal vitamin B12 concentrations (216pmol/l) than the placebo group (111pmol/l) ³⁶. The vitamin B12 supplementation dosage in this study was over four times that of our research due to the persistent poor status in their population. However, both the 12mcg and 50mcg dosage consumed in our study and Duggan et al study respectively, showed to be proficient in improving maternal concentrations of vitamin B12 in these women, which was expected since they were both well above the established RDA of 2.8mcg/day ⁶⁰. Yet, in contrast to most research assessing this relationship, our study was performed on women from developed countries where vitamin B12 rich foods are commonly consumed and readily available. Thus, less than 5 % of the women in our total sample were below the WHO cut off for adequate vitamin B12 status (<220pmol/l). The adequate maternal vitamin B12 status of women in our study, allowed us observe higher concentration of vitamin B12 in the breast milk, as maternal vitamin B12 is thought to be used for the mother needs prior to be expressed in the milk in vitamin B12 deplete

women. The elevated concentration of vitamin B12 in our breast milk samples made it easier to observe changes that occurred in our breast milk vitamin B12 concentration analysis.

4.1.2 Breast Milk Vitamin B12 Outcomes Eight weeks Postpartum

A significant difference was found between the vitamin B12 breast milk concentrations in supplemented and unsupplemented groups ($p < 0.0001$) (**Table 3.5**). **Vitamin B12** supplementation was being regularly consumed from 22 weeks gestation to study completion (eight weeks postpartum). Comparative research for these results are limited, as only a few studies have been published assessing the efficacy of supplementation on breast milk vitamin B12 concentrations³⁶. In the Indian RCT described above, a significant difference was also documented between the vitamin B12 supplemented and placebo group at six weeks postpartum, before vitamin B12 supplementation was halted. Yet, once supplementation was stopped after six weeks postpartum, the difference between breast milk vitamin B12 concentrations of the two groups was no longer significant between the two groups. Breast milk vitamin B12 concentrations at eight weeks in our unsupplemented group were lower than those in the deficient placebo population at six weeks in the Duggan et al., study; 74pmol/l vs. 87pmol/l respectively. This is alarming as women in our population were in good vitamin B12 status and had access to vitamin B12 foods. The lower values found in our study may implicate a reassessment of the current AI present using current laboratory measurements for breast milk in which are not at as high of risk of over estimating. However, in our study, we were not able to obtain infant serum in our unsupplemented population, therefore we cannot determine how it was reflected in infant status. In order to better understand breast milk vitamin B12 concentrations, its relationship with maternal serum vitamin B12 has to be tested.

In our study, breast milk vitamin B12 concentrations between women in the supplemented and unsupplemented groups were significantly correlated with maternal serum vitamin B12 concentrations. These findings were in contrast to the earlier documented research assessing correlations between maternal serum and breast milk vitamin B12, which observed no significant association between the two ⁵⁹. However previous methods used for the measurement of breast milk vitamin B12 have been recently identified as unreliable as compared to current methods used such as the IMMULITE chemiluminescence machine used in our study. When similar laboratory measurement techniques were used in a recent cross sectional evaluation of lactating Guatemalan women, maternal serum vitamin B12 concentration seemed to be the primary indicator of breast milk vitamin B12 concentration as they observed significantly higher ($p < 0.05$) breast milk vitamin B12 concentrations in women whose serum vitamin B12 concentrations were above 220pmol/l compared to those below 150pmol/l ³. It is generally accepted that maternal serum vitamin B12 impacts vitamin B12 concentrations in the breast milk and infant serum, however, at what concentrations are these associations are strongest is still unknown ⁶.

4.2 Transfer of B12 From the Mother to the Infant

The main determinant of vitamin B12 concentrations of breast milk is still under debate. The most recent hypothesis attributes maternal dietary vitamin B12 as the main determinant of breast milk vitamin B12 concentrations²⁵ since we do not have dietary data in our study we cannot support or refute this idea. However, the Food and Agriculture Organization states that per capita, New Zealand habitants consume more meat on average than Canadians, suggesting that at least in theory the New Zealand group had adequate access to dietary vitamin B12 ⁶¹.

