

Vitamin B12: A secondary analysis of status, biomarkers and other determinants in young and elderly women.

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## Abstract

**Background/Aim:** Vitamin B12 has multiple functions within the human body, including roles as a co-factor for both the one carbon metabolism cycle and methylmalonyl CoA mutase. Vitamin B12 deficiency can have serious lifelong consequences. The aim of this study is to explore the associations between markers of vitamin B12 status and other biochemical, dietary and physical measures.

**Methods:** Three data sets composed of either young omnivore women (n=65; age  $24.5 \pm 4.4$  y; mean  $\pm$  SD), randomly selected young women (n=305; age  $22.5 \pm 3.9$ ) and elderly women (n=44; age  $80.5 \pm 7.6$ ) were examined. Associations between vitamin B12 biomarkers and other selected biomarkers of nutritional status (i.e. serum folate, erythrocyte folate), lifestyle factors such as; dietary intake, alcohol intake, oral contraceptive pill (OCP) use (in the non-elderly groups), and other factors such as BMI were examined using mixed effects regression, accounting for study clusters.

**Results:** Serum vitamin B12 stratification (low, medium or high) was not associated with likelihood of being classed as having a raised methylmalonic acid (MMA) and/or homocysteine (tHcy) (p=0.316). Younger women who used the OCP had serum vitamin B12 concentrations that were 72.3 (SE=12.4) pmol/L lower than non-users (p<0.001), there was however no associations between OCP use and tHcy (p=0.669) or MMA (p=0.595) concentrations. Serum vitamin B12 concentration (pmol/L) was related positively to both serum folate (nmol/L) ( $\beta=0.018$  95% CI: 0.009, 0.026, p<0.001) and erythrocyte folate (nmol/L) ( $\beta=0.456$  95% CI: 0.164, 0.747, p<0.01). Vitamin B12 was marginally associated with the intake of protein (p<0.05) and negatively associated with alcohol intake (p<0.001).

**Conclusions:** The lack of an association between serum vitamin B12 stratifications and metabolite cut-offs suggest that you are not more likely to have raised metabolites in conjunction with any of the three serum vitamin B12 cut-offs. The lack of negative metabolite consequences in those who use the OCP suggest that the decreased serum vitamin B12 is possibly inconsequential. This, however, suggests that in populations who are frequent users of the OCP, serum vitamin B12 may not be suitable as a primary measure of vitamin B12 status. Alcohol use in Australia females is above the global average and suggests that this group are at increased risk of decreased vitamin B12 status due to alcohol intake. Serum vitamin B12 proved less than ideal throughout this study as a primary marker of vitamin B12 status. It was unable to show concordance with MMA and tHcy cut-offs and demonstrated it was unsuitable as a primary marker in OCP users. This suggests that it is time for a new primary marker of status with holo-transcobalamin II being the likely successor. Research needs to be focused on this marker of status in the future to determine the factors which affect it so we can be better suited to use it as a primary marker of status.

## Preface

The proposal for this project was developed and supervised by Professor Samir Samman (Department of Human Nutrition, University of Otago). Dr Jill Haszard (Department of Human Nutrition, University of Otago), was a co-supervisor for the project, and was involved in statistical analysis and interpretation of results. Dr Fiona O’Leary (Discipline of Nutrition & Metabolism, School of Molecular Bioscience, The University of Sydney), Dr Flavia Fayet-Moore (Discipline of Nutrition & Metabolism, School of Molecular Bioscience, The University of Sydney), and Dr Jennifer McArthur (Discipline of Nutrition & Metabolism, School of Molecular Bioscience, The University of Sydney) completed the primary studies of which the data were used for this secondary analysis. As part of the thesis, the candidate:

- Cleaned and transformed the data set to give uniform results for secondary analysis.
- Undertook secondary analysis of the combined data set using Stata V14.0.
- Interpreted the outputs of secondary analysis and developed methods of accurately displaying these results.
- Was responsible for the write up of the thesis.

Both Professor Samman and Dr Haszard were responsible for providing feedback for work completed by the candidate. Professor Samman, Dr Haszard, Dr O’Leary, Dr Fayet-Moore, and Jennifer McArthur all provided feedback on abstracts for conference submission.

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## Chapter One: Introduction

In the early 1900's pernicious anaemia was a perplexing and harmful medical diagnosis that would not see a potential treatment until 1926. Minot and Murphy announced they had cured 45 patients of their pernicious anaemia by persuading them to consume a diet containing up to half a pound of beef liver per day (1). The source of treatment in beef liver was termed vitamin B12 and the discovery of its complete structure eventuated in 1956 by a team lead by Dorothy C. Hodgkin (2). She would later receive the Nobel Prize in Chemistry for her work in determining the structures of important biochemical substances.

In the present we have three main markers of vitamin B12 which are commonly used to determine an individual's vitamin B12 status (3-5). The three main measures are serum vitamin B12, homocysteine (tHcy), and methylmalonic acid (MMA). While serum vitamin B12 is considered the primary marker of status, tHcy and MMA are considered to be appropriate for monitoring change following vitamin B12 supplementation/treatment. The three markers have also been combined in a possible method for determining a subclinical deficiency state (3).

This being said, all three markers of status have their own flaws. Serum vitamin B12 measures vitamin B12 in sera which is both; metabolically available (holo-transcobalamin II) and metabolically unavailable (holohaptocorrin) (4, 5). This opens up the possibility for holohaptocorrin to falsely infer deficiency or inversely mask true deficiency.

Homocysteine is also affected by many other factors other than vitamin B12. Folate and renal status among others affect tHcy levels more than do vitamin B12; as such levels of this marker must be further interpreted before classification of vitamin B12 deficiency (4, 5). Methylmalonic acid can be falsely increased in the elderly and those with renal

impairments (4). While these are the main causes of variation, they account for only a small proportion of variation.

The reliance of serum vitamin B12 as our primary measure of status is a potentially negative tendency given its uncertainties. New techniques have given us the ability to accurately measure holo-transcobalamin II (holo-TC II). While this method is now more available we need to further understand the factors which affect it, requiring ongoing research.

Currently there is no gold standard measure of vitamin B12 deficiency, a factor which limits our ability to recognise and diagnose deficiency. As such it is important to determine how the markers of status have been used to determine deficiency in the past and how they could potentially be used in the future.

Outside of the biomarkers of status it is important to determine which factors may be affecting vitamin B12 status in our study population. Factors of particular importance include use of an oral contraceptive pill (OCP) and alcohol intake. It is also important to decide if associations between vitamin B12 levels and other factors are authentic or spurious.

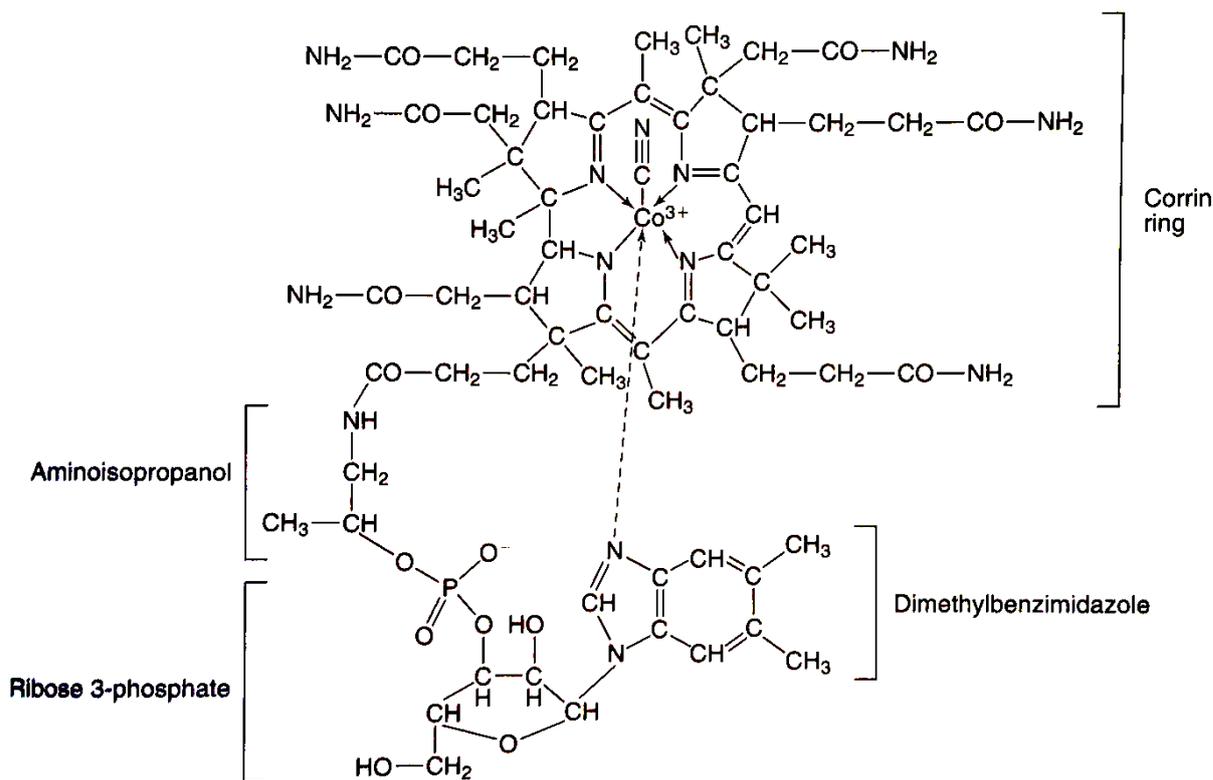
## Chapter Two: Literature Review

### 2.1 Background

#### 2.1.1 History

Early in the previous century, hospitals around the world began to experience an increase in the recognition of a perplexing form of anaemia which carried dire consequences for those affected (6). The outlook for these individuals despite the occasional slight improvement in treatment, was by no means positive (6). This anaemia had aptly been named “pernicious anaemia” because of its harmful and at the time deadly effect (6). In 1908, Richard C. Cabot (7), analysed the time of survival in 1200 such patients and found that survival following the onset of said disease was generally from one to three years. His results also showed that in only six patients of the 1200, was permanent recovery witnessed (7). It wasn't until 1926 that the medical world was stunned by a potential treatment. Minot and Murphy (1) announced that they had effectively eliminated the anaemia in a group of 45 patients by persuading them to eat for some months, a diet that contained up to half a pound of beef liver daily.

Following the announcement of Minot and Murphy, purified beef liver extracts were produced (6) with continuing improvement until in 1948 when the source of activity in pernicious anaemia treatment present in beef liver was discovered and published. The source of activity was identified as crystalline cyanocobalamin by Karl Folkers and associates (8), and then again only a few weeks later by Smith and Parker (9). The active substance was termed “Vitamin B12.” Following its isolation, exhaustive research over a decade by many investigators resulted in the discovery of the complete structure of vitamin B12. This was completed in 1956 by Dorothy C. Hodgkin's group (2) and she was later awarded the Nobel Prize in Chemistry for her efforts in 1964. The structure of vitamin B12 is outlined in Figure 1.

