THE PREVALENCE AND TREATMENT OF METFORMIN ASSOCIATED
VITAMIN B₁₂ DEFICIENCY IN PATIENTS WITH TYPE 2 DIABETES
MELLITUS IN NEW ZEALAND

BY

SYLVAN HAEUSLER

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With an ever increasing incidence of type 2 diabetes in the New Zealand population, continual assessment must be made to ensure the ongoing evaluation of treatment options to ensure patient care is not compromised. Previous research has shown higher prevalence of Vitamin B12 deficiency in patients with type 2 Diabetes Mellitus who have had long term metformin treatment. An investigation into patients attending secondary care clinics at the Diabetes Clinic at Wellington Regional Hospital as well as primary health care patients has explored the prevalence of this interaction in the New Zealand population as well as assessed the factors that may contributed to such an effect. It was found that there is a high prevalence of decreased serum Vitamin B12 level in patients with type 2 Diabetes exposed to metformin treatment. Factors such as age and ethnicity were seen to be key predictors of this interaction and have shown a unique view of how the population of New Zealand may be affected.

Treatment of decreased serum Vitamin B12 levels was seen to be effective through both sublingual and intramuscular methods with variations in both short and long term effects. The use of sublingual treatment administration of Vitamin B12 supplementation is unique in the scope of metformin induced Vitamin B12 deficiency and has shown potential to be a therapeutic route for patients affected by such an interactions. Current views on the interaction between metformin and serum Vitamin B12 levels are currently under debate in regards to physiological relevance and clinical outcomes. This paper provides a first look at the effect of this interaction in a New Zealand population and evaluates treatment options for such. Furthermore a proposal is made for an alternate mechanism of metformin action on Vitamin B12 levels which may aid understanding of observations made in both in this paper and previous studies.

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1.5 – List of Abbreviations

In order of appearance

DM	Diabetes Mellitus
UK	United Kingdom
VDR	Virtual Diabetes Registry
β	beta
α	alpha
UKPDS	United Kingdom Prospective Diabetes Study
DCCT	Diabetes Control and Complications Study
HbA1c	haemoglobin A1c
EDIC	Epidemiology of Diabetes Intervention and Complications
ADA	American Diabetes Association
AMP	adenosine monophosphate
AMPK	AMP activated protein kinase
ATP	adenosine triphosphate
LKB1	liver kinase B1
CREB	cAMP response element-binding
TORC2	transducer of regulated CREB protein 2
Mtor	mammalian target of rapamycin
Cbl	cobalamin
Me	methyl
OH	hydroxide
CN	cyanide
FDA	Food and Drug Administration

Hcy	homocysteine
MMA	methylmalonic acid
DNA	deoxyribonucleic acid
GIF	gastric intrinsic factor
TCII	transcobalamin II
CoA	co-enzyme A
RNA	ribonucleic acid
SAM	S-adenosylmethionine
tHcy	total homocysteine
S-LANSS	self-report Leeds Assessment of Neuropathic Symptoms and Signs
NZSSD	New Zealand Society for the Study of Diabetes
PHO	Primary Health Organization
SD	standard deviation
ANOVA	analysis of variance
CI	confidence interval
LSD	least significant difference
NZ	New Zealand
Q-Q	quantile-quantile
MNSI	Michigan Neuropathy Screening Instrument
NHMRC	National Health and Medical Research Council
PPI	proton pump inhibitor

2.0 Introduction

2.1 – Diabetes

The number of people with type 2 diabetes is expected to double between the years 2000 and 2030 (J. E. Shaw, Sicree, & Zimmet, 2010). A combination of population growth, population aging, and increased rates of obesity as urbanization and commercialization sweeps the globe has increased the incidence of diabetes in recent years. It is estimated that some 382 million people globally now have some form of diabetes, of which approximately 46% of cases are undiagnosed (International Diabetes Federation, 2013). This steep increase in incidence has led to a high mortality in many countries, up to 76% of all deaths under the age of 60 in some African countries as well as a large financial burden on health care systems; costing the United States of America \$245 billion dollars alone in the last year and an estimated £1 million each and every hour in the UK (Diabetes UK, October 2008). The current trend of incidence suggests that the number of patients suffering from diabetes in the year 2035 will be above 590 million worldwide with developing countries increasing in incidence at higher rates than those in most developed countries (International Diabetes Federation, 2013).

Globally type 2 diabetes makes up more than 90% of confirmed diabetes cases diagnosed (Zimmet, Alberti, & Shaw, 2001). While traditionally the disease was not seen in younger patients, this has been observed to be a changing trend. Increased rates of childhood obesity are resulting in increasing incidence of type 2 diabetes in young people (Farsani, van der Aa, van der Vorst, Knibbe, & de Boer, 2013). High levels of heritability (Rathmann, Scheidt-Nave, Roden, & Herder, 2013) coupled with a lack of patient education has led to families and communities being stricken by the epidemic. The ease of access to high sugar and fat containing low cost food has fuelled this rise in many developed as well as developing countries (Hu, 2013).

Diabetes surveillance in New Zealand has been greatly strengthened by the development and utilisation of the Virtual Diabetes Register. The Virtual Diabetes Register (VDR) allows for the pooling of information through various health system levels to form a comprehensive over view of

the prevalence across the country. The VDR filters information gathered from hospital admissions, outpatient services, pharmaceutical dispensaries, pathology tests, and primary health organization enrolment data to allow analysis on a nationwide level. Figures released in December 2012 identified a total of 225,731 patients who had been identified with diabetes by the VDR. Of this entire population there are increased numbers of diabetes cases within certain ethnic groups suggesting increased risk rates with people of Pacific identity having a prevalence of type 2 diabetes of up to 10% in adults (*A Portrait of Health: Key results of the 2006/2007 New Zealand Health Survey.*, 2008). According to Ministry of Health data the incidence of diabetes in New Zealand is still increasing. Current estimates of prevalence of diabetes in New Zealand were up to 7.0% in 2008/2009 (Coppell et al., 2013) with a pre-diabetes prevalence of 25.5%. It is believed that part of the recent increase in incidence is a result of increased detection. Diagnosis has been seen to increase the ratio of diagnosed to undiagnosed patients with specific improvements in Maori populations, much improvement is still needed with some populations reporting an undiagnosed rate of 6.4% of the entire population (Coppell et al., 2013). The prevalence of pre-diabetes in the population with high risk groups reaching 35.8%, in obese men, is of great concern for the potential further explosion of diabetes. A large pre-diabetic population coupled with an earlier onset of diabetes type 2 in youth populations is certain to lead to increased burden on the health sector in the coming years and requires constant and vigilant monitoring combined with proactive and preventative care strategies.

The disease state of diabetes highlights the need for constant homeostasis within the body and the damage that can result if these checks and balances are not constantly maintained at optimal levels. Glucose is derived from the digestion of food containing carbohydrate. As the glucose is absorbed into the blood stream the pancreas monitors the blood glucose level to ensure that it does not become too high (after a meal) or too low (during a fasting period). This function is predominantly carried out through the secretion of two opposing hormones, insulin and glucagon. As glucose levels rise as a result of food digestion it enters the β cells in the pancreatic islets. The influx of glucose signals the β cells to release the hormone insulin into the blood stream which is then transported to cells throughout the body and promoting cellular uptake of glucose. Muscle cells use the glucose for energy in biochemical reactions. Other target sites such as in the liver also result in further downstream mechanisms resulting from insulin interaction. Insulin uptake by the liver

results in the increased absorption of glucose from the blood and conversion of this glucose into glycogen which acts as an energy storage molecule. During fasting periods when blood glucose levels are low, the pancreas releases glucagon via α cells. This hormone enters the blood stream and acts on target cells in the liver, this signals the liver cells to break glycogen down into glucose and the molecules are released back into the blood to maintain a supply of glucose to organs that require a constant supply such as the brain.

Diabetes is the result of the inability of the body to maintain blood glucose levels at optimal levels. Glucose derived from food digestion cannot be absorbed by target cells and continues to rise as continual glucose is absorbed from the diet. The kidney has limited ability to reabsorb filtered glucose, such that above a plasma concentration of approximately 10mmol/L, there is loss of glucose in the urine. This results in an osmotic gradient which leads to increased water loss and polyuria. Higher urine production leads to thirst as excessive amounts of water are expelled from the body. The excess glucose in the urine also increases the risk of urinary infections as bacteria are supplied with an ample source of energy in which to thrive. High circulating glucose concentrations also results in other symptoms of uncontrolled diabetes such as blurred vision as the osmotic effect causes fluid shifts from the lens in the eye distorting its shape. High blood glucose also impairs the function of white blood cells and leads to slow healing of soft tissue infections. As the body is not able to derive energy from the circulating glucose molecules, patients with untreated type 1 diabetes can often feel tired or lethargic as their body attempts to find energy sources to maintain body functions. One way the body does this is by breaking down fat stores in the body resulting in weight loss.

Type 1 diabetes is characterised by the autoimmune destruction of the β cells in the pancreas which are responsible for the synthesis and release of insulin (Battaglia, 2014). In type 2 diabetes the β cells are still initially functional and still release insulin. However the insulin responsive tissues such as liver, skeletal muscle and adipose, do not respond as efficiently to insulin to promote glucose uptake into cells. In addition, the beta cell function declines and progressively generates less insulin. One proposed mechanism that leads to these changes is excess deposition of fat in the non-adipose tissues, particularly liver and muscle. This results in impairment of insulin signalling and glucose

uptake, but also reduced switching off of glycogen breakdown in the liver and release of glucose into the circulation. The pancreas eventually fails to keep up with the required insulin production and loses function itself. Both of type 1 and type 2 diabetes result in excess blood glucose levels and the effects described above.

The increase of glucose concentrations in the blood if not treated can lead to both microvascular and macrovascular complications. Damage to the blood capillaries through microangiopathy can lead to the development of many complications. Endothelial cells lining capillaries are constantly exposed to increased glucose concentrations and increased glucose uptake results in the formation of excess glycoproteins on the cell surface. This leads to the thickening and weakening of the basement membrane. This process has been attributed to the development of cardiomyopathy, nephropathy, neuropathy and retinopathy. These impairments can lead to visual symptoms, chronic kidney disease, and commonly the loss of sensation in the peripheral nervous system (Nathan & Grp, 2014). Additionally, some research in type 1 diabetes suggests that the autoimmune response, responsible for the pancreatic destruction, may also contribute to some of these microvascular complications (Ichinose, Kawasaki, & Eguchi, 2007).

Vascular abnormalities are not limited to capillaries; macrovascular complications of diabetes are a common cause of fatality in patients with diabetes. The development of atherosclerosis, subsequent plaque rupture and overlying clot formations in the large vessels of the body increase the likelihood of ischemia and subsequent cardiovascular events such as myocardial infarction, stroke or peripheral vascular disease. Patients with type 2 diabetes may be more susceptible to macrovascular disease because of additional risk from hypertension and dyslipidaemia associated with obesity. Intensive treatment of glucose levels in diabetes, aiming for near-normal glucose concentrations has seen reduction in both micro and macrovascular complications and improve outcomes for those with diabetes (Rensing et al., 2011; Rosenson, Fioretto, & Dodson, 2011).

The evidence for the benefits of good glycaemic control are numerous and convincing. Many large scale studies have evaluated the many facets of diabetes management and pathogenesis in order to form a comprehensive picture of the treatment effects. The United Kingdom Prospective Diabetes Study (UKPDS) is one such study that has shown significant improvements in health following a strict glycaemic control program. The randomized multicentre intervention trial studied the effects of treatment such as diet, insulin and metformin on complications of diabetes, associated morbidity and mortality. The study has shown that intensive glycaemic control resulted in decreases of diabetic microvascular complications (Mooradian & Chehade, 2000; R. C. Turner & Holman, 1996; Wright, Cull, Macleod, Holman, & Grp, 2006). While initial results from the study showed only modest improvements in cardiovascular disease risk, this was seen to be more significant following analysis of metformin treatment.

By the three month time period the patients randomized to metformin treatment had shown reductions in fasting blood glucose, similar to insulin and sulfonylureas, but also showed improvements in fasting plasma insulin and no weight gain. Furthermore the number of hypoglycaemic episodes was much lower than those in sulphonylureas or insulin therapy. Evaluation of diabetes related complications showed metformin treatment had a lower risk of any diabetes-related endpoint, diabetes related-death, all-cause mortality, myocardial infarction (39%) and all macrovascular diseases when compared to diet alone treatment. UKPDS showed that metformin is as effective as sulfonylureas and insulin in glycaemic control, but without weight gain and a lower risk of hypoglycaemia (Group, 1995).

The Diabetes Control and Complications Trial (DCCT) was a large scale assessment of the effects of a tight glycaemic management program on the complications of type 1 diabetes (Nathan & Grp, 2014). The study aimed to compare intensive treatment of patients with type 1 diabetes in order to maintain a median HbA1c of 7%, to conventional treatment of patients with maintenance of HbA1c of 9%. The results from DCCT provided evidence for the benefits of aiming for near normal glycaemic control. However the trade-off of tight glycaemic control was an increased rate of hypoglycaemia and weight gain. The results have shown that intervention with strictly decreased glucose level results in improvements in all areas of microvascular and cardiovascular complications of type 1 diabetes. Presence of early stage microvascular complications were reduced by 35-76% in patients exposed to intensive glycaemic control. Major beneficial effects of intensive control were

seen in, retinopathy, nephropathy, and neuropathy. Measurements of atherosclerosis have revealed less atherosclerosis in the intensive treatment group, corresponding to decreases in clinical presentations of fatal and nonfatal myocardial infarctions and stroke, a reduction of 58% in cardiovascular related disease events after 18 years of follow-up from the beginning of the DCCT. However the observational study, The Epidemiology of Diabetes Interventions and Complications (EDIC) which was used concurrently with the DCCT, has shown that the differences in the HbA1c of each of the treatment groups deteriorated in the later periods of patient monitoring. This phenomenon was labelled “metabolic memory” and was seen to effect all microvascular complications of diabetes and relate to glycation of proteins (Monnier et al., 1999).

American Diabetes Association (ADA) guidelines for the management of type 2 diabetes state the initial pharmacological intervention suggested is in the form of metformin ("Executive summary: Standards of medical care in diabetes--2013," 2013). If noninsulin monotherapy does not alleviate the increased glucose levels in patients after 3 months then a second agent such as a glucagon-like peptide 1 receptor agonist is indicated. The initiation of insulin is required in markedly symptomatic patients with newly diagnosed type 2 diabetes or as an outcome of disease progression beyond the control of oral agents.

This front line role of metformin is similar in many guidelines set by healthcare agencies. New Zealand and Australian standards both recommend commencement of metformin treatment if no contradictions are present and inadequate improvements due from diet or lifestyle changes.

2.2 – metformin

Metformin has been used to combat high blood glucose since middle age Europe where its active ingredient was extracted from the French lilac plant. It belongs to the biguanide class of oral antidiabetic drugs. However its physiological benefits stretch further than its antihyperglycaemic properties. A range of additional affects such as improvements in endothelial function, haemostasis and oxidative stress, insulin resistance, lipid profiles, and fat redistribution accompany the primary glycaemic function of metformin (Aguayo Rojas & Gomes, 2013). While the biochemical mode of action of metformin is still not completely understood, its utilization is vast and it is still considered to be the first-line oral antidiabetic treatment for patients with type 2 diabetes (Bouchoucha, Uzzan, & Cohen, 2011).

The action of metformin on glucose metabolism is dependent on the presence of insulin. The main site of metformin action is thought to be in the liver where the drug suppresses glucose production. It promotes glucose uptake by tissues via the glucose transporters, and inhibits hepatic gluconeogenesis (Bouchoucha et al., 2011). It is thought that the main mode of action for the antihyperglycaemic property of metformin occurs via the activation of adenosine monophosphate (AMP) – activated protein kinase (AMPK). AMPK is activated in the brain by metabolic stresses that inhibit ATP production such as ischemia, hypoxia, glucose deprivation, metabolic inhibitors (such as metformin), as well as catabolic and ATP consuming processes.

More in-depth analysis of biochemical regulations affected by metformin has shown that these effects are mediated by the activation of an upstream kinase, liver kinase B1 (LKB-1), this in turn regulates the downstream kinase adenosine monophosphatase co-activator. This allows the transduction of regulated CREB protein 2 (TORC2), resulting in its inactivation and thus the down regulation of transcriptional events that promote synthesis of gluconeogenic enzymes. Inhibition of mitochondrial respiration has also been proposed to contribute to the reduction of gluconeogenesis as it directly reduces the energy supply required for this process (R. J. Shaw et al., 2005).

Further to its kinase interactions there are numerous additional actions of metformin such as alterations to small intestine glucose metabolism and small bowel absorption that alter the absorption profiles of some vitamins (Qureshi, Ainsworth, & Winocour, 2011).

Metformin has consistently shown improvements not only in glycaemic control but also in cardiovascular management (Rojas & Gomes, 2013). Evidence for the ongoing utilization of metformin as a front line anti-diabetic drug in both obese and non-obese patients is strongly supported (Ito et al., 2010). The progression of type 2 diabetes often results in an increased dose of drug being required at later disease stages, however even small dosages can improve HbA1c and diabetic outcomes.

The most common side effects of metformin treatment are gastrointestinal – including nausea, vomiting and diarrhoea. These usually minor side-effects are observed in approximately 30% of patients, and lead to treatment discontinuation in 5% of patients (Rojas & Gomes, 2013). The gastrointestinal side effects are commonly addressed by using slow titration of dosages to wean patients onto the drug. Commonly, but not universally, people become more tolerant to these side effects over time. There have also been associations with the potentially serious and sometimes fatal side-effect of lactic acidosis, although there is much controversy over the real risk conferred by metformin.

Further studies into alternate applications of metformin treatment and observation of additional physiological outcomes of the use of the drug have resulted in advantageous outcomes for reducing incidence and mortality in patients suffering from a variety of carcinomas (Zhang, Li, Tan, Chen, & Wang, 2013; Riedmaier, Fisel, Nies, Schaeffeler, & Schwab, 2013). Studies into the effect of metformin in cancer patients has suggested that the glycaemic control of metformin, which involves the absorption of glucose by tissue such as large muscle, may aid in the reduction of tumours as it limits the amount of sugar available to the uncontrolled proliferating cells which are characteristic of carcinoma growth. Investigation has suggested that the use of metformin can directly inhibit cancer cells by initiating the LKB1/AMPK/mTOR axis which works to regulate energy metabolism as well as protein synthesis within the cells. A meta-analysis identified four different cancer types which showed marked improvement in incidence and mortality if patients had been treated with metformin. The risk of developing liver, pancreatic, colorectal and breast cancers were reduced by 78%, 46%, 23% and 6% respectively. Mortality was reduced in liver cancer to

0.23 and breast cancer reduced to 0.63 (Zhang, Li, Tan, Chen, & Wang, 2013). metformin has also been seen to provide decreased likelihood of hepatocellular carcinoma development in patients suffering from chronic hepatitis C virus(Harris & Smith, 2013).

2.3 – Vitamin B12

Vitamin B12, also referred to as cobalamin (Cbl), is one of eight B group vitamins and is thought to play a large role in the formation of blood as well as key roles in brain and nerve function. The vitamin has several different structural forms depending on method of synthesis and processing. Hydroxocobalamin is the most commonly formed structure synthesized by bacteria; it is also the form in which the compound is found in the intramuscular injection commonly used to treat deficiencies. The extraction of hydroxocobalamin in pharmacological manufacturing can result in the compound being converted to cyanocobalamin through the change of a ligand group. This form of the vitamin is considered semi-synthetic and is commonly used due to its low cost and high stability. Conversion of both hydroxocobalamin and cyanocobalamin to the human physiological form adenosylcobalamin and methylcobalamin occurs in the body in order for bioavailability of the vitamin to take place.

The key structural changes that occur between the different forms of Vitamin B12 occur in the upper axial ligand (β -axial ligand). Derivatives of physiological importance, adenosylcobalamin (co-enzyme B12) and methylcobalamin have a 5-deoxy-5-adenosine or methyl group as the upper axial ligand respectively. Cyanocobalamin contains a cyanide group in the β -axial ligand (Takahashi-Iniguez, Garcia-Hernandez, Arreguin-Espinosa, & Flores, 2012).

Figure 2.3.1 – Chemical structure of cobalamin, R group represents either, 5'-deoxyadenosyl, Me, OH, or CN depending on form of cobalamin

Vitamin B12 is produced by bacteria and fungi. While there are certain bacteria in the human large bowel which produce the vitamin, this form is not absorbed and therefore humans need to obtain the vitamin through dietary sources. The sources which are highest in concentration of Vitamin B12 are animal products such as meat and eggs with varying levels depending on source. Meat sources such as shellfish and beef have the highest concentration of Vitamin B12 with upwards of 70 micrograms per serving, about 1,000 % of the daily value recommended by the United States Food and Drug Administration (FDA). Other sources such as fish and fortified cereals have much lower levels of Vitamin B12 (between 2 and 6 micrograms per serving) whilst sources such as milk and eggs have lower range levels (1.2 and 0.6 micrograms per serving respectively) (*Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline*, 1998; Montville et al., 2013). This creates a problem for people who adhere to a strict vegetarian diet and supplementation via intramuscular injection and/or oral supplements may be required. Certain algae and seaweeds have been proposed as sources of Vitamin B12 however the bioavailability of the vitamin from these sources is still disputed (Kwak, Park, & Cho, 2012) . In a

western country the average non-vegetarian diet includes around 5 to 8 micrograms of cobalamin daily. This is higher than the recommended daily allowance of 2.4 micrograms for adults, although this is higher in pregnant or lactating women (2.6 and 2.8 micrograms respectively). A strict vegetarian however may consume only 0.5 micrograms a day and therefore be at the risk of deficiency. Due to our large natural stores of the vitamin it may take an extended period of time for a deficiency to develop however. The mode of absorption, trafficking and metabolic exchange that take place with Vitamin B12 highlight the rarity and essential nature of the compound (Gueant & Alpers, 2013). Although the compound is seen to be carefully monitored throughout its metabolic pathway through the body system, it has been identified as being involved in only two important metabolic processes in the human body. These include the metabolism of Homocysteine (Hcy) and Methylmalonyl-CoA (MMA). In a broader sense, B12 is involved in the formation of red blood cells, to sustain function in the nervous system, DNA synthesis and cell proliferation.