Therefore, the significantly larger breast milk and maternal serum vitamin B12 concentration we determined in our supplemented group may provide support that vitamin B12 supplementation consumption plays an important role in vitamin B12 concentrations and the transfer of vitamin B12 to the breast milk. Another factor more important than how vitamin B12 is consumed may be the time of consumption, since serum vitamin B12 seems to be well correlated with breast milk concentrations.

In order to determine how concentration of maternal serum vitamin B12 affect the transfer of vitamin B12 to the infant and breast milk, maternal serum concentrations were divided into three groups (<310pmol/l, 310-624pmol/l and >624pmol/l) within our study. A correlation between breast milk and infant serum vitamin B12 was only observed in the middle concentration group ranging from (310 -634pmol/l).

A study in 1997 assessing vitamin B12 deficient lactating Guatemalan women observed no inverse association between vitamin B12 concentrations in breast milk and urinary methylmalonic acid (a functional indicator of vitamin B12) in infant serum ⁵. Over ten years later, another analysis in a similar population also observed no significant relationship between vitamin B12 concentrations in infant serum and breast milk ³. In both of these studies maternal serum vitamin B12 concentrations during pregnancy and lactation were below the 310pmol/l ^{3,5}. Conversely, a Danish study that evaluated the association between infant serum and breast milk vitamin B12 concentrations in women with an average maternal serum vitamin B12 concentration of 400pmol/l found a significant correlation ($p=0.005$, $r=0.58$) between the two. These findings indicate that there may be a stepwise relationship between maternal serum vitamin B12 concentrations and those of the infant status.

The lack of association outside of the middle maternal serum vitamin B12 concentration group in our study may be due – in part - to two possible explanations. Firstly, when maternal serum concentrations are low during pregnancy all available vitamin B12 is transferred to the infant through placental transfer, or utilized by the mother during lactation and therefore, vitamin B12 is not available for transfer to breast milk³⁸. Secondly in cases where there are extremely high concentrations of maternal serum vitamin B12, there may be a threshold in how much vitamin B12 can be transferred to breast milk. This phenomenon is only an observation as our sample size could be improved and we are the first of the limited studies completed on this topic to observe this trend. These results may indicate that maternal serum vitamin B12 concentration is the greatest indicator of infant vitamin B12 status given its effects on both breast milk and in uterine concentrations.

4.3 Limitations

The cross sectional study design of our study does not allow us to state causation from our results. Due to the nature of secondary analyses, we were only able to evaluate vitamin B12 concentration from the blood and breast milk samples obtained from these previous conducted studies. Ideally it would be preferable to have larger and more equal distributed population sizes than the 136 and 50 used from the Canadian and New Zealand populations, respectively. In addition, our samples were gleaned from populations residing in different countries (Canada and New Zealand), which may weaken the strength of results comparing our supplemented and unsupplemented populations. Some may argue that different environments and lifestyles in these countries may affect uniformity of participants involved.

Another limitation of secondary analyses is the lack of control over the collection of entry characteristics. Those collected for the samples used were not all relevant for vitamin B12 analysis. Following an evaluation of the participant characteristics of samples obtained, we noted our population to be primarily of European descent and well educated. These findings prevented us from observing any variations that may have been present across ethnics groups and/or educational status. It is also possible that higher education confers a better understanding of the importance of supplementation and proper nutrition practices, thus lowering the populations' risk of vitamin B12 deficiency.

4.4 Future Directions

Very few studies have evaluated the efficacy of vitamin B12 supplementation on improving mothers and their infant's vitamin B12 status during pregnancy and the subsequent lactation period. Our study will therefore contribute to the growing body of knowledge on vitamin B12 status in these cohorts, which will hopefully stimulate future RCTs that will enable future studies to determine causation. It is recommended that moving forward, RCTs collect all necessary baselines demographic characteristics such as the child's birth weight and dietary consumption, as both seen to be associated with vitamin B12 status, in order to address all possible confounding factors that may affect the relationship between maternal serum, infant serum and breast milk. As mentioned above, during our study a paper was published describing a trial, which evaluated the effect of supplementation during pregnancy and lactation ³⁶. Promising improvements in the vitamin B12 concentrations of the supplemented women and their infants in this study were seen.