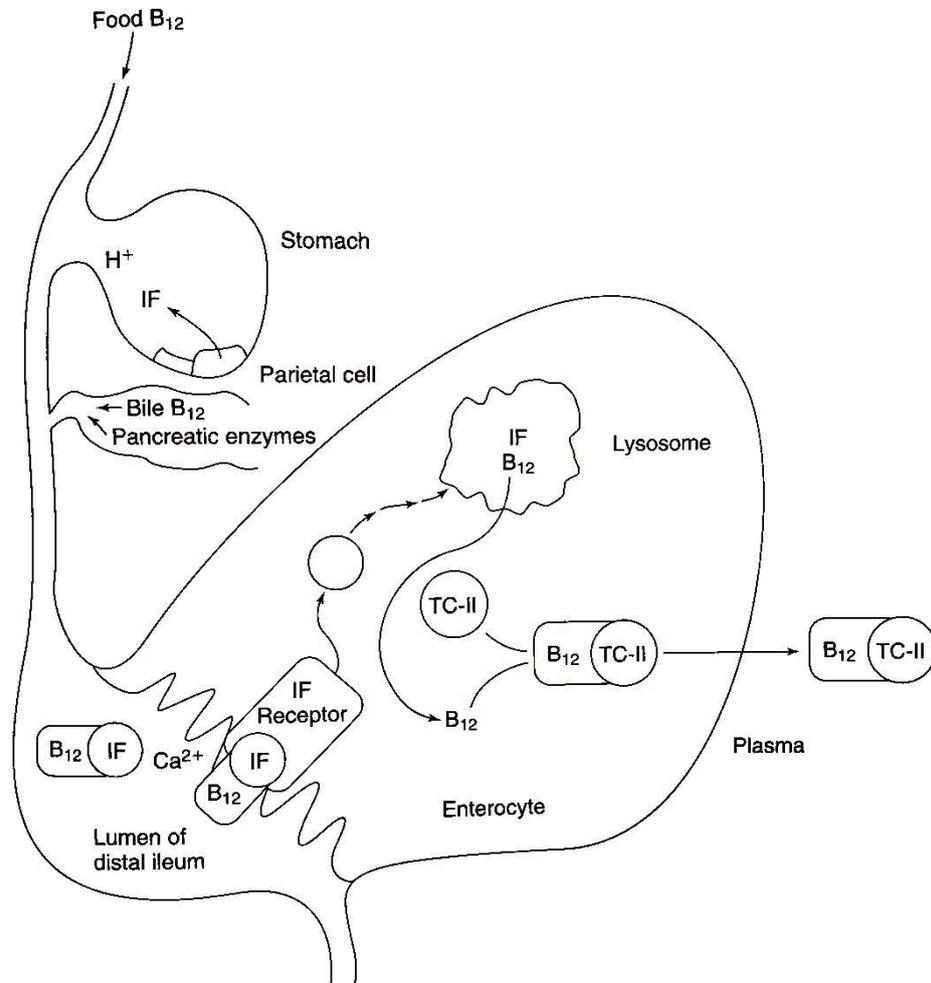


**Figure 1 Structure of Vitamin B12 (10)**

### 2.1.2 Physiology and Function

Stepping forward in time, our understanding of vitamin B12 and the way it is processed and absorbed has progressed remarkably. The mechanism by which vitamin B12 is absorbed into the body is complex and is outlined in Figure 2. Vitamin B12 under normal circumstances is introduced to the body bound to protein following the ingestion of food. In the stomach vitamin B12 is cleaved from protein by gastric pepsin (11) produced in the parietal cells. The parietal cells also produce and secrete intrinsic factor (IF), another essential factor related to vitamin B12 absorption (12). Unbound vitamin B12 within the stomach is then preferentially bound by haptocorrin (TC I) (11). Degradation of TC I occurs in the upper intestine by pancreatic proteases as it leaves the stomach (13). The rereleased vitamin B12 is now available to be bound by IF forming an IF-vitamin B12 complex (13). This complex travels through the intestine to the ilium, where it is taken up by the IF receptor, cubilin, located on gut epithelial cells (14). The IF-vitamin B12

complex is degraded in the ileal cell endosome releasing vitamin B<sub>12</sub>, which eventually reaches the abluminal surface of the ileal cell and enters the bloodstream attached to transcobalamin II (TC II) (15).



**Figure 2 Absorption of vitamin B<sub>12</sub> process (10)**

Vitamin B<sub>12</sub> has two known functions in the human body, including serving as a cofactor in the methylation of homocysteine to methionine, and as a cofactor in the rearrangement of L-methylmalonyl-coenzyme A to succinyl-coenzyme A (4).

## 2.2 Biochemical Assessment of Vitamin B12 Status

Methods of diagnosing vitamin B12 deficiency fall into one of two categories, these being: measures of vitamin B12 amounts (i.e. serum vitamin B12 or holo-TC II); or measures of functional metabolic status, such as vitamin B12 metabolite biomarkers (i.e. MMA or tHcy) (4). Currently no diagnostic gold standard for vitamin B12 status exists (4). See Table 1 for collated information on vitamin B12 status measures.

**Table 1 Biomarkers of Vitamin B12 status with cut-offs for deficiency**

<b>Biomarker</b>	<b>Biopsy Marker</b>	<b>Gold Standard Measurement Method</b>	<b>Cut-off of Deficiency</b>	<b>References</b>
<b>Serum Vitamin B12</b>	Blood Sera	Automated, Competitive-binding immune chemiluminescence	< 148 pmol/L	12, 13, 19
<b>MMA</b>	Blood Sera	Gas chromatography- mass spectrometry	> 270 nmol/L	12, 19
<b>tHcy</b>	Blood Plasma	Enzyme Immunoassay	> 10 µmol/L	12, 13, 22
<b>Holo-TC II</b>	Blood Sera	2-step sandwich microparticle enzyme immunoassay	< ~40 pmol/L	29, 30

### 2.2.1 Serum Vitamin B12

Serum vitamin B12 is the measure of total vitamin B12 in the sera of an individual. This includes both holo-haptocorrin and holo-TC II (5, 16). Measurement of serum vitamin B12 is currently the most commonly used method for evaluating status (5), there is however much disagreement about the reliability of this method and some studies have shown systematic overestimates of vitamin B12 status in individuals with established deficiency (as measured by serum vitamin B12), often with pernicious anaemia (17-19).

The earliest employed methods of serum vitamin B12 analysis relied on microorganisms. The growth of these organisms was proportional to the content of vitamin B12 in an individual's sample, allowing the determination of the content of vitamin B12 in the sample (20). The current test for serum vitamin B12 is widely available, as well as being relatively cheap (5). It uses an automated method and competitive-binding immune chemiluminescence (5).

It is suggested by some that a serum vitamin B12 level of <148 pmol/L is sensitive enough to diagnose 97% of patients with deficiency (5), and this appears to be a similar cut-off that the majority of studies have employed since cut-offs were impossibly low in the 1950's (21). Carmel reported (4) that some investigators raised the cut-off of deficiency from 148 to 258 pmol/L in order to ensure no case of deficiency be missed (4). The gross over-diagnosis of this change was substantial and MMA and tHcy results of these patients suggest that two thirds of the newly classified deficient patients had suspect results. Furthermore, very few of the remaining one third had clinical deficiency (4).

As only approximately 20-30% of the vitamin B12 in serum is in the holo-TC II form (16), the serum vitamin B12 test may mask true deficiency or falsely infer a deficient state. The high amounts of holo-haptocorrin which are largely metabolically unavailable can mask the true amount of holo-TC II, conversely a low holohaptocorrin concentration may imply deficiency when levels of the available holo-TC II are sufficient for metabolic processes (5, 16, 21). This fact renders the serum vitamin B12 test potentially unreliable.

### **2.2.2 Methylmalonic Acid**

The conversion of L-methylmalonyl-CoA to succinyl-CoA relies on the interaction of methylmalonyl-CoA mutase and its cofactor vitamin B12 (in the form of 5'-deoxyadenosylcobalamin). In the absence of vitamin B12, D-methylmalonic acid (MMA)

accumulates in the body (4, 21). It is for this fact that MMA can be used as an indicator for vitamin B12 status.

Analysis of MMA began in the late 1950's (21) when it became possible to look at the amount of MMA in pooled samples of urine. Moving forward in time, more sensitive methods of MMA analysis appeared and made it possible to measure small amounts of serum with increasing accuracy and precision (22, 23). Currently, MMA can be measured reliably by gas chromatography-mass spectrometry (21).

Reference cut-offs for MMA vary widely but the most commonly applied cut-off is ~270 nmol/L, with many laboratories defining cut-offs based on two or three standard deviations from the mean (~270 or 370 nmol/L respectively). While 270 nmol/L is the most common cut-off, there is much variation with levels ranging from 210 - 370 nmol/L (21).

Many experts currently see MMA as the best metabolic test available for confirmation of vitamin B12 deficiency, with normal MMA levels providing compelling evidence to disprove deficiency (4). There are however limitations of MMA measurements which occur in older patients (>65) and those with renal impairments. In these individuals levels can be falsely increased. These two factors are the main causes of variation but still only explain a small proportion of variation. Still, high levels of MMA can usually be explained by vitamin B12 deficiency, especially if coupled with symptoms of deficiency.

### **2.2.3 Total Homocysteine**

Increased plasma tHcy, like MMA is a marker of vitamin B12 deficiency and indicates impaired methionine synthase activity (4). Furthermore, elevated tHcy concentrations are weakly associated with an increase in cardiovascular disease (CVD) risk. However, causality remains uncertain (24, 25). Like MMA, tHcy is used for monitoring changes following vitamin B12 therapy (21) and it has comparable sensitivity to MMA (4, 21).

Questions are raised about tHcy's ability to be considered as a biomarker for vitamin B12 status however due to other factors affecting levels. Both folate status and renal status affect tHcy levels more than that of vitamin B12 (26), and there are many other factors which raise tHcy, such as vitamin B6 status and hyperthyroidism (5).

Nevertheless, cut-offs for tHcy are defined and plasma levels of tHcy lower than 10  $\mu\text{mol/L}$  are considered optimal (4). Many laboratories however use cut-offs of 12 to 14  $\mu\text{mol/L}$  in adult men and 10 to 12  $\mu\text{mol/L}$  in premenopausal women (4). A recent review by Hunt et al. states that the cut-off used also depends on the individual technique utilised to measure tHcy, and suggests that a cut-off of 15  $\mu\text{mol/L}$  should be regarded as high (5).

#### **2.2.4 Holo-Transcobalamin II**

Holo transcobalamin II is the complex of the serum transport protein TC II with attached vitamin B12 (27). Transcobalamin II has an important role in the blood, but small quantities are also found in other fluids (i.e. milk, cerebrospinal fluid, semen) (28).

Vitamin B12 is rapidly delivered to the tissues of the body by Holo-TC II as it has a relatively short plasma half-life of around 90 minutes (compared to the holo-haptocorrin I half-life of 9-10 days) (29).

It was first postulated in 1983 (30) that the measurement of holo-TC II may result in a more accurate measure of vitamin B12 status than its predecessor, total vitamin B12. It was not until 2002 that reliable methods of analysis surfaced in the forms of an enzyme-linked immunosorbent assay (31) and a radioimmunoassay (32). Holo-TC II assays are now more accurate and a fully automated method developed in 2008 (33) has become commercially available.