The journey of Vitamin B12 from intake to absorption follows the binding of the compound to several different protein structures and the involvement of numerous digestive enzymes. Vitamin B12 initially binds to haptocorrin following proteolytic digestion by pepsin in the low pH environment of the stomach (Gherasim, Lofgren, & Banerjee, 2013). Haptocorrin are Vitamin B12 binding proteins that are produced in the salivary glands; however the actual binding does not occur until pepsin frees the vitamin from the proteins in the food. The Vitamin B12 – haptocorrin complex is referred to as “holo-R-protein” which remains bound until degradation by pancreatic proteases released into the upper small bowel. This allows the Vitamin B12 to be bound to gastric intrinsic factor (GIF), forming cobalamin-IF. This new complex travels to the distal ileum where recognition of its receptor which are selective for cobalamin-IF and not holo-R-protein allows the uptake of the vitamin complex (Alpers & Russell-Jones, 2013). The receptors are located on the enterocytes in the ileum thus uptake of Vitamin B12 is through these cells. While the exact processes that occur within these enterocytes are not fully understood, the cobalamin appears bound to transcobalamin II (TCII) in the portal blood. The TCII-cobalamin complex is rapidly cleared due to specific binding sites on numerous cells. Internalization of TCII-cobalamin leads to dissociation of the complex and conversion of the cobalamin into its physiologically active forms. Both transcobalamin and intrinsic factor bind cobalamin in a base-on conformation, however the binding mode for haptocorrin depends on the lower axial ligand, this allows for the clearance of

analogous to Vitamin B12 from the blood through selective binding to the haptocorrin transport protein (Gherasim et al., 2013).

The two forms of cobalamin that are found in the human body both have differing physiological roles. Adenosylcobalamin acts as a coenzyme of methylmalonyl-CoA mutase, the main function of this protein is the conversion of methylmalonyl-CoA to succinyl-CoA. Methylcobalamin works as a co-enzyme of methionine synthetase which catalyses the movement of the methyl group of the cobalamin molecule to homocysteine thus forming methionine, methylcobalamin is regenerated by further methyl group transfer. Methionine undergoes further biochemical processing to form adenosyl-methionine, a methyl group donor responsible for the methylation of many proteins, neurotransmitters and nucleic acids (Gherasim et al., 2013). The metabolism of methionine synthase requires a balance of carbon molecules which can lead either towards synthesis of DNA and RNA or the methylation of substrate compounds by S-adenosylmethionine (SAM). The role of Cbl in this function may therefore alter some epigenetic properties of an individual through specific methylation of DNA, RNA, histone and gene expression which may result in a variety of physiological differences (Gnimpieba, Eveillard, Gueant, & Chango, 2011).

Clinical presentations of Vitamin B12 deficiency are difficult to identify as being a unique symptom of the condition. One of the most common presentations is a feeling of fatigue or “general tiredness”; this relates to the role that Vitamin B12 plays in the regulation of sleep patterns. Further long term presentations may include neuropathy of the feet as well as (in very deficient cases) the development of anaemia. A key problem area in the identification of Vitamin B12 deficiency is the overlapping symptomatic expressions with diabetes. Unexplained fatigue may be attributed to low sugar levels as a result of medication or alterations in diet or eating habits and therefore may be misinterpreted as other problems. Neuropathy as a result of Vitamin B12 deficiency is particularly difficult to identify as it mimics that of diabetic neuropathy and can take a long period of time to develop. The currently used biochemical markers for diagnosis of Vitamin B12 deficiency are under much debate. The traditional serum Vitamin B12 assessment has been suggested to not be sufficient in predicting the presence of Vitamin B12 deficiency (Obeid et al., 2013). The current use of serum Vitamin B12 level relies on several different range points for Vitamin B12 status. Serum Vitamin B12 level under 148 pmol/L are said to be deficient, low/borderline levels are interpreted from

levels between 148-220 pmol/L (Mazokopakis & Starakis, 2012). Normal ranges of Vitamin B12 serum range from 220- 800 pmol/L, levels that exceed this are rarely suspicious in cause, although excessive B12 serum may be a marker of myeloproliferative disorders. Analysis of biochemical markers of methylmalonic acid (MMA) and total homocysteine (tHcy) are said to be more definitive at obtaining a correct diagnosis of deficiency with levels $>0.28 \mu\text{mol/L}$ and $>15 \mu\text{mol/L}$ respectively being seen as elevated levels and markers of cobalamin deficiency however the cost of such assessments is often a barrier (Mazokopakis & Starakis, 2012).

The development of Vitamin B12 deficiency can be triggered by numerous different pathways and identification of the causative source of deficiency is crucial to treatment administration. Some factors that may lead to eventual deficiency in Vitamin B12 include; gastric abnormalities (pernicious anaemia, bariatric surgery etc.), small bowel disease, diet (strict vegetarian, vegan), agents that block absorption (neomycin, metformin etc.), impaired utilization (congenital transcobalamin II deficiency), and biological competition (bacterial overgrowth etc.) (Mazokopakis & Starakis, 2012). While the body usually has a massive store of Vitamin B12 ($\approx 2500\mu\text{g}$) this store can be slowly depleted via any of the above avenues leading to either a decreased or deficient state.

Developments of megaloblastic changes have been observed in Vitamin B12 deficient environments, however the mechanism for this is not precisely known. Due to the disruption of DNA synthesis proliferating cells have been observed in cell cycle arrest in epithelial, peripheral blood, and most strikingly in bone marrow cells as a result of Vitamin B12 deficiency. An increase in mean corpuscular volume is seen to be the earliest sign of megaloblastosis followed by irregularities in the cell shape (Briani et al., 2013).

Vitamin B12 plays a crucial role in the nervous system where it acts as a coenzyme for methyl malonyl-CoA mutase reaction, a required step in myelin synthesis. Loss of myelin formation can lead to several central and peripheral nervous system pathogenic states such as neuropathy, neuropsychiatric abnormalities, myelopathy and possible optic nerve atrophy. The development of

neuropathy due to Vitamin B12 deficiency usually begins with sensory impairment in the lower limbs. This is commonly seen to correspond with a loss of vibratory sensation, diminished proprioception and loss of cutaneous sensation (Senol, Sonmez, Ozdag, & Saracoglu, 2008). Neuropsychiatric syndromes associated with Vitamin B12 deficiency include chronic fatigue syndrome (Dommissie, 1991), cognitive impairment and depression (Kibirige, Wekesa, Kaddu-Mukasa, & Waiswa, 2013).

2.4 – metformin induced Vitamin B12 deficiency

The association between metformin treatment and impaired Vitamin B12 status dates back to the 1970s when the interaction was first proposed (Stowers & Smith, 1971), and estimates of prevalence were stated to be close to 30%. Since then the prevalence of metformin associated Vitamin B12 deficiency has been reported in many different studies, however the ranges of incidence in such populations vary by a large amount. While many studies estimate the rate of prevalence to be around 10-20% of treated patients (Calvo Romero & Ramiro Lozano, 2012; Leung et al., 2010), other studies have reported levels as low as 5% of treated patients (Reinstatler, Qi, Williamson, Garn, & Oakley, 2012) and levels higher than 30% (Nervo et al., 2011). Data available in New Zealand in regards to Vitamin B12 deficiency is not metformin specific but rates in elderly patients (aged 65 and above) suggest that acquired Vitamin B12 deficiency could be as high as 35% (Green, Venn, Skeaff, & Williams, 2005). As there are multiple factors affecting the risk of acquiring the deficiency it is imperative to correctly identify the key influences in attempt to minimize a compounding effect.

The mechanism for metformin induced Vitamin B12 deficiency is still to be definitively deduced. Some of the earliest studies to suggest the relationship were published in the 1970's (Stowers & Smith, 1971; Tomkin, 1973), with data from over 100 patients being compiled into a report that highlighted not only the significance of duration of therapy required to lower the Vitamin B12 levels, but also suggested a link between therapeutic dose and overall decrease in the vitamin level.

Stowers *et al* in the British Medical Journal in 1971 said:

“We would like to support the recommendation of Dr. Tomkin and others that all patients on long-term therapy with metformin should have an annual estimation of serum vitamin B12, for it is otherwise quite easy to miss cases of classical macro cytic anaemia and/or signs of subacute degeneration of the cord” (Stowers & Smith, 1971)

The current evidence supports a mechanism in which metformin interaction is isolated to calcium channel interactions and an impairment of calcium-dependent membrane activity in the ileum (Bauman, Shaw, Jayatilleke, Spungen, & Herbert, 2000) which leads to malabsorption of Vitamin B12 bound to intrinsic factors. Studies of the association of metformin and Vitamin B12 effect have yielded numerous related findings (Reinstatler et al., 2012), as well as many conflicting views of mechanisms. A measurable reduction in Vitamin B12 levels can occur as quickly as 3-4 months after the initiation of the metformin therapy (Bauman et al., 2000), while symptomatic deficiency may take as long as 5 -10 years to manifest. Many papers agree on the prevalence of lowered B12 levels, reported at between 10 – 20% of patients taking metformin (Calvo Romero & Ramiro Lozano, 2012; Leung et al., 2010; Liu, Dai, & Jean, 2006; Mazokopakis & Starakis, 2012). However there have been several theories into the exact mode of this malabsorption (Musarrat, Kalathil, & Varughese, 2008; Nervo et al., 2011).

Due to the variety of pathogenic causes for a deficiency in Vitamin B12 there is no best fit mode of treatment for the deficiency. The most common method for treatment of Vitamin B12 deficiency is the use of intramuscular Hydroxocobalamin injections which are used in a loading regimen followed by a maintenance administration for long term treatment (Mazokopakis & Starakis, 2012). However there is a requirement for a less invasive and clinically reliant administration of treatment in order to address the large number of patients requiring supplementation. The investigation into efficacy of oral and sublingual routes of medication has resulted in comparable treatment success to that of the parenteral route for some cases, while others have shown insufficient corrections in serum Vitamin B12 level (Reinstatler et al., 2012). Due to the proposed mechanism of interaction of metformin on absorption of Vitamin B12 the use of sublingual supplementation has been gaining favour. Sublingual treatment has been shown to be just as effective as oral doses in patients not being treated with metformin (Delpre, Stark, & Niv,

1999; Sharabi, Cohen, Sulkes, & Garty, 2003; Yazaki, Chow, & Mattie, 2006) on both serum Vitamin B12 level as well as biomarkers of Vitamin B12 functionality. With the current evidence of Vitamin B12 malabsorption occurring at the intrinsic factor level (Bauman et al., 2000) it is thought that the use of sublingual Vitamin B12 supplementation should be more effective than oral administration in patients being treated with metformin; there is no current literature that has directly assessed this however. Using studies focused on patients following gastric bypass surgery as models for malabsorption of Vitamin B12 it has been seen that intramuscular injections are more effective in treatment than oral routes, however again the information on the sublingual route is not available. (Majumder, Soriano, Cruz, & Dasanu, 2013; Shakeri-Leidenmuhler et al., 2013).

2.5 – Previous Research

Metformin associated serum Vitamin B12 deficiency has been evaluated thoroughly and the association between the two is well documented, however the mechanisms of interaction and potential clinical outcomes of such are still in dispute. Evidence has been given to support the theory that the metformin interaction is isolated to calcium channel interactions and an impairment of calcium-dependent membrane activity in the ileum (Bauman et al., 2000). Calcium-dependent channels allow the GIF bound Vitamin B12 to be taken up for further processing; the suggestion that metformin inhibits the action of these channels would create a barrier for the GIF bound B12 and thus lead to malabsorption. However the study showed that while malabsorption decreased after calcium supplementation, there was no corresponding increase in serum Vitamin B12 level. The effect of calcium was not trialled independent of metformin and therefore may have an increasing effect on B12 absorption as opposed to a correcting of a metformin induced interaction. Even though the use of calcium would reverse the malabsorption it does not increase serum levels and is also potentially causes an increase in cardio vascular risk and therefore is not favoured, especially in patients with an already elevated risk level.

In a contrasting perspective, Obeid.R *et al.* (2013) suggest that while patients with type 2 diabetes may show a normal extracellular Vitamin B12 level, the intracellular markers used to measure the biochemical reactions in which Cbl takes place were abnormal compared with a control population (Obeid et al., 2013). It is further suggested that the use of metformin in type 2 diabetes does not lead to malabsorption of the vitamin, but rather “cures” Vitamin B12 resistance which leads to the lowered intracellular concentrations of metabolites which indicates a lower biochemical usage of the vitamin. With this influx of Vitamin B12 into the cell the extracellular measurements (serum etc.) are seen to drop. This suggested that there is no lack or inhibition on the metabolic pathways that Cbl is required for. The evidence provided for these claims was based on the red blood cell extracts and related metabolites of Cbl action (MMA, SAM/SAH ratio). It was observed that the metabolic markers for Vitamin B12 were lower in a population with diabetes (not on metformin) compared to a control population. The extra cellular markers were seen to be comparable or higher in the diabetic population. The population of patients with diabetes and taking metformin was seen to have a lower extracellular measurement (holotranscobalamin, serum Vitamin B12) but individuals seemed to have an improved cell content and metabolic profile of

Vitamin B12 (lower MMA, better methylation index) (Obeid et al., 2013). It is through this process of thought that the study suggests that Vitamin B12 congestion in blood is associated with intracellular deficiency, highlighted by elevated total homocysteine, leading to further evidence for the theory that there is cellular Vitamin B12 resistance in patients with type 2 diabetes. While there is currently no evidence for the claim that metformin uncovers the Vitamin B12 status in patients with type 2 diabetes, it is suggested that mechanisms such as changes to cellular membrane composition or alterations to the dynamics of glycosylation processes may hold the answer. The study concludes by claiming that metformin treatment is related not only to low serum Vitamin B12 but also to improvements in the intracellular Vitamin B12 content and markers for metabolism.

An *in vivo* animal trial carried out by Greibe. E *et al* (2013) aimed to explore the potential for alternate absorption patterns for vitamin B12 caused by metformin exposure (Greibe, Miller, Foutouhi, Green, & Nexø, 2013). This study incorporated the use of a study population of 18 Sprague Dawley rats. The rats were injected subcutaneously with either metformin or a saline control for 3 weeks. Following this exposure period the amount of Vitamin B12 was located and regionalized using radio-labelled B12. The results from the study reinforce what studies (Obeid et al., 2013) before have shown, that metformin treatment reduces serum Vitamin B12. It was found that a 22% reduction in Vitamin B12 levels was seen in rats exposed to metformin therapy for just 3 weeks. However the mechanism of reduction of serum levels is not investigated thoroughly. This lowering of Vitamin B12 in the blood was thought to be attributed to an increase of Vitamin B12 in the livers of treated rats which showed a 36% increase in vitamin content compared to control rats. It is suggested that this result may be consistent in humans with diabetes and taking metformin, where the serum level of Vitamin B12 is decreased due to an increased absorption proficiency and displacement of Vitamin B12 to the liver. Furthermore the study shows that the total amount of absorbed Vitamin B12 in both rat populations (metformin vs. control) were comparable, further suggesting that there is no absence of absorption due to metformin but rather a redistribution of the vitamin. Kidneys in rats treated with metformin contained 36% less B12 than the control population, this was again attributed to increase in liver absorption. While this study brings to light some very interesting ideas and hypotheses regarding the mechanism of metformin and Vitamin B12 interaction, it has several very crucial limitations that need to be considered. While the use of animal subjects in such a trial is obviously a practical issue, the translation of such findings into human individuals may not mimic such observations as the pathogenesis of diabetes may be highly

variable between species. As suggested previously, if patients with type 2 diabetes have a cellular resistance to Vitamin B12 this study may fall short of addressing the reality of Vitamin B12 status in humans with diabetes. Most crucial to the application of this study was the mode of administration bypassing the gastric system. This is a key factor as it is thought that much of the metformin interaction occurs in the small bowel and/or the ileum (Bauman et al., 2000), thus the subcutaneous route may mask the potential malabsorption effect of metformin treatment.

Vitamin B12 is suggested as a crucial factor in the maintenance of neuronal health and function and that deficiencies caused via avenues such as metformin treatment may corrupt the neuronal abilities of an individual. A study carried out by Chen *et al* (2012) explored the relationship between metformin use and altered neuronal function (Chen, 2012). A study group of 202 patients with type 2 diabetes was split between those taking metformin for a period longer than 6 months and a non-metformin group. Once again the metformin population yielded a lower serum Vitamin B12 level than that of the non-metformin group. Through several tests for neuropathy: history, vibration perception, s-LANSS (self-assessed Leeds Assessment of Neuropathic Symptoms and Signs) neuropathic pain etc. it was seen that there was no significant difference between the metformin and the non-metformin patients in regard to neuropathy. The author claims that diabetic peripheral neuropathy is clinically indistinguishable from Vitamin B12 related neuropathy, however the two study populations were all sourced from a general population who had all previously been diagnosed with diabetic neuropathy. This poses a boundary in the ability to perceive any differences in the neuropathic profiles of each of the populations as any increase or decrease in Vitamin B12 neuropathy may be masked by the primary diagnosis of diabetic neuropathy. Furthermore it was seen that the metformin group had an overall lower ferritin level with significant difference to the control group ($p=0.03$). The study does make several suggestions in regards to the mechanism of Vitamin B12 reduction in metformin patients; alterations in bowel motility, bacterial overgrowth and possible inhibition of B12 absorption are all noted.

While the above studies illustrate differing opinions on the mechanism of Vitamin B12 alteration in metformin treated patients, there is much to be gained from this exploration into the kinetics of interaction. The potential for irreversible neuronal damage resulting from preventable loss of the vitamin as well as the increased risk of megaloblastic-anaemia (Callaghan & Hadden, 1980) make it crucial that the prevalence for metformin induced Vitamin B12 malabsorption in

New Zealand be addressed. While Medsafe guidelines (Medsafe, 2009) and other studies (Mahajan & Gupta, 2010) suggest annual screening for B12 deficiency for all patients on metformin, this is thought to be rarely followed in practice.

Clinically significant deficiency of Vitamin B12 is not flagged until the patient has a serum level of <148 pmol/L, however it has been observed that levels under 220 pmol/L should be considered as suboptimal and that may be indicative of levels that may lead to an inhibition of biological function (Mazokopakis & Starakis, 2012). For this reason a level of 220 pmol/L should be considered as eligible for treatment, this is further supported by evidence suggesting that serum level of Vitamin B12 do not accurately reflect a deficient amount of the vitamin (Obeid et al., 2013) thus false negatives for suboptimal Vitamin B12 may arise. Furthermore as with many health issues, an approach of preventative treatment should be adopted in which patients who are identified as borderline B12 (148 – 220 pmol/L) may develop a deficiency if the factors that have led to such a level are continued, i.e. metformin treatment.

There is still much dispute about the clinical outcomes of the observed serum Vitamin B12 decrease associated with long term metformin treatment. There is no available literature on the prevalence of such an association in a New Zealand population. Previous studies have rates of prevalence with vast differences depending on the study population and therefore the assessment of a New Zealand population will allow further understanding of factors that may be affecting the development of this deficiency. While studies completed have shown the differences between sublingual, oral and intramuscular treatment options for the treatment of Vitamin B12 deficiency (Yazaki et al., 2006; Delpre, Stark, & Niv, 1999; Sharabi, Cohen, Sulkes, & Garty, 2003; Yazaki, Chow, & Mattie, 2006), there is not a large amount of literature regarding the use of these treatments in the metformin associated Vitamin B12 deficiency. With the current mechanism of metformin interaction relying on malabsorption of the vitamin during digestion, the route of sublingual treatment may show results that differ from previous sublingual study which does not address such a mechanism and therefore exhibit results that may understate the role a sublingual treatment and the evasion of the digestive tract.

An evaluation of the levels of metformin associated Vitamin B12 deficiency in a New Zealand population will allow assessment on the prevalence as well as the factors affecting this decreased vitamin state. A direct comparison of sublingual based treatment against the traditional intra muscular route of administration will allow for investigation into the potential means of correcting a decreased serum Vitamin B12 level following long term metformin treatment in patients with type 2 diabetes mellitus. More in depth research also needs to be conducted into the outcomes of metformin related Vitamin B12 deficiency to assess whether the treatment of this is required in order to maintain regular Vitamin B12 functionality.

This study will address the two main issues that have not been investigated in detail. A clinical audit and prevalence assessment will help identify the proportion of patients who are being treated with metformin over a long period of time who have developed a Vitamin B12 deficiency. This information will aid in knowledge of factors contributing towards increased risk factors of such a deficiency and aims to allow for a better understanding of how population demographics affect any differences in the rate of prevalence. Furthermore this study will draw direct comparisons of two different treatment options aiming to treat the acquired deficient state. This will allow not only the development of knowledge in this area but will also assess potential treatment options for the future treatment of patients afflicted by the interaction.

3.0 Methods

This study addresses the previously observed and explored effect of long term metformin treatment on serum Vitamin B12 level for patients who have Type 2 Diabetes Mellitus. Through analysis of patients attending clinical appointments at Wellington Regional Hospital as well as assessment of patients in a primary care environment the investigation will add further information to our understanding of this association.

The main objectives of the research are:

- A: To identify the prevalence of lowered Vitamin B12 serum levels (<220 pmol/L) in patients who have Type 2 Diabetes in both a secondary care outpatient population and in a primary healthcare environment.
- B: To evaluate the effectiveness of two treatment methods for patients identified with a lowered serum Vitamin B12 level in a randomised controlled trial.

Ethics approval for the study was obtained from the Central Health and Disability Ethics Committee (reference: 12/CEN/65/AM02).

The screening phase was undertaken as a clinical audit and therefore did not require individual patient consent. All patients taking part in the randomised controlled trial gave written informed consent to take part.

Funding for the project was obtained through a University of Otago research grant.

3.1 – Study 1 – Prevalence assessment

To evaluate the prevalence of lowered Vitamin B12 levels (serum Vitamin B12 < 220pmol/L) in patient population in both secondary care and primary care environments and evaluate any relationships between factors of; duration of metformin treatment, dosage of metformin, patient demographics.

3.1.1 – Screening

All patients attending a Diabetes Clinic at Wellington Regional Hospital and Kenepuru Hospital between February 12th 2013 and February 12th 2014 were assessed for eligibility for investigation into prevalence of decreased serum Vitamin B12 level. Patients attending the clinic before August 1st 2013 were also considered for *Study 2 – Intervention Study*, as detailed later. The flow of patients through the stages of screening and participation in the prevalence study is shown in *Figure 3.1.1*

Patients investigated for prevalence were assessed on the following:

Inclusion Criteria:

- Diagnosis of Type 2 Diabetes
- Treatment with metformin for a minimum duration of 12 months.