In order to build on this current study, future studies should span the period prior to pregnancy to the end of lactation. Attaining results during all of these periods will help determine which periods most influence vitamin B12 concentrations in the breast milk. Understanding this relationship will allow for more precise recommendations on vitamin B12 supplementation during pregnancy to prevent deficiencies at birth; which otherwise could go unnoticed due the developmental delay of vitamin B12 deficient symptoms during infancy.

As stated above, the exact effects of supplementation on vitamin B12 concentrations during pregnancy and lactation are not fully understood due to the limited knowledge on maternal-infant vitamin B12 transfer and ambiguous cut offs used for this specific vitamin. Therefore, it is important that future studies focus on the mechanisms, which elucidate the transfer of vitamin B12 from mother to infant. When these mechanisms are understood the true effect of vitamin B12 supplementation will be easier to determine.

Chapter 5: Conclusion

The objective of this study was to evaluate the relationship of vitamin B12 concentrations in breast milk, infant serum (Canadian only) and maternal serum and their concentration compared between supplemented and unsupplemented populations. Very little research has been published on this topic to date; therefore, a goal of this study was to inform and encourage an increase of experimental research in this field.

A recent RCT in India showed that vitamin B12 supplementation improved maternal serum B12 concentrations which in turn improved breast milk and infant status. However, this study took place in India where deficiency is common. Results of our study between maternal serum and breast milk vitamin B12 showed that even in predominantly vitamin B12 replete population associations are still present.

In our exploratory research a significant association between maternal serum and breast milk B12 was found in the study populations at all concentrations, this allowed us to reject our null hypothesis. The sample size used in this study was modest, and infant serum was only obtained from the supplemented population, therefore we cannot examine the effect of maternal B12 supplementation on B12 transfer to infants. Yet, the lack of association between breast milk and infant serum at high and low concentrations has led us to speculate that infant serum at the eight week postpartum period may be dependent on maternal serum vitamin B12 during pregnancy in addition to breast milk B12 concentration during lactation. To note, results may have differed if infant B12 concentrations were assessed at a later period, such as six month postpartum, when infant B12 stores are normally depleted. To our knowledge no research has yet to be conducted to evaluate this possibility.

Maternal B12 supplementation is important throughout pregnancy and lactation. Our results showed significantly higher concentrations in the breast milk of women who consumed 12mcg/day of vitamin B12 supplementation throughout this period. It is important to note both our populations in the study were in good vitamin B12 status, which potentially provides support to the efficacy of supplementations efficacy. Unfortunately, this study could not add any insights that could be used to advise on the precision of current cut offs used for this vitamin, although we can concede the importance of good maternal B12 status throughout pregnancy and lactation will have a direct effect on breast milk B12 content.

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Appendices

Appendix A

A.1 Introduction Letter



March 6th, 2014

Dear «Name»,

Thank you for your participation in the vitamin **D in Pregnancy and Lactation Study**. We attached an abstract published on this work in case you are interested.

The reason we are contacting you is that we would like to measure vitamin B12 and folate (B vitamins) in the frozen blood and breast milk samples that you provided during the vitamin D study. However, when you originally consented to participate we did not indicate that we would measure folate and B12. As such, we are requesting your permission (re-consent) to measure these other vitamins.

Why do we want to do this? A lack of vitamin B12 during early life can lead to anemia (blood problems) and serious neurological complications. Fortunately this is not very common in Canada. However, in some emerging countries such as India this is a serious problem. One potential way to improve vitamin B12 may be to give the mother a vitamin B12 supplement to increase the amount of vitamin B12 in her breast milk.

To determine if this might work we hope to use blood and breast milk samples collected from the study you participated in and a similar study in New Zealand. In your study most women took a supplement that contained vitamin B12, whereas, in New Zealand most women did not. We hope to show that the women who took a supplement have higher B12 levels in their blood and breast milk and their babies have higher blood B12 at 8 weeks after birth

What do I need to do for this study? Read this letter and decide if you want to participate. Please indicate your consent and sign the form attached and return it in the stamped addressed envelope.