As with other measures of vitamin B12 status, reference limits vary and a standard level is not available. One study evaluating laboratory performance (34) compared available data

from 8 studies. These studies reported lower limits of healthy holo-TC II ranging from 11-41 pmol/L (34). The authors summarised that a lower limit of ~40 pmol/L was most likely the best estimate of a healthy status (34). The fully automated method developed in 2008 however identified a 95% CI cut-off of only 19 pmol/L (33).

Low concentrations of holo-TC II can indicate one of two things; firstly that there is impaired transfer of holo-TC II from the ileal cell into the bloodstream (vitamin B12 malabsorption), and secondly that there is insufficient availability of vitamin B12 for tissues (metabolic insufficiency) (35). As both of these indications have merit (36), the analysis of holo-TC II is rendered unspecific for diagnosing one problem over the other.

#### 2.2.5 Summary

The above information highlights the lack of clarity available in the classification of vitamin B12 status. Without specific cut-offs, diagnosis of deficiency without symptoms becomes more difficult. Using combinations of vitamin B12 markers has been suggested by both Carmel & Sarrai (3) and Bailey et al (37). In the past with Carmel & Sarrai (3) suggesting that a composite criteria be utilised, in which serum vitamin B12 < 148 pmol/L, or 148-258 pmol/L and MMA > 0.3 µmol/L, or tHcy > 13 µmol/L (female) and > 15 µmol/L (male) be used to indicate inadequate vitamin B12 status. Bailey et al. opted to use both serum vitamin B12 and MMA results to analyse results from the NHANES data (37). Another recent clinical review by Hunt et al. (5) outlined the clinical diagnosis and management of vitamin B12 deficiency including a serum vitamin B12 cut-off for deficiency of <148 pmol/L or a tHcy >15 µmol/L which may be indicative of vitamin B12 deficiency.

## 2.3 Nutrition and bioavailability

### 2.3.1 Food sources

Vitamin B12 is synthesised by certain bacteria and archaea (38). Animals that ingest said microorganisms then absorb and concentrate the produced vitamin B12 in their tissues (39). Plants do not provide a naturally occurring bioactive form of vitamin B12, some plant foods may contain added vitamin B12 while others such as mushrooms contain vitamin B12 analogues which are inactive in humans and as such provide no nutritional benefit (39). However some studies have shown that dried green and purple algae (nori) may contain vitamin B12 which may be active (39), while other edible algae contain only trace or no vitamin B12 (39). Furthermore, certain types of Japanese seaweeds have prevented deficiency in vegans (40). Excluding nori, active vitamin B12 is found only in foods originating from an animal source (41). See appendix A for a compiled list of vitamin B12 containing foods, including a more in depth analysis of vitamin B12 containing foods.

### 2.3.2 Bioavailability

Currently there appears to be limited information on the bioavailability of vitamin B12 in humans. What is known however is that in the average human the absorption of vitamin B12 has been shown to have varying results based on the type and quantity of protein that is consumed (41). Vitamin B12 from whole foods shows us that there appears to be different absorption rates with certain foods having much higher absorption than others, for example, studies show that chicken (42) and fish (43) have relatively high absorption ( $\geq 61\%$  and  $\geq 30\%$  respectively) when compared to another common vitamin B12 source such as eggs (3.7-9.2%) (44). The main limiting factor in vitamin B12 absorption is an IF-mediated system which limits the amount of vitamin B12 that can be absorbed from a single oral dose to around 1.5-2.0  $\mu\text{g}$  (20). The limiting factor is likely to be the number of ileal receptors rather than available IF, as this is produced in abundance by the body (20).

Appendix B gives an adapted insight into bioavailability of certain animal products from O'Leary & Samman (45).

## 2.4 Vitamin B12 Requirements

The current Nutrient Reference Values for Australia and New Zealand (46) state that for both male and female adults, the RDI of vitamin B12 is 2.4 µg/day. The RDI for pregnant females is slightly higher (2.6 µg/day) to account for foetal and placental needs. The RDI for lactating women is slightly higher again (2.8 µg/day) to account for the average amount of vitamin B12 secreted in breast milk per day. These levels mirror those of the United States RDA values for vitamin B12 set by the Institute of Medicine (47). The US recommendations state that the RDA is based on the amount needed for the maintenance of normal serum B12 levels and haematological status (i.e. the prevention of megaloblastic anaemia).

The elderly are the population who are the most susceptible to vitamin B12 deficiency (48, 49). Their susceptibility lies in their greater development of gastritis, food-bound cobalamin malabsorption and pernicious anaemia (20, 48, 50) rather than B12 restricted diets. As such, both US and Australia/New Zealand reference publication suggest that the elderly should meet the majority of their RDA/RDI by consuming; foods rich in vitamin B12, foods fortified with vitamin B12 or a vitamin B12 containing supplement (47).

One study however, suggests the need for a change in the daily requirements of vitamin B12 (51). This study concluded that a daily intake of 4-7 µg/day is more appropriate than the current recommendations for maintaining optimal vitamin B12 and biomarker status. This is backed up by the knowledge that clinical features of deficiency can manifest in the absence of anaemia or low serum vitamin B12 levels (52, 53). The European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies recently suggested an

adequate intake reference value of 4 µg/day based on data from different biomarkers of status (54).

There is at this time insufficient evidence to set an upper limit of vitamin B12 intake. It is thought that this may result from the gastrointestinal tracts limited ability to absorb vitamin B12, effectively preventing excess intake. This does not account for vitamin B12 supplements given by injection or sublingual routes however, as these methods bypasses the gastrointestinal limiting factor.

## 2.5 Deficiency State

The most common clinical expression of vitamin B12 deficiency presents in the form of megaloblastic anaemia, which affects all blood cells (not just red-blood cells). Symptoms of megaloblastic anaemia are vast including but not limited to; fatigue, muscle weakness and nausea (20). The role of vitamin B12 in the causes of megaloblastic anaemia is complex and outside of the scope of this study, and as such will not be discussed. An expert understanding can be gained in the work of Chanarin (20).

Prolonged vitamin B12 deficiency has long been known for its role in the development of subacute combined degeneration of the spinal cord (55). This degenerative disease leads to progressive parathesis, incoordination, impaired sensation, and other neurological issues through its effect on myelin sheaths in the spinal cord (4, 56). Neurological abnormalities will respond to vitamin B12 therapy if caught early enough (4). In cases where deficiency is left untreated for an extended period, only partial response can be achieved with the possibility of complete irreversibility (4).

Currently, elevated tHcy concentrations are considered a risk factor for CVD (24, 25). The majority of research into CVD and tHcy focuses on the effect of folate with or without the

addition of vitamins B12 and B6, whereas investigations into the effects of vitamin B12 on CVD risk are limited (45).

## **2.6 Deficiency Causes**

There are a multitude of reasons why vitamin B12 deficiency may develop in an individual. These causes have varying levels of severity and can be categorised into severe malabsorption, mild malabsorption, and dietary deficiency (57). Deficiency develops over an extended period of time due to the relatively large vitamin B12 stores in the body. Daily losses are approximately  $\sim 1 \mu\text{g/day}$  (4). With a body store of around  $2500 \mu\text{g}$  (4), deficiency develops over many years rather than days. Deficiency will develop more quickly in those who have both malabsorption issues and dietary deficiency.

### **2.6.1 Severe Malabsorption**

The most common cause of severe malabsorption is due to the effects of pernicious anaemia (4, 57). This anaemia is an autoimmune gastritis caused by the destruction of gastric parietal cells (4). As parietal cells are the source of IF production, this causes a major problem in the ingestion of vitamin B12. Without the required IF, vitamin B12 can only be absorbed by passive diffusion, which accounts for approximately 1-3% (58) in a healthy individual. Similar effects are seen when there is total/partial gastrectomy, or gastric bypass/bariatric surgery (57). These procedures can result in the loss of gastric acid necessary for the cleaving of food bound vitamin B12 and like pernicious anaemia the loss of IF (59). Intestinal causes of severe malabsorption are related to disorders of the ileum. Total or partial resection of the ileum results in total or partial loss of the IF-receptor cubilin, which results in the loss or limiting of the ability to absorb the IF-vitamin B12 complex from the gastrointestinal contents (59). Imerslund-Gräsbeck is a hereditary cause of vitamin B12 malabsorption. This syndrome arises from the mutation of the gene for

cubilin or the gene for amnionless (required for the endocytosis of the IF- vitamin B12 complex) (4) .

### **2.6.2 Mild Malabsorption**

Atrophic gastritis is a condition which develops gradually over time due to the inflammation of the stomach lining (45). This extended inflammation eventually destroys cells in the stomach lining, which decreases the secretion of both gastric acid, necessary for releasing protein bound vitamin B12, and IF. Atrophic gastritis prevalence increases with age and is common in the elderly (45).

Another common cause of mild malabsorption results from the use of medications. Extended periods of metformin use are associated with low vitamin B12 levels (4), the mechanism for this is still poorly documented however. Other common medications include those used to block stomach acid secretion in the event of gastric reflux or stomach ulcer production, such as proton pump inhibitors and H<sub>2</sub> receptor antagonists (4, 57). These drugs result in a decreased ability to cleave food bound vitamin B12 resulting in a decrease in the amount of vitamin B12 available to bind with IF.

### **2.6.3 Dietary Deficiency**

Dietary deficiency results from the avoidance of foods containing vitamin B12. This typically arises in vegans who eat no meat or animal products and to a lesser extent vegetarians (although this subgroup can still get vitamin B12 from animal products such as dairy products and eggs) (45). These individuals can instead meet their requirements by consuming fortified foods such as yeast or by taking supplements (45). Infant vitamin B12 deficiency can result from breast feeding in mothers who are themselves deficient, thus limiting the amount of vitamin B12 secreted in the breast milk (57).

#### **2.6.4 Other Factors Affecting Vitamin B12 Levels**

In the past, studies have shown lower concentrations of vitamin B12 in OCP users compared to non-users (60, 61). Theories for this change include a false indication of deficiency (62) and a redistribution of vitamin B12 among transporters (63).

Following an 8 week crossover intervention with red wine and vodka, Gibson et al (64) showed that a two week alcohol intervention significantly decreased serum vitamin B12 levels and increased plasma tHcy. In 2014, the World Health Organisation (WHO) global status report on alcohol and health (65) reported an alcohol intake per capita (aged 15+) consumption (in litres of pure alcohol) of 12.2 L for Australia. It also reported a breakdown of males and females per capita with females consuming 7.2 L/year or 15.6 g/day (pure alcohol). Both the per capita and female per capita results were above the global average alcohol intake of 6.2 L per year. These results highlight the increased alcohol intake in the Australian population and may prove an issue for vitamin B12 levels. Multiple studies have found an inverse relationship between BMI and vitamin B12 concentrations in children (66, 67). Of interest is determining if this relationship holds true into adult years and why such a relationship may exist.