Exclusion Criteria:

- Diagnosis of Type 1 diabetes
- Known existing megaloblastic anaemia
- Existing Vitamin B12 treatment or supplementation

Diagnosis of Type 2 Diabetes Mellitus was in accordance to the position statement released by the New Zealand Society for the Study of Diabetes (NZSSD) ("NZSSD Position Statement on the

diagnosis of, and screening for, Type 2 Diabetes," 2011) which has been adopted by the Ministry of Health New Zealand which is as follows:

- *In **symptomatic** individuals an $HbA1c \geq 50$ mmol/mol (and, if measured, a fasting blood glucose of ≥ 7.0 mmol/l or a random glucose ≥ 11.1 mmol/l) is sufficient to establish the diagnosis of diabetes*
- *In **asymptomatic** individuals the same criteria apply but, to confirm the diagnosis of diabetes, a confirmatory test (preferably $HbA1c$) is needed on a separate occasion.*

If the patient met the inclusion criteria they were considered as **Level 1 Eligible**. All patients who were **Level 1 Eligible** were included in the prevalence study and a serum Vitamin B12 level sought from the laboratory records. All information obtained from screening is kept on a central database which relies on the unique NHI number for each patient.

- If the obtained value for serum Vitamin B12 from within the previous 3 months was less than 220 pmol/L, the patient was approached with an offer to join the intervention study and deemed to be **Level 2 Eligible**
- If the obtained value for serum Vitamin B12 from within the previous 3 months was 220 pmol/L or higher the patient was included in the prevalence evaluation but deemed to be not eligible for the intervention study.
- If there was no value for serum Vitamin B12 from within the previous 3 months then a blood test was requested by the doctor assessing the patient and the patient was added to a recall list labelled **Recall Yellow** to follow up the request.

3.1.2 – Primary Health Screening

In order to increase recruitment and diversify the demographics included in the prevalence study, four primary health providers were approached by the research team. Through the practice management system (Medtech) an initial screen was conducted on the patient database of each practice. The software screening relied on the matching of Ministry of Health read codes used for diagnoses and medications with several input criteria. The input criteria used for this screening were as follows:

- Diagnosis of type 2 Diabetes
- Prescription of metformin Hydrochloride between 12/02/2012 and 12/02/2014

This screen produced a list of patients who have been coded with a diagnosis of Type 2 Diabetes and who had received a prescription for metformin within the last year. These individuals were posted an invitation of study which included an explanation about the observed association between metformin and Vitamin B12. Patients who expressed interest were investigated in order to ensure that there was no current Vitamin B12 treatment and that they had been taking metformin for a minimum of 12 months. If a patient was eligible and still expressed interest in the study a blood test form was sent to the patient in order to evaluate their serum Vitamin B12.

All patients who were deemed to be **Level 1 Eligible** were assessed for the following demographic data in order to assess factor affecting serum Vitamin B12 level:

- Age
- Gender
- Ethnicity
- Dosage of metformin (mg/day)
- Duration of metformin treatment (years)

3.1.3 – Follow up – Level 1 Eligibility

Level 1 Eligible patients who did not have a previous serum Vitamin B12 level within the last 3 months and who were thus allocated to **Recall Yellow** were periodically reviewed to obtain a serum Vitamin B12 level. Patients who were on the **Recall Yellow** list for 1 month and still did not yield a serum Vitamin B12 level were sent a recall letter notifying them of the observed association of metformin use and serum Vitamin B12 deficiency. This letter included a blood test form for a Vitamin B12 test and these patients were then placed on the **Recall Orange** list. The **Recall Orange** list was periodically reviewed to obtain Vitamin B12 levels from patients, however no further contact with the patient was made. Any patient who produced a serum Vitamin B12 level during the review period was allocated to either **Level 2 Eligible** (if serum Vitamin B12 <220 pmol/L) or deemed non eligible (if serum Vitamin B12 $\geq$ 220 pmol/L).

3.1.4 – Follow up – Level 2 Eligibility

Patients who were identified as being **Level 2 Eligible** were invited to take part in the intervention study via their clinic physician, or via the researcher in the case of patients screened through primary health recruitment who had already expressed interest. If no answer was passed onto the research team in regards to the patients desire to take part, the patients were added to **Recall Red** list. This list worked much the same as the previous recall list in which a patient who had not notified their decision to participate were sent a follow up letter after one month. This letter advised of the association between metformin and serum Vitamin B12 deficiency and that the patient had been found to have a suboptimal Vitamin B12 level. The patient was invited to take part in the study via a response form. Once this invitation was sent, no further offer of invitation was made to the patient.

Figure 3.1.1 – Flow chart of patient screening and recruitment

3.1.5 – Phlebotomy

Patients who had been identified as **Level 1 Eligible** and who did not have a current serum Vitamin B12 level were approached by their physician for a test to be done. The samples were analysed at either the CCDHB laboratory or if taken in the community by Aotea Pathology.

The **CCDHB laboratory** uses the Roche Cobas e601 and competitive electrochemiluminescence (ECLIA) method for evaluation of serum Vitamin B12 level. The method is said to be accurate between the ranges of 22.0 – 1476 pmol/L (CV = $\pm 5\%$)

Aotea Pathology uses the Roche Modular E170 and the same competitive electrochemiluminescence immunoassay (CV = $\pm 5\%$).

As both of the test centres use the same method of assessment for serum Vitamin B12, and only differ in the type of analyser (also compatible) the results from both centres are claimed to be of equivalent accuracy. Full procedure is seen in Appendix A

3.2 – Study 2 - Intervention study

A randomised control study of intramuscular injections of Hydroxocobalamin 1mg vs. sublingual treatment with Methylcobalamin 1mg/day, in patients with Type 2 Diabetes Mellitus who are being treated with metformin and have a current serum Vitamin B12 level of <220 pmol/L.

Patients identified during *Study 1 – Prevalence Study* as having a decreased serum Vitamin B12 level who were seen before August 1st 2013 were invited to participate in an intervention study. The time frame allows for results of the 6 month trial to be available by study completion.

3.2.1 – Clinic Visit 1

Level 2 Eligible patients who accepted an invite to participate in the intervention study were booked an appointment with the research team in the Endocrine, Diabetes and Research Centre. After obtaining written informed consent further information was obtained.

Inclusion criteria:

- Type 2 Diabetes diagnosis
- Treatment with metformin for 12 months or longer
- Vitamin B12 of < 220 pmol/L

Exclusion criteria:

- Existing treatment for B12 deficiency
- Folate deficiency or related anaemia
- Pregnancy or lactation
- Cobalamin allergy
- Breach of Medsafe guidelines for safe administration of intramuscular injection of Hydroxocobalamin

Primary outcome measure was serum Vitamin B12 level at baseline, 3 month and 6 month time points.

Secondary outcome measures were prevalence of decreased serum Vitamin B12 level, and Michigan Neuropathy Screening scores at baseline and 6 months.

If all the inclusion criteria and none of the exclusion criteria were met, and the patient was willing to participate in the study, the patient was deemed **Level 3 Eligible** and was included in the intervention study. Once patients were deemed eligible details are collected including key variable information, date of initial diagnosis, date of initial commencement of metformin, current metformin dosage, ethnicity, as well as additional medical information as seen in Appendix B. The patient was also required to sign a consent form. The Michigan screening tool is also used in order to evaluate the presence, if any, of neuropathy in the patient. The protocol for this screening tool is seen in Appendix C. This tool was used due to its ease of application and its acceptance as an adequate assessment of symptoms of neuropathy, verification and score boundaries are seen in Al-Geffari *et al* (2012).

Randomization was carried out using an internet based randomization tool (randomizer.org) to generate a random order sequence in the values of 1 or 2 to correspond to treatment groups. The sequence obtained was used in the order of patient recruitment. The investigators were not blinded to the randomization but the sequence was not altered during patient recruitment.

Treatment 1 patients received a 1mg intramuscular injection of Hydroxocobalamin¹ following CCDHB protocol. Treatment 2 patients were provided a 3 month course of sublingual Methylcobalamin supplements of a 1000 mcg/day dosage². Patients also completed the University of Otago Food Questionnaire obtained from the Human Nutrition Department at Otago school of Medicine in order to assess if their background dietary intake at baseline. Appendix D.

¹ Hydroxocobalamin ABM – Hydroxocobalamin acetate
Solution for injection 1mg/mL – Ampoules of 1 mL containing 1 mg hydroxocobalamin acetate per mL equivalent to 0.96 mg of hydroxocobalamin per mL. Other ingredients: sodium chloride, sodium acetate, acetic acid, water for injections.
<http://www.medsafe.govt.nz/profs/datasheet/h/HydroxocobalaminABMinj.pdf>

² Bronson Sublingual B12 1000mcg – Methylcobalamin
Bronson Sublingual B12 provides this vitamin as Methylcobalamin. Extensive research studies show that Methylcobalamin is much more biologically active than cyanocobalamin
<http://www.healthpost.co.nz/supplements-and-natural-health/vitamins/vitamin-b/sublingual-b12-1000mcg--methylcobalamin-nhb12>

Due to the different treatment methods blinding of investigators and/or patients was not possible. However as the primary outcome variable was objective serum Vitamin B12 level it was not expected that blinding of patients or investigators would cause bias in this regard.

If the patient desired, a letter was sent to their GP informing them of their involvement in the study as well as information about the research project. Patients were provided with travel compensation as per the information sheet as seen in Appendix E.

3.2.2 – Clinic Visit 2

After 10 weeks from their first clinic visit patients were contacted to arrange a time for their second clinic visit. Patients were also sent a blood test form to assess their serum Vitamin B12 in order to evaluate the effect of the treatment to which they were assigned. The results from this blood test are intended to be available before the patients visit the clinic. At the second clinic visit the patients were told of their updated B12 level. If the B12 level of the patient had risen to above 220 pmol/L the patient was not provided additional treatment regardless of treatment group. If the patients latest B12 was still below 220 pmol/L they received a further treatment course of the same treatment to which they are allocated.

If the patient desired, a letter was sent to their GP letting them know of the development of the study and the patient's current Vitamin B12 level.

3.2.3 – Clinic Visit 3

At 23 weeks patients were contacted to arrange a time for their third clinic visit. Patients were once again sent a blood test form to assess their latest serum Vitamin B12 level. The Michigan screening tool was re-implemented to assess any potential change to the patients score as a result of the treatment. Patients were informed of their final B12 level and the pattern of change in their Vitamin B12 level was discussed with them. Any questions that the patient had concerning the study were addressed and the patient was discharged from the care of the researchers.

If the patient desired, the results were sent to their GP and the patient was discharged back into their GPs care.

Figure 3.2.1 – Patient flow chart for intervention for all comprehensively eligible patients

3.3 – Statistical Analysis

All analysis of data was conducted through the use of IBM software SPSS 20.0. The primary outcome results are presented as means. Assessment for significance between means was tested through application of t –test. Univariate Analysis of Variance (ANOVA) was used to assess between group interactions. Correlation was assessed using Pearson (for normal distribution, linear correlation) and Spearman (for non-normal, non-linear correlation) tests as specified. Continuous variables were log transformed wherever possible before running tests that require normal distribution of the data. All tests were two-sided and p values of <0.05 were considered to be statistically significant. Post hoc assessment of multiple interactions was conducted by LSD as indicated. Logistic regression analysis was used for comparison of prevalence data due to the dichotomous property of the outcome variable. Stepwise forward multiple regression analysis was applied to identify variables that predict serum Vitamin B12 level. Graphical representation of statistical significance was allocated in the following manner:

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

4.0 Results – Prevalence of Vitamin B12 Deficiency

Study 1. Prevalence of low Serum Vitamin B12 level in people with Type 2 DM on metformin therapy for at least 12 months.

4.1 – Study population

A total of 19,788 patients were screened from four Primary Health Providers in the Wellington region (Island Bay Medical Centre, Newtown Medical Centre, Karori Medical Centre Ltd., Newlands Medical Centre) and the Diabetes Outpatient Clinic at the Endocrine, Diabetes and Research Centre at Wellington Regional Hospital.

Screening through Primary Health Providers using Medtech 20 software identified 720 **Level 1 Eligible** patients (confirmed diagnosis of Type 2 Diabetes Mellitus (DM) and treatment with metformin for a period of 12 months or more). Of these 720 patients, 108 were deemed ineligible by their general practitioners for reasons such as pregnancy, currently breastfeeding, significant mental health issues, complicating comorbidities (such as anaemia). The remainder were approached for investigation into prevalence of decreased Serum Vitamin B12 level. A total of 612 invitations to study were sent out to the remaining eligible primary health care patients. Of the initial 612 initiations that were sent out, 117 patients indicated interest in the study and were contacted with further information and provided a blood test form. From which 98 patients completed the required blood test for Serum Vitamin B12.

Screening through the Diabetes Centre at Wellington Regional Hospital resulted in the assessment of 1090 patients attending clinic. Of these 305 were deemed **Level 1 Eligible** and considered for serum Vitamin B12 level investigation. Of the initial secondary health population, 226 patients had a reported Vitamin B12 level in the previous 3 months or provided a current Serum Vitamin B12 level during the study period.

Figure 4.1.1 – CONSORT Diagram of study to assess prevalence of lowered Serum Vitamin B12 level

The two study populations were similar in demographic composition but different in overall population size. Significant difference was seen in the variable of age ($p = 0.008$) where the patients recruited from the secondary healthcare environment were seen to be significantly younger than those from the primary healthcare environment (mean difference of 3.52 years). Both populations had a higher percentage of men than women. (Table 4.1.1)

Table 4.1.1 – Demographics of patient populations included in prevalence study. Mean (SD)

	Primary Health n = 98	Secondary Health n = 226	Total Population n = 324
Age (years)	63.93 (10.28)	60.41 (11.23)*	61.48 (11.06)
Men - n (%)	57 (58%)	118 (52%)	175 (54%)
Duration of metformin treatment (years)	7.74 (4.33)	7.72 (4.15)	7.72 (4.20)
Dosage of metformin (mg/day)	1852.04 (696.27)	1998.67 (767.20)	1954.32 (748.43)
Serum Vitamin B12 (pmol/L)	348.53 (177.56)	357.48 (185.08)	354.77 (182.62)

* $p < 0.01$

In order to assess the factor of ethnicity in the prevalence of serum Vitamin B12 deficiency the New Zealand Ministry of Health ethnic identifiers were used. These are based on a numerical code for each ethnic group and are used nationally. Due to patient numbers the ethnic groups were combined for all ethnicities that had 10 or fewer individuals who identified with it. This resulted in 6 different ethnic categories, here reported in order of coding: 11 – NZ European, 12 – Other European, 21 – NZ Maori, 31 – Samoan, 43 – Indian, All Others. The composition of different ethnicities present in each population was skewed greatly towards individuals who identified as NZ European; nearly half of each treatment group population was comprised of this ethnicity. There was no significant difference between treatment groups in regards to the proportions of different

ethnic groups; however there were differences between the numbers of patients in each ethnic group.

Table 4.1.2 – Ethnicity composition of study population. Number of patients and percentage of group population

Ethnicity	Primary Health n = 98	Secondary Health n = 226	Total n = 324
11 - NZ European	45 (45.9%)	99 (43.8%)	144 (44.4%)
12 - Other European	10 (10.2%)	22 (9.7%)	32 (9.9%)
21 - NZ Maori	13 (13.3%)	23 (10.2%)	36 (11.1%)
31 - Samoan	6 (6.1%)	13 (5.8%)	19 (5.9%)
43 - Indian	9 (9.2%)	28 (12.4%)	37 (11.4 %)
Other	15 (15.3%)	41 (18.1%)	56 (17.3%)

Screening through the Diabetes Clinic at Wellington Regional Hospital also provided informative statistics on patient numbers visiting the secondary health service (Table 4.1.3). Of the unique individuals attending clinic during the study period 27.96% were seen to be eligible for investigation of serum Vitamin B12.

Table 4.1.3 – Eligibility of patients attending Diabetes Clinic at Wellington Regional Hospital. Number of patients and percentages of screened population

Criteria	Number of Patients (% of secondary health screened population)
Diabetes Diagnosis	1091 (100%)
Type 2 Diabetes	533 (48.85%)
metformin Use	368 (33.73%)
Treatment Over 12 Months	305 (27.96%)
Reported Vitamin B12 Level	226 (20.71%)

Figure 4.1.2 – Eligibility of patients attending Diabetes Clinic at Wellington Regional Hospital. Percentage of screened secondary health patients

In total data for patients with type 2 DM receiving metformin therapy for more than 12 months were available for 324 patients (32.86 % of the total invited eligible population). This included 98 from primary care (15.86% of eligible) and 226 from secondary care (74.10 % of eligible).

Table 4.1.4 – Descriptive statistics for serum Vitamin B12 level across the study population

Parameter	Measurement (pmol/L)
Minimum	80.00
Maximum	1450.00
Mean	354.77
Std Deviation	182.62

Serum Vitamin B12 level were not seen to follow a normal distribution with a skewed pattern towards the left. Peak distribution is found to be within one standard deviation of the 220pmol/L value of decreased Vitamin B12 status. X –axis reference line has been added to show this level graphically:

Figure 4.1.3 – Distribution of serum Vitamin B12 level for entire study population

Analysis of distribution of serum Vitamin B12 level suggest a log normal distribution

Figure 4.1.4 – Q-Q plot for serum Vitamin B12 distribution showing log correction of data

Vitamin B12 levels for total study population were placed into 3 different categories based on clinical relevance of serum levels: Vitamin B12 Deficiency level (<150 pmol/L), Lowered Vitamin B12 level ($150 - 219$ pmol/L), and Normal Vitamin B12 level (≥ 220 pmol/L).

Table 4.1.5 – Vitamin B12 levels in total study population $n = 324$. Number of patients and percentages of population

Vitamin B12 Level	Number of Patients
Deficient (<150 pmol/L)	16 (4.9%)
Lowered ($150 - 219$ pmol/L)	48 (14.9%)
Normal (≥ 220 pmol/L)	260 (80.2%)

Figure 4.1.5 – Serum Vitamin B12 level in total population of eligible patients in accordance to above boundaries

A total of 64 patients from the total population of 324 patients showed decreased serum Vitamin B12 level, corresponding to a prevalence of 19.75% for a decreased serum Vitamin B12 level (<220 pmol/L).

Figure 4.1.6 – Prevalence of decreased Vitamin B12 level in total population. Percentage of study population

The values for serum Vitamin B12 were assessed on the variable of patient population source, whether the patient was assessed through a Primary Health Provider or whether they were a patient of the Diabetes Centre at Wellington Regional Hospital/Secondary Health Care. The patient population were found to be comparable between population portion of deficient and lowered serum Vitamin B12 status ($p = 0.519$, $p = 0.619$ respectively)

Table 4.1.6 – Number and percentage of patients in each serum Vitamin B12 level group according to population group

Vitamin B12 Level	Number of Patients	
	Primary Health n = 98	Secondary Health Care n = 226
Deficient (<150 pmol/L)	6 (6.1%)	10 (4.4%)
Lowered (150-219 pmol/L)	15 (15.3%)	33 (14.6%)
Normal (≥ 220 pmol/L)	77 (78.6%)	183 (81.0%)

Figure 4.1.7 – Reported Vitamin B12 levels in patients investigated through Primary Health Care according to above boundaries

Figure 4.1.8 – Reported Vitamin B12 levels in patients investigated through Secondary Health according to above boundaries

Analysis of decreased serum Vitamin B12 level requires assessment of patients who have a serum level of below 220 pmol/L. The two patient populations were found to have a comparable level of prevalence for decreased serum Vitamin B12. Differences in prevalence between the two populations was seen to not be statistically significant ($p = 0.626$).

Table 4.1.7 – Number of patients and percentage of populations with decreased Vitamin B12 (<220 pmol/L) for each population group, showing percentage for prevalence of Vitamin B12 status

	Patient Population	
	Primary Healthcare n = 98	Secondary Healthcare n = 226
Number of patients (%)	21 (21.4%)	43(19.0%)

Potential predictor variables were evaluated in order to investigate potential factors effecting serum Vitamin B12 level and the rate of prevalence of decreased levels of serum Vitamin B12.

4.2 – Effect of metformin Dosage on serum Vitamin B12 level and prevalence of lowered level

Metformin is available in either 500mg or 850mg doses. The maximum daily dose is 3000mg. Therefore the data for dosage is not continuous but determined by multiples of the available tablet strengths. Data for dosage of metformin was taken as mg/day; this allows for assessment between populations regardless of specific treatment regimens.

Figure 4.2.1 – Q-Q plot of metformin dosage data shows lack of variability between dosage levels

Figure 4.2.2 – Histogram of dosage of metformin data shows lack of normal distribution

The mean dosage of metformin (mg/day) was slightly higher in the secondary health patient population, however this not seen to be significant ($p = 0.224$). Again there was no difference in serum Vitamin B12 level between populations ($p = 0.560$)

Table 4.2.1 – Mean dosage of metformin and mean serum Vitamin B12 level for different populations

	Mean (SD)	
	Primary Health n = 98	Secondary Health n = 226
Dosage (mg/day)	1852.04 (696.27)	1998.67 (767.20)
Serum Vitamin B12 (pmol/L)	348.53 (177.57)	357.48 (185.08)

Correlation between categorized dosage of metformin and serum Vitamin B12 level was seen with Pearson's co-efficient of -0.178 ($p = 0.001$), however due to the abnormal distribution of the metformin dosage data this should be interpreted with caution.

Figure 4.2.3 –Observed linear regression of metformin dosage and serum Vitamin B12 level

Metformin doses are dependent on pharmaceutical availability of drug; therefore distribution of values is not natural. An assessment of different ranges of drug dosage was carried out to further test the variable.

Metformin dosages were assigned to six different categories dependant on the gram/day value. The general decrease in serum Vitamin B12 level as metformin dose increased was reflected in ANOVA assessment ($p = 0.036$) (Table 4.2.1). Post hoc (LSD) analysis of the difference between separate categories (Table 4.2.2) showed significant differences between the serum Vitamin B12 level of patients in the following interactions:

- $<1000\text{mg/day}$ vs. $\geq 3000\text{mg/day}$ ($p = 0.011$)
- $1000\text{-}1499\text{mg/day}$ vs. $\geq 3000\text{mg/day}$ ($p = 0.008$)
- $1500\text{-}1999\text{mg/day}$ vs. $\geq 3000\text{mg/day}$ ($p = 0.046$)

Table 4.2.2 – Categorization of metformin dosage with mean serum Vitamin B12

metformin Dosage (mg/day)	Serum Vitamin B12 (pmol/L)(SD)
<1000 n = 14	445.93 (226.86)
1000-1499 n = 64	389.98 (192.21)
1500-1999 n = 36	379.50 (192.95)
2000-2499 n = 119	341.26 (156.96)
2500-2999 n = 24	334.79 (168.25)
≥ 3000 n = 67	319.97 (197.84)

Figure 4.2.4 - Categorization of metformin dosage with mean serum Vitamin B12 level

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

As there was an observed interaction between the grouped metformin dosages and serum Vitamin B12 an analysis of the effect of grouped metformin dosage on the rate of prevalence of lowered serum Vitamin B12 serum was conducted.

Logistic regression shows significant effect of metformin dosage on prevalence of decreased serum Vitamin B12 level (<220 pmol/L) ($p = 0.031$).