If you have any questions about this study before or during participation, or if you experience any adverse effects, you can contact Dr. Tim Green

Do I have to participate? No, your participation is entirely voluntary, so it is up to you to decide whether or not you allow us to use your stored blood and breast milk sample. If you choose not to allow us to use your samples, or do not answer our request for re-consent we will not use them

Thank you for considering this request. Please contact me if you have any questions

Sincerely,

A handwritten signature in black ink, appearing to read 'Tim J. Green', with a stylized, flowing script.

Dr. Tim J. Green
Food, Nutrition and Health

A.2 Letter of Consent



The Relationship Between Maternal, Infant and Breast Milk Vitamin B12 Concentrations Assessed at 8 Weeks Postpartum

Consent Form

Principal Investigator	Tim Green, PhD Food, Nutrition, and Health University of British Columbia
Co-investigators	Philip Chebaya, BSc Yvonne Lamers, PhD David Kitts, PhD Food, Nutrition, and Health University of British Columbia Dr. Sheila Innis, PhD Department of Paediatrics University of British Columbia Dr. Peter von Dadelszen, PhD, MD Department of Obstetrics and Gynaecology University of British Columbia
Sponsor	Canadian Institute of Health Research
Site	University of British Columbia
Contact Numbers	If you have any concerns or complaints about your rights as a research participant and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the University of British Columbia Office of Research Services by email or by phone at

What is the overall design of the study? It is a secondary analysis meaning we will be using blood and breast milk samples in which we previously collected from you for the vitamin D study. These samples will be tested for their vitamin B12 content. They will then be compared with another group of previously collected samples from New Zealand in which did not receive prenatal supplementation. The two groups will be compared to determine the efficacy of B12 supplementation on infant and maternal blood as well as breast milk B12 concentrations. Results from this study will help address the prevalence in B12 deficiency in emerging countries.

Will my information remain 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 at the University of British Columbia Women's and Children's Research Ethics Board 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 have been assigned a unique study number as a subject in this study. Only this number is 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 is 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.

Who is conducting the study? Researchers in the Food, Nutrition, and Health at the University of British Columbia and Department of Obstetrics & Gynaecology are conducting this study.

What will the study cost me? Since all samples have already been collected, there is no cost to you and no reimbursement will be provided.

What are the risks and benefits to me of participating? There are no risks as samples are already collected. There are no direct benefits to you; however, the information we obtain may provide information on how to improve vitamin B12 levels in babies, especially those in emerging countries.

Participant Consent

- I have read and understood the subject information and consent form.
- I have been able to ask questions and have had satisfactory responses to my questions.

- I have had sufficient time to consider the information provided and to ask for advice.
- I understand that I am completely free at any time to refuse to participate or withdraw from this study at any time, and this will not change the quality of care I will receive.
- I have been advised how to contact the lead investigator to ask questions.
- I understand that all of the information collected will be kept confidential and that the result will only be used for scientific objectives.
- I understand that participation in this study is voluntary and that I am completely free to refuse to participate.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I have read this form and I freely consent to participate in this study.

☐ I consent to the use of my stored blood & breast milk samples for vitamin B12 analysis.

Printed Name of Participant

Signature

Date

Printed Name of Principal Investigator

Signature

Date

A.3 Introduction Letter



Dear 'Name'

My name is Philip Chebaya and I am Master's Student at the University of British Columbia. First of all I would like to thank you for your participation in the vitamin D pregnancy study. I am contacting you to request permission to measure vitamin B12 concentrations in your blood and breast milk samples you previously provided. vitamin B12 is essential in ensuring a healthy pregnancy and infant. Since all samples have already been collected, your participation in the study would only require your signature for consent.

Attached you will find consent forms which will provide further information on the importance of vitamin B12 and your participation in this study.

Thanks again for your consideration

Sincerely

Philip Chebaya
FNH Building UBC
2205 East Mall V6T 1Z4
Vancouver BC