#### **2.7 Conclusion**

Since pernicious anaemia was first cured in the early 1900's, our understanding of vitamin B12 has progressed dramatically. We now understand its role in the body and have a greater appreciation of the consequences of deficiency. While our knowledge of vitamin B12 has progressed, we are still lacking a gold standard measure of deficiency. Of the three common markers used for vitamin B12 deficiency, each has both positive and negative aspects of its use including a lack of clearly defined cut-offs of deficiency. Neurological impairment as a result of vitamin B12 deficiency is a serious consequence of a lack of understanding and one that we should endeavour to avoid at all costs. It is

because of such serious outcomes that we must continue to drive the evolution of understanding in the area of vitamin B12 through ongoing research. A greater understanding of deficiency markers and the interconnected relationships of markers is required in the search for clearly defined cut offs and the potential development of a gold standard method for diagnosing vitamin B12 deficiency.

## Chapter Three: Objective statement

Currently there is no gold standard measure of vitamin B12 status. We have multiple markers of status including both direct and functional markers which have different factors affecting their reliability. There are many dietary factors as well as other factors which are associated with vitamin B12 status. This study is focused on compiling three data sets from studies completed in women from Sydney, Australia. From here we are interested in using the compiled data with aims to:

1. Assess the Vitamin B12 status of younger and older Australian women.
2. Determine the association of folate status on vitamin B12 biomarkers.
3. Determine the association of the OCP on vitamin B12 status.
4. Explore the association between total protein intake and vitamin B12 status.
5. Explore the association between total energy intake and vitamin B12 status.
6. Explore the association of alcohol on vitamin B12 status.
7. Assess associations of vitamin B12 biomarkers with BMI, smoking, and diet.

## Chapter Four: Methods

For our secondary analysis we used data from three studies based in Sydney, Australia. The three studies have been given abbreviations owing to a particular characteristic of each, namely; elderly participants (ELD), omnivores (OMN), and randomly selected participants (RND), respectively. The methods for our study are broken into two main sections, discussing both the designs and procedures of the primary studies we have used and the statistical methods employed during the analysis of the combined data set. A full breakdown of the ELD, OMN and RND studies individual methods have been published by O’Leary et al. (68), McArthur et al. (69), and Fayet et al. (70) respectively.

### 4.1 Study Designs

#### 4.1.1 Recruitment

Recruitment of participants differed between the three studies, with different methods being employed as well as a range of participants being recruited. The ELD study was completed in a 48-bed sub-acute geriatric rehabilitation unit; this was also a part of a larger study examining nutritional status and drug-interactions on vitamin B12 status. Both the OMN and RND studies recruited participants through various methods at the University of Sydney campus. Inclusions for the studies were newly admitted, English patients aged 60+ without dementia (ELD), and females aged 18-35 (OMN and RND). Exclusion criteria for the ELD study included; having a condition affecting the absorption of vitamin B6, vitamin B12 or folate, untreated hypothyroidism, active neoplasm, renal impairment, or were taking B vitamin supplements/injections. Exclusions for the OMN and ELD studies were similar to each other, with both studies excluding individuals who were pregnant/lactating, or were consuming nutritional supplements or medications (excluding the oral contraceptive pill). Differences between the two studies were exclusions in the OMN study of individuals who; were vegetarian or had a reported major illness, and in the

RND study of individuals who; had a diagnosed eating disorder, were elite athletes, or were not enrolled as students. Approval for the ELD study was given by the Calvary Health Care Sydney Ethics Committee, while approval for the OMN and RND studies was given by the University of Sydney Human Ethics Committee.

#### **4.1.2 Nutrient analysis**

Dietary analysis was completed in all three studies using a food frequency questionnaire (FFQ). All three FFQs were based on the Blue Mountains Eye Study semi-quantitative food frequency questionnaire. An FFQ which was validated in an elderly population using weighed diet records (71). This FFQ was not validated for use in the ELD study as it was assumed the population in which it was validated for (71) was similar enough to the target population to not require revalidation. For the FFQ to be used in the OMN and RND studies however, it needed to be validated in a similar population due to the overwhelming differences between the two study populations and the Blue Mountains Eye Study population. This was completed in a prior validation study by Fayet et al (72) and showed that the FFQ was an appropriate tool for use in a younger female population.

Dietary data analysis was completed in two ways; firstly the ELD study used FoodWorks Xyris software (2005 Professional Edition) which utilised the Australian Food and Nutrient Database (AUSNUT99). Missing nutrient data was added manually based on The British Tables of Food Composition. For both the OMN and RND studies, the average daily nutrient intake was calculated using purpose built software (Microsoft Access 2007) which was linked to the Australian Food Composition database (NUTTAB 2006) (73).

#### **4.1.3 Blood sampling and testing**

In all three studies, blood samples were collected from an antecubital vein while subjects were in a fasted state (10-12 hours). Collection of samples occurred between the hours of

0600 and 0830 for the ELD study and between 0730 and 0930 for the OMN and RND studies. Samples were collected into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA), which were either EDTA-coated or untreated, dependent on the analysis to be run.

In all three studies; serum vitamin B12, serum folate and erythrocyte folate were analysed using an automated system (UniCel DxI Immunoassay System, Beckman Coulter Inc., Brea, CA). Both the ELD and RND studies used the same methods for determining tHcy and MMA. High performance liquid chromatography (Homocysteine HPLC Kit, BioRad, Munich, Germany) with fluorescence detection (Waters, Milford, MA, USA) was used to determine serum tHcy. Serum MMA was measured in both studies by liquid chromatography-tandem mass spectrometry with electrospray ionization as previously described (74, 75).

## **4.2 Statistical Methods**

All statistical analysis was completed using Stata V14.0. Diet energy was reported in two forms between the three groups (kcal and kJ); as such the ELD group diet energy was converted from kcal to kJ using a conversion factor of 4.184. All men from the ELD group (n=11) were excluded to give continuity between the three groups and to give clearer application for any results found. Further exclusions included two individuals with multiple skewed results and a replacement of one individual's MMA result with a missing value due to being twice the next highest value. Histograms and box plots of variables (age, BMI, serum folate, erythrocyte folate, serum vitamin B12, serum tHcy, serum MMA, dietary energy, and dietary protein) for each group were plotted to assess normality, and determine any apparent skew or identify outliers within the data sets. Variables which were determined visually to be skewed were log transformed for statistical testing

purposes. Potential outliers were further investigated to determine if they were acceptable or required exclusion from the data set because of likely error.

Descriptive statistics for demographic, biochemical and some dietary intake variables were calculated for each of the groups (mean(SD) for continuous and n(%) for categorical).

Oneway ANOVA with an F-test and chi-squared tests determined if continuous variables were different between the groups.

New categorical variables were created to assess the vitamin B12 status of the data set.

Multiple cut-offs were utilised from two different sources (3, 5). Two methods were utilised to assess serum vitamin B12 levels, the first used a cut-off of 148 pmol/L (5). The second method used three different categories for serum vitamin B12 being; >258 pmol/L, 148-258 pmol/L, and <148 pmol/L (3). Both sources used a different tHcy cut-off, being 13  $\mu$ mol/L or 15  $\mu$ mol/L (3, 5). Only one source gave a cut-off for MMA which was 0.3  $\mu$ mol/L (3). A final cut-off for serum folate (10 nmol/L) at which tHcy levels are optimised was also included for further analysis (76). Table 2 outlines the cut-offs used and the tables throughout the thesis that utilised each cut-off.

Mixed-effects maximum likelihood (ML) regressions with study group as a random effect were performed on folate biomarkers (serum folate and erythrocyte folate) vs vitamin B12 biomarkers (serum vitamin B12, serum tHcy and serum MMA) as well as vitamin B12 biomarkers (serum vitamin B12, serum tHcy, serum MMA) vs other variables (dietary energy, dietary protein and BMI) to test for associations; giving a coefficient, p-value and 95% CI. Tests including transformed data were back transformed to give OR, p-value and 95% CI. Fixed lines were predicted and incorporated into twoway scatter plots where appropriate.

**Table 2 Deficiency measure cut-offs and location utilised**

Measure Concerned	Cut-off used	Tables used in	Reference
<b>Serum Vitamin B12</b> (pmol/L)	>258		
	148-258	5, 7	Carmel & Sarrai (3)
	<148		
	≥148	4,6	Hunt et al. (5)
<b>tHcy</b> (µmol/L)	<148		
	≤13	5	Carmel & Sarrai (3)
	>13		
<b>MMA</b> (µmol/L)	≤15	4, 6, 7	Hunt et al. (5)
	>15		
	≤0.3	5, 7	Carmel & Sarrai (3)
<b>Serum Folate</b> (nmol/L)	>0.3		
	≥10	6	Selhub et al. (76)
	<10		

Tabulation of participants based on their vitamin B12 deficiency status and their OCP use status was performed to determine the differences in status of users' vs non-users. Vitamin B12 deficiency status was determined using the same serum vitamin B12 cut-offs (5) published by Hunt et al. Only serum vitamin B12 was used to determine deficiency this time however, as OCP participants came from both the OMN and RND studies. Due to the lack of tHcy and MMA data in the OMN group, it was decided that using only serum vitamin B12 would provide greater clarity in the results, rather than splitting the OCP users into groups and adding extra variables where available.

Mixed-effects ML regressions with study group as a random effect were performed on vitamin B12 biomarkers (serum vitamin B12, serum tHcy, serum MMA) vs alcohol intake. Due to the skewed nature of alcohol intake, data was log transformed first.

## Chapter Five: Results

A combined total of 411 participants from three previous studies were included in the final analysis of our study. Table 3 outlines descriptive characteristics as well as selected biochemical and dietary data about the participants included in our secondary analysis sorted into the three groups. Box plots of vitamin B12 markers (serum vitamin B12, tHcy and MMA) can be seen in Appendix C.

**Table 3 Descriptive characteristics of study groups**

	<b>ELD (n=43)</b>	<b>OMN (n=63)</b>	<b>RND (n=305)</b>	<b>P-value*</b>
<b>Age</b>	80.5 (7.6)	24.5 (4.4)	22.5 (3.9)	<0.001
<b>BMI (kg/m<sup>2</sup>)</b>	27.3 (7.2)	21.9 (2.8)	21.5 (2.8)	<0.001
<b>OCP use n (%)</b>	-	21 (33)	69 (23)	0.084
<b>Smoker n (%)</b>	1 (2)	2 (3)	28 (9)	0.114
<b>Serum folate (nmol/L)</b>	16.5 (7.5)	22.0 (9.3)	23.3 (10.3)	<0.001
<b>Erythrocyte folate (nmol/L)</b>	1130 (476)	853 (342)	823 (297)	<0.001
<b>Serum vitamin B12 (pmol/L)</b>	263 (110)	260 (123)	223 (105)	0.008
<b>Serum tHcy (µmol/L)</b>	15.0 (5.3)	-	7.2 (2.3)	<0.001
<b>Serum MMA (µmol/L)</b>	0.26 (0.13)	-	0.17 (0.08)	<0.001
<b>Dietary energy (kJ/day)</b>	8124 (1994)	12324 (5211)	9616 (3491)	<0.001
<b>Dietary protein (g/day)</b>	86 (21)	142 (54)	111 (46)	<0.001
<b>Alcohol (g/day)</b>	-	7.8 (11.9)	6.4 (9.5)	0.353

Results reported as mean ± (SD) unless stated \*p-values determined using an F-test for continuous variables, chi-squared test for OCP and a Fishers exact test for smoker. ELD = Elderly, OMN = Omnivores, RND = Random.