Categorization was again implemented to further analyse interactions at between different levels of dosage (Table 4.2.3). Categorized dosage of metformin treatment was shown to be significant through logistic regression ($p = 0.033$). Chi square analysis showed no specific group comparison showed significant difference in prevalence of decreased Vitamin B12 status (<220 pmol/L).

Table 4.2.3 - Categorization of metformin dosage with rate of prevalence of decreased serum Vitamin B12 level (<220 pmol/L)

metformin Dosage (mg/day)	Prevalence of lowered level
<1000 n = 14	7%
1000-1499 n = 64	14%
1500-1999 n = 36	19%
2000-2499 n = 119	19%
2500-2999 n = 24	25%
≥ 3000 n = 67	27%

Figure 4.2.5 - Categorization of metformin dosage with rate of prevalence of decreased serum Vitamin B12 level (<220 pmol/L)

Odds ratio calculation shows increase in chance of lowered serum Vitamin B12 level (<220 pmol/L) with increase in dosage of metformin for most steps of 500mg/day

Table 4.2.4 – Odds ratio for decreased serum Vitamin B12 status (<220pmol/L) for each 500 mg increase in dosage of metformin

metformin Dosage (mg/day)	Comparative metformin Dosage (mg/day)	Odds Ratio of decreased Vitamin B12 status (cases assessed)
<1000 n = 15	1000-1499 n = 63	2.04 (78)
1000-1499 n = 63	1500-1999 n = 35	1.75 (98)
1500-1999 n = 35	2000-2499 n = 114	0.96 (149)
2000-2499 n = 114	2500-2999 n = 24	1.39 (138)
2500-2999 n = 24	≥3000 n = 66	1.12 (90)

4.3 – Effect of duration of metformin treatment on serum Vitamin B12 level and prevalence of lowered level

The distribution of data for the duration of metformin treatment was seen to be very irregular. The data did not match normal or log normal distribution expectations or assumptions.

Figure 4.3.1 – Q-Q plot of duration of metformin treatment showing non-normal distribution

Figure 4.3.2 – Log normal distribution of duration of metformin treatment showing insufficient log transformation of data to normality

Figure 4.3.3 – Distribution of duration of metformin treatment

Analysis of correlation between duration of metformin treatment and serum Vitamin B12 level resulted in significant linear regression between the two variables by Pearson Correlation. Pearson Correlation Coefficient = -0.112 ($p = 0.044$)

Figure 4.3.4 – Scatter of data for duration of metformin treatment and serum Vitamin B12 level showing linear correlation

Categorization of duration of metformin treatment was carried out to allow for irregular distribution of data. Data were assigned to one of five different time periods for duration of metformin treatment: 1.0-2.9 years, 3.0-4.9 years, 5.0-6.9 years, 7.0-8.9 years and ≥ 9.0 years.

Difference between groups was seen to be significant (ANOVA $p = 0.075$). Post hoc (LSD) analysis identified significant difference between the following duration groups:

- 3.0-4.9 years vs. ≥ 9.0 years ($p = 0.048$)
- 5.0-6.9 years vs. ≥ 9.0 years ($p = 0.038$)

Table 4.3.1 – Mean serum Vitamin B12 level (pmol/L) by categorized duration of metformin use

Duration of metformin treatment (years)	Serum Vitamin B12 (pmol/L)(SD)
1.0 – 2.9 n = 49	361.59 (135.37)
3.0 – 4.9 n = 51	391.18 (192.22)
5.0 – 6.9 n = 52	402.96 (258.43)
7.0 – 8.9 n = 52	328.54 (174.88)
≥ 9.0 n = 120	327.01 (152.28)

Figure 4.3.5 – Mean serum Vitamin B12 level (pmol/L) with 95% confidence interval by categorised duration of metformin serum use

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

As there was an observed interaction between categorical duration of metformin treatment and serum Vitamin B12 level, an analysis of prevalence was undertaken to investigate a translation of altered serum Vitamin B12 level to decreased Vitamin B12 status.

Through logistic regression no significant difference was seen between the rate of prevalence of lowered serum Vitamin B12 level and categorical duration of metformin treatment ($p = 0.081$).

Chi square analysis showed no specific group comparison showed significant difference in prevalence of decreased Vitamin B12 status (< 220 pmol/L).

Table 4.3.2 - Categorization of duration of metformin for prevalence of lowered serum Vitamin B12 level (<220 pmol/L)

Duration of metformin treatment (years)	Prevalence of lowered level
2.0 – 2.9 n = 48	14%
3.0 – 4.9 n = 50	20%
5.0 – 6.9 n = 52	13%
7.0 – 8.9 n = 51	19%
≥9.0 n = 116	25%

Figure 4.3.6 - Categorization of metformin dosage with rate of prevalence of decreased serum Vitamin B12 level (<220 pmol/L)

Assessment of odds ratio across different categories of duration of metformin treatment shows trend towards incremental increase in odds of prevalence towards the longer period of metformin treatment.

Table 4.3.3 – Odds ratio for decreased Vitamin B12 status (<220 pmol/L) by categorized duration of metformin use relative to

Duration of metformin treatment (years)	Comparative Duration of metformin treatment (years)	Odds Ratio of decreased Vitamin B12 status (cases assessed)
1.0 – 2.9 n = 48	3.0 – 4.9 n = 50	1.75 (98)
3.0 – 4.9 n = 50	5.0 – 6.9 n = 52	0.66 (102)
5.0 – 6.9 n = 52	7.0 – 8.9 n = 51	1.57 (103)
7.0 – 8.9 n = 51	≥9.0 n = 116	1.37 (167)

4.4 – Effect of ethnicity on serum Vitamin B12 level and prevalence of lowered level

There were various interactions between the variable of ethnicity and that of serum Vitamin B12 level. Ethnicity as an independent variable was seen to significantly correlate with the variables of serum Vitamin B12 level and age (ANOVA $p < 0.0005$ and $p = 0.001$ respectively).

Table 4.4.1 – Ethnicity dependent presentation of mean values of data for entire study population

Ethnicity	Mean level (SD)			
	Serum Vitamin B12 (pmol/L)	metformin Dosage (mg/day)	Duration of metformin Treatment (years)	Age (years)
NZ European n = 144	315.89 (131.68)	1911.81 (751.18)	7.71 (4.34)	62.71 (9.76)
Other European n = 32	333.09 (166.53)	1714.06 (642.48)	6.82 (3.89)	67.46 (12.25)
NZ Maori n = 36	429.67 (196.77)	2181.94 (782.41)	7.69 (4.38)	59.14 (10.20)
Samoan n = 19	499.90 (203.39)	2007.89 (568.69)	8.22 (3.64)	60.40 (8.19)
Indian n = 37	364.03 (284.24)	1987.74 (739.41)	7.83 (4.13)	56.63 (11.73)
Other n = 56	363.64 (169.59)	2014.64 (811.77)	8.05 (4.09)	59.96 (12.70)

There were numerous significant differences in mean serum Vitamin B12 level when considering ethnicity as a predictor variable. These are expressed in (Figure 4.4.1)

Figure 4.4.1 – Differences in mean serum Vitamin B12 level for different ethnic categories (CI = 95%)

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

There were numerous significant differences in mean age when considering ethnicity as a predictor variable. These are expressed in (Figure 4.4.2). As can be seen, a majority of these are due to the significantly older Other European population.

Figure 4.4.2 – Differences in mean age for different ethnic categories (CI = 95%)

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

As there was significant interaction between the variables of ethnicity and serum Vitamin B12 level, further analysis was undertaken to evaluate whether this influence translated into a difference in prevalence of decreased serum Vitamin B12 level (<220 pmol/L) across the population (Table 4.4.2)

Linear regression shows that the prevalence of low serum B12 (<220 pmol/L) shows no significant differences across all ethnic populations ($p = 0.963$).

Chi square analysis showed no specific group comparison showed significant difference in prevalence of decreased Vitamin B12 status (<220 pmol/L), the largest mean difference, between Samoan and Indian groups, having a p value of 0.07.

Table 4.4.2 – Prevalence of lowered serum Vitamin B12 (<220 pmol/L) dependent on patient ethnicity for the entire study population

Ethnicity	Prevalence (%) of low serum Vitamin B12 level (<220 pmol/L) within ethnic groups	Group population
NZ European	22%	144
Other European	19%	32
NZ Maori	11%	36
Samoan	5%	19
Indian	27%	37
Other	21%	56
Total	20%	324

Figure 4.4.3 - Prevalence of lowered serum Vitamin B12 (<220 pmol/L) dependent on patient ethnicity for the entire study population

4.5 – Effect of age on serum Vitamin B12 level and prevalence of lowered level

Linear regression did not identify age as a predictor of serum Vitamin B12 level ($p = 0.064$). However a correlation assessment using Spearman test resulted in a co-efficient value of -0.121 ($p = 0.030$) suggesting that there may be a non-linear correlation between the two variables with a decrease in serum Vitamin B12 with an increase in age.

Logistic regression identified increase in age as a predictor of lowered serum Vitamin B12 status (<220 pmol/L) ($p = 0.047$).

Figure 4.5.1– Scatter plot of data on age vs. serum Vitamin B12 level (pmol/L)

Categorization of age of patient was conducted to assist in analysis. Patients were allocated into the following age groups: 30-50 years, 50-70 years, 70-90 years. There was no overall significant prediction of serum Vitamin B12 by categorized age ($p = 0.120$). Post hoc (LSD) analysis showed significant difference between serum Vitamin B12 level between patients of 30-50 years of age and 70-90 years of age ($p = 0.040$).

Table 4.5.1 – Mean serum Vitamin B12 (pmol/L) dependent on age group categorization

Age range (years)	Mean serum Vitamin B12 level (<220 pmol/L) (SD)	Group population (% of study population)
30-50	382.96 (158.97)	49 (15.12%)
50-70	357.18 (195.19)	199 (61.42%)
70-90	330.32 (160.39)	76 (23.46%)

Figure 4.5.2 – Mean serum Vitamin B12 level (pmol/L) according to categorized age groups

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

Logistic regression suggests that there is an influence of categorized age and prevalence of decreased serum Vitamin B12 level (<220 pmol/L) ($p = 0.038$). Chi square assessment showed significant difference between the age groups of 30-50 years vs. 70-90 years ($p = 0.019$)

Table 4.5.2 – Prevalence of lowered serum Vitamin B12 (<220 pmol/L) dependent on age group categorization

Age range (years)	Prevalence (%) of low serum Vitamin B12 level (<220 pmol/L) within age group	Group population (% of study population)
30-50	8%	49 (15.12%)
50-70	21%	199 (61.42%)
70-90	25%	76 (23.46%)

Figure 4.4.3 - Prevalence of lowered serum Vitamin B12 (<220 pmol/L) dependent on categorised age of patient

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

Table 4.5.3 – Odds ratio for decreased Vitamin B12 status (<220 pmol/L) by categorized age of patient

Age of patient (years)	Comparative age of patient (years)	Odds Ratio of decreased Vitamin B12 status (cases assessed)
30-50 n = 49	50-70 n = 199	2.19 (245)
50-70 n = 199	70-90 n = 76	1.285 (275)

Patients who were 65 years or older were seen to have a lower mean serum Vitamin B12 level and higher rate of prevalence for a lower serum Vitamin B12 level (Table 4.5.1). These differences however were not statistically significant (Linear regression $p = 0.138$ and Chi square $p = 0.114$ respectively).

Table 4.5.4 – Proportion of elderly patients and the apparent effect on mean serum Vitamin B12 level and prevalence of decreased level

Age Parameter	Mean serum Vitamin B12 level (pmol/L)(SD)	Prevalence of lowered level (<220 pmol/L)(% of population)
<65 years n = 201	363.26 (182.15)	17%
≥65 years n = 123	340.90 (183.31)	24%

Age was seen to be correlated with dosage of metformin (mg/day) with Pearson correlation coefficient of -15.75 ($p < 0.0005$) in linear regression. Age was also seen to be correlated with duration of metformin treatment (years) with Pearson correlation coefficient of 0.259 ($p < 0.0005$).

This interaction between age of patient, dosage of metformin and duration of metformin treatment may interfere with results from serum Vitamin B12 level investigation as dosage of metformin and duration of metformin use are seen to impact serum Vitamin B12 level. The association between these three variables is illustrated below (Figure 4.5.3).

Figure 4.5.3 – Association between patient age and dosage of metformin and corresponding mean serum Vitamin B12 level (pmol/L)

Adjustment for age in linear regression of dosage of metformin and serum Vitamin B12 indicated that the correlation remains significant ($p < 0.0005$)

Figure 4.5.4 – Association between patient age and duration of metformin treatment and corresponding mean serum Vitamin B12 level (pmol/L)

Adjustment for age in linear regression of duration of metformin and serum Vitamin B12 indicated that the correlation is insignificant ($p = 0.051$)

5.0 Results – Randomized Control Trial

Study 2. Treatment of decreased serum Vitamin B12 (<220 pmol/L) using a randomized control trial

5.1 – Population Overview

Of the 19 patients enrolled in the randomized control trial, a total of 17 patients completed the study by the end of the study period. Of these patients; 7 had been randomized to the 1 mg Hydroxocobalamin intramuscular injection group (Treatment 1) and 10 had been randomized to the Bronsons Sublingual Methylcobalamin B12 Supplement 1000mcg/day group (Treatment 2).

Figure 5.1.1 – CONSORT diagram of intervention study

The demographic composition of the two treatment groups was comparable with no significant differences in any of the key variables. The most significant difference between the groups was in the variable of dosage of metformin where patients in Hydroxocobalamin injection treatment group had a higher mean dosage than those in Methylcobalamin sublingual treatment group = (0.224). As the two populations have comparable compositions any differences in outcome variables can be likely to be attributed to the treatment provided.

There was a disproportionate number of male patients in both treatment groups, however there was no difference between the genders in regards to clinical measurements.

Table 5.1.1 – Demographics of treatment group populations

Variable	Treatment Group		Total
	Hydroxocobalamin Injection n = 7	Methylcobalamin Sublingual n = 10	
Age (years)	65.60 (8.61)	61.50 (8.22)	63.19 (8.37)
Gender – n (male) (%)	6 (85.71%)	6 (60%)	12 (70.59%)
Healthcare source – n (primary) (%)	4 (57.14%)	6 (60%)	10 (58.82%)
Dosage of metformin (mg/day) (SD)	2328.57 (949.94)	1905 (403.08)	2079.41 (689.90)
Duration of metformin treatment (years) (SD)	12.36 (5.49)	10.64 (5.50)	11.35 (5.39)
Baseline serum Vitamin B12 (pmol/L) (SD)	157.43 (42.20)	176.70 (36.51)	168.76 (38.90)
Insulin treatment – n (treated) (%)	3 (42.86%)	4 (40%)	7 (41.18%)

Within the study population the baseline serum Vitamin B12 level ranged from a minimum of 80 pmol/L to a maximum of 219 pmol/L, mean value 168.76 pmol/L (SD = 38.90). Patients were randomized to treatment groups without baseline serum Vitamin B12 level stratification.

Mean Serum Vitamin B12 level at baseline for patients was seen to not be significantly different between treatment groups ($p=0.333$).

Of the 3 patients who were classed as clinically deficient (Serum Vitamin B12 <140 pmol/L); 2 were in Hydroxocobalamin injection treatment group and 1 was in Methylcobalamin sublingual treatment group.

Figure 5.1.2 – Illustration of serum Vitamin B12 status of entire patient population patients and commencement of study according to parameters outlined by Mezakopakis et al 2013

Study protocol required retreatment of patients if serum Vitamin B12 level at 3 months was less than 220 pmol/L. Two patients were hence retreated at 3 months and therefore the results for serum Vitamin B12 at 6 months were omitted from the statistical analysis due to increased treatment compared to the rest of the study population.

Serum Vitamin B12 level for the patients recruited in the randomized control trial were recorded at Month 3, and Month 6.

5.2 – Raw data of Randomized Control Trial

Serum levels changed in Hydroxocobalamin injection treatment group and Methylcobalamin sublingual treatment group significantly ($p = 0.003$, $p < 0.005$ respectively) at the 3 month time period compared to baseline levels.

Serum Vitamin B12 level at 3 months were seen to be significantly different between the two treatment groups (ANOVA $p = 0.014$).

Serum levels changed in Hydroxocobalamin injection treatment group and Methylcobalamin sublingual treatment group significantly ($p = 0.006$, $p < 0.0005$ respectively) at the 6 month time period compared to baseline levels.

Serum Vitamin B12 at 6 months were insignificantly different between treatment groups ($p = 0.588$)

Table 5.2.1 – Mean serum Vitamin B12 level (pmol/L) for treatment groups at baseline and different time points

Treatment Group		Serum Vitamin B12 (pmol/L)		
		Baseline	3 Month	6 Month
Hydroxocobalamin Injection	Mean	157.43	282.14	248.20
	Std Deviation	42.20	135.25	26.44
	N	7	7	5
Methylcobalamin Sublingual	Mean	176.70	406.60	268.60
	Std Deviation	36.51	80.77	59.97
	N	10	10	10

Figure 5.2.1 – Graphical representation of raw serum Vitamin B12 level (pmol/L) at each time point during the study period. Red line represents the 220 pmol/L level which is considered to be the lower limit of normal serum Vitamin B12 level.

* = p<0.05, ** = p<0.005, *** = p<0.0005.

Figure 5.2.2 – Graphical representation of mean Serum Vitamin B12 level (pmol/L), illustration of peak differences between treatment groups over the duration of the study period (CI not shown)

5.3 – Baseline Correction of serum Vitamin B12 level

As the mean baseline Serum Vitamin B12 level of patients were not stratified between treatment groups further analysis of the B12 levels was made assuming a 0 pmol/L level baseline and exploring the change in serum Vitamin B12 level between treatment groups.

Both of the treatment groups showed increased mean values for Serum Vitamin B12 level at the 3 month time point. Hydroxocobalamin injection treatment group exhibited a mean increase of 124.71 pmol/L (SD 118.77). Methylcobalamin sublingual treatment group exhibited a mean increase of 229.90 pmol/L (SD 96.93)

Hydroxocobalamin injection treatment group had an insignificant mean decrease from the 3 month time point to 70.20 pmol/L (SD 30.37) above baseline at the 6 month time point ($p = 0.084$).

Methylcobalamin sublingual treatment group had a significant mean decrease from the 3 month time point to 91.90 pmol/L (SD 54.85) above baseline at the 6 month time period ($p = 0.002$).

Change in serum Vitamin B12 level at 3 months were seen to be significantly different between the two treatment groups ($p = 0.009$) after correction for baseline.

Change in serum Vitamin B12 level at 6 months were seen to be not significantly different between the two treatment groups ($p = 0.057$) after correction for baseline.

Table 5.3.1 – Mean change in serum Vitamin B12 level (pmol/L) from baseline at 3 month and 6 month time points

Treatment Group		Serum Vitamin B12 level (pmol/L)	
		3 month time point	6 month time point
Hydroxocobalamin Injection	Mean	124.71	70.20
	Std. Deviation	118.77	30.37
	N	7	5
Methylcobalamin Sublingual	Mean	229.90	91.90
	Std. Deviation	96.93	54.85
	N	10	10

Figure 5.3.1 – Graphical representation of change in serum Vitamin B12 level (pmol/L) between treatment groups after baseline correction

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

Figure 5.3.2 – Graphical representation of changes in Serum Vitamin B12 level (pmol/L) between treatment groups over study period after baseline correction (CI not shown)

Due to the single outlying patient *SCK015* who was randomized to Hydroxocobalamin injection treatment group the data analysis has potentially been skewed. Statistical analysis has suggested the case to be an abnormal result.

Analysis of this case found a Cooks distance of 0.839 suggesting that the case may be an outlier and can therefore be omitted with caution.

Figure 5.3.3 – Box plot of serum Vitamin B12 level (pmol/L) at 3 month time point for both treatment options showing presence and confirmation of SCK015 outlier

Revised results for mean change in serum Vitamin B12 from baseline at 3 months indicated significant difference between treatment groups ($p < 0.0005$)

Difference in mean change of serum Vitamin B12 from baseline was significant between the treatment groups at the 6 month time point ($p = 0.042$)

Difference in corrected serum Vitamin B12 level for Hydroxocobalamin injection treatment group from the 3 month time point to the 6 month time point was still insignificant ($p = 0.139$)

Table 5.3.2 – Corrected mean change in serum Vitamin B12 level (pmol/L) from baseline at 3 month and 6 month time points

Treatment		3 months (pmol/L)	6 months (pmol/L)
Hydroxocobalamin Injection	Mean	80.83	65.50
	Std. Deviation	27.43	32.91
	N	6	4
Methylcobalamin Sublingual	Mean	229.90	91.90
	Std. Deviation	96.93	54.85
	N	10	10

Figure 5.3.4 – Corrected chart of change in Vitamin B12 levels (pmol/L) from baseline for treatment groups when single identified potential outlier is excluded from analysis.

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

5.4 – Analysis of $\Delta B12$ over 3 month time period

In order to further assess the effect of the two treatment options on serum Vitamin B12 level, an analysis of short term changes in serum Vitamin B12 level was conducted. This was done by evaluating the difference in serum Vitamin B12 level from the result 3 months previous:

3 month $\Delta B12$ = serum Vitamin B12 at 3 months – baseline serum Vitamin B12 level

6 Month $\Delta B12$ = serum Vitamin B12 at 6 months – serum Vitamin B12 at 3 months

There was no significant difference between the 3 month $\Delta B12$ and 6 month $\Delta B12$ for Hydroxocobalamin injection treatment group ($p = 0.118$)

There was significant difference between the 3 month $\Delta B12$ and 6 month $\Delta B12$ for Methylcobalamin sublingual treatment group ($p < 0.0005$)

The difference in 3 Month $\Delta B12$ between the treatment groups was seen to be not significant ($p = 0.063$)

The difference in 6 Month $\Delta B12$ between the treatment groups was seen to be not significant ($p = 0.345$)

Table 5.4.1 – $\Delta B12$ (pmol/L) for each time point between the different treatment groups

Treatment		$\Delta B12$ at 3 months (pmol/L)	$\Delta B12$ at 6 months (pmol/L)
Hydroxocobalamin Injection	Mean	124.71	-79.6
	Std. Deviation	118.77	124.56
	N	7	5
Methylcobalamin Sublingual	Mean	229.90	-138.00
	Std. Deviation	96.93	100.94
	N	10	10

Figure 5.4.1 – Representation of $\Delta B12$ (pmol/L) levels for the study period for each treatment group.

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

Once again the results for the patient *SCK015* were excluded to evaluate any potentially masked correlation between treatment and $\Delta B12$.