As shown in Table 3, some of the listed variables are missing where they weren't measured in the respective study, namely serum tHcy and serum MMA (OMN group), and alcohol (ELD group). The ELD group has significantly higher ( $p<0.001$ ); age, BMI and erythrocyte folate and significantly lower ( $p<0.001$ ) serum folate than the other two groups. As well as this the ELD group has significantly higher ( $p<0.001$ ); tHcy and MMA than the RND group.

## 5.1 Biomarkers

The first area of interest centres on biomarkers and the methods of determining vitamin B12 deficiency. Tables 4 and 5 represent the participants' vitamin B12 status based on two different methods of assessing deficiency. The first, a recently published review from Hunt et al. (5) recommends the use of serum vitamin B12 or tHcy levels. The second, proposed by Carmel and Sarrai (3), uses serum vitamin B12, or a combination of serum vitamin B12 with either MMA or tHcy.

**Table 4 Vitamin B12 deficiency using criteria from Hunt et al. (5) clinical review of deficiency**

Serum vitamin B12 (pmol/L)	Serum tHcy ( $\mu\text{mol/L}$ )	
	$\leq 15$	$> 15$
<b><math>\geq 148</math> (n)</b>	244	20*
ELD	20	19
RND	224	1
<b><math>&lt; 148</math> (n)</b>	75*	3*
ELD	3	2
RND	72	1

\*indicates possible vitamin B12 deficiency. ELD and RND groups only included

Table 4 categorises participants into one of two categories; adequate vitamin B12 status with a serum vitamin B12  $\geq 148$  pmol/L and a tHcy  $\leq 15$   $\mu$ mol/L, or vitamin B12 deficient with a serum vitamin B12  $< 148$  pmol/L and/or a tHcy  $> 15$   $\mu$ mol/L. Based on the serum vitamin B12 cut off, 78 participants (22.8%) are classed as having vitamin B12 deficiency. By combining the two criteria we are able to identify a further 20 participants (8%) in the adequate serum vitamin B12 classification with raised tHcy that would have been missed had we only used a serum vitamin B12 cut-off for defining deficiency, these individuals require further investigation for potential vitamin B12 deficiency. Table 5 categories participants into one of three categories; adequate vitamin B12 status (with a serum vitamin B12  $\geq 258$  pmol/L or a serum vitamin B12 of 148-258 in combination with a tHcy  $\leq 13$   $\mu$ mol/L and a MMA  $\leq 0.3$   $\mu$ mol/L), sub-clinical vitamin B12 deficiency (with a

**Table 5: Vitamin B12 deficiency/sub-clinical deficiency using Carmel & Sarrai composite criteria (3)**

Serum vitamin B12 (pmol/L)	tHcy $\leq 13$ + MMA $\leq 0.3$	tHcy $> 13$ + MMA $\leq 0.3$	tHcy $\leq 13$ + MMA $> 0.3$	tHcy $> 13$ + MMA $> 0.3$
<b>&gt;258 (n)</b>	89	7	5	2
<b>ELD (n)</b>	7	7	1	2
<b>RND (n)</b>	82	0	4	0
<b>148-258 (n)</b>	133	9 <sup>†</sup>	9 <sup>†</sup>	4 <sup>†</sup>
<b>ELD (n)</b>	7	8	2	4
<b>RND (n)</b>	126	1	7	0
<b>&lt;148 (n)</b>	62 <sup>*</sup>	5 <sup>*</sup>	9 <sup>*</sup>	1 <sup>*</sup>
<b>ELD (n)</b>	1	3	0	1
<b>RND (n)</b>	61	5	9	0

tHcy and MMA both measured in  $\mu$ mol/L. \*indicates vitamin B12 deficiency, †indicates sub-clinical vitamin B12 deficiency. ELD and RND groups only included.

serum vitamin B12 of 148-258 pmol/L in combination with a tHcy  $> 13$   $\mu$ mol/L and/or a MMA  $> 0.3$   $\mu$ mol/L), or vitamin B12 deficiency (with a serum vitamin B12  $< 148$

pmol/L). Table 5 classes 77 participants (22.9%) with deficiency. Similarly to table 4, by combining cut-off measures we are able to further identify individuals with adequate serum vitamin B12 levels that may have vitamin B12 deficiency based on metabolite levels and require further investigation. By using a MMA and tHcy cut-off in combination with a moderate serum vitamin B12 level (148-258 pmol/L) we are able to identify 22 individuals (14% of the moderate serum vitamin B12 level group) with sub-clinical deficiency. In total this cut-off criteria gives a total of 99 individuals (29.5%) with concerning vitamin B12 status.

Table 6 reports the percentage of individuals with vitamin B12 deficiency based on either serum vitamin B12 levels or tHcy levels. Based on these two measures, 55% of the ELD group and 25% of the RND group have potential vitamin B12 deficiency. When we exclude individuals whom have folate deficiency based on a low serum folate without a low serum vitamin B12 level these numbers drop to 39% and 24% respectively.

Table 7 shows the percentage of individuals with optimal MMA and/or tHcy levels stratified by serum vitamin B12 levels. From this table we can see that of the individuals with a low serum vitamin B12, 83% have optimal MMA and tHcy levels. This is consistent with both the optimal and moderate serum vitamin B12 groups, which have 88% and 86% of individuals respectively with optimal MMA and tHcy levels. A Pearson  $\chi^2$  test showed that there were no significant differences between the three groups ( $p=0.316$ ). When OCP users are excluded from this analysis a Pearson  $\chi^2$  again shows that there were no significant differences between the three groups ( $p=0.214$ ).

Figure 3 shows the combined graphs of both, serum and erythrocyte folates vs serum vitamin B12, serum tHcy and serum MMA. All serum vitamin B12 and serum tHcy results vs serum folate and erythrocyte folate

**Table 6 Vitamin B12 deficiency based on serum vitamin B12 and serum tHcy with accounts for serum folate status**

	<b>low serum vitamin B12<sup>1</sup></b>	<b>high tHcy<sup>2</sup></b>	<b>low serum vitamin B12<sup>1</sup> and/or high tHcy<sup>2</sup></b>	<b>high tHcy<sup>2</sup> with normal serum folate status<sup>3</sup></b>	<b>low serum vitamin B12<sup>1</sup> and/or high tHcy<sup>2</sup> with normal serum folate status<sup>3</sup></b>
<b>ELD % (n)</b>	11 (5)	48 (21)	55 (24)	30 (13)	39 (17)
<b>RND % (n)</b>	24 (73)	0 (2)	25 (74)	0 (1)	24 (73)
<b>Total % (n)</b>	23 (78)	7 (23)	29 (98)	4 (14)	26 (90)

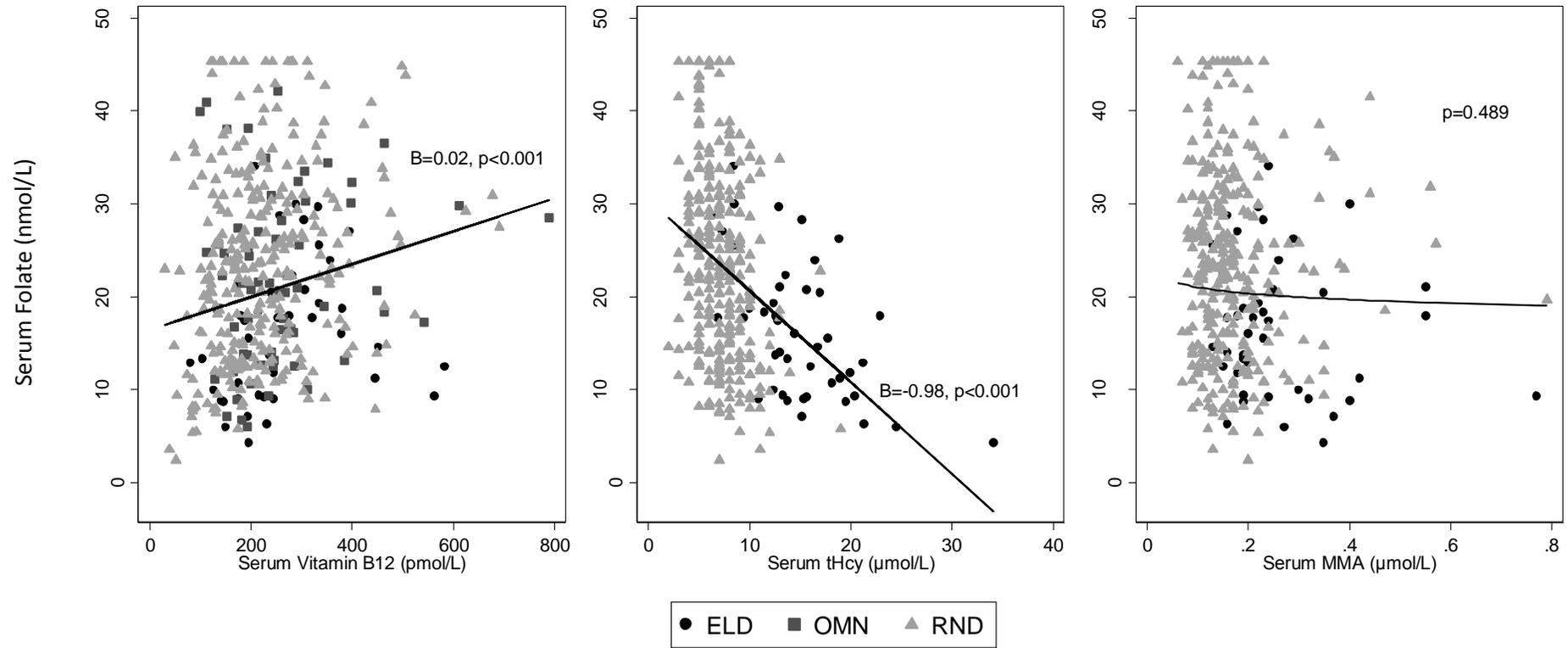
<sup>1</sup>serum vitamin B12 < 148 pmol/L <sup>2</sup>serum tHcy > 15 µmol/L <sup>3</sup>serum folate ≥ 10 nmol/L

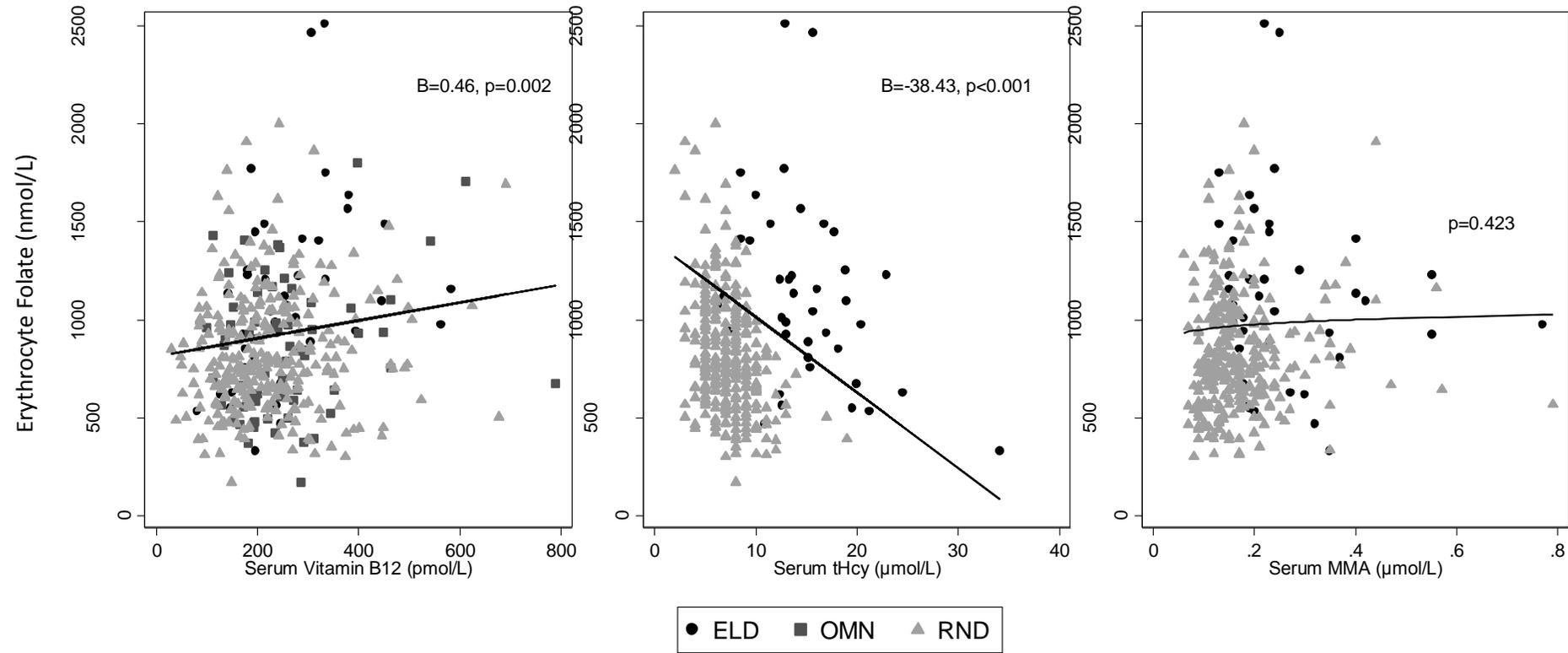
**Table 7 Concordance between MMA levels and tHcy levels stratified by serum vitamin B12 status**

	<b>Optimal Serum Vitamin B12<sup>1</sup></b>	<b>Moderate Serum Vitamin B12<sup>2</sup></b>	<b>Low Serum Vitamin B12<sup>3</sup></b>
<b>Optimal MMA<sup>4</sup> &amp; tHcy<sup>5</sup> % (n)</b>	88 (91)	86 (134)	83 (64)
<b>Optimal MMA<sup>4</sup> and high tHcy<sup>5</sup> % (n)</b>	5 (5)	5 (8)	4 (3)
<b>Optimal tHcy<sup>5</sup> and high MMA<sup>4</sup> % (n)</b>	5 (5)	6 (9)	13 (10)
<b>High MMA<sup>4</sup> and high tHcy<sup>5</sup></b>	2 (2)	3 (4)	0 (0)

<sup>1</sup>serum vitamin B12 > 258 pmol/L <sup>2</sup>serum vitamin B12 148-258 pmol/l <sup>3</sup>serum vitamin B12 < 148 pmol/L <sup>4</sup>optimal serum MMA ≤ 0.3 µmol/L, high serum MMA > 0.3 µmol/L <sup>5</sup>optimal serum tHcy ≤ 15 µmol/L, high tHcy > 15 µmol/L. P-value = 0.316

**Figure 3** Linear relationships between serum folate and erythrocyte folate with serum vitamin B12, serum tHcy and serum MMA





were shown to be statistically significant. Serum MMA was not significantly associated with either serum folate ( $p=0.489$ ) or erythrocyte folate ( $p=0.423$ ).

Serum vitamin B12 (pmol/L) was positively associated with both serum folate (nmol/L) ( $\beta =0.018$  95% CI: 0.009, 0.026,  $p<0.001$ ) and erythrocyte folate (nmol/L) ( $\beta =0.456$  95% CI: 0.164, 0.747,  $p=0.002$ ). Serum tHcy ( $\mu\text{mol/L}$ ) however was negatively associated with both serum folate (nmol/L) ( $\beta =-0.983$  95%CI: -1.246, -0.720,  $p<0.001$ ) and erythrocyte folate (nmol/L) ( $\beta =-38.428$  95%CI:-49.929, -26.927,  $p<0.001$ )

## 5.2 Oral Contraceptive Pill Use

The next area of interest we focused on was that of OCP user and the associations that may exist with vitamin B12 levels. Table 8 categories participants as having an adequate vitamin B12 status or as being vitamin B12 deficient (using a serum vitamin B12 cut-off of 148 pmol/L (5)). The table shows that 37 (42%) of the OCP users are classed as deficient based on serum vitamin B12 concentrations compared to 44 (16%) non-users of the OCP. Figure 4 displays the boxplots of serum vitamin B12 concentrations (pmol/L) separated by OCP user status; this clearly shows a lower median serum vitamin B12 concentration in OCP users vs non-users. OCP use is associated with a mean decrease in serum vitamin B12 concentration of 72.3 pmol/L (95% CI: -93.9,-44.5,  $p<0.001$ ). Neither tHcy ( $p=0.669$ ) or MMA ( $p=0.595$ ) was affected by OCP use.

## 5.3 Lifestyle factors

The next area of interest focuses on lifestyle factors that may be associated to vitamin B12 status including; dietary factors and BMI. Table 9 highlights the associations between markers of vitamin B12 status and total protein intake, total energy intake and BMI. From the table we can see that total energy does not have any statistically significant associations with any of the three markers of status. Total protein however, has a positive

association with serum vitamin B12 ( $p=0.001$ ). BMI also has a negative association with serum vitamin B12 which is not statistically significant ( $p=0.051$ ).

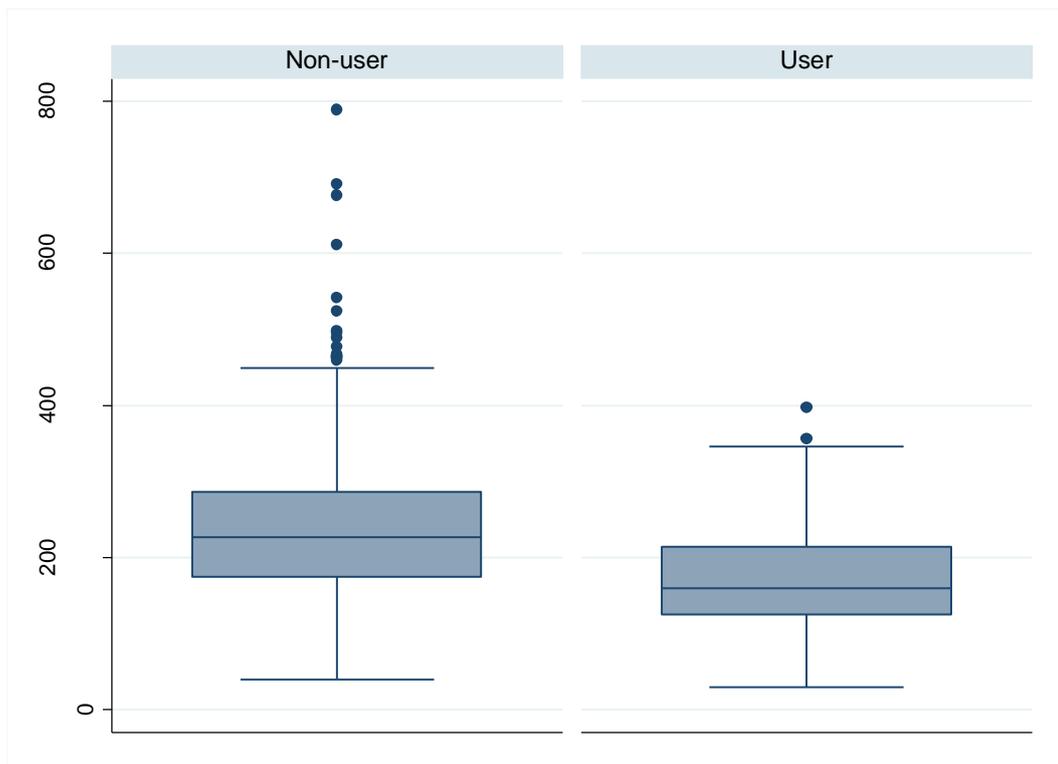
Serum vitamin B12 was also significantly negatively associated with alcohol intake ( $p<0.001$ ), where for a 10% higher alcohol intake, this was related to a lower serum vitamin B12 by 1.9 pmol/L. Alcohol intake was not associated with serum tHcy but a 10% higher consumption of alcohol was associated with a 0.6% increase in serum MMA ( $p=0.005$ ).

**Table 8 Serum vitamin B12 deficiency stratified by oral contraceptive use**

Serum vitamin B12 (pmol/L)	OCP user	
	No	Yes
$\geq 148$	224 (84)	52 (58)
$< 148$	44 (16)	37 (42)

Reported as n (%), only OMN and RND groups included

**Figure 4 Box plot of serum vitamin B12 concentration sorted by oral contraceptive pill user status**



**Table 9 Associations between serum vitamin B12, serum tHcy, and serum MMA with energy and protein intakes and BMI**

	Serum vitamin B12 <sup>†</sup>		Serum tHcy <sup>x*</sup>		Serum MMA <sup>x*</sup>	
	Regression coefficient (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
<b>Energy (MJ)</b>	2.282 (-0.591, 5.155)	0.120	0.992 (0.981, 1.004)	0.191	0.987 (0.974, 1.000)	0.055
<b>Protein (10g)</b>	3.844 (1.533, 6.154)	0.001	0.992 (0.983, 1.000)	0.063	0.990 (0.980, 1.001)	0.053
<b>BMI (kg/m<sup>2</sup>)</b>	-2.864 (-5.743, 0.016)	0.051	1.002 (.992, 1.011)	0.710	1.004 (0.992, 1.016)	0.494

<sup>†</sup>Measured in ELD, OMN, and RND groups. <sup>x</sup>Measured in ELD, RND groups \*Skewed data which has been log transformed. Results are the reverse transformed OR and 95% CI.

## Chapter Six: Discussion

Our results showed that an association between different serum vitamin B12 strata and raised metabolites was not present. Use of the OCP was associated with a decrease in serum vitamin B12 levels; a decrease was also seen with increased alcohol consumption. Folate was shown to have a positive association with serum vitamin B12 levels.

Our results highlighted the difficulty associated with classifying people as deficient. Of the two methods we employed (3, 5) in our analysis to determine the number of individuals with deficient vitamin B12 levels, we came across differences. While the number of individuals with potentially problematic vitamin B12 status was similar, Carmel and Sarrai's method distinguished between those with vitamin B12 deficiency and those with sub-clinical deficiency using measures of serum vitamin B12, tHcy and MMA in the diagnostic phase (3). Hunt et al. however, only looked at deficiency, and used only serum vitamin B12 and tHcy as measures of deficiency (5).