Revised values for $\Delta B12$ at 3 month time point were significantly different between the treatment groups (p value = 0.003),

Differences between treatment groups were not significant for $\Delta B12$ at 6 month time point (p value = 0.051)

Corrected difference in $\Delta B12$ for Hydroxocobalamin between 3 and 6 month time points was seen to be significant ($p = 0.014$)

Table 5.4.2 Revised values for $\Delta B12$ (pmol/L) at time points for each treatment group

Treatment		$\Delta B12$ at 3 months (pmol/L)	$\Delta B12$ at 6 months (pmol/L)
Hydroxocobalamin Injection	Mean	80.83	-24.75
	Std. Deviation	27.43	25.11
	N	6	4
Methylcobalamin Sublingual	Mean	229.90	-138.00
	Std. Deviation	96.93	100.95
	N	10	10

Figure 5.4.2 – Modified graph of $\Delta B12$ (pmol/L) during study period after exclusion of potential outlier from assessment

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

5.5 – Treatment of Vitamin B12 deficiency

Evaluation of the effect of each of the treatment options in alleviation of decreased serum Vitamin B12 level ($<220\text{pmol/L}$) was carried out. Patients were assessed on whether their level for Serum Vitamin B12 at 3 and 6 months was above 220 pmol/L or not. If the level was above this level the patient Vitamin B12 was deemed “resolved”. In some instances patients who were “resolved” at 3 months had levels that were below 220 pmol/L at 6 months, these patients were therefore no longer considered “resolved” at the 6 month time point. The patients who were not “resolved” at 3 months received an additional treatment; these results were omitted from the population at the 6 month time period. This method allows for the analysis of long term resolution.

Hydroxocobalamin injection treatment group had success rate of 71.4% patient resolution rate at 3 months. Of those patients who were resolved at 3 months, resolution at 6 months was 80.0%.

Methylcobalamin sublingual treatment group had a 100% patient resolution rate at 3 months.

Resolution at 6 months was 75% of the group population.

Difference between resolution rates of treatment groups was assessed using Chi square tests. Resolution rate at 3 months was not significantly different between groups ($p = 0.154$). Resolution rate at 6 months was seen to not be statistically significant ($p = 1.000$).

Table 5.5.1 – Resolution of decreased serum Vitamin B12 level in treatment groups

Treatment Group	Number of patients resolved – n (% of group population)	
	3 Months	6 Months
Hydroxocobalamin Injection	5 (71.4%)	4 (80.0%)
Methylcobalamin Sublingual	10 (100%)	8 (80.0%)
Total	15 (88.2%)	12 (70.6%)

Figure 5.5.1 – Illustration of Vitamin B12 levels of each patient at 3 months showing treatment groups, red line shows 220 pmol/L level which is considered to be level of resolution of lowered Vitamin B12 level

Figure 5.5.2 – Illustration of Vitamin B12 levels of each patient at 6 months showing treatment groups, red line shows 220 pmol/L level which is considered to be level of resolution of lowered Vitamin B12 level

5.6 – Neuropathy and symptomatic Vitamin B12 status

Using the Michigan Neuropathy screening Instrument (MNSI) neuropathy is defined operationally:

- Score of ≥ 7 on patient questionnaire for neuropathic symptoms
- Score of > 2 on the examination for neuropathic evaluation

Mean scores for questionnaire for neuropathy evaluation yielded no patients that would be classed as neuropathic by the above guidelines at baseline or at the 6 month time period. Mean scores for the entire population of 2.09 at baseline (SD 2.11) and 2.20 at 6 month time point (SD 1.85) showed no significant difference after treatment ($p = 0.535$).

No difference in mean score between the two treatment groups at baseline or at the 6 month time point for questionnaire score ($p = 0.244$ and $p = 0.673$ respectively)

Mean scores for examination for neuropathy evaluation yielded 2 patients that would be classed as neuropathic by the above guidelines at baseline, both were still classed as displaying neuropathy at the 6 month time period. Mean scores for the entire population of 0.94 at baseline (SD 1.73) and 1.37 at 6 month time point (SD 2.49) and showed no significant difference after treatment ($p = 0.307$).

No difference in mean score between the two treatment groups at baseline or at the 6 month time point ($p = 0.804$ and $p = 0.945$ respectively)

No difference was seen between the 3 month or 6 month scores for either the questionnaire or the examination for either treatment group assessed independently.

Table 5.6.1 – Scores for patient questionnaire according to MNSI guidelines

Treatment		Baseline Michigan Score	6 Month Michigan Score	Δ Michigan Score
Hydroxocobalamin Injection	Mean	1.36	1.83	0.17
	Std. Dev	2.17	2.75	0.29
	N	6	3	3
Methylcobalamin Sublingual	Mean	2.60	2.06	-0.31
	Std. Dev	2.01	1.70	1.09
	N	10	8	8

Table 5.6.2 – Scores for patient examination according to MNSI guidelines

Treatment		Baseline Michigan Score	6 Month Michigan Score	Δ Michigan Score
Hydroxocobalamin Injection	Mean	1.08	1.30	0.08
	Std. Dev	2.18	2.64	0.38
	N	6	3	3
Methylcobalamin Sublingual	Mean	0.85	1.62	0.56
	Std. Dev	1.53	2.84	1.32
	N	10	8	8

Table 5.6.3 – Number of patients exhibiting neuropathy according to MNSI guidelines

Treatment Group	Number of patients exhibiting neuropathy (% of group population)	
	Baseline	6 Month
Hydroxocobalamin Injection	1 (16.67%)	1 (16.67%)
Methylcobalamin Sublingual	1 (10.00%)	1 (10.00%)

The presence of general fatigue was assessed as a symptom of Vitamin B12 deficiency in patients from the study population. There was no significant difference between the number of patients in each group who reported the symptom ($p = 0.913$)

Table 5.6.4 – Number of patients with the symptom of general fatigue in each treatment group

Treatment Group	Number of patients claiming general fatigue (% of group population)
Hydroxocobalamin Injection	4 (57.1%)
Methylcobalamin Sublingual	6 (60.0%)

Using logistic regression there was no correlation between serum Vitamin B12 and the presence of the symptom of general fatigue in patients from the study population ($p = 0.427$).

5.7 – Potential confounding factors

Additional factors of age, ethnicity, gender, healthcare environment, co-treatment with insulin, dosage and duration of metformin treatment were assessed for predictability of serum Vitamin B12 at any time point. All were found to have no effect on any of the serum Vitamin B12 level results or the prevalence results at any time point (data not shown).

The factor of dosage of metformin was seen to effect serum Vitamin B12 level at 3 months and prevalence of decreased Vitamin B12 status at 3 months ($p = 0.012$ and $p = 0.010$ respectively)

Linear regression modelling of dosage of metformin (mg/day) vs. serum Vitamin B12 level resulted in a linear correlation coefficient of -0.640 ($p = 0.006$)

Figure 5.7.1 – Scatter of the suggested relationship between dosage of metformin and serum Vitamin B12 level (pmol/L) at 3 month time point

The dosage of metformin was not found to be correlated to baseline serum Vitamin B12 level or serum Vitamin B12 level at the 6 month time point ($p = 0.487$ and $p = 0.728$ respectively)

Factor of Omeprazole (Losec) usage was found to significantly decrease serum Vitamin B12 level at the six month time point ($p = 0.041$) and was found to increase prevalence of decreased serum Vitamin B12 level at the six month time period ($p = 0.009$)

Losec treatment increases odds ratio of decreased serum Vitamin B12 status (<220 pmol/L) at the six month time period by a factor of 4.000

Table 5.7.1 – Effect of Losec treatment on mean serum Vitamin B12 level (pmol/) of study population

	Mean serum Vitamin B12 level (pmol/L)(SD)		
	Baseline	3 month	6 month
Losec treatment (n = 5)	158.80 (42.81)	339.80 (130.19)	222.50 (56.72)
No Losec treatment (n = 12)	172.92 (38.35)	361.83 (121.63)	276.09 (42.99)

Table 5.7.2 – Effect of Losec treatment on prevalence of decreased serum Vitamin B12 level of study population

	Prevalence of decreased serum Vitamin B12 level (<220 pmol/L) (n) (% of population)	
	3 month	6 month
Losec treatment	1 (20%)	3 (75%)
No Losec treatment	1 (8%)	0 (0%)

6.0 Discussion

6.1 – Prevalence of lowered serum Vitamin B12 level

Although there are recommendations for the annual screening of Vitamin B12 status in patients with type 2 diabetes on long term metformin treatment; it has been observed in this audit that this is not a common practice. The front line usage of metformin as a therapeutic agent was evident from the review of patients visiting the Diabetes Clinic at Wellington Regional Hospital (*Table 4.1.3*), suggesting a high number of patients potentially at risk of metformin associated Vitamin B12 deficiency. American Diabetes Association (ADA) guidelines for 2013 recommend the commencement of metformin as the initial pharmacological agent for type 2 diabetes when there are no contraindications (Amer Diabet, 2013). New Zealand Ministry of Health Primary Care Handbook 2012 recommends metformin as a primary intervention agent for glycaemic control once lifestyle modifications have been deemed ineffective (*New Zealand Primary Care Handbook 2012.*, 2012). The National Health and Medical Research Council (NHMRC) for the Australian Government encourage the use of metformin as a safe and effective treatment of patients with type 2 diabetes (Colaguiru, 2009). With these recommendations in mind it is imperative to fully investigate any interactions that a pharmaceutical agent may have in order to prevent wide spread impairment.

There are several factors that may be relevant in the comparison of populations from the two different healthcare environment populations studied. Patients who are seen in the Diabetes Clinic (secondary healthcare) are often more complex, with associated comorbidities or diabetes complications, and often having poor glycaemic control resulting in the referral to a specialist service. This may lead to a higher level of Vitamin B12 deficiency owing to potential factors such as poor diet and maintenance of health. It is also quite possible that once referred to specialist care the patients are exposed to a more comprehensive health care environment that will consider and address factors that may be over looked in a primary health care setting. However these factors did not appear to have a significant influence on serum Vitamin B12 level, there was no difference between primary and secondary care. Age was seen to be significantly different between the two population groups, with the secondary care group being older (*Table 4.1.1*). Increasing age has been

associated with lower Vitamin B12 status in some patients (Green et al., 2005) and may cause confounding interaction with some of the comparisons between the population groups.

The screening in both Primary Health Providers as well as the Diabetes Clinic shows that there is a limited amount of Vitamin B12 monitoring occurring with over 20% of patients reviewed through the Diabetes Clinic having no reported serum Vitamin B12 (*Figure 4.1.5*). Only one of the four medical centres approached for recruitment had a regular serum Vitamin B12 screening program for patients with type 2 diabetes who are taking metformin. Medsafe guidelines for the treatment of patients with Type 2 Diabetes with metformin state that there is an investigated association of Vitamin B12 deficiency with long term treatment (Medsafe, 2009). Whether this is being regularly considered by primary health providers is not known. ADA guidelines only recommend Vitamin B12 testing in two scenarios; the diagnosis of patients who exhibit neuropathy, where prolonged metformin use is mentioned as a contributing factor, and in patients with type 1 diabetes who exhibit signs and symptoms of potential additional autoimmune pathogenesis (Amer Diabet, 2013). These recommendations may result in patients missing screening owing to delayed onset of neuropathic symptoms in patients with type 2 diabetes taking metformin. NZ Primary Care Handbook has no mention of potential Vitamin B12 deficiency associated with metformin use (*New Zealand Primary Care Handbook 2012.*, 2012). The Australian National Evidence Based Guideline for Blood Glucose Control in Type 2 Diabetes released by the NHMRC does not mention potential vitamin B12 deficiency as a result of metformin usage (Colaguiru, 2009).

The overall level of prevalence that was observed for impaired Vitamin B12 (serum Vitamin B12 <220 pmol/L) of 19.56% of patients (*Figure 4.1.6*) is similar to results from other studies (Mazokopakis & Starakis, 2012; Reinstatler et al., 2012). The lack of observable difference between both serum Vitamin B12 level and prevalence of decreased Vitamin status between the two investigated populations (*Table 4.1.6*) may be due to an absence of regular screening thus no difference in health care environment is influential on such values. Previous reports have shown that long term treatment of patients with Type 2 Diabetes with metformin can result in a lowered level of Vitamin B12 in anywhere from 5 – 22.6 % of the treated population (de Groot-Kamphuis et al., 2013) with one study claiming a 36.8% prevalence rate (Nervo et al., 2011). The vast range of prevalence rates are observed in countries of varying economic status. This may suggest that the decreased Vitamin B12 status is therefore more attributed to other factors, such as some form of dietary impairment, as opposed to metformin interactions. The Reinstatler *et al* study was based on

a population within the United States and reported a biochemical Vitamin B12 deficiency in only 5.8% of patients with type 2 diabetes taking metformin compared to only 2.4% of patients with type 2 diabetes who were not taking metformin. However there was also a reported rate of 3.3% prevalence of deficient biochemical Vitamin B12 in patients who did not have type 2 diabetes and who were not taking metformin. This may suggest that while the prevalence of metformin associated deficiency is higher and statistically significant there are additional factors which may influence the prevalence.

This is in stark contrast to the Nervo *et al* study which was based on a population in Brazil. While the range for deficiency in this study was slightly higher (250 pmol/L vs 220 pmol/L) the prevalence of decreased serum B12 in the Brazilian population was seen to be at a level of 36.8% of assessed patients. Differences between the two aforementioned studies are also seen in the effect of vitamin supplementation in the population. While supplementation with vitamin B12 in the study based on an American population did not result in significant alteration to prevalence, the Brazilian based population suggested an increase in Vitamin B12 intake is positively correlated to serum Vitamin B12 status. This supports the notion that the impairment is linked more closely to malnutrition of Vitamin B12 in the diet of the populations.

The distribution of serum Vitamin B12 level obtained in the screening was not that of normal distribution, but rather skewed towards the lower serum Vitamin B12 level with a tail to the higher levels (*Figure 4.1.3*). This in itself may suggest an increased risk of serum Vitamin B12 level lowering or may suggest a number of patients taking supplementary Vitamin B12 that was not noted in the screening phase. The peak of the distribution was seen to fall lower than would be expected of normal distribution and only slightly higher than the 220 pmol/L level of lowered Vitamin B12 status. According to the National Health and Nutrition Examination Survey (*Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 2012*, 2012) carried out in America between the years 2003 to 2006, the average serum Vitamin B12 level in the study population was 500 pg/mL (approximately 370 pmol/L). This value is seen to be higher than the mean serum Vitamin B12 level found in this study (354.77 pmol/L) (*Table 4.1.4*). As the survey was carried out on a study population intended to be a representative of national standards it can therefore be argued that the treatment of metformin, or arguably the presence of type 2 diabetes, in the population of this study did not have a significant effect on serum Vitamin B12 level. Furthermore prevalence data from the same survey suggests that there is a 2% prevalence of

Vitamin B12 deficiency (defined as $B12 < 148\text{pmol/L}$) in the national population. Applying the same standard for the deficient status to this study the prevalence would be 4.7%. Although metformin may be a factor in this it cannot be concluded that this is due solely to the metformin treatment, or whether type 2 diabetes is a more pronounced influence on serum Vitamin B12 level, or dietary/supplement factors (Obeid et al., 2013).

Unfortunately there is scarce literature on Vitamin B12 status in a general population in New Zealand. One study which aimed to evaluate the Vitamin B12 status of an elderly population in New Zealand (age >65) claimed there is a 35.54% prevalence of lowered serum Vitamin B12 level ($<221\text{ pmol/L}$) (Green et al., 2005). This level reflects the much researched association of age and decreased Vitamin B12 status, and suggests that the levels that were shown in this study may not be solely metformin associated but reflect more on the age of the patients involved. The prevalence of lowered serum Vitamin B12 level ($<220\text{ pmol/L}$) in our study in the elderly population (age ≥ 65) was compared with the population in the Green *et al.* study. It was observed that prevalence of decreased serum Vitamin B12 status in metformin treated patients in our study was lower than those in the Green *et al.* study. This comparison must be interpreted with caution as there are other factors that may affect the serum Vitamin B12 of these patients which were not addressed in this study (diet, drug interactions etc.). Furthermore as the patients recruited into the Green *et al.* study were non-institutionalized there may have been a level of malnutrition in regards to nutritional intake as has been observed in such populations (McElnay et al., 2012). However it is interesting to note that our sample population had a better Vitamin B12 status than that of a population without diabetes type 2 or metformin treatment

While there are some apparent reductions in Vitamin B12 serum as a result of metformin treatment, the natural presence of lowered Vitamin B12 levels in older populations and prevalence of deficiency in such patients may account for a large proportion of these cases. An analysis published in the American society of nutrition highlights this (Allen, 2009). The argument is made that, according to the national health and nutrition examination survey, the presence of Vitamin B12 deficiency is no higher in the tested population of metformin treated patients who have type 2 diabetes than in a standard population. Much like the presence of comparable rates of Vitamin B12 deficiency ($< 148\text{ pmol/L}$) the comparison between levels deemed to be borderline ($148 - 221\text{ pmol/L}$) bring about startling similarities. Evidence is cited that up to 20% of people assessed are within the range of “borderline” serum Vitamin B12 level. This figure is higher than that of the

population in this study (19.75%) (*Figure 4.1.6*). Of notable mention is the trend towards decreased serum Vitamin B12 in this study in patients taking metformin, a large portion of the population is seen to be located just above the cut off for Vitamin B12 deficiency and thus may still be at risk (<220 pmol/L) (*Figure 4.1.3*).

6.1.1 – metformin Dosage

The analysis of dosage of metformin on the serum Vitamin B12 level has shown results supported by previous research (de Groot-Kamphuis et al., 2013). An increase in the dosage of metformin correlates with an apparent decrease in serum Vitamin B12 (*Figure 4.2.3*). Linear regression suggests that for every milligram increase in metformin dosage, serum Vitamin B12 level are lowered by 0.040 pmol/L; in common dosage quantities this would suggest a dosage difference of 1000mg would result in an expected linear reduction of serum Vitamin B12 by approximately 40 pmol/L. This is evident in categorical comparison of metformin dosage (*Figure 4.2.4*). However it is not known by these results how this deficit would accumulate over time.

The mechanism of metformin inhibition of absorption is not fully understood. The current theory of inhibition is based on interference with intrinsic factor binding in the gut. It would be assumed that an increase in concentration of the inhibiting substance (metformin) would result in an increase in inhibition of said absorption. As metformin is excreted unchanged in urine with an elimination half-life of approximately 5 hours (Graham et al., 2011) it would be logical for the relationship between metformin and Vitamin B12 malabsorption to be somewhat linear. The daily intake of Vitamin B12 in the body, approximately 2.4 micrograms, is much lower than natural body stores, of close to 2500 micrograms (Mazokopakis & Starakis, 2012). Thus even with partial malabsorption caused by an external agent the effect would take a long period of time to develop clinical important deficiency. The apparent association between increased dosage of metformin and decreased serum Vitamin B12 level was observed in this study. Through linear regression there is a significant decrease of serum Vitamin B12 with increase in dosages (*Figure 4.2.3*); this can also be seen in a categorical dosage comparison (*Figure 4.2.4*). The reduction in serum Vitamin B12 level translates to an increase in prevalence of decreased Vitamin B12 status (through logistic regression); however no specific difference was seen between any two of the categories of metformin dosage. This may be due to the reasonably low populations in the groups of metformin dosages as on

appearance the prevalence levels seem to increase strongly with an increase in dosage of metformin. Odds ratio analysis exhibits this trend, showing increases in the risk rate of lowered serum Vitamin B12 level as doses of metformin are increased, with the most significant increase seen in the increase of dosage from below 1000 mg/day to between 1000 and 1500 mg/day (*Table 4.2.4*). The values for this increased odds ratio did not match that of a previous study (de Groot-Kamphuis et al., 2013) which claimed a value of 1.081 per 100 mg increase in daily metformin however this is based on a level of <150 pmol/L for decreased serum Vitamin B12 status.

A correlation was observed between the dosage of metformin prescribed and age (*Figure 4.5.3*). This correlation suggests that with an increase in age, a decrease in dosage of metformin is observed. This may be due to several different clinical reasons; as patients age treatment focus may tend to shift from preventing long term hyperglycaemic complications to preventing short term hypoglycaemic episodes. While metformin does not cause hypoglycaemia it may influence an episode in patients taking insulin. Renal impairment results in reduced excretion of metformin and thus reducing dosages once renal function begins to decline is common practice. Renal impairment is common in the elderly and is further increased with the development of diabetic nephropathy. The dose of metformin is often maximized in patients who are nearing the point of requiring insulin therapy; however as the additional benefits of doses greater than 2000 mg per day is marginal, after the introduction of insulin the dose is often reduced from 3000 mg per day to 2000 mg per day to minimize side effects. Adjustment for this factor into the regression model of dosage of metformin and serum Vitamin B12 level did not alter the significance of the model however.

6.1.2 – Duration of metformin Treatment

Duration of metformin treatment influences serum Vitamin B12 level. Due to the large natural stores of Vitamin B12 in the human body of around 2500 µg, and the reasonably low daily intake (≈2.4 µg) it is thought that a long period of malabsorption is required to cause clinically important Vitamin B12 deficiency (Mazokopakis & Starakis, 2012). The time point at which metformin use becomes a factor in the risk of Vitamin B12 deficiency and some form of clinical presentation is commonly thought to be between 5 – 10 years of treatment (Mazokopakis & Starakis, 2012). However there have been observations made on the effect of metformin on Vitamin B12 from as early as one year from commencement of treatment (Chen, 2012). The results from this study have

shown that there is an apparent but not statistically significant correlation between duration of metformin treatment and both serum Vitamin B12 level and prevalence of decreased Vitamin B12 status (*Figure 4.3.4*). Post-hoc analysis highlighted several duration ranges which were statistically different, the Pearsons correlation showed significance of duration of metformin treatment ($p = 0.044$). Durations of treatment of above nine years started to suggest linear decreases in serum Vitamin B12 level however with low numbers in some categories these changes were not statistically significant. A larger population study may help to clarify this relationship. As stated previously metformin excreted unchanged in the urine and has a reasonably short half-life within the body. Therefore current views on metformin interference with intrinsic factor binding leading to malabsorption of cobalamin would be a cumulative process as internal body stores are used and not replaced. Furthermore as the interaction between metformin and the cubulin receptor is said to work through competitive inhibition there would still be some cobalamin being absorbed dependent on the level of dietary intake.

A correlation between duration of metformin use and serum Vitamin B12 is observed in this study which supports previous findings of association; however the relationship between the two variables becomes more complex when confounding variables are considered. While the duration of metformin treatment was seen to correlate with serum Vitamin B12 level, it was also seen to correlate with age of the patient (*Figure 4.5.4*). This follows from logical reasoning that the longer a patient is on metformin, the older the patient is likely to be, however it creates a confounding effect on our analysis of the influence of the long term impacts metformin use may have on serum Vitamin B12. The associations drawn from this paper which are thought to be related to duration of metformin treatment may simply be the observation of the age related malabsorption that has been observed in other studies. This was tested with age adjustment of the regression model for the relationship between duration of metformin treatment and serum Vitamin B12 level. This resulted in a reduction of the significance of duration of metformin to an insignificant level ($p = 0.051$).

It is important to note a limitation of this study was the ability to collect accurate data for duration of metformin treatment. The data were collected as the first documentation of metformin treatment in an individual patient according to available databases. For patients in a primary health care environment this was the database of the medical centre they were currently enrolled in, for secondary health care patients it was the Diabetes Clinic database. Neither of these are robust

sources of information for this purpose. While electronic patient transfer aims to maintain medical notes of patients moving from one medical centre to the next, there is a possibility of incomplete records being used. Furthermore information regarding metformin duration obtained from diabetes clinic database is limited to the patient attendance to a secondary health service at which time metformin use was noted. This has likely resulted in many patients having much lower reported metformin duration than is actually the case and therefore caution is advised when interpreting the results and correlations made.

6.1.3 – Ethnicity

One of the unique aspects to this study is the consideration of ethnic differences in the New Zealand population, a factor that has not yet been explored in previous literature. The method of analysis between ethnic groups was based on grouping of the New Zealand Ministry of Health ethnic categories. This allowed a more statistically viable assessment of the effect of ethnicity on serum Vitamin B12 level. All categories in which there were more than 15 individuals who identified to be within the category were maintained as separate groups. This resulted in the five groups as defined in the results. All other individuals who identified as other categories were combined in a singular “Other” category (*Table 4.4.1*). The significant differences between the ethnic groups were seen to not only be in the serum Vitamin B12 level but also in the age variable where the Other European population was significantly older than all other population groups. Higher mean serum Vitamin B12 level was seen in NZ Maori and Samoan populations compared to the remaining groups (*Figure 4.4.1*). However this did not translate to a significant decrease in prevalence of Vitamin B12 deficiency. This may be due to small numbers of, particularly in the Samoan group, in the deficient category and a larger sample size may have helped clarify this.

The cause for a higher mean Vitamin B12 level in NZ Maori and Samoan populations compared to both NZ Europeans and Other European populations is not easy to distinguish. Previous research into micronutrient levels in different ethnicities did however show similar results (Hinds, Johnson, Webb, & Graham, 2011). Hinds *et al* has shown evidence that an “American Black” population has a significantly higher mean Vitamin B12 level than their “American White” counterparts. This may suggest that there is a genetic or lifestyle factor that predisposes people of Caucasian descent from yielding lower Vitamin B12 levels than those of other ethnical backgrounds.

Conversely non-European individuals may not be relatively protected from Vitamin B12 deficiency, again either through dietary or genetic factors.

For example a potential explanation for the difference in mean Vitamin B12 levels between the mentioned ethnicities may be elucidated from the 2008/2009 New Zealand Adult Nutrition Survey. This data shows that levels of Vitamin B12 consumption via the dietary groups of red meat and seafood are higher in the Maori population than that in the NZ European population (*A Focus on Nutrition: Key findings of the 2008/09 New Zealand Adult Nutrition Survey*, 2011). A similar pattern of dietary preference can be seen in the Pacific population, of which the Samoan ethnic group is part of, in which a higher proportion of Vitamin B12 is derived from fish and seafood compared to that of NZ Europeans. Vitamin B12 is at a much higher concentration in meat, fish, and shellfish as opposed to milk, which is the primary source for European populations (9.4 µg/100g, 9.9µg/100g, 52.4µg/100g and 0.4µg/100g respectively) (Watanabe, Yabuta, Tanioka, & Bito, 2013). Thus greater intake may explain the higher levels of Vitamin B12 in Maori and Samoan populations. The inclusion of some form of dietary assessment such as a food frequency questionnaire with the specific food items may have provided more insight into this.

6.1.5 – Future Research

In order to better understand the role that ethnicity may play on serum Vitamin B12 level of New Zealand patients with Type 2 Diabetes being treated with metformin a greater population size should be considered to ensure comprehensive demographic consideration. With known predictor variables such as age being influential in this analysis it is recommended that a younger population group be saturated to allow comparative analysis. More thorough investigation needs to be carried out on the variable of ethnicity on both serum Vitamin B12 and prevalence of decreased Vitamin B12 status. This should consider the factor of dietary intake as aforementioned in order to assess not only ethnic dietary differences but also age related nutritional comparisons. The use of a healthy control population, and/or a group without diabetes who are taking metformin for another reason, such as woman with polycystic ovarian syndrome, to assess the effects of metformin on a population of patients who do not have type 2 diabetes would further lend information towards the understanding of the potential interactions of metformin and Vitamin B12 absorption.

6.1.6 – Concluding statements

The results of the prevalence aspect of this research has given new insight into both the prevalence of Vitamin B12 deficiency in New Zealand as well as suggested factors that may impact the likelihood of a patient developing said deficiency.

While correlations have been seen between factors of metformin treatment (dosage and duration), it is not clear whether the factor of metformin treatment is the most influential in regards to a decreased Vitamin B12 status in a New Zealand population base. Prevalence of decreased serum Vitamin B12 level was comparative to populations in population of similar economic development where there was no metformin treatment present. Coupled with the high prevalence of age related vitamin malabsorption, and high levels of elderly patients in this study, it is difficult to ascertain that the observed deficiency was significantly caused by metformin interaction without an appropriate control population, or a prospective randomised controlled design. The role of ethnicity in Vitamin B12 status is important in the New Zealand population with marked differences between the ethnic groups. Whether this is related to genetic, lifestyle or dietary factors is uncertain.

Much evidence has been put forth to suggest that the use of serum Vitamin B12 level as a measure of physiological Vitamin B12 functionality is insufficient, thus proposing that any observable serum Vitamin B12 deficiency that may be linked to metformin treatment may not necessarily lead to clinical implications. This study further supports the previous findings of prevalence rates of serum Vitamin B12 deficiency but does not provide insight into the outcomes of such an effect through assessment of any markers of Vitamin B12 functionality.

6.2 – Randomized Control Trial

The randomised controlled study has given evidence that the treatment of patients with decreased serum Vitamin B12 level with both Hydroxocobalamin injections and Methylcobalamin sublingual supplementation is effective in correcting an impaired Vitamin B12 status. Both treatments resulted in significant improvements in not only serum Vitamin B12 level (*Table 5.3.1*) but also showed marked decreases in prevalence of low Vitamin B12 levels (*Table 5.5.1*). While both treatments were effective in the management of decreased Vitamin B12 levels, there were significant differences in the level of therapeutic impact in both short and long term instances (*Figure 5.3.2*).

The current treatment method of choice for Vitamin B12 deficiency is intra muscular Hydroxocobalamin injections. These have been shown to improve both biochemical markers for B12 deficiency and also improve physiological functions of Vitamin B12 deficiency (Castelli & Kragie, 2013; Glass, Skeggs, & Lee, 1966; Hvas & Nexø, 2006; M. R. Turner & Talbot, 2009). The administration of a single dose of 1mg Hydroxocobalamin was seen to correct serum Vitamin B12 status in a majority of the patients treated in this study. Furthermore the corrected levels were seen to remain in the normal range for up to 6 months post-treatment. This long term effect of Hydroxocobalamin treatment is thought to be due to the increased retention of the cobalamin form compared to other B12 vitamins.

The current treatment protocol for Vitamin B12 deficiency dictates that a loading period of Hydroxocobalamin should be used in order to raise baseline levels before a periodic maintenance regimen should be initialised. One study suggests a daily 1mg administration of Hydroxocobalamin daily for one week followed by 1 mg once weekly for 4 weeks, subsequent maintenance of B12 is achieved through 1 mg administration every 2-3 months (Mazokopakis & Starakis, 2012). While this regimen would lead to an increased rate of Vitamin B12 correction, results from our study suggest that this protocol would lead to over correction in decreased levels of Vitamin B12 caused by metformin treatment. Evidence for this can be seen not only in the success of treatment with a single Hydroxocobalamin treatment in 71.4% of patients, but also in the maintenance of the resolved Vitamin B12 levels in all but one of the patients at the 6 month time period (*Table 5.5.1*).

There is little information about the possible consequences of elevated serum Vitamin B12 level, and the masking of other biomarkers is possible with excessive Vitamin B12 in the blood.

6.2.1 – Treatment factors

Methylcobalamin treatment exhibited a quicker increase in serum Vitamin B12 level (*Table 5.3.1*). This steeper increase in blood levels of B12 may be caused by several factors in the treatment; the mode of treatment, the increased dosage of B12 as compared to the comparative treatment group, and finally the binding affinity and clearance of Methylcobalamin compared to Hydroxocobalamin.

The use of sublingual delivery is a growing trend in vitamin supplementation. The method evades the potentially damaging first pass of the liver and allows a more direct absorptive path into the capillaries and thus the rest of the blood. This is of higher importance in the case of metformin induced Vitamin B12 malabsorption as the proposed method of interaction of metformin is that of interference with intrinsic factor absorption of Vitamin B12 during digestion (Bauman et al., 2000). The use of sublingual treatment therefore would evade such a mechanism and should allow for direct Vitamin B12 absorption. The dosage regimen for the sublingual treatment group involved a single 1000mcg sublingual tablet taken once a day for 30 days. This is a higher dosage than that of the single 1mg injection administered to the other treatment group and could explain a much higher treatment response. Although the response is markedly less than that of the Hydroxocobalamin when considering increase in serum Vitamin B12 level pmol/mcg of treatment provided. Methylcobalamin is one of the physiologically active forms of cobalamin; there is evidence to suggest that the uptake cobalamin in this form has a different affinity than that of Hydroxocobalamin due to binding affinity of transcobalamin (Gherasim et al., 2013).

The assessment of sublingual treatment as a comparable treatment to oral supplementation has resulted in corrections of serum and functional marker of Vitamin B12 status (Yazaki et al., 2006). Methylcobalamin has been shown to be equally as effective in sublingual treatment as in oral administration in an elderly population (age 50 – 80). Results from this study support the observation of increased serum Vitamin B12 level with treatment of Methylcobalamin however it cannot be asserted whether there was an increase in the physiological effect of Vitamin B12 as functional biomarkers were not assessed. Intestinal malabsorption of Vitamin B12 in elderly population may mimic the malabsorption of Vitamin B12 in patients being treated with metformin

due to the phase of absorption and interaction of intrinsic factors that are influenced (Bauman et al., 2000; Yazaki et al., 2006); therefore there may be physiological improvement in an impaired functionality of Vitamin B12 deficiency caused by metformin treatment with Methylcobalamin sublingual supplementation. Other administration routes for the treatment of Vitamin B12 deficiency have shown effective increases in of serum Vitamin B12 level which would avoid the oral route of administration such as intranasal administration (Lonterman et al., 2000). Again such routes are seen to increase serum Vitamin B12 level but an absence of physiological functionally testing has not addressed the issue of the translation ability of serum levels in diagnosis of Vitamin B12 deficiency.

6.2.2 – Long term benefits

The differences in the long term effects of the two treatment groups may be attributed to the different clearance and excretion properties of the two cobalamin vitamers (*Table 5.4.1*). Research into the clearance of Hydroxocobalamin has been scarce since the 1960's when the drug was extensively investigated as a replacement to cyanocobalamin as a treatment option for patients suffering from pernicious anaemia and Vitamin B12 deficiencies. In a detailed assessment of Hydroxocobalamin clearance following both 500mcg and 1000mcg injections the levels of serum Vitamin B12 were analysed at 5, 24, 48, 72 hour post injection time points, as well as at 1, 2, 3, and 4 week post injection time points (Hertz, Kristensen, & Hoff-Jorgensen, 1964). These data highlighted a significant short term increase in B12 which decreases significantly by 72 hours. Measurements of urinary excretion of B12 found that 27% of the 1000mcg dose of Vitamin B12 was removed from the body within the first 72 hour period. According to this pattern Vitamin B12 retention, the time points for measurements of Vitamin B12 for patients in this study will have missed the initial peak level of serum Vitamin B12 level. While this may not be ideal for complete analysis of the treatment protocol, it may be argued that the initial increase of Vitamin B12 in the blood does not provide extensive therapeutic advantage to the patient as the clearance is rapid.

Further study into the mechanism of Hydroxocobalamin has shown a high affinity of binding to liver cells which have been identified as a location of high Vitamin B12 storage. Vitamin B12 bound to transcobalamin II is rapidly cleared from plasma and preferentially distributed to hepatic parenchymal cells where it remains as an active coenzyme. This transport of cobalamin from the

blood and into an intracellular storage state may explain the large discrepancy between the measured amount cobalamin excretion and the serum Vitamin B12 level after injection with Hydroxocobalamin. It has also been suggested that there is preferential movement of cobalamin to liver storage by TC-II in the presence of serum albumin when the cobalamin is in the form of Hydroxocobalamin (Begley, Colligan, & Chu, 1993). This may suggest a higher preferential storage and slow usage of Vitamin B12 in the Hydroxocobalamin form, this would also aid in explanation of the lower response peak of Vitamin B12 in the serum but a longer effect tail as the vitamin is used. This is comparative to the rapid decline in serum Vitamin B12 level in the patients assigned to the Methylcobalamin sublingual supplement group. Whilst receiving treatment, over the first 3 months, the results showed large increases in serum Vitamin B12, all patients were resolved at the 3 month time point. However the retention of this treatment effect was much lower than that of Hydroxocobalamin (*Table 5.4.2*). This may be due to the specificity of the serum Vitamin B12 assay which does not distinguish between bound and unbound Vitamin B12 it may be that the high increase in serum levels is due to unbound, and non-functional free Vitamin B12, which seem to be rapidly removed from the blood once treatment is ceased. This has resulted in two patients returning to a deficient Vitamin B12 status (<220 pmol/L) by the end of 3 months of observation (*Table 5.5.2*).

6.2.3 – Cellular considerations

Cellular uptake of cobalamin requires the presence of transcobalamin as the binding of the complex to the transcobalamin receptor is structurally specific. Unbound transcobalamin (apo-transcobalamin) in the blood can bind to free Vitamin B12 molecules for transport of the molecule to cells that require its functionality. Free Vitamin B12 in the blood is thought to be not internalized due to the absence of an uptake mechanism that does not rely on some form of transcobalamin binding (Quadros, 2010). Serum Vitamin B12 assays measure the level of total Vitamin B12 in the blood, both bound and unbound forms, free forms, and therefore may not represent the measure of functionally available Vitamin B12 in the body (Hvas & Nexø, 2006). The presence of circulating haptocorrin in the blood may bind free forms of Vitamin B12 thus making it inaccessible for transcobalamin binding and cellular uptake. Supplementation of Vitamin B12 in patients with Vitamin B12 deficiency has been seen to increase both serum levels and functional biomarkers, total homocysteine, of Vitamin B12 activity (Deshmukh et al., 2010) in certain populations while only

significantly improving serum Vitamin B12 in other populations (Favrat et al., 2011). This may be due to the main causative factor of Vitamin B12 in each of the population structures, malnutrition vs. malabsorption. Treatment of decreased Vitamin B12 in elderly patient populations with oral supplementation has been to increase both serum Vitamin B12 and specific holotranscobalamin levels in deficient patients (Albers et al., 2012) suggesting that impairment in absorption of Vitamin B12 can be corrected by increased dietary supplementation of the vitamin.

It has been suggested that metformin increases liver preferential storage of Vitamin B12 (Greibe, Miller, et al., 2013), coupled with the higher affinity of Hydroxocobalamin to bind to hepatic parenchymal cells via preferential trafficking (Begley et al., 1993), may suggest that the decreased serum Vitamin B12 level response to Hydroxocobalamin compared to Methylcobalamin is due to increased hepatic storage of the vitamin as opposed to a more deficient state or lower total Vitamin B12 in the body.

6.2.4 – Potential Confounding Factors

There were several interactions noted during the study period that were seen to have a potential influence on the outcome measures:

Final decreased Vitamin B12 status prevalence in this study was seen to be significantly correlated to the co-treatment of patients with Omeprazole; an increased rate of relapse into cobalamin deficient states was seen in patients who were also treated with the drug. Research into the use of proton pump inhibitors (such as Omeprazole) and their effect on cobalamin levels have yielded mixed results (Marcuard, Albernaz, & Khazanie, 1994; Schenk, Festen, Kuipers, Klinkenberg-Knol, & Meuwissen, 1996).

It is proposed that the use of PPI decreases the ability of Vitamin B12 cleavage from dietary proteins and thus inhibiting the facilitation of intrinsic factor binding (Bradford & Taylor, 1999). While some studies suggest direct correlation between proton pump inhibitors (PPIs) and a decreased Vitamin B12 state (Hirschowitz, Worthington, & Mohnen, 2008) these results are not limited to interactions with the administration of the PPIs but suggest an unobserved further interaction to cause the deficient state.

Common conclusions are drawn that may suggest that the combination of old age and treatment with PPIs may have a synergistic effect on reducing absorption of Vitamin B12 but the sole interaction of PPI does not influence this enough to consider it as a separately contributing factor. Results from this intervention study might suggest a more direct correlation between serum Vitamin B12 and PPI treatment. This interaction was only observed after some form of intervention and Vitamin B12 supplementation and was not seen to correlate to baseline Vitamin B12 levels or prevalence of decreased Vitamin B12 status.

Such a selective interaction is unusual in this study as both the treatment options provided the therapeutic administration of Vitamin B12 to the blood without passage through the gut absorptive pathway which is the location of PPI interaction with malabsorption (Bradford & Taylor, 1999). This would suggest that the patients exhibiting a deficient state at the 6 month time period not only have very low retention of introduced Vitamin B12, but also may suggest that the effect of PPI treatment may have effects on serum cobalamin levels and not just malabsorption in the gut. While this is an untested theory it has been observed in this study that all three of the patients that were in lowered Vitamin B12 status at the end of the 6 month time point were also being treated with PPIs, and no patients not being treated with PPIs were in such a state at the said time point. Furthermore at the 3 month time point, of the two patients that were found to have not been resolved of a decreased Vitamin B12 status one was being treated with PPIs, the other was a strict vegetarian. A much larger study, with a factorial design including PPI use would be required to specifically test these hypotheses.

Only one patient was still in a deficient Vitamin B12 state after an initial and 3 month treatment. While the patient made significant improvement in serum Vitamin B12 level after each of the treatments (Hydroxocobalamin injection) it is thought that a combination of a low baseline serum Vitamin B12 level (80 pmol/L, the lowest of the study population) and strict vegetarianism were to be attributed to the lack of Vitamin B12 resolution. This case further raises the issue of synergistic effects of numerous factors leading to a deficient state. It can be argued that it was the lack of dietary intake of Vitamin B12 by the patient that may have contributed largely to the development of a decreased Vitamin B12 status; not helped by some form of malabsorption caused by metformin treatment.

No significant symptoms of Vitamin B12 deficiency were observed in the study population through analysis of symptomatic assessment (Michigan Neuropathy Screening Instrument) (*Table 5.6.3*). Of the study population only one patient from each group was seen to meet the criteria for neuropathy (Al-Geffari, 2012), of which one of the two had previously documented and treatment of diabetic neuropathy. The consideration of the symptom of general fatigue was difficult to assess as while there was a high number of patients claiming to experience the symptom (*Table 5.6.4*) there was no specific correlation with the presence of the symptom and serum Vitamin B12 level. The difficulty is whether the qualitative measurement of general fatigue is applicable in a population seen to be of an older age with type 2 diabetes. The potential for additional factors affecting the outcome of this is high and there may be no interaction with metformin treatment on the presence or absence of such a symptom.

6.2.5 – Future Research

Further study should be aimed at correctly assessing the presence of an impaired state of Vitamin B12 in patients being treated with metformin to determine if the apparent deficiency in serum levels is functionally relevant. This has been shown to be viable through assessment of MMA and tHcys levels. Investigation into the retention of free cobalamin in the blood compared to that of intestinally ingested cobalamin will aid in the understanding of the efficacy of sublingual treatment against oral medications. Further exploration of retention dependent on the form of cobalamin administered may allow for a better understanding of the potential preferential trafficking of different vitamers to the storage organs such as the liver.

Assessment of additional time points may provide a more concise picture of the action of the treatments and capture an alternate peak shape which may have passed before the first time point at three months. Similarly a longer observation period post treatment would allow more investigation into the retention and excretion of the different cobalamin forms and observation of return to baseline levels of Vitamin B12 levels of each patient. As evidence from this study has shown, the rate of decrease of serum Vitamin B12 post treatment differs significantly between the treatment options (*Table 5.4.2*) and would be of interest in further examination.

6.2.6 – Concluding statements

The evidence from this study has shown that decreased serum Vitamin B12 level in patients with type 2 diabetes who are exposed to long term treatment of metformin can be alleviated through treatment with either Hydroxocobalamin injections or Methylcobalamin sublingual supplements. There is an apparent difference in the mode and shape of treatment effect between the treatment groups however and this may lead to preferred treatment through one option as opposed to the other. While the sublingual treatment showed rapid increase in serum Vitamin B12 level in a short period of time, the benefit of the treatment was seen to decrease rapidly once supplementation ceased. Treatment with Hydroxocobalamin showed a more modest short term increase in serum Vitamin B12 level but had a more consistent long term profile. It may be suggested that a combination of both treatment options be considered; an initial three month treatment period of sublingual supplementation to build up serum levels followed by maintenance of corrected levels with periodic Hydroxocobalamin injections.

Results from neuropathy screening suggest that which has been previous suggested; decreased Vitamin B12 status in metformin treated patients is not based on levels of physiologically functional Vitamin B12 and is therefore not clinically detrimental. While the number of patients exhibiting neuropathic symptoms was low, it can be suggested that this was due to the sample size of patients assessed, and as no control group was considered it is difficult to rule out the absence of neuropathy caused by decreased Vitamin B12 levels associated with metformin treatment.

6.3 – An Alternate Hypothesis

6.3.1 – Asymptomatic deficiency

Of concern is the low number of published findings that link the decrease of serum Vitamin B12 level seen in metformin treated patients to direct physiological impairment of Vitamin B12 functionality. As mentioned before recent papers (de Groot-Kamphuis et al., 2013; Obeid et al., 2013) have suggested that the lowered status of Vitamin B12 as a result of metformin treatment is not a true deficiency and thus explain the apparent absence of the well documented symptoms of Vitamin B12 deficiency through other routes of pathogenesis. These two papers both put forth evidence that whilst the classic lowered serum levels are still seen in the test populations, intracellular and functional Vitamin B12 levels were not altered after metformin exposure. Investigation is needed to explain why the traditionally low serum Vitamin B12 level which would usually indicate a deficient state of cobalamin are being found to be asymptomatic.