These methods were taken a step further by combining Carmel and Sarrai's three cut-offs of serum vitamin B12 with the tHcy cut-off of Hunt. We developed this method further by adding folate status as a further stratification. In the ELD group, who had significantly lower serum folate levels than the larger RND group, the addition of folate changed the outcome of the diagnosis of deficiency drastically. By excluding individuals with moderate to high serum vitamin B12 levels with low tHcy levels in combination with low serum folate levels, the percentage of individuals in the ELD group with potential vitamin B12 deficiency dropped from 55% to 39%.

Folate levels and renal status have been shown to be a bigger influence on tHcy levels than vitamin B12 (4, 26). Folate deficiency is also known to be associated with chronic alcohol intake due to interference with folate absorption (77). A folate deficient diet is also

common in those who abuse alcohol. Folate deficiency is also seen in the elderly due to intestinal malabsorption (78). In populations with relatively good folate, tHcy may serve as a viable option as a metabolic marker of vitamin B12 deficiency, but as this cannot be guaranteed, it calls into question the usefulness of tHcy as a primary marker of deficiency like that of the methods outlined by Hunt et al. Of interest is the lack of an MMA diagnostic cut-off inclusion in the Hunt et al. clinical review (5). It is often concluded that MMA is more superior to tHcy in relation to vitamin B12 deficiency diagnosis despite having its own challenges (26).

Further to the questions raised about tHcy as a biomarker, our results call into question the usefulness of serum vitamin B12 as a biomarker of deficiency. For some time now, serum vitamin B12 has been queried about its usefulness as a primary measure of vitamin B12 status. Our results showed that regardless of vitamin B12 stratification (optimal, moderate or low levels), the percentage of individuals with both optimal MMA and tHcy levels remained the same (88%, 86% and 83% respectively). We would expect that as serum vitamin B12 levels decrease from optimal to moderate and then again to low that there would be a decrease in the percentage of individuals with optimal MMA and tHcy levels. This does not however happen in our population, suggesting that of the individuals with low serum vitamin B12 levels, the majority do not have any metabolic issues present. Further raising the question, should serum vitamin B12 be considered the primary marker of deficiency.

Holo-transcobalamin II has been touted as a potential measure of vitamin B12 status. This transporter promotes cellular uptake of vitamin B12 and approximately 20 to 30% of plasma vitamin B12 is attached to this transporter at any given time (4). By measuring holo-TC II in comparison to serum vitamin B12 we are able to determine the amount of vitamin B12 which is biologically available for cellular uptake. Assays for holo-TC II are

now accurate, while a fully automated method has been available for some time (33). This measure has also been shown to be advantageous over serum vitamin B12 (16, 21).

Oral contraceptive pill use was significantly associated with a decrease in mean serum vitamin B12 concentration of 72.3 pmol/L in individuals who took part in the OMN and RND studies. However, there were no metabolite (tHcy or MMA) increases associated with OCP use. The implications of said decrease could be potentially great if it were to be a true deficiency. Oral contraceptives are a relatively common medication in young women nowadays, and a 72.3 pmol/L decrease in serum vitamin B12 levels could have major consequences. Past studies have found similar results showing that the OCP decreases serum vitamin B12 concentrations significantly (60, 61, 79, 80). One recent study (81) also observed a 45.3 pmol/L reduction in serum vitamin B12 concentrations ( $p=0.036$ ) in oral contraceptive pill users.

There is evidence to suggest that the change in serum vitamin B12 in OCP users is due to a redistribution of the vitamin among transporters (62, 63). Shojania and Wylie (63) showed that absorption and urinary excretion of vitamin B12 remains normal in individuals using the OCP. Their findings also showed that OCP use was significantly associated with a lower total vitamin B12 binding capacity of serum and a decrease in transcobalamin I (haptocorrin) levels (63), which could explain the decrease in serum vitamin B12 levels. Shojania and Wylie suggest a decrease in transcobalamin I levels and an increase in transcobalamin III levels as the mechanism responsible for decreased serum vitamin B12 levels (63). Gardyn et al. (82) also showed that in women using the OCP with low vitamin B12 levels, 60% had decreased transcobalamin I blood levels. In this group, deficiency was shown to be false based on normal urinary MMA and plasma tHcy levels. Furthermore, serum vitamin B12 levels returned to normal following OCP cessation for one month (82). Many studies hypothesise that the change in serum vitamin B12 is

harmless and point to biomarker (tHcy & MMA) evidence to show that this decrease has not caused a change in the metabolic processes associated with vitamin B12 with one study also showing that the decreased vitamin B12 levels did not affect bone mineral density as would be expected (80).

Ultimately, our study confirms that an association between OCP use and lower serum vitamin B12 levels does exist, but that this is not an indicator of a true deficiency based on metabolite markers of vitamin B12 status. This does not however mean we can rest easy, it is important that we acknowledge that pernicious anaemia is still a possibility in women of reproductive age, and as such, a low serum vitamin B12 level in women using the OCP should not be discounted based on the use of this medication. Of interest would be the furthered research of OCP users looking into the relatively new marker of vitamin B12 status, holo-TC II.

Interestingly, serum and erythrocyte folate levels were both related to serum vitamin B12 levels. This is an unexpected discovery as while vitamins B12 and folate have interrelated pathways in the body, they are by no means related in the diet. As previously stated, vitamin B12 originates from animal products, while the main dietary sources of folate include; green leafy vegetables, legumes, some fruits, as well as fortified cereals and cereal products (83). As expected an inverse relationship between both serum and erythrocyte folate and tHcy levels was present, due to folates role in the methionine synthase metabolic process. While folate and vitamin B12 are not related in terms of where they come from in the diet, they have been linked in previous studies. Bailey et al (37) showed that in the NHANES data set, serum folate and erythrocyte folate concentrations increased with increasing vitamin B12 status. Two studies have showed that when individuals were vitamin B12 deficient, higher serum folate levels were associated with increasing tHcy and MMA concentrations (84, 85), suggesting a potential worsening of enzymatic function as

folate levels increased. Another study combining data from three cohorts has shown that in elderly individuals with low vitamin B12 status, increased folate levels are associated with impaired cognition (86). These findings raise questions about food fortification with folate, and beg the question of whether fortification may result in an associated rise in serum vitamin B12; or for those with established vitamin B12 deficiency, will be detrimental for metabolite levels.

Alcohol intake was found to be negatively associated with serum vitamin B12 concentrations, where a 10% increase in alcohol consumption was associated with a decrease in serum vitamin B12 concentration of 1.9 pmol/L ( $p < 0.001$ ). This is a concerning result given the population in which our participants have come from. Alcohol intakes were measured only in the OMN and RND group. As previously stated, Australian females drink in excess of the global average alcohol intake of 6.2 L of pure alcohol per year. The report also stated an average per day intake of 15.6 g/day in Australian females. With the OMN and RND studies having relatively low average daily intakes of 7.8 g/day and 6.4 g/day respectively, this suggests that the possible ramification of alcohol intake on vitamin B12 levels in Australian women could be far greater than seen in our study. The relationship between alcohol and vitamin B12 status has previously been explored with multiple studies showing a negative association between alcohol intake and vitamin B12 status (64, 87) with both Gibson et al. and Laufer et al. showing a decrease in serum vitamin B12 and an increase in tHcy. A mechanistic explanation for the relationships have yet to be identified, although it has been postulated that increasing alcohol consumption is related with decrease in dietary adequacy of B vitamins.

A positive association between protein intake and serum vitamin B12 was shown in our study, with a 10g increase in dietary protein intake being associated with a 3.8 pmol/L increase in serum vitamin B12 concentration. Restriction of animal food group intakes is a

known determinant of low vitamin B12 intake (70, 88) due to vitamin B12 being located primarily in animal products. There appears to be little/no evidence on overall protein intakes association with serum vitamin B12. An association between animal protein and vitamin B12 status would be expected as we know dietary vitamin B12 is introduced to the body bound to animal protein, a factor which is important in individuals who follow a vegan (and to a lesser extent vegetarian) diet pattern (88).

## **6.1 Strengths and Limitations**

The strength of our study lies in the inclusion of three data sets. This has given us a large sample size and increases the power with which we have to draw conclusions from results we have found. By combining the three data sets we have included a range of ages and broadened the characteristics of our sample population.

Between our three data sets there are differences in our groups that make the combination of the data a limitation. We have to be aware that these intragroup differences are present when making conclusions about our results. Further limitations lay within the study designs themselves. It was assumed that the Australian Blue Mountains eye study FFQ was appropriate for the elderly group without validation in the population. This FFQ was originally validated for a healthy population, whereas the population used in the ELD study were sourced from hospital admissions. The difference in measures used between the studies has also proved a limitation. As the studies were completed independently, measures across all three do not align; this has resulted in some tests omitting sections of the data set due to a lack of data. The main limitation of this lay in the lack of tHcy and MMA data in the OMN study. Total protein was also not separated into animal protein and plant protein which would give better understanding of vitamin B12 in terms of total

animal protein in comparison to plant based protein intakes. Our sample is also a convenience sample and this may be a limitation.

## 6.2 Conclusion

Our study has highlighted issues surrounding serum vitamin B12 and its ability to identify those with deficiency. We know that serum vitamin B12 is a fairly poor measure of the ability to deliver vitamin B12 to tissues within the body. Serum vitamin B12 measures both holo-TC II and the r-binders within blood. Holohaptocorin (r-binder) is relatively biologically unavailable to tissues compared to holo-TC II and can mask true deficiency when looking at serum vitamin B12 levels. We have seen multiple instances throughout our study where serum vitamin B12 has proved inadequate; namely when the OCP is involved. Furthermore, we have seen that there are no differences in metabolite levels (tHcy and MMA), regardless of serum vitamin B12 status.

The diagnosis of vitamin B12 deficiency is an area inundated with discrepancy. Without an ideal diagnostic test we will continue to struggle to identify those individuals with vitamin B12 deficiency. It is my opinion that the future of vitamin B12 and a potential gold standard criteria does not lie in the continued utilisation of serum vitamin B12. In previous decades it has become far easier to measure levels of holo-TC II in the blood. This measure has the potential to give us a greater insight into the body's ability to meet metabolic process requirements of vitamin B12, rather than measuring total vitamin B12 which includes both the metabolically available/unavailable sections of this vitamin.

In order to consider holo-TC II as a future primary measure of vitamin B12 status, this should be our main area of concern for future research. We must investigate the grey areas that currently cloud this measure and determine the factors which may affect its levels

within the blood such as OCPs. Only by furthering our understanding of holo-TC II can we hope to utilise its full potential.

By furthering our ability to identify vitamin B12 deficiency, we give ourselves the greatest possibility of preventing the potential issues of deficiency. As well as this, it is important that in the age of folate fortification we mitigate the effects of high folate intakes that have been shown in this discussion.

## Chapter Seven: Application of research to dietetic practice

From this study we can see that serum vitamin B12 is less than ideal as a primary marker of vitamin B12 status. Until the issues with serum vitamin B12 are rectified or a new marker of status becomes the norm, it is important that we examine serum vitamin B12 results further before deciding that they are correct. Our study has shown that serum vitamin B12 may give either a false positive or a false negative when it comes to diagnosing vitamin B12 deficiency. As such, it is important to analyse each patient with a fresh outlook; using all the available information to determine if the serum vitamin B12 levels fit with the assessment of the patient. Without clinical symptoms of deficiency it is important to look at metabolite levels to get a better picture of what is going on within the body. It is also important to consider other factors that may be influencing metabolite levels such as folate or renal status.