6.3.2 – Displacement vs. malabsorption

A potential mechanism for this phenomena can be drawn from results in a study mentioned previously in which a population of rats were intravenously administered a 3 week regimen of metformin treatment (Greibe, Miller, et al., 2013). The study mimicked previous reductions in plasma Vitamin B12, however showed that rather than a decreased absorption of cobalamin, the results suggest a displacement of cobalamin into the liver of the treated rats. Results show 36% higher liver content in the liver of the metformin treated population compared to the saline, control, population. This was coupled with a 34% decrease in cobalamin found in the kidney of the cases. Total cobalamin detected in the rats was comparable between the two populations and suggested that there was no presence of malabsorption from dietary Vitamin B12 sources. This mechanism of displacement would explain the state in which a patient being treated with metformin has a decreased serum level of Vitamin B12 but no markers of traditional deficiency. As the liver is known to be the main storage organ for Vitamin B12 the increased uptake would allow continual physiological functionality of the vitamin.

A key weakness in this study however is the mode of delivery of metformin into the body of the rats. As the drug is administered subcutaneously it avoids the potential site of interaction in the

gut where Vitamin B12 is proposed to be interfered with. During cobalamin absorption the vitamin binds to cubulin receptors which are required for the calcium dependent channelling of the molecule into the ileum. However it is believed that competitive metformin binding to this B12-cubulin complex leads to membrane potential alterations and repulsion of calcium ions. As the channel is calcium instigated the interaction leads to malabsorption (Gilligan, 2002). This interaction is supported by results from calcium supplementation which is seen to reverse the malabsorption in treated patients (Bauman et al., 2000) by reactivating the calcium dependent channels. However the results from the Greibe *et al* paper may still hold true via oral administration of metformin but have a less severe increase on preferential absorption by the liver due to a tandem malabsorption affect which has been commonly suggested. This would explain the presence of correlations between both drug dosage and duration of treatment as seen in this study and previous literature whilst explaining the continual functionality of Vitamin B12. Current assessment of Vitamin B12 levels are through either serum levels or metabolite levels. As there have been no studies found which have assessed the possibility of increased internal Vitamin B12 storage in the liver of patients being treated with metformin the mechanism could be a plausible reason for absence of symptoms in treated patients.

This theory is supported by evidence put forth which suggested an increased ability to absorb circulating serum Vitamin B12 in patients treated with metformin (Obeid et al., 2013). This theory claims that lowered levels of serum Vitamin B12 in patients being treated with metformin is indicative of an increased intracellular level of B12, this is supported by an absence in changes in physiological functions of Vitamin B12 (Greibe, Trolle, Bor, Lauszus, & Nexø, 2013). Further evidence of this is noted in a study which highlights the absence of typical Vitamin B12 deficiency symptoms in patients with metformin induced deficiency (de Groot-Kamphuis et al., 2013). A study on the effect of metformin on cobalamin status of women with polycystic ovary syndrome further supported this claiming that while serum Vitamin B12 level dropped significantly with introduction of metformin treatment, measures of physiological cobalamin (holotranscobalamin) were not altered (Greibe, Trolle, et al., 2013). It may be suggested that a decrease in serum Vitamin B12 in type 2 diabetic patients being treated with metformin is due to increased uptake of TCII-B12 complexes for both cellular use and organ storage, therefore not leading to a deficient B12 state but rather a lower circulating measured Vitamin B12 concentration.

The mechanism of interaction that would lead metformin causing such a displacement effect on Vitamin B12 is not elucidated to in the Greibe *et al* paper. A potential mechanism may involve an increase in affinity to haptocorrin binding by cobalamin. As the protein has corresponding cell surface receptors located only on reticuloendothelial storage cells(Herbert, Fong, Gulle, & Stopler, 1990) this may lead to increase in Vitamin B12 deposit in the liver. As the mechanism of metformin function on inhibition of gluconeogenesis is believed to primarily liver based there may be a direct influence on liver uptake of circulating cobalamin after metformin exposure. In order to understand such a mechanism further study must be conducted into the possibility of Vitamin B12 storage in the liver as a cause of lowered serum Vitamin B12. Furthermore, assessment of serum Vitamin B12 level have been shown to be inadequate in their own for the diagnosis of Vitamin B12 deficiency and evidence has been seen that such information be interpreted with caution. The assessment of patients in this study was based on serum levels alone primarily because of funding restrictions, but also as this is the measure analysed routinely in primary care.

6.3.3 – Evidence from study results

Increased liver uptake of cobalamin also leads to a further understanding of the overall shape of serum Vitamin B12 in patients who were treated with the Hydroxocobalamin injection. The increase at the 3 month time point was followed by a decrease of only 14.3% over the following 3 months during which time there was no repeated treatment of patients. This decrease had a mean value of 24.75 pmol/L over the 3 month observation period (*Table 5.4.2*) and may be attributed to the natural utilization of Vitamin B12 by the liver as it secretes the vitamin into bile in an enterohepatic cycle, in which up to 60% is not reabsorbed (Gherasim et al., 2013; Kanazawa & Herbert, 1983). Longer observation of patients following the administration of Hydroxocobalamin would allow a higher understanding of the residual effect of Hydroxocobalamin absorption and distribution throughout the body.

6.3.3 – Diagnosis of deficiency

There are contradicting beliefs on the relevance of serum Vitamin B12 level in patients with Type 2 Diabetes who are being treated with metformin. Obeid *et al* suggest that the serum level of the vitamin itself is not sufficient enough in assessing the bioavailability and chemical activity of Vitamin

B12 as the lowered serum level of the vitamin does not necessarily reflect any functional disadvantage in the patient (M. R. Turner & Talbot, 2009). The study suggests that only through assessment of Vitamin B12 metabolites (holotranscobalamin and methylmalonic acid) and intracellular levels can a full understanding and evaluation of metformin influence be achieved (Obeid et al., 2013). Strong evidence is given to show higher methylation indices in patients treated with metformin which would indicate higher level of Vitamin B12 functionality in these patients. This theory is supported by work by de Groot-Kamphuis *et al* which highlights that although there is an observed Vitamin B12 deficiency (measured only through serum level assessment) in patients treated with metformin, there are no clinical consequences of such deficiency in the patient population (de Groot-Kamphuis et al., 2013).

Conversely, studies published recently have completely contradictory results to the above mentioned. Wile *et al.* exhibited that metformin treatment for a period of longer than three months was associated with decreased serum Vitamin B12 level (as seen in many studies), but also in higher homocysteine and methylmalonic acid (MMA) levels, which would suggest an inhibited Vitamin B12 functionality (Wile & Toth, 2010). These observations were linked to clinical consequences with increased neuropathy scores. However it is key to note that the recruitment for said study was based on a requirement of existing peripheral neuropathy as well as metformin treatment. This selection criterion may lead to external factors contributing towards the presence of altered biochemical markers as the clinical significance of Vitamin B12 deficiency was a selection criteria as opposed to a measured outcome.

A further study conducted by Sato *et al* (Sato, Ouchi, Funase, Yatnauchi, & Aizawa, 2013) attempts to draw a similar association between metformin treatment and a change in the biochemical markers of Vitamin B12 functionality. Once again there was an associated decrease in serum Vitamin B12 in the metformin treated patients compared to a non-metformin treated population. This association continued with altered ratios of homocysteine (HC):B12; however there was no significant correlation found directly between metformin treatment and increased homocysteine levels. This suggests that while the mentioned ratio of HC: B12 changed, this result was due to the lowering of serum Vitamin B12 and not a significant increase in homocysteine. The study continues to draw significant correlations between the markers of HC and impaired physiological functions of retinopathy and renal function (eGFR) however fails to relate the HC

increase exclusively to metformin treatment. Furthermore the study relies on a metformin treated population which is comprised of patients with a significantly higher mean body mass index (BMI), 26.1 ± 4.2 vs. 24.3 ± 3.0 ($p = 0.02$), than the non-metformin population. The factor of BMI has been shown to increase levels of HC independent of treatment (Park & Georgiades, 2013; Sanlier & Yabanci, 2007), and therefore any physiological differences between the two populations may be as a result of this association.

In the presence of true Vitamin B12 deficiency the risk of developing hyperhomocystemia has been documented in populations with type 2 diabetes and without subdivision of treatment with metformin (Mahalle, Garg, Kulkarni, & Naik, 2013). In these particular studies the drop in serum Vitamin B12 status was attributed to both the presence of a diabetes state and the high prevalence of Vitamin B12 deficiency in the national populations. Dietary Vitamin B12 deficiency has been a documented problem in India due to vegetarianism, and leads to development of hyperhomocystemia.

Singh, A *et al* exhibited differences in neuropathy which were attributed to impaired serum Vitamin B12 level in a metformin treated population compared to a non-metformin treated population (Singh, Kumar, Karmakar, & Jha, 2013). The use of the Toronto Clinical Neuropathy Screening System allowed for assessment of neuropathy in the study populations. While there were significant results found between the groups, these were limited to a slight increase in number of patients expressing mild neuropathy in the metformin group. There was no significant difference in moderate or severe neuropathy findings between the populations.

6.3.4 – Potential implications

Through the observation of both confounding prevalence rates and the potential mechanism of Vitamin B12 displacement and not malabsorption it is proposed that metformin may not have clinical significance on Vitamin B12 functionality. It has been shown that while there is an increased prevalence of decreased serum Vitamin B12 level in metformin treated patients, this increase may not be attributed solely to metformin use but rather a combination of factors. The influences of age and nutritional intake have both shown much stronger correlations with Vitamin B12 status. Furthermore evidence has been shown and discussed which suggests that a decrease in serum Vitamin B12 that arises as a result of metformin treatment may not be clinically detrimental owing

to the absence of both symptomatic expression and biochemical functionality impairment in patients showing decreased serum levels. It has been proposed that while there may be a level of malabsorption of Vitamin B12 due to metformin interaction, the change in serum levels may also be caused by a displacement of Vitamin B12 into storage sites where it remains functional.

Further studies should aim to address this apparent displacement model and assess the functionality of Vitamin B12 in patients expressing decreased serum levels in order to ascertain if there is physiological indication of deficiency. This could avoid the potentially unnecessary treatment of many patients who have apparent Vitamin B12 deficiency, but normally functioning levels of the vitamin. This may also alleviate the need for monitoring of Vitamin B12 levels in those taking metformin and would reduce the burden both personally and financially on patients and the health system.

7.0 – References

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8.0 – Appendices

Appendix A – Serum Vitamin B12 assay protocol

Vitamin B12 3.2.doc**VITAMIN B12****Principle**

Competitive electrochemiluminescence immunoassay

Specimen Requirements¹

Serum (SST) (BIOHAZARD)		√
Plasma Na Heparin or K ₃ -EDTA plasma (Preferably fasting)		√
Stability	2 – 8°C	48 hours
	-20°C (Protected from light)	2 months

- Samples must be free from cells and particulate matter.
- Freeze only once and mix thawed samples prior to analysis.
- **Protect serum from light**
- Do not use heat-inactivated samples.
- Patient samples, calibrators and controls must be brought to room temperature (20-25°C) before analysis.
- Azide–stabilised samples, calibrators and controls are unsuitable for analysis.

Reagent Handling

Reagents in kit are ready to use. Store kit upright in order to ensure complete availability of microparticles during automatic mixing prior to use. Open bottle caps manually before use. Allow cooled reagents to equilibrate to room temperature before placing onto reagent carousel. Avoid the formation of foam.

WARNING:

Pre-Treatment 2 reagent (grey cap) contains Sodium Hydroxide (CORROSIVE), and Sodium Cyanide (TOXIC). Refer to Section 1: Safety.

Performance Specifications¹

Sensitivity	Analytical: 22 pmol/L
	Functional: Not stated
Measuring Range: 22 – 1476 pmol/L	

Dilution Protocols

Specimen pre-dilution is not required (see Results Management)

Analytical Specificity¹

The following cross-reactivity was found:

Cobinamide dicyanide 200ng/mL 0.024%

Interferences and Limitations¹

Interferent	Level of interferent below which there is < 10% interference
Haemoglobin	<10 g/L
Bilirubin	<1112 µmol/L
Lipaemia	<15 g/L
Biotin	<205 nmol/L

Other:

- In patients receiving therapy with high biotin doses (i.e. >5mg/day) no sample should be taken until 8 hours after the last biotin administration.
- In rare cases, interference due to extremely high titres of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur.
- Samples with extremely high total protein concentrations (e.g. patients suffering from Waldenstrom's macroglobulinaemia) are not suitable for use in this assay, since they may lead to the formation of protein gel in the assay cup. The critical protein concentration is dependant upon the individual sample composition

Reference Interval²

- Vitamin B12: Unisex 130 – 830 pmol/L.

Results Management

- Results <44 pmol/L - autp report using <B12.
- Results >1475 pmol/L – auto report >B12.

B12 results less than 250 pmol/L are held on verification screen for manual release.

Confirm QC results are satisfactory (for each reagent pack in use) before releasing results.

If QC results are within specification, release results ≥ 90 pmol/L.

If result is < 90 pmol/L - check previous result(s) where available. If previously low, then release current result.

If this is the first low B12 result for a patient - check MCV. If MCV ≥ 98 , release result. Otherwise repeat B12 assay. If repeat result is within 20 pmol/L, add comment `conf` to the report.

Interpretation

Nutritional and macrocytic anaemias can be caused by a deficiency of Vitamin B12. This deficiency can result from diets devoid of meat and bacterial products, from alcoholism or from structural/functional damage to digestive or absorptive processes (forms of pernicious anaemia). Malabsorption is the major cause of deficiency, through pancreatic deficiency, gastric atrophy or gastrectomy, intestinal damage, loss of intestinal Vitamin B12 binding protein (intrinsic factor), production of autoantibodies directed against intrinsic factor, or related causes. Vitamin B12 is necessary for normal metabolism, DNA synthesis and red blood cell regeneration. Untreated

deficiencies will lead to megaloblastic anaemia and irreversible central nervous system degeneration.

Normal absorption of B12 requires a non-vegetarian dietary source, a normal stomach to produce intrinsic factor, and a normal terminal ileum to absorb the B12/IF complex. A healthy person with replete body stores has enough B12 to last 3-6 years if no more is ingested.

Low B12 levels causes include:

- vegetarian diet
- *drugs* - oral contraceptives, metformin, methotrexate, colchicine, slow K, anticonvulsants, cimetidine, triamterene
- pregnancy - B12 levels are often low without tissue deficiency
- low B12 binding protein, transcobalamin I, which can be measured, but the expense is seldom warranted
- pernicious anaemia
- Intrinsic factor/parietal cell antibodies.
- malabsorption
- inflammatory bowel disease: Crohn's disease, or ulcerative colitis in the terminal ileum
- previous gastrectomy or ileectomy
- primary folate deficiency
- Diphyllbothrium fish tapeworm infestation (loss of ingested B12)

Further investigation and treatment of low B12

If haematological abnormalities are present - raised MCV, anaemia, neutropenia, thrombocytopenia, oval macrocytes and hypersegmented neutrophils in the blood film - the underlying cause must be identified

If there are no haematological abnormalities and the patient seems healthy, there is less urgency. The level may be normal for that person or it may indicate early deficiency. It is a matter of clinical judgement and patient choice whether to investigate further at once; or follow-up in 6. The elderly often have low B12 and folate levels and may benefit from supplements.

Elevated B12 levels include:

- oral or parenteral vitamin B12 supplement
- haematological disorders: myeloproliferative disorders, leukaemias, high white cell counts
- liver disease, chronic renal failure, severe CHF

References

1. Roche package insert 04761707001
2. Aotea Pathology: Data on file

Appendix B – Baseline Questionnaire for patients in randomised control trial

Metformin and Vitamin B12 Study Baseline Questionnaire

Metformin and Vitamin B12 Study

Study ID: NHI:

Date:

Surname:..... First Name:

Preferred name:.....Title: Mr / Mrs / Miss / Ms (delete what does not apply)

Date of birth:

Sex :

Full Address:

.....

.....Postcode.....

Phone: Home

Work

Mobile

E mail address:

Ethnicity: Tick all that apply

New Zealand European ☐

Maori ☐

Samoan ☐

Cook Island Maori ☐

Tongan ☐

Niuean ☐

Chinese ☐

Indian ☐

Other _____

Name of General Practitioner:

Medical Centre:

.....

.....

.....Postcode

What year were you diagnosed with diabetes?

Metformin and Vitamin B12 Study Baseline Questionnaire

What year was metformin started?

Exclusion checklist: (If “Yes” ticked the person is ineligible)

Yes No

Existing treatment for B12 deficiency

☐ ☐

Folate deficiency or related anaemia

☐ ☐

Pregnancy or lactation

☐ ☐

Cobalamin allergy

☐ ☐

Other reasons why taking part would be practically difficult

☐ ☐

Relevant Medical Conditions:

Yes No

Gastroparesis

☐ ☐

Inflammatory bowel disease

☐ ☐

Thyroid disease

☐ ☐

.....
.....
.....
.....

Current medications and doses:

Diabetes treatment:

.....
.....
.....

Other prescribed treatment:

.....
.....
.....
.....

Metformin and Vitamin B12 Study Baseline Questionnaire

Are you currently taking antibiotics?

(if so, must take the vitamin B12 tablets *at a different time of day* to the antibiotic)

Are you currently taking any of the following medications: Y N

Oral contraceptives	☐	☐
Chloramphenicol	☐	☐
Colchicine	☐	☐
Ranitidine (Zantac)	☐	☐
Phenobarbital	☐	☐
Omeprazole (Losec) /pantoprazole (somac)	☐	☐

(these medications can also lower B12 status)

Have you experienced ongoing unusual fatigue or tiredness
over the last three months? ☐ ☐

Do you drink alcohol? ☐ ☐

If yes – how many units per week:

Do you smoke? ☐ ☐

If yes – how many packs per week

Study Forms:

Michigan neuropathy screening questionnaire ☐

Appendix C – Michigan Neuropathy Screening Instrument

Patient Version

MICHIGAN NEUROPATHY SCREENING INSTRUMENT

A. History (To be completed by the person with diabetes)

Please take a few minutes to answer the following questions about the feeling in your legs and feet. Check yes or no based on how you usually feel. Thank you.

1. Are you legs and/or feet numb?	☐ Yes	☐ No
2. Do you ever have any burning pain in your legs and/or feet?	☐ Yes	☐ No
3. Are your feet too sensitive to touch?	☐ Yes	☐ No
4. Do you get muscle cramps in your legs and/or feet?	☐ Yes	☐ No
5. Do you ever have any prickling feelings in your legs or feet?	☐ Yes	☐ No
6. Does it hurt when the bed covers touch your skin?	☐ Yes	☐ No
7. When you get into the tub or shower, are you able to tell the
hot water from the cold water?	☐ Yes	☐ No
8. Have you ever had an open sore on your foot?	☐ Yes	☐ No
9. Has your doctor ever told you that you have diabetic neuropathy?	☐ Yes	☐ No
10. Do you feel weak all over most of the time?	☐ Yes	☐ No
11. Are your symptoms worse at night?	☐ Yes	☐ No
12. Do your legs hurt when you walk?	☐ Yes	☐ No
13. Are you able to sense your feet when you walk?	☐ Yes	☐ No
14. Is the skin on your feet so dry that it cracks open?	☐ Yes	☐ No
15. Have you ever had an amputation?	☐ Yes	☐ No

Total: _____

MICHIGAN NEUROPATHY SCREENING INSTRUMENT

B. Physical Assessment (To be completed by health professional)

1. Appearance of Feet

Right

a. Normal ☐ 0 Yes ☐ 1 No

b. If no, check all that apply:

Deformities ☐

Dry skin, callus ☐

Infection ☐

Fissure ☐

Other ☐

specify: _____

Left

Normal ☐ 0 Yes ☐ 1 No

If no, check all that apply:

Deformities ☐

Dry skin, callus ☐

Infection ☐

Fissure ☐

Other ☐

specify: _____

Right

Absent

☐ 0

Present

☐ 1

2. Ulceration

Left

Absent

☐ 0

Present

☐ 1

3. Ankle Reflexes

Present	Present/ Reinforcement	Absent
☐ 0	☐ 0.5	☐ 1

3. Ankle Reflexes

Present	Present/ Reinforcement	Absent
☐ 0	☐ 0.5	☐ 1

4. Vibration perception at great toe

Present	Decreased	Absent
☐ 0	☐ 0.5	☐ 1

4. Vibration perception at great toe

Present	Decreased	Absent
☐ 0	☐ 0.5	☐ 1

5. Monofilament

Normal	Reduced	Absent
☐ 0	☐ 0.5	☐ 1

5. Monofilament

Normal	Reduced	Absent
☐ 0	☐ 0.5	☐ 1

Signature: _____

Total Score _____ /10 Points

How to Use the Michigan Neuropathy Screening Instrument

History

The history questionnaire is self-administered by the patient. Responses are added to obtain the total score. Responses of “yes” to items 1-3, 5-6, 8-9, 11-12, 14-15 are each counted as one point. A “no” response on items 7 and 13 counts as 1 point. Item #4 is a measure of impaired circulation and item #10 is a measure of general aesthenia and are not included in scoring. To decrease the potential for bias, all scoring information has been eliminated from the patient version.

Physical Assessment

For all assessments, the foot should be warm ($>30^{\circ}\text{C}$).

Foot Inspection: The feet are inspected for evidence of excessively dry skin, callous formation, fissures, frank ulceration or deformities. Deformities include flat feet, hammer toes, overlapping toes, halux valgus, joint subluxation, prominent metatarsal heads, medial convexity (Charcot foot) and amputation.

Vibration Sensation: Vibration sensation should be performed with the great toe unsupported. Vibration sensation will be tested bilaterally using a 128 Hz tuning fork placed over the dorsum of the great toe on the bony prominence of the DIP joint. Patients, whose eyes are closed, will be asked to indicate when they can no longer sense the vibration from the vibrating tuning fork.

In general, the examiner should be able to feel vibration from the hand-held tuning fork for 5 seconds longer on his distal forefinger than a normal subject can at the great toe (e.g. examiner’s DIP joint of the first finger versus patient’s toe). If the examiner feels vibration for 10 or more seconds on his or her finger, then vibration is considered decreased. A trial should be given when the tuning fork is not vibrating to be certain that the patient is responding to vibration and not pressure or some other clue. Vibration is scored as 1) present if the examiner senses the vibration on his or her finger for < 10 seconds, 2) reduced if sensed for ≥ 10 or 3) absent (no vibration detection.)

Muscle Stretch Reflexes: The ankle reflexes will be examined using an appropriate reflex hammer (e.g. Trommer or Queen square). The ankle reflexes should be elicited in the sitting position with the foot dependent and the patient relaxed. For the reflex, the foot should be passively positioned and the foot dorsiflexed slightly to obtain optimal stretch of the muscle. The Achilles tendon should be percussed directly. If the reflex is obtained, it is graded as present. If the reflex is absent, the patient is asked to perform the Jendrassic maneuver (i.e., hooking the fingers together and pulling). Reflexes elicited with the Jendrassic maneuver alone are designated “present with reinforcement.” If the

reflex is absent, even in the face of the Jendrassic maneuver, the reflex is considered absent.