As well as this, it is important to recognise that individuals using oral contraceptive pills may be falsely diagnosed as vitamin B12 deficient. This however does not mitigate the fact that women of reproductive age using OCPs are still at risk of inadequate vitamin B12 intakes or malabsorption issues such as pernicious anaemia. As such, it is important that we do not dismiss all OCP users with low serum vitamin B12 levels. We must be sure that the decrease in serum vitamin B12 is caused by the OCP as opposed to a true deficiency.

Another area of concern is in individuals who drink excessive amounts of alcohol. While these individuals are putting themselves in danger of a number of health consequences, it is important to remember the potential result this pattern of alcohol consumption may have on their vitamin B12 status.

This research adds to the growing evidence that we may be off track with diagnosing vitamin B12 deficiency, this research suggests that it is time we look into other avenues of

diagnosing deficiency. Holo-transcobalamin II is the likely successor to the primary diagnostic stage of vitamin B12 deficiency. Before this can happen however, we must endeavour to further our understanding of this biomarker of status. We are still unable to utilise holo-TC II to the fullest of its potential as we are still lacking in understanding of the factors which affect it. As such, we must continue research in this area if we are to have a hope of finding a better alternative to serum vitamin B12 as the primary marker of vitamin B12 status.

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## Appendix A: Vitamin B12 content of whole foods

Vitamin B12 content per 100g of whole foods.

Food	Vitamin B12 content ( $\mu\text{g}/100\text{g}$ )	References
<b>Beef Liver</b>	83 $\mu\text{g}$	36
<b>Lean Beef Cuts</b>	0.7-1.5 $\mu\text{g}$	37
<b>Chicken Meat</b>	Trace-1.0 $\mu\text{g}$	38, 39
<b>Lamb Meat</b>	1.6-1.8 $\mu\text{g}$	39
<b>Milk</b>	0.3-0.4 $\mu\text{g}$	33, 39
<b>Eggs</b>	0.9-1.5 $\mu\text{g}$	33, 39, 41, 42
<b>Shellfish</b>	$\geq 6.0$ $\mu\text{g}$	44
<b>Fish</b>	$\sim 3.0$ -159 $\mu\text{g}$	33, 36, 46

### Vitamin B12 in animal products

#### Meat

Currently the United States Department of Agriculture (USDA) database reports the vitamin B12 contents of cooked beef liver to be approximately 83  $\mu\text{g}/100\text{g}$  (89). Quite differently, lean cuts of beef have relatively less B12, containing 0.7-1.5  $\mu\text{g}/100\text{g}$  (90). This difference is not limited to beef, offal tends to have higher concentrations of B12 than its lean meat counterparts in all animals (89). Chicken meat has relatively small amounts of B12 when compared to beef, with only trace-1.0  $\mu\text{g}/100\text{g}$  (45, 91). Lamb on the other hand is comparable to beef having 1.6-1.8  $\mu\text{g}/100\text{g}$  (91).

#### Milk

Milk is a major part of many individuals' diets, and while the vitamin B12 content (0.3-0.4  $\mu\text{g}/100\text{g}$ ) of various milk varieties is not high (39, 91), it is still a significant contributor of vitamin B12 for many individuals. The intake of dairy products in general populations is high (92) and as such the overall amount of B12 consumed from these products is significant.

### Egg

Vitamin B12 content of the whole egg is around 0.9-1.5 µg/100g (39, 91, 93). Within the egg however the majority of the B12 is contained within the yolk (91, 94). Vitamin B12 consumption from eggs is again generally considered to be large because of the large and growing consumption of eggs around the world (95).

### Shellfish

Currently various varieties of shellfish are consumed around the world. Edible shellfish that siphon large amounts of vitamin B12 producing microorganisms have been shown to be outstanding sources of Vitamin B12, with concentrations having been shown to equal or exceed 6 µg/100g (96).

### Fish

Fish (as well as shellfish) are another source of vitamin B12 which is becoming increasingly important around the world. Between 2006 and 2011 the annual consumption of fish and aquaculture increased from 114.3 million tonnes to 130.8 million tonnes and is expected to continue rising (97). Current USDA data shows that for general fish (salmon, trout, tuna etc.) a vitamin B12 content of ~ 3.0-11.0 µg/100g. There is evidence however to show that the content of skipjack tuna ranged from 10-159 µg/100g depending on the type of muscle consumed (39). Similar results were also found in another study by Nishioka (98) looking at the vitamin B12 content of yellow fin tuna.

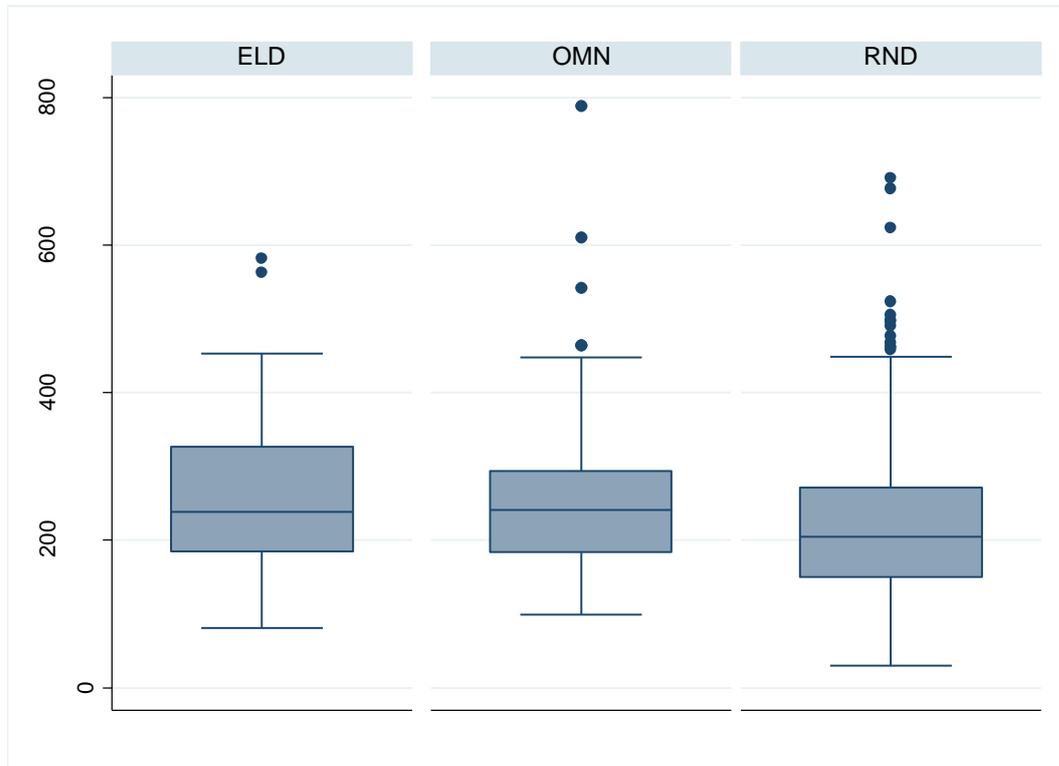
## Appendix B: Bioavailability

*Bioavailability of vitamin B12 in different animal products, adapted from O'Leary & Samman (45)*

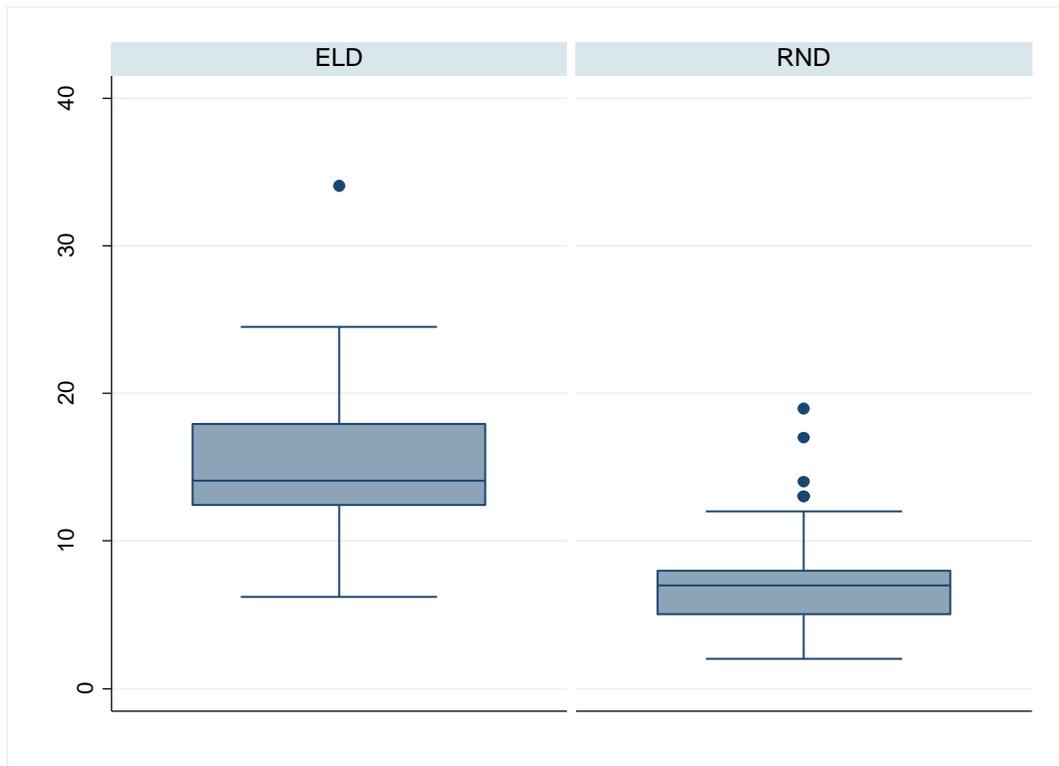
Food type	Subjects	Vitamin B12 Content	% Absorption, mean (range)
Mutton	3 healthy young subjects	0.9 µg per 100g portion	65 (56-77)
	2 healthy young subjects	3.03 µg per 200g portion	83
	2 healthy young subjects	5.11 µg per 300 g portion	53
Liver Pate	6 healthy young subjects	38 µg per serve	9.1 (5.1-19.5)
	4 older subjects	38 µg per serve	4.5 (2.4-6.0)
	5 subjects with pernicious anaemia	38 µg per serve	1.8 (0-3.7)
Chicken	3 healthy young subjects	0.4-0.6 µg in 100g portion	65 (58-74)
		0.8-1.3 µg in 200g portion	63 (48-76)
		1.3-1.9 µg in 300g portion	61 (49-75)
Fish	3 healthy young subjects	2.1 µg in 50g portion	42
		3.1 µg in 100g portion	38
		9.2 µg in 200g portion	42
		13.3 µg in 300g portion	30
Eggs: boiled, scrambled, fried	18 healthy young subjects	0.9-1.4 µg in 100g portion	3.7-9.2

## Appendix C: Box plots of vitamin B12 markers

### C.1 Box plot of serum vitamin B12 concentrations sorted by group



## C.2 Box plot of serum tHcy sorted by group



### C.3 Box plot of serum MMA sorted by group