Monofilament Testing: For this examination, it is important that the patient's foot be supported (i.e., allow the sole of the foot to rest on a flat, warm surface). The filament should initially be prestressed (4-6 perpendicular applications to the dorsum of the examiner's first finger). The filament is then applied to the dorsum of the great toe midway between the nail fold and the DIP joint. Do not hold the toe directly. The filament is applied perpendicularly and briefly, (<1 second) with an even pressure. When the filament bends, the force of 10 grams has been applied. The patient, whose eyes are closed, is asked to respond yes if he/she feels the filament. Eight correct responses out of 10 applications is considered normal: one to seven correct responses indicates reduced sensation and no correct answers translates into absent sensation.

Appendix D – Otago School of Medicine Food Questionnaire

Study Number

Food?

Questionnaire 1

Different eating patterns have an effect on people's health. To help us understand these eating patterns we would like you to think about all the foods you have had over the **last year** and answer the following questions about the foods you **usually** eat. This food questionnaire measures how often, **on average**, you have eaten a specified amount of food over the past 12 months.

If you have any queries regarding this questionnaire please contact:

YOUR DIET LAST YEAR

For each food there is an amount shown, either "one serving", or an example of a serving such as "a slice" or "a teaspoon". Please put a tick (✓) in the box to indicate how often, on average, you have eaten a specified amount of food over the last 12 months. Answer each question as best you can. Estimate if you are not sure. A guess is better than no answer! When you are finished the questionnaire please check every page to see if you have missed any questions. Please complete the questionnaire using a blue or black pen. The rest of this page shows examples of how to fill in the questionnaire. These questions are all about the food you have eaten over the last year.

Example 1:

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Apples (1 fruit)	☐	☐	☐	☒	☐	☐	☐

When asked how often I eat "Apples" I usually have 3 apples every week. The serving size is one piece of fruit. Therefore I have 3 servings per week and should place a tick in the "2-4 per week" box.

Example 2:

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
White bread (1 slice/roll)	☐	☐	☐	☐	☒	☐	☐

When asked how often I eat "White bread (1 slice/roll)" I usually have 3 slices of bread and 2 rolls every week. The serving size is one slice or roll. Therefore I have 5 servings per week and should place a tick in the "5-6 per week" box.

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

DAIRY PRODUCTS AND EGGS	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Cream or sour cream (1 tablespoon)	☐	☐	☐	☐	☐	☐	☐
Low fat, low sugar yoghurt (1 small pottle) e.g. Delite, Fresh 'n' Fruity lite	☐	☐	☐	☐	☐	☐	☐
Low fat yoghurt, fromage frais (1 small pottle) e.g. Fresh 'n' Fruity, Meadow Fresh	☐	☐	☐	☐	☐	☐	☐
Full fat or Greek yoghurt (1 small pottle) e.g. Cyclops, Puhoi Valley	☐	☐	☐	☐	☐	☐	☐
Dairy desserts (1 small pottle) e.g. chocolate mousse	☐	☐	☐	☐	☐	☐	☐
Cheese (1 matchbox size) e.g. cheddar, mild, brie, edam	☐	☐	☐	☐	☐	☐	☐
Cottage/feta Cheese (2 tablespoons) e.g. Tararua, Country Goodness	☐	☐	☐	☐	☐	☐	☐
Quiche (1 slice)	☐	☐	☐	☐	☐	☐	☐
Eggs as boiled, fried, scrambled etc (1 egg)	☐	☐	☐	☐	☐	☐	☐
Low fat mayonnaise (1 tablespoon) e.g. ETA Lite n' Free	☐	☐	☐	☐	☐	☐	☐
Full fat mayonnaise (1 tablespoon) e.g. Best Foods real mayonnaise, Heinz	☐	☐	☐	☐	☐	☐	☐
French dressing (1 tablespoon)	☐	☐	☐	☐	☐	☐	☐
Salad dressing (1 tablespoon) e.g. Pams creamy salad dressing, coleslaw dressing	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

The following list refers to **DAIRY PRODUCTS AND FATS** that you put on bread or cooked vegetables

Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
--------------------------------------	---------------	---------------	--------------	--------------	-------------	---------------------

Butter (1 teaspoon)

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Reduced fat butter (1 teaspoon)
e.g. Mainland Country soft lite, Taranua
super soft lite

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Olive oil spread (1 teaspoon)
e.g. Olivio or Olivani

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Polyunsaturated margarine (1 teaspoon)
e.g. Flora, Gold 'n' Canola

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Other margarines (1 teaspoon)
e.g. Pams, MeadowLea

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Reduced fat spread (1 teaspoon)
e.g. Flora ultra lite

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Cholesterol lowering fat spreads
(1 teaspoon) e.g. Pro-active, Logicol

☐	☐	☐	☐	☐	☐	☐
--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------	--------------------------

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

FRUIT

For seasonal fruits marked*, please estimate your average consumption when these are in season

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Apples (1 fruit)	☐	☐	☐	☐	☐	☐	☐
Pears (1 fruit)	☐	☐	☐	☐	☐	☐	☐
Oranges (1 fruit), mandarins (2 fruit)	☐	☐	☐	☐	☐	☐	☐
Grapefruit (half)	☐	☐	☐	☐	☐	☐	☐
Banana (1 fruit)	☐	☐	☐	☐	☐	☐	☐
Grapes (1 handful)	☐	☐	☐	☐	☐	☐	☐
Melon (1 slice)	☐	☐	☐	☐	☐	☐	☐
*Peaches, plums, apricots (1 fruit)	☐	☐	☐	☐	☐	☐	☐
*Strawberries, raspberries, other berries (1 handful)	☐	☐	☐	☐	☐	☐	☐
Kiwifruit (1 fruit)	☐	☐	☐	☐	☐	☐	☐
Tinned fruit (1/2 a cup)	☐	☐	☐	☐	☐	☐	☐
Dried fruit (1 heaped tablespoon) e.g. raisins, prunes	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

VEGETABLES

Fresh, frozen or tinned

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Onions (handful)	☐	☐	☐	☐	☐	☐	☐
Garlic (1 clove)	☐	☐	☐	☐	☐	☐	☐
Mushrooms (handful)	☐	☐	☐	☐	☐	☐	☐
Capsicum (handful)	☐	☐	☐	☐	☐	☐	☐
Bean sprouts (handful)	☐	☐	☐	☐	☐	☐	☐
Beetroot (handful)	☐	☐	☐	☐	☐	☐	☐
Sweetcorn (handful)	☐	☐	☐	☐	☐	☐	☐
Tomatoes (handful)	☐	☐	☐	☐	☐	☐	☐
Watercress/ Puha (handful)	☐	☐	☐	☐	☐	☐	☐
Coleslaw (handful)	☐	☐	☐	☐	☐	☐	☐
Green salad, lettuce, cucumber, celery (handful)	☐	☐	☐	☐	☐	☐	☐
Avocado (half)	☐	☐	☐	☐	☐	☐	☐
Carrots (handful)	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

VEGETABLES

Fresh, frozen or tinned

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Cabbage (handful)	☐	☐	☐	☐	☐	☐	☐
Spinach/Silverbeet (handful)	☐	☐	☐	☐	☐	☐	☐
Broccoli (handful)	☐	☐	☐	☐	☐	☐	☐
Brussel sprouts (handful)	☐	☐	☐	☐	☐	☐	☐
Pumpkin/Kumara (handful)	☐	☐	☐	☐	☐	☐	☐
Baked beans (handful)	☐	☐	☐	☐	☐	☐	☐
Peas (handful)	☐	☐	☐	☐	☐	☐	☐
Green beans, broad beans, runner beans (handful)	☐	☐	☐	☐	☐	☐	☐
Lentils, chickpea, pulses (handful) e.g. red kidney bean	☐	☐	☐	☐	☐	☐	☐
Marrow, courgettes (handful)	☐	☐	☐	☐	☐	☐	☐
Cauliflower (handful)	☐	☐	☐	☐	☐	☐	☐
Parsnips, turnips, swedes (handful)	☐	☐	☐	☐	☐	☐	☐
Leeks (handful)	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

MEAT AND FISH

(half a cup)

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Lamb: roast, steak, chops, mince, casserole or stew	☐	☐	☐	☐	☐	☐	☐
Beef: roast, steak, mince, casserole or stew	☐	☐	☐	☐	☐	☐	☐
Beef burgers	☐	☐	☐	☐	☐	☐	☐
Chicken or other poultry: roast, steak, chops, mince, casserole or stew	☐	☐	☐	☐	☐	☐	☐
Pork: roast, steak, mince, chops, casserole or stew	☐	☐	☐	☐	☐	☐	☐
Bacon	☐	☐	☐	☐	☐	☐	☐
Ham, cured meats, salami, chorizo	☐	☐	☐	☐	☐	☐	☐
Corned beef, spam, luncheon meats	☐	☐	☐	☐	☐	☐	☐
Sausages	☐	☐	☐	☐	☐	☐	☐
Savoury pies e.g. meat pie, pork pie, pastries, steak and kidney pie, sausage roll	☐	☐	☐	☐	☐	☐	☐
Liver, liver pate, liver sausage	☐	☐	☐	☐	☐	☐	☐
Fried fish in batter (one piece) as in fish and chips	☐	☐	☐	☐	☐	☐	☐
Fish fingers, fish cakes and crumbed fish e.g. Birdseye oven bake	☐	☐	☐	☐	☐	☐	☐
White fish, fresh or frozen e.g. hoki, cod, sole, gurnard, tarakihi	☐	☐	☐	☐	☐	☐	☐
Other fish e.g. tuna, mackerel, salmon, sardines	☐	☐	☐	☐	☐	☐	☐
Shellfish e.g. crab, prawns, mussels, oysters	☐	☐	☐	☐	☐	☐	☐
Meat substitutes e.g. tofu, soymeat, textured vegetable protein, veggieburger	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

BREAD AND SAVOURY BISCUITS

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
White bread (1 slice/roll)	☐	☐	☐	☐	☐	☐	☐
Wholemeal bread (1 slice/roll)	☐	☐	☐	☐	☐	☐	☐
Wholegrain bread (1 slice/roll)	☐	☐	☐	☐	☐	☐	☐
Cream crackers, savoury biscuits (1 biscuit) e.g. Huntley and Palmers	☐	☐	☐	☐	☐	☐	☐
Crispbread, rice crackers (1 biscuit) e.g. Ryvita, Cruskits, Vitawheat	☐	☐	☐	☐	☐	☐	☐
Naan, poppadoms, flour tortillas (1 piece)	☐	☐	☐	☐	☐	☐	☐

SOUPS, SAUCES, AND SPREADS

Vegetable soup (bowl)	☐	☐	☐	☐	☐	☐	☐
Meat soups (includes meat and vegetable soups) (bowl)	☐	☐	☐	☐	☐	☐	☐
Tomato sauce (1 tablespoon)	☐	☐	☐	☐	☐	☐	☐
Sauces (1 tablespoon) e.g. white sauce, cheese sauce, gravy	☐	☐	☐	☐	☐	☐	☐
Pickles, chutneys (1 tablespoon)	☐	☐	☐	☐	☐	☐	☐
Jam, marmalade, honey (1 tablespoon)	☐	☐	☐	☐	☐	☐	☐
Marmite, vegemite (1 teaspoon)	☐	☐	☐	☐	☐	☐	☐
Peanut butter (1 teaspoon)	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

SWEETS AND SNACKS

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Cakes (one cup) e.g. chocolate, banana, carrot, fruit, sponge	☐	☐	☐	☐	☐	☐	☐
Muffin, slices, pastries (one), brownie e.g. scones, croissants, doughnuts	☐	☐	☐	☐	☐	☐	☐
Fruit pies, tarts, crumble (one cup)	☐	☐	☐	☐	☐	☐	☐
Milk puddings (one cup) e.g. rice custard, trifle	☐	☐	☐	☐	☐	☐	☐
Ice cream (one cup)	☐	☐	☐	☐	☐	☐	☐
Chocolate sweet biscuits (two) e.g. Tim Tams, Toffee Pops.	☐	☐	☐	☐	☐	☐	☐
Plain sweet biscuits (two) e.g. Nice, Gingernuts	☐	☐	☐	☐	☐	☐	☐
Reduced fat biscuits (two) e.g. Weight Watchers	☐	☐	☐	☐	☐	☐	☐
Muesli bars, cereal bars (one)	☐	☐	☐	☐	☐	☐	☐
Chocolate snack bars (one) e.g. Mars, Crunchie	☐	☐	☐	☐	☐	☐	☐
Dark chocolates, single or squares (one)	☐	☐	☐	☐	☐	☐	☐
White or milk chocolates, single or squares (one)	☐	☐	☐	☐	☐	☐	☐
Lollies, toffees, mints (small packet)	☐	☐	☐	☐	☐	☐	☐
Chips, packet snacks (1 small packet) e.g. Corn chips, cheezels	☐	☐	☐	☐	☐	☐	☐
Nuts (1 handful) e.g. Brazil, walnuts, peanuts, cashews	☐	☐	☐	☐	☐	☐	☐
Seeds (1 tablespoon) e.g. Sunflower, pumpkin	☐	☐	☐	☐	☐	☐	☐
Sugar added to tea, coffee, cereal (1 teaspoon)	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

CEREAL
(One bowl)

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Porridge, oats	☐	☐	☐	☐	☐	☐	☐
High fibre breakfast cereals e.g. Bran Flakes, Fibre Plus, All Bran	☐	☐	☐	☐	☐	☐	☐
Sugar topped cereals e.g. Cocopops, Crunchy Nut Cornflakes, Froot Loops	☐	☐	☐	☐	☐	☐	☐
Breakfast cereals e.g. Cornflakes, Ricies, Special K, Weetbix	☐	☐	☐	☐	☐	☐	☐
Toasted muesli	☐	☐	☐	☐	☐	☐	☐
Natural Muesli	☐	☐	☐	☐	☐	☐	☐

POTATOES, RICE AND PASTA
(One cup)

Potato: Boiled, mashed, instant, baked, roasted	☐	☐	☐	☐	☐	☐	☐
French fries, hot chips, wedges, hashbrowns	☐	☐	☐	☐	☐	☐	☐
Potato salad	☐	☐	☐	☐	☐	☐	☐
White rice-based dishes e.g. Stir-fry, curry, savoury sauce	☐	☐	☐	☐	☐	☐	☐
Brown rice-based dishes e.g. Stir-fry, curry, savoury sauce	☐	☐	☐	☐	☐	☐	☐
Lasagne, moussaka	☐	☐	☐	☐	☐	☐	☐
White pasta-based dishes e.g. macaroni cheese, spaghetti bolognaise, instant noodles	☐	☐	☐	☐	☐	☐	☐
Wholemeal pasta-based dishes e.g. macaroni cheese, spaghetti bolognaise, instant noodles	☐	☐	☐	☐	☐	☐	☐
Pizza (one slice)	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

FOOD AND AMOUNTS

AVERAGE USE IN THE LAST TWELVE MONTHS

DRINKS

	Never or less than once per month	1-3 per month	Once per week	2-4 per week	5-6 per week	1 every day	2 or more every day
Green Tea (1 cup)	☐	☐	☐	☐	☐	☐	☐
Herb/Fruit Tea (1 cup) e.g. Healtheries Sleep Tea, Twinings Peach and Raspberry	☐	☐	☐	☐	☐	☐	☐
Black tea (1 cup)	☐	☐	☐	☐	☐	☐	☐
Coffee: instant, ground, espresso (1 cup) e.g. short black, latte, cappuccino	☐	☐	☐	☐	☐	☐	☐
Coffee decaffeinated (1 cup)	☐	☐	☐	☐	☐	☐	☐
Cocoa, Hot Chocolate (1 cup) e.g. Cadburys, Pams	☐	☐	☐	☐	☐	☐	☐
Low fat hot chocolate (1 cup) e.g. Jarrah, Nestle Double Blend	☐	☐	☐	☐	☐	☐	☐
Milo, Horlicks, Ovaltine (1 cup)	☐	☐	☐	☐	☐	☐	☐
White wine (1 small glass)	☐	☐	☐	☐	☐	☐	☐
Red Wine (1 small glass)	☐	☐	☐	☐	☐	☐	☐
Beer, lager or cider (1 standard drink)	☐	☐	☐	☐	☐	☐	☐
Port, sherry, vermouth, liqueurs (1 standard drink)	☐	☐	☐	☐	☐	☐	☐
Spirits (1 nip) e.g. gin, brandy, whisky, vodka	☐	☐	☐	☐	☐	☐	☐
Standard fizzy soft drinks (1 cup) e.g. Coca Cola, lemonade	☐	☐	☐	☐	☐	☐	☐
Diet fizzy soft drinks (1 cup) e.g. Diet coke, Spite zero	☐	☐	☐	☐	☐	☐	☐
Fruit juice (100%) (1 cup) e.g. orange, apple juice	☐	☐	☐	☐	☐	☐	☐
Standard Cordial (1 cup) e.g. Raro, Refresh	☐	☐	☐	☐	☐	☐	☐
Diet cordial (1 cup)	☐	☐	☐	☐	☐	☐	☐
Smoothies (1 cup)	☐	☐	☐	☐	☐	☐	☐

Please check that you have put a tick (✓) on EVERY line

YOUR DIET LAST YEAR, continued

2. Are there any OTHER foods which you ate more than once a week over the last year?

No ☐ → **GO TO** question 3 Yes ☐ **if yes, please list below**

Food	Serving Size	How many times per week?
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

3. Have you drunk milk or milk substitute over the past year? (including in hot drinks)

No ☐ → **GO TO** question 6 Yes ☐ → **GO TO** question 4

4. What type of milk or milk substitute did you most often use over the last year?

Select one only

☐ Full cream (blue top)	☐ Milk powder
☐ Trim (green top)	☐ Calci-trim (yellow top)
☐ Light milk (light blue top)	☐ Soya
☐ other (please specify) _____	

5. About how much milk did you drink each day, including milk with tea, coffee, cereals etc?

☐ Less than half a cup	☐ More than one cup
☐ Half a cup	☐ More than two cups
☐ One cup	

6. Have you eaten meat in the last year?

No ☐ → **GO TO** question 8 Yes ☐ → **GO TO** question 7

7. What did you do with the visible fat on your meat?

☐ Did not eat meat with visible fat	☐ Ate some of the fat
☐ Trimmed all visible fat and did not eat	☐ Ate most of the fat

8. We would like to summarise what you have told us. During the course of last year, on average how many times **a week** did you eat the following foods?

Food Type	Times/week	Serving size
Vegetables (excluding potatoes but including baked beans)	<input type="text"/> <input type="text"/>	1/2 cup
Salads (e.g. green salad, mesclun)	<input type="text"/> <input type="text"/>	1 cup
Fruit	<input type="text"/> <input type="text"/>	1 medium/2 small
Fish, seafood	<input type="text"/> <input type="text"/>	1/2 cup cooked
Meat, meat products and meat dishes (including bacon, ham and chicken)	<input type="text"/> <input type="text"/>	1/2 cup cooked

Thank you for completing this questionnaire!
Please check that you have answered every question.

Appendix E – Information sheet for patients in randomised control trial

Information Sheet

The administration of vitamin B12 to people with T2DM on long term metformin therapy

Principal investigator-

Dr Amber Parry Strong, Post-Doctoral Research Fellow, Endocrine, Diabetes and Research Centre, Wellington Hospital, Wellington

Co-Investigators

Dr Jeremy Krebs, Clinical Leader, Endocrine, Diabetes and Research Centre, Wellington Hospital, Wellington;

Sylvan Haeusler, Masters Student, Endocrine, Diabetes and Research Centre, Wellington Hospital, Wellington;

You are invited to take part in the above study. Your participation is entirely voluntary (your choice). You do not have to take part in this study, and if you choose not to take part this will not affect any future care or treatment. If you do agree to take part in the study, you are free to withdraw from the study at any time, without having to give a reason, and this will in no way affect your future or continuing health care.

About the study

You have been selected because a blood test taken for the Diabetes Clinic at Wellington Hospital showed you have low levels of vitamin B12, and you take the diabetes medication Metformin.

The aim of this study is to compare the usual treatment of low vitamin B12 (an injection) with vitamin B12 tablets in people taking Metformin.

There are studies showing that taking the diabetes medication Metformin can stop people from absorbing vitamin B12 from food, and so over some time of taking this medication, vitamin B12 levels can become too low. The main symptom of low vitamin B12 levels is tiredness. At the moment there is an injection funded to increase vitamin B12 levels again, but we would like people to have the choice of an injection or tablets, and we need to find out whether the tablets work just as well as the injection. This study will help us to know if tablets are a good treatment for people with low vitamin B12 levels who take Metformin.

What will happen during the study

To do this we would like you to come in for one visit to either have the injection or collect your tablets, depending on which arm of the study you end up in. Whether you get the tablets or the injection will be completely random, like the toss of a coin. At your visit we will also ask some questions about symptoms you may have been experiencing and check for neuropathy. You will then do another blood test three months later to see if your B12 levels have increased enough. If they haven't, you will have another course of treatment.

Benefits, Risks and Safety

The major benefit of this study is to further our knowledge of which options are effective in treating low vitamin B12 levels in people who take Metformin. Your GP will be informed of your participation and results of the investigations will be forwarded onto your GP for any action.

There is little risk to taking part in the study. We require some of your time and the inconvenience of a blood test. Any adverse outcome should be covered by ACC compensation provisions.

There will be no direct cost for taking part in the study. We expect to be able to cover parking and some transport costs but there is no payment for taking part in the study. The vitamin B12 injection or tablets will be provided to you for free.

If you require additional information on the trial you can contact Dr Jeremy Krebs or Dr Amber Parry strong at the Endocrine, Diabetes and Research Centre, Wellington Hospital, Wellington. Phone 04 8062458.

If required an interpreter can be provided.

If you have any queries regarding your rights as a participant in this study, you may wish to contact an independent health and disability advocate:

Free phone : 0800 555 050

Free fax: 0800 2 SUPPORT (0800 2787 7678)

Email: advocacy@hdc.org.nz

Compensation

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation, and Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Injury Prevention, Rehabilitation, and Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

You are also advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.

Confidentiality

All details will be kept in a confidential way as per Capital and Coast DHB policy. All subjects will be given a unique identifying code and only this will be used as a marker for analysis. No material that could personally identify you will be used in any reports on this study will be used in any of the final reports.

We expect publication of results in international journals and presentations at national or international conferences. Participants are able to get copies of reports and presentations if they wish. There will be a delay between data collection and publication of results.

This study has received ethical approval from the Central Region Ethics Committee, which reviews national and multi regional studies